Immunomagnetic separation and typing of a food-borne pathogen: *Campylobacter jejuni*

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Declaration

I declare that this thesis was composed by myself and the research presented is my own, except where otherwise stated.

Simon Andrew Needham
2006

Dedicated to Florence Needham
15th April 1911- 4th November 2005

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I would like to thank Bruce Ward, my supervisor, for his encouragement and advice over the course of my project. Thanks also go to Andrew Coulson. David Leach and Graeme Reid, who have acted as my second supervisor throughout this project. Andreas Merdes and Maggie Chambers gave invaluable technical advice and support during the monoclonal experiments; and without which the experiments would not have been possible.

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Lastly, I must thank Jeff Needham, my father for providing the funding for this project and enabling me to continue my education.

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Abbreviations

Ab	Antibody
Ac	Acetyl
ACMSF	Advisory Committee on the Microbiological Safety of Food
AFLP	Amplified fragment length polymorphism
Ag	Antigen
Am	Acteimidoyl
Amp	Ampicillin
Bac	2,4-diacetmido-2,4,6-trioxy-D-glucopyranose
BSA	Bovine serum albumin
CHM	Complete hybridoma medium
Cmr	Chloramphenicol
CMP-NeuNAc	CMP-N-acetylneuraminic acid
CPS	Capsular polysaccharide
EDTA	Disodium ethylenediaminetetraacetate
ELISA	Enzyme linked immuno-sorbent assay
EtBr	Ethidium bromide
EtOH	Ethanol
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
G-ManHep	L-glycero-D-manno-heptose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GBS	Guillian-Barré syndrome
GlcNAc	N-acetylglucosamine
GSL	Glycosphingolipid
Glu	Glucose
HAT	hypoxyanthine-aminopterin-thymidine
Hex	Hexose
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HS:	Heat stable serotype or Penner serotype
IFA	Immuno-fluorescent assay
IMS	Immunomagnetic separation
IPTG	Isopropyl β-D-thiogalactopyranoside
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
LB	Luria-Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LT-PCR	Long template polymerase chain reaction
mAb	Monoclonal antibody
ManDAc	N-acetylmannosamine
MCM	Myeloma culture media
MFS	Miller-Fisher syndrome
MLEE	Multi-locus enzyme electrophoresis

MLST	Multi-locus sequence typing
NCTC	National Collection of Type Cultures
Neu5Ac	N-acetylneuramic acid
NeuNAc	N-acetylneuramic acid
PAGE	Polyacrylamide gel eletrophoresis
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis
RFLP	Restriction fragment length polymorphism
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SedHep	Sedoheptulose-7-phosphate
SFM	Serum free medium
ST	Sequence type
Tricine	N-tris(hydroxymethyl)methylglycine
TIGR	The Institute for Genomic Research
Tris	Tris(hydroxymethyl)aminomethane
UDP-GlcNAc	UDP-N-acetyl-D-glucosamine
USDA	United States Department of Agriculture
VNC	Viable-but-non-culturable
X-gal	5-Bromo-4-chloro-indoyl-β-D-galactoside

Abstract

Campylobacter jejuni is acknowledged as the most common cause of bacterial enteritis in the world. In addition its association with a number of autoimmune diseases makes reliable isolation and typing of major importance.

PEB3, a cell envelope protein, was identified as a target for use in an immunomagnetic isolation system (IMS). *Peb3* was cloned into *Escherichia coli* and expressed as a His-tagged construct (His.PEB3). Rabbits were then challenged with the purified construct to produce polyclonal antisera. Isolation of *C. jejuni* from a mixed culture (*E. coli* or *Arcobacter* spp. and *C. jejuni*) with polyclonal antisera was attempted but failed to capture whole *C. jejuni* cells.

C. jejuni expresses two cell envelope associated polysaccharides: a short chain lipooligosaccharide (LOS) and a long chain lipid-linked polysaccharide, capsular polysaccharide (CPS). The Penner serotyping system has been shown to use CPS as its major discriminatory determinant. A novel molecular based typing system was devised based upon restriction fragment length polymorphism (RFLP) using the CPS locus as a discriminatory determinant. Primers for use in long template PCR (LT-PCR) were designed against the genes, *kpsD*, *kpsS*, *gmhA2* and *cj1430*. The theoretical maximum amplicon length obtainable in LT-PCR is ~40 kb; however, this is dependent on a number of factors including the %GC of the target amplicon. The low %GC of *C. jejuni* DNA prevented the amplification of the entire locus length (~41 kb) in one reaction and by experimentation the maximum amplifiable length was determined to be 25 kb. This maximum length was only achieved sporadically and the maximum length that could be reliably amplified was ~15 kb. Because of this limitation only the amplicon *kpsS-gmhA2* was used in the typing scheme.

Amplification was only achieved with the type strain, *C. jejuni* NCTC 11168. This is believed to be due to difficulties in amplifying low %GC targets with the Expand™ 20 kb plus long template system (Roche).

Introduction

1.1 General Background

Campylobacter has been long recognized as a veterinary pathogen (Skirrow, 1987); *C. fetus* is a cause of spontaneous abortion in cattle and sheep. Other members of the genus have been implicated in a variety of diseases in domestic animals including enteritis. Although, the first isolation was in 1833 (Nachamkin and Blaser, 2000) the characterisation of human *Campylobacters* did not begin in depth until 1957 (King, 1957). The link between *Campylobacter* and human enteritis was finally confirmed in 1977 by Skirrow, who used a mixture of vancomycin, polymyxin B and trimethoprim to inhibit other intestinal organisms in faeces obtained from patients with diarrhoea (Skirrow, 1987).

The family Campylobacteraceae (part of the epsilon proteobacteria) has two other members, *Arcobacter* and *Sulfurospirillum*. *Arcobacter* spp. are becoming of increasing interest as they have been implicated in enteritis in humans and livestock, as well as, spontaneous abortion in livestock (Wesley, 1997). Sulfurospirilla have been isolated from oxidized marine sediment (Finster *et al.*, 1997) and are not known to be pathogenic. The Campylobacteraceae are also closely related to *Helicobacter* and *Wolinella* (family Helicobacteraceae). Like *Campylobacter* and *Arcobacter* spp., the genus *Helicobacter* is a known pathogen of humans; *H. pylori* has been linked to formation of gastric ulcers (Wesley, 1997). Reclassification of members of Campylobacteraceae and Helicobacteraceae is ongoing, meaning that the previously non-pathogenic genus *Wolinella* now contains *W. rectus* (formally *C. rectus*), which is associated with gingivitis and peridonitis (Lai *et al.*, 1992). Figure 1.1 shows the phylogeny of the Campylobacteraceae and some of the diseases caused by its members.

The campylobacters form the largest genus (fifteen species) and most are important veterinary or medical pathogens. Of greatest importance, medically, are *C. jejuni* and *C. coli*; accounting for approximately 57 % and 3 % of food poisoning cases within England and Wales during 2004 (Health Protection Agency, http://www.phls.co.uk/infections/topics_az/campy/data_ew.htm).

1.2 *C. jejuni* as the causative agent in food-poisoning

C. jejuni is the primary cause of bacterial enteritis worldwide, annually causing 3 to 4 times more cases than *Salmonella* (see Figure 1.2). The number of reported cases of *C. jejuni* steadily increased during the 1980's reaching a peak in 2000. This is partially due to increased recognition by health services and better surveillance. Since 2000 the number of reported cases of both *C. jejuni* and *Salmonella* has fallen due to the increased emphasis on food hygiene education by the government. *C. jejuni* is a commensal of both chickens and pigs; consumption of improperly cooked chicken products is the major route of infection. Other routes include the consumption of unpasteurised milk, untreated water and contact with infected animals. During April and June 2001, the Advisory Committee on the Microbiological Safety of Food (ACMSF) sampled chicken products throughout the UK and found that 50 % were contaminated with *Campylobacter* c.f. *Salmonella* – 5.7 % (ACMSF report, 2004). Of major concern is *C. jejuni*'s ability to assume a 'viable but non-culturable' (VNC) state, brought about by stress such as starvation. A number of bacteria are known to form VNC states including *Salmonella enterica* (Asakura *et al.*, 2002), *Salmonella typhi* (Cho and Kim, 1999),

Listeria monocytogenes (Besnard *et al.*, 2000), *Vibrio cholera* (Shukla, 1995), *H. pylori* (Velazquez and Feirtag, 1999) and *E. coli* (Arana *et al.*, 1997). Importantly, the VNC state of *C. jejuni* retains its infectivity (Stern *et al.*, 1994); meaning that sentinel surveillance at food processing facilities may miss contaminated meat. Studies of *C. jejuni* VNC state cells has shown them to be more susceptible to a number of common disinfectants containing quaternary ammonium compounds or amphoterics used in the food industry. It is thought that this is due to degradation in cell membrane integrity during the transition from culturable to VNC states (Rowe *et al.*, 1998).

The infectious dose varies considerably, between 500 cells and 5 x 10^6 cells, depending on both the immune status of the individual and the manner in which the dose is ingested (Kothary and Babu, 2001). Infection is also characterized by flu-like symptoms prior to the onset of diarrhoea, stomach cramps and occasionally vomiting. The flu-like symptoms are brought about by a severe inflammatory reaction during the initial infection stage. Diarrhoea is caused by the subsequent invasion of the epithelial cells by the *C. jejuni* and destruction of the epithelial lining. Like *Salmonella* and *E. coli* the lipopolysaccharide/lipooligosaccharide of *C. jejuni* is toxic and thought to play an important role in the development several associated diseases (see below). Several toxins are produced by some strains but their contribution to the pathophysiology of *C. jejuni* is uncertain (Wasenaar and Blaser, 1999).

While not fatal in itself *C. jejuni* infection can lead to a number of extremely painful, debilitating and potentially life threatening diseases. Guillian-Barré syndrome (GBS) and its subset, Miller-Fisher syndrome (MFS), cause flaccid

paralysis and degradation of the autonomic nerve system. Hospitalisation is often required as the paralysis lasts for months and can lead to collapse of the respiratory system. Both GBS and MFS are caused by the demyelinization of nerves by the immune system. It has been shown that mimics of the gangliosides present on the surface of the nerves are present in some strains of *C. jejuni* (see 1.5.1). Other examples of *C. jejuni* associated disease are Henoch-Scholein purpura (potentially fatal), Irritable Bowel syndrome and Reactive Arthritis (both chronic and debilitating). The financial cost of *C. jejuni* enteritis is estimated at £ 587 per patient in the UK (Sockett and Pearson, 1987).

The epidemiology of *C. jejuni* infections is interesting because it follows a seasonal distribution (Nylen *et al.*, 2002) and can be accounted for by different cooking practices during the summer (e.g. barbecuing (Allerberger *et al.*, 2003)). Cases tend to be sporadic in nature as person to person transmission is rare. Outbreaks of a large scale have been recorded and are usually due to an infected person improperly handling food (Fitzgerald *et al.*, 2001).

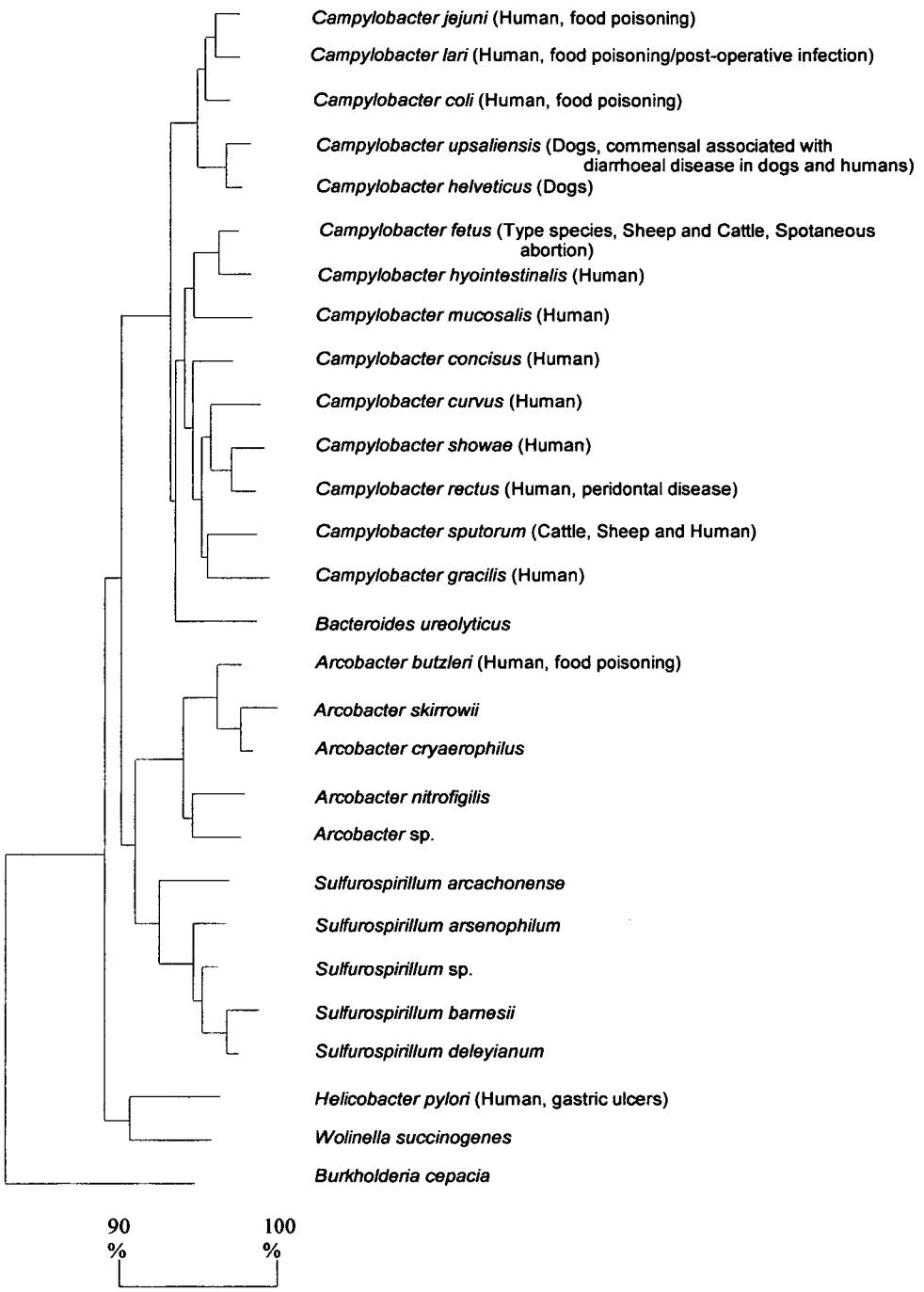


Figure 1.1 Phylogenetic tree of the family Campylobacteraceae, based upon 16S rRNA sequence homology; including the close relatives *Helicobacter pylori* and *Wolinella succinogenes*. Hosts are shown and associated diseases (if known and confirmed). *Burkholderia cepacia* is included as an outgroup organism (adapted from Nachamkin and Blaser, 2000)

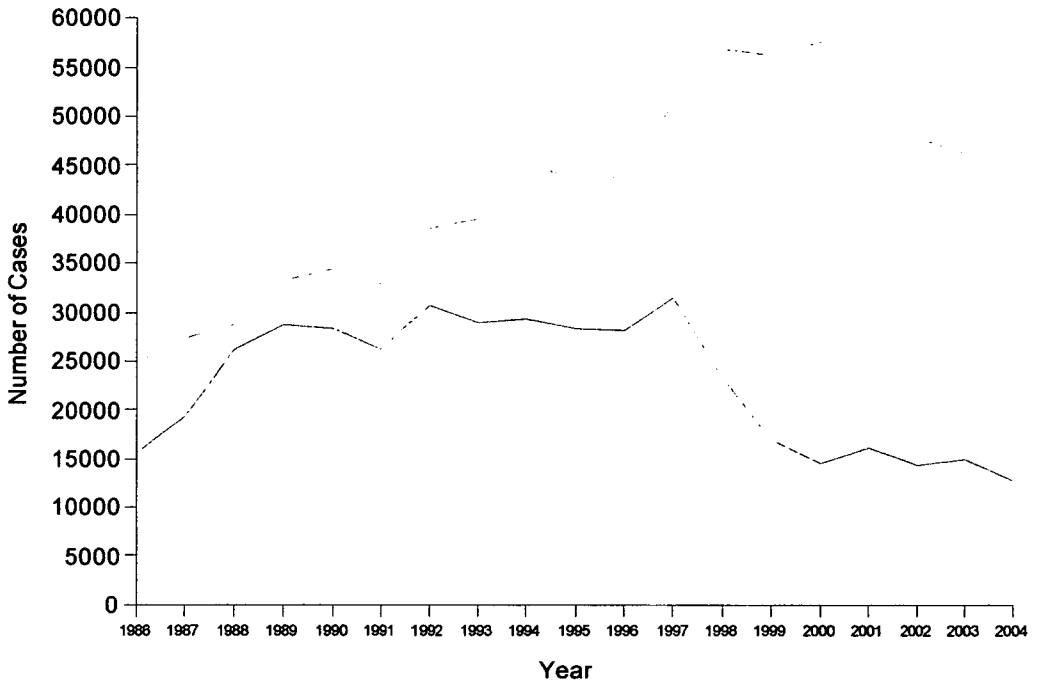


Figure 1.2 Graph showing the number of isolations reported to the Health Protection Agency (in England and Wales) of *Campylobacter* spp. (blue line) and *Salmonella* spp. (red line) during the period 1986 to 2004.

1.3 *C. jejuni* genomics

C. jejuni NCTC 11168 was the second pathogenic organism to be sequenced by the Sanger Institute and was completed in 2000 (Parkhill *et al.*). The decision to sequence this strain was made because it was hoped that the genome sequence would explain some interesting observations on the capability of *C. jejuni* to produce toxins (Wassenaar and Blaser, 1999). The genome size was determined to be 1.64 Mb and encoded 1654 putative genes, making it one of the densest genomes sequenced (see Table 1.1 for a comparison with other related bacteria). It was also unusual as no insertion sequences or phage associated sequences were found.

A second strain of *C. jejuni*, RM1221, was sequenced by The Institute for Genomic Research (TIGR) and completed in 2004 (Fouts *et al.*, 2005). It was hoped that comparison of the two genomes would show differences that influenced host range as NCTC 11168 was a human isolate and RM 1221 was isolated from a chicken carcass. However, the genomes showed an extremely high degree of synteny (see Table 1.1), the only differences being the presence of 4 phage associated sequences in RM 1221 and differing capsular polysaccharide (CPS) gene complements. Both genomes include homopolymeric tracts of guanidine and cytidine thought to be important in protein expression as mechanisms for phase variation. The tracts are clustered within the loci for lipooligosaccharide (LOS), CPS and flagella biosynthesis, all of which are important recognition factors for the host.

Species	***Wolinella succinogenes***	***Helicobacter pylori***	***C. jejuni***	***C. jejuni***	***C. coli***	***C. lari***	***C. upsaliensis***
Strain	DSM 1740	26695	NCTC 11168	RM 1221	RM 2228	RM 2100	RM 3195
Host	Bovine	Human	Human	Avian	Avian	Avian	Canine/Feline
Genome Size (bp)	2 110 355	1 667 867	1 641 481	1 777 824	~1.68 Mb	~1.5 Mb	~1.66 Mb
GC content (%)	48.5	39	30.6	30.3	31.4	29.6	34.5
Open reading frames							
Predicted number	2 046	1 552	1 654	1 882	~1 771	~1 558	~1 793
Flexible genome pool							
Reported plasmids	0	0	0	0	1	1	2
Phages and phage-like elements	1	0	0	4	Unknown	Unknown	Unknown
IS elements	2	2	0	0	5	0	0
Genomic islands	2	1	0	0	Unknown	Unknown	Unknown

Table 1.1 Comparison of *C. jejuni* sequenced strains to related bacteria. *C. coli*,*C. lari* and *C.upaliensis* are currently being sequenced and so all data is based on the most current contigs available.

1.4 Outer membrane proteins of *C. jejuni*

Outer membrane proteins of *C. jejuni* have been studied by several methods as a means to elucidating its pathophysiology and identifying possible vaccine candidates (Blaser *et al.*, 1983 and 1984. Dunn *et al.*, 1987). In 1991, Pei, Ellison and Blaser purified four outer membrane proteins (named PEBs) that had been previously identified by immunoblotting of glycine extracts from *C. jejuni* whole cells (Blaser and Duncan, 1984). Each of the PEB proteins were of molecular weight, PEB1 28 kDa, PEB2 29 kDa, PEB3 30 kDa and PEB4 31 kDa and dominant proteins in glycine extracts. However, the function of these proteins remained unknown.

Both PEB1 and PEB4 were identified as being able to bind to the surface of HeLa cells (Kervella *et al.*, 1993). The same study showed that PEB4 is not surface exposed and so therefore, its ability to bind to eukarytotic cells is a secondary effect. PEB1 was shown to be surface exposed and later shown to be a major binding factor for *C. jejuni* cells (Pei *et al.*, 1998).

1.5 Sugar structures in *C. jejuni*

1.5.1 Lipopolysaccharide or Lipooligosaccharide

The schemata of a Gram-negative cell wall is shown in Figure 1.3. The inner membrane comprises a lipid bilayer with proteins embedded or associated with either the periplasmic or cytoplasmic surfaces. The outer membrane consists of two leaflets; the inner leaflet is composed of unmodified lipids (as in the inner

membrane) and the outer leaflet of modified lipids (mainly lipid-A). The outer membrane also has proteins embedded and associated with its periplasmic surface. The major constituent of the outer membrane's outer leaflet is lipopolysaccharide (LPS). LPS is a polysaccharide chain embedded in the outer membrane by lipid-A. The polysaccharide chain consists of an inner core which has a constant structure, an outer core that is moderately variable and an O-chain made of repeating units of polysaccharides. A number of mucosal pathogens, including *C. jejuni*, produce a structurally related compound LOS; which lacks the polymeric O-chain.

The biosynthetic pathway for LOS and LPS is identical and conserved across the Gram negative bacteria. Lipid-A was thought to be essential for all Gram negative bacteria, but it has been shown that *Neiserria meningitidis* is able to survive without (Bos and Tommassen, 2004). LPS is manufactured in the cytoplasmic leaflet of the inner membrane. The first step in synthesis is the addition of a 14-acyl chain to UDP-GlcNAc by LpxA. The acetate side chain is then removed by LpxC and replaced by a 14-acyl chain by LpxD to form UDP-2,3-diacylglucosamine. In *E. coli* and *Salmonella* LpxH then cleaves the UDP-side chain at its pyrophosphate bond to form 2,3-diacylglucosamine (see Figure 1.4). An orthologue of LpxH does not exist in *C. jejuni*, however, it is likely that there is another as yet unidentified enzyme. A dissaccharide is then produced by LpxB linking UDP-2,3-diacylglucosamine and 2,3-diacylglucosamine. The 4' position of the dissaccharide is then phosphorylated by LpxK to produce Lipid IV_A. Before Lipid-A sysnthesis can proceed any further, the first inner core residue 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), must be attached. The number of Kdo residues attached is species specific; *C. jejuni* attaches one residue, *E. coli* attaches two residues and *Chlamydia trachomatis* attaches three

residues. Kdo is attached by KdtA (also called WaaA) and the KdtA amino acid sequence is conserved across the Gram negative bacteria. The mechanism which controls the number of attached Kdo residues is not known. The final stage of Lipid-A synthesis is the attachment of two acyl chains to the disaccharide (see Figure 1.5). These terminal acyltransferases require Kdo in their substrate for activity. The length of each acyl chain is species specific.

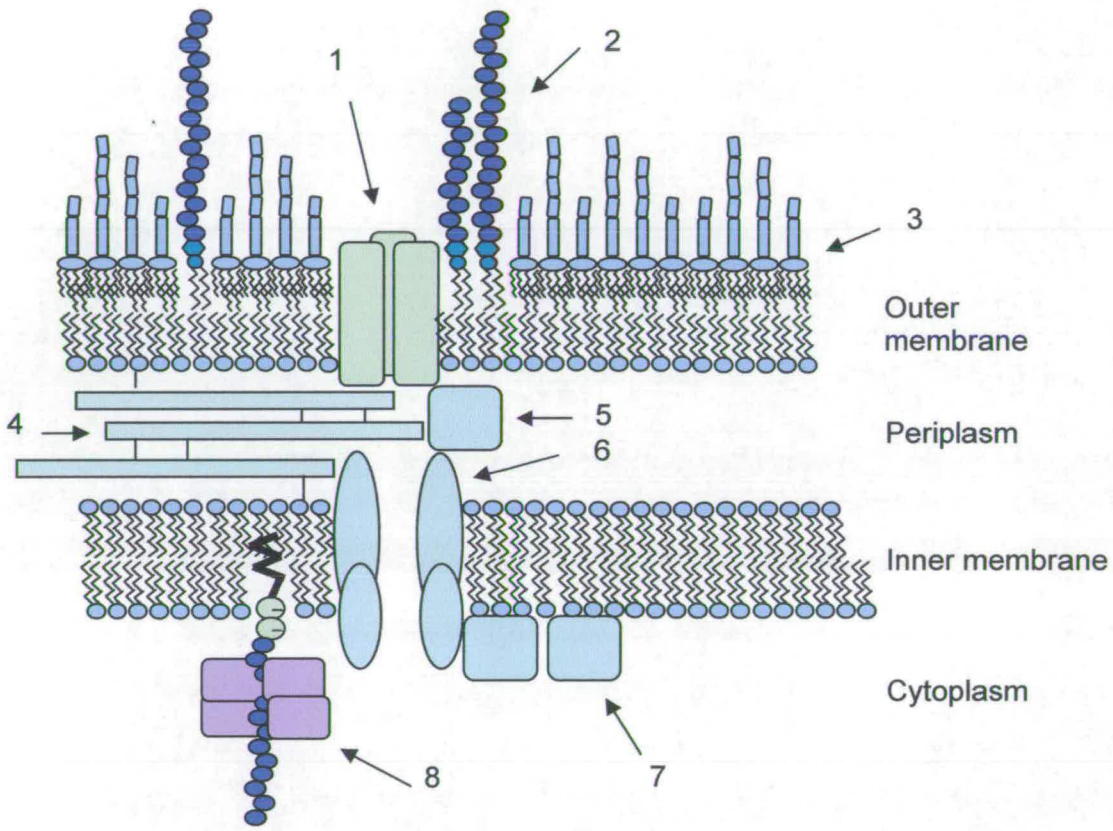


Figure 1.3 Schematic of the Gram negative cell wall showing 1-Porin 2- Capsular polysaccharide 3-Lipopolysaccharide 4-Peptidoglycan 5-Periplasmic protein 6-Transmembrane protein 7-Membrane associated protein 8-Cytoplasmic proteins

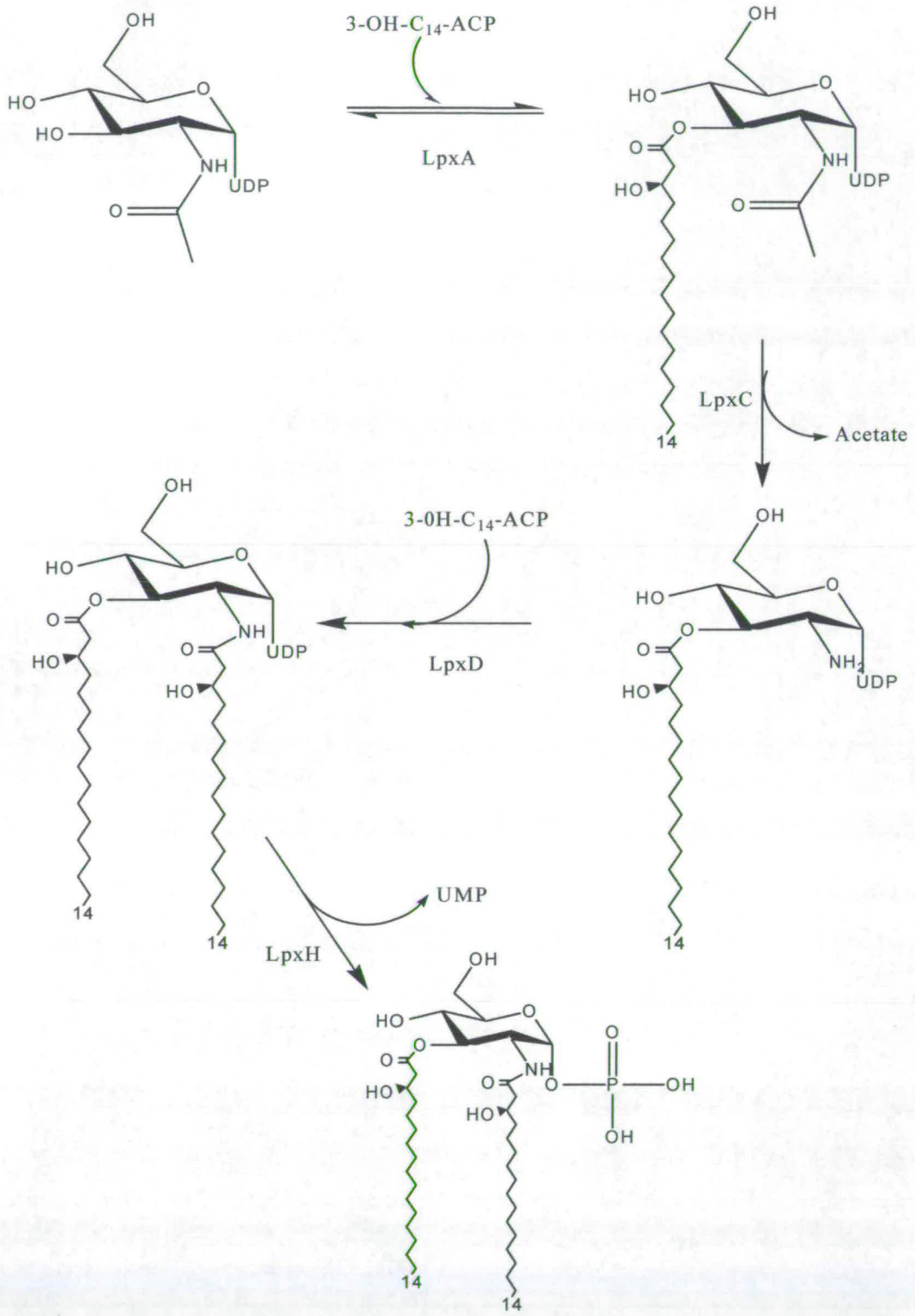


Figure 1.4 The first four steps of lipid-A synthesis in *E. coli*. Initially, a 14-carbon acyl chain is added to UDP-GlcNAc by LpxA. The acetate side chain is the removed by LpxC to allow the addition of a second 14-carbon acyl chain by LpxD. Finally, the pyrophospate bond in the UDP is cleaved by LpxH.

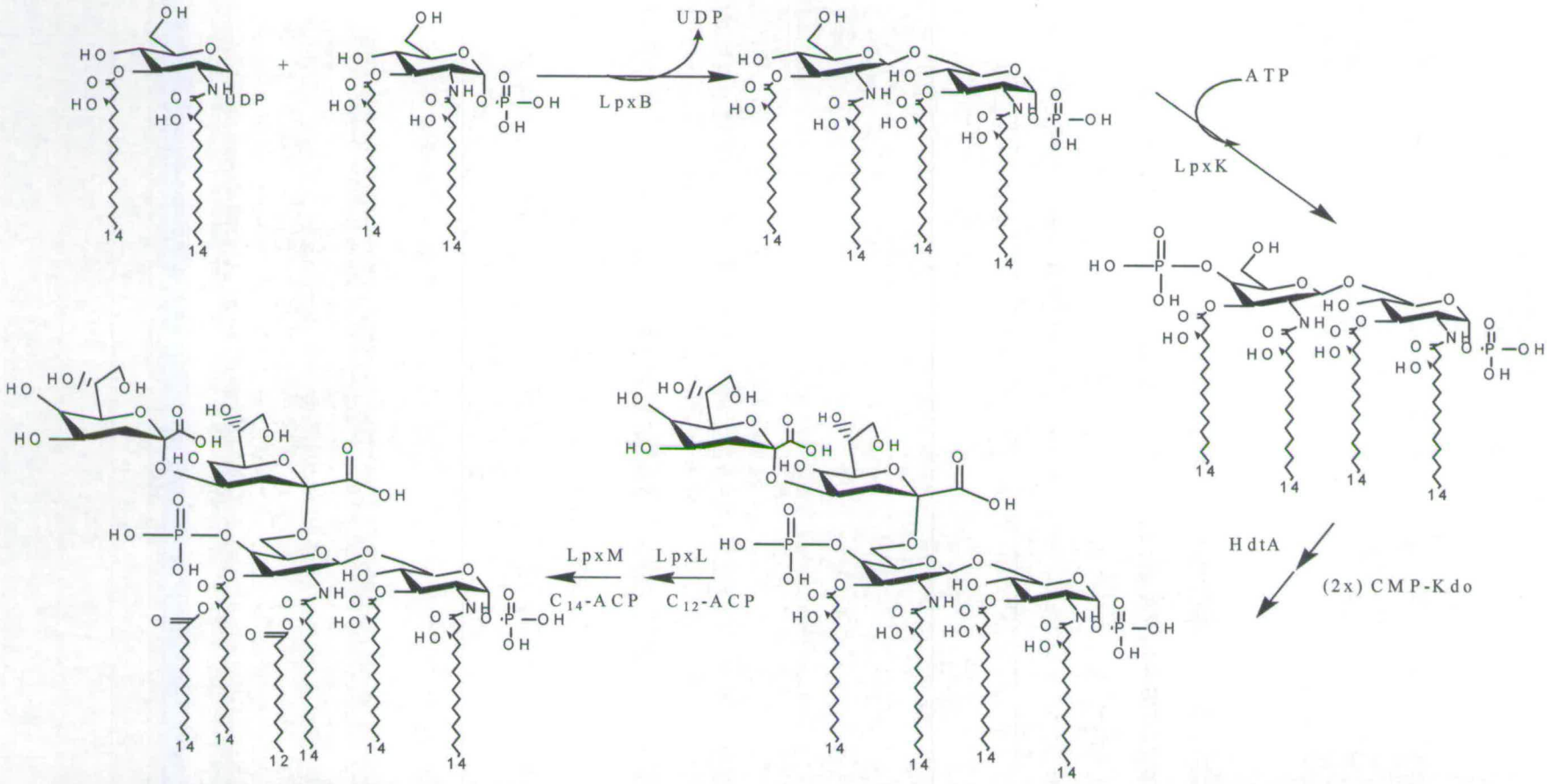


Figure 1.5 The final six steps in Lipid-A biosynthesis by E. coli. A diasccharide is formed by LpxB and phosphorylated by LpxK. Two Kdo residues are then added to the disaccharide by HdtA. The final steps are the addition of two acyl chains by LpxL and LpxM.

Synthesis of the core saccharide proceeds sequentially, beginning with the addition of L-glycero-D-manno-heptose (G-ManHep) by WaaC (see Figure 1.6). A second G-ManHep residue is then attached by WaaF to complete the inner core. The length, order of residues, number of side chains and position of side chains is strain specific and is determined by both gene complement and by regulation of expression. The LOS biosynthetic locus in *C. jejuni* was located by Wood *et al.* (1999), they probed a chromosomal library of *C. jejuni* NCTC 11168 and NCTC 11351, with a ^{32}P-labelled fragment of a *wbaP* (WbaP initiates O-chain synthesis *Salmonella*). A single plasmid from each strain library, positive for *wbaP* by Southern hybridization, was sequenced and a partial LOS locus was obtained. Further analysis of the locus from eight strains by Gibert *et al.* (2002) found three structurally related loci (see Figure 1.7). The differences between the loci classes, explains the extremely variable structure of *C. jejuni* LOS. Importantly, all three classes contain *cst* which is responsible for the attachment of N-acetylneuramic acid (NeuNAc). NeuNAc is not commonly found in bacteria and is responsible for the structural similarity between *C. jejuni* LOS and mammalian gangliosides.

Gangliosides are a sialic acid containing subset of glycosphingolipid (GSL), found on the surface of neurons. GSLs are found on the surface of every cell and their function has not yet been determined, but they are thought to play roles in morphogenesis, cell differentiation (Hakomori, 1984) and cell adhesion (Philips *et al.*, 1990 and Walz *et al.*, 1990). During an immune response to *C. jejuni* infection the structural similarity of the LOS and gangliosides can lead to an autoimmune reaction. In particular, anti-GM(1) has been linked with the onset of GBS and

MFS (see 1.2).

Phase variation plays an important role in the structure of the expressed LOS. *C. jejuni* OH4384 possesses a class B locus (see Figure 1.7) and therefore two copies of *cst*. The expression of *cst-I* can be switched on/off by strand slippage in a homopolymeric tract that will cause premature termination of translation. OH4384 can therefore change the number of and position of NeuNAc residues by switching *cst-I* on and off. If *cst-I* is on, a long LOS which mimics GT1a is produced. When *cst-I* is switched off, *cst-II* is switched on and produces a short LOS that mimics GD1 (see Figure 1.8 (Gilbert *et al.*, 2000)).

Once synthesis of the LOS molecule is complete, it is transported across the membrane by an ABC transporter (MsbA in *E. coli*). The mechanism by which LOS is transported across the perplasmic space is unknown. Two hypotheses have been suggested (see Figure 1.9); the first is that a soluble chaperone binds the lipid-A and allows passage across (Bos and Tommassen, 2004) and the second, that transport occurs at a site of direct contact between the two membranes (Bayer junctions (Leive, 1977)). The final step is for a flippase (Imp in *E. coli*) to flip the LOS from the inner leaflet to the outer leaflet.

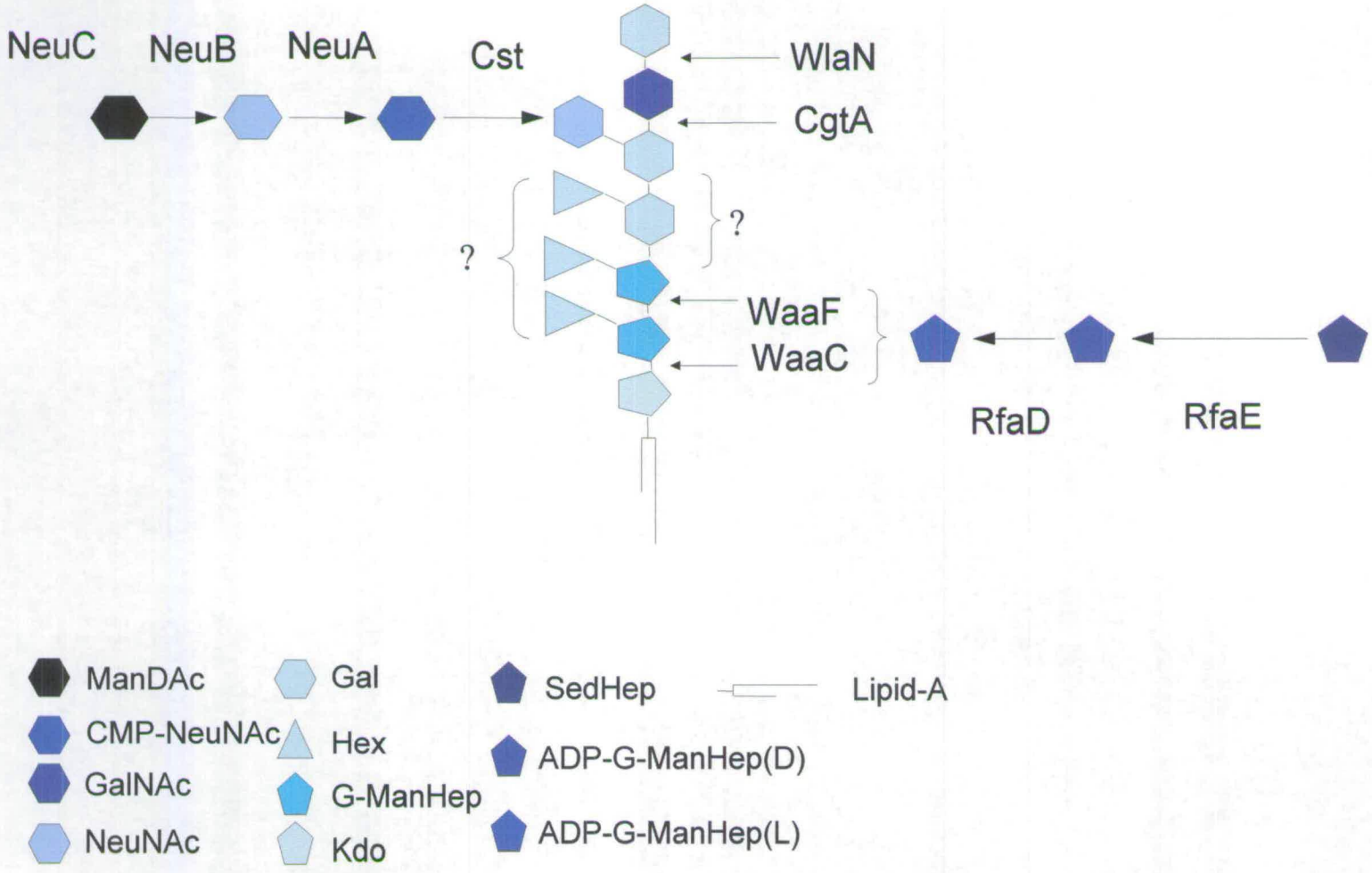


Figure 1.6 Putative synthetic pathway for the LOS inner and outer core in *C. jejuni* NCTC 11168; showing the synthetic pathways for the precursors of G-ManHep and NeuNAc (adapted from Linton *et al.*, 2002)

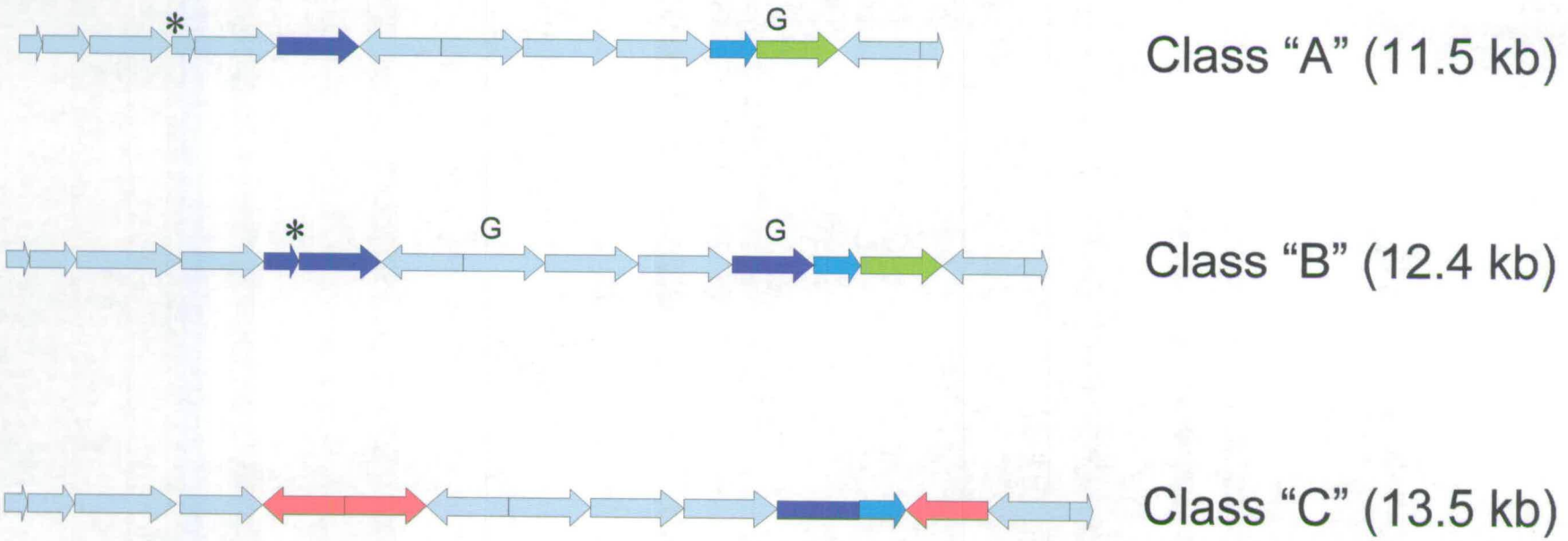


Figure 1.7 Schematic showing the 3 classes of LOS loci arrangements found in *C. jejuni*. Genes are colour coded to indicate function. The * indicates where a premature translation stops are observed in some strains. The G indicates the position of a poly(G) tract. The genes *orf10* (dark green) and *orf11* (olive green) are unique to class "A" and "B" loci. Three genes (*orf14, 15* and *16*) are unique to class "C" loci. *Cst* (dark blue) is found in all three classes but class "A" only has one copy, class "B" two copies and in class "C" it is as an in-frame fusion with CMP-Neu5Ac synthase, *orf10* (adapted from Gilbert *et al.*, 2002).

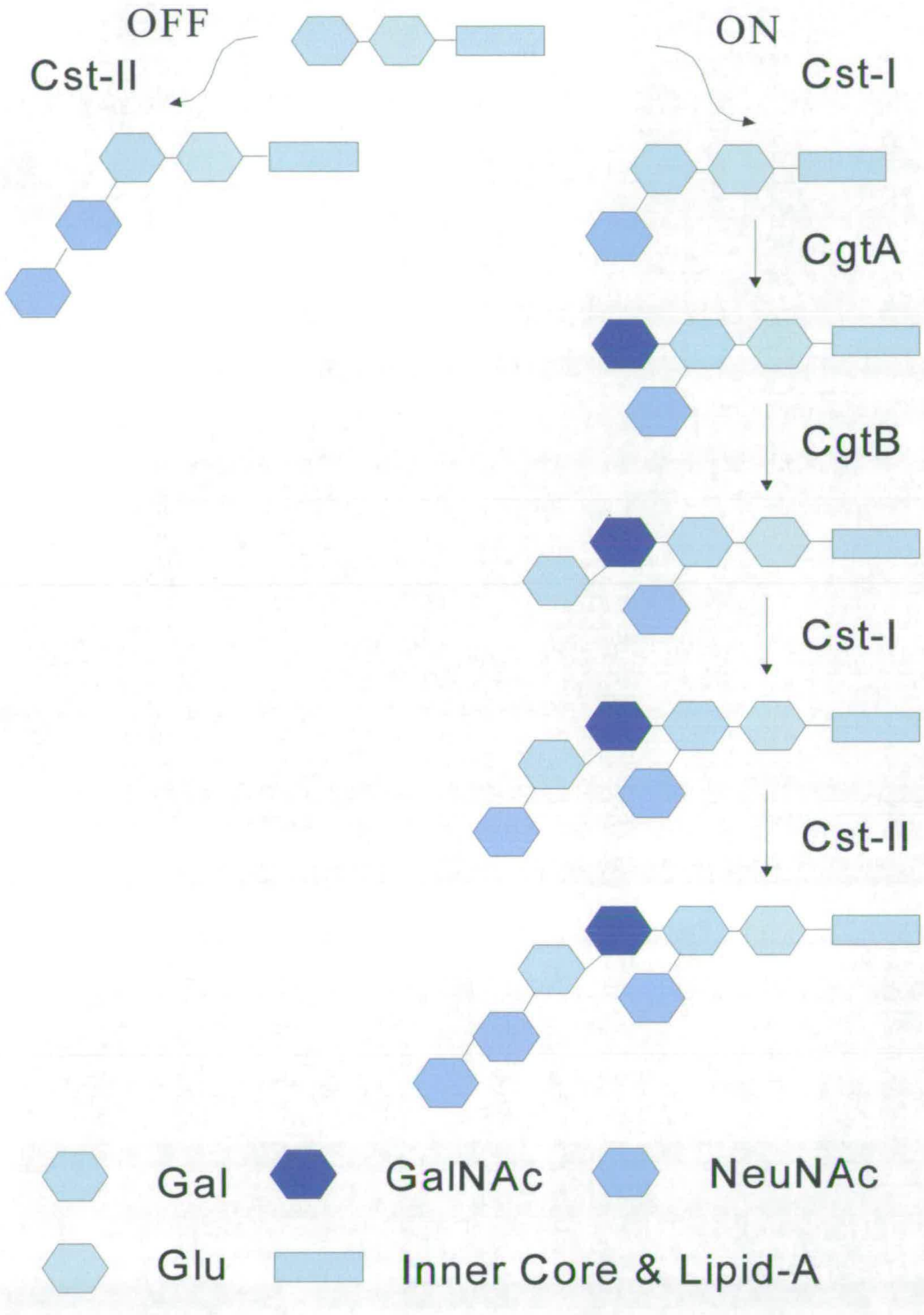


Figure 1.8 Schematic to show the effect of turning expression of *cst-I* on/off on the LOS structure produced by *C. jejuni* OH4384 (adapted from Gilbert *et al.*, 2000).

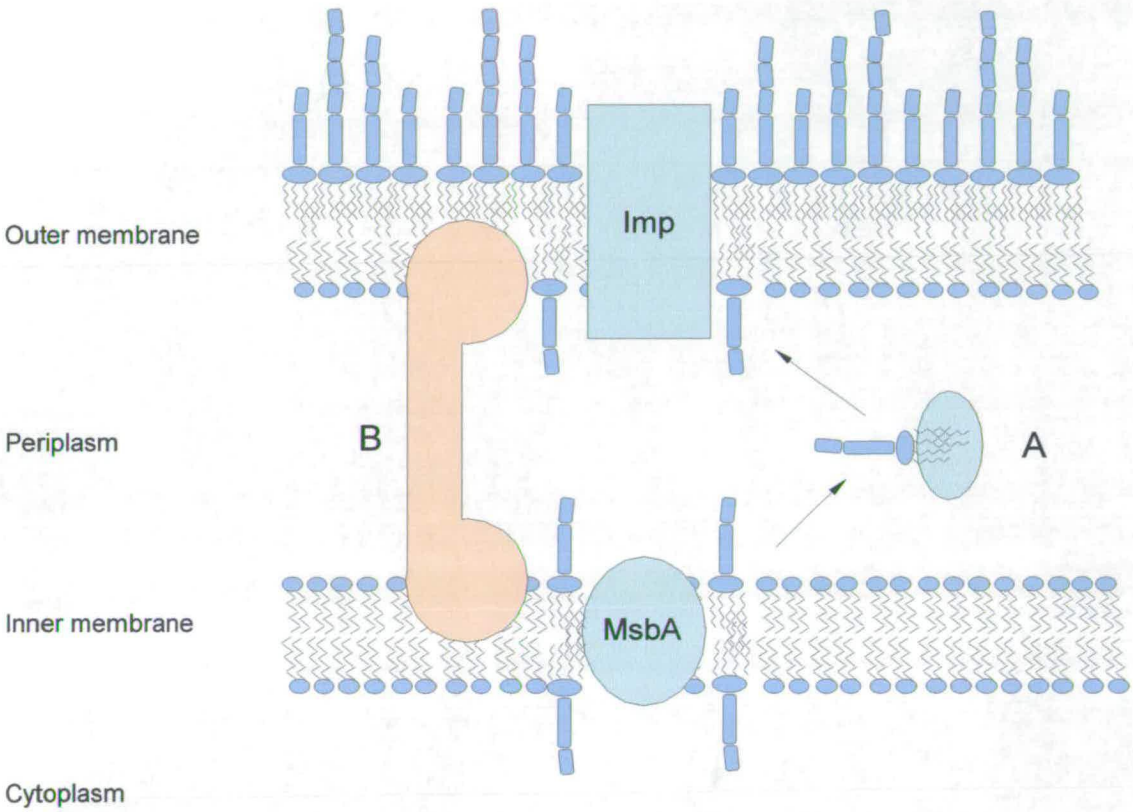


Figure 1.9 Proposed methods for the transport of LOS from the cytoplasm to the outer leaflet of the outer membrane. A: The completed LOS molecule is transported across the inner membrane by MsbA. A soluble chaperone then transports the LOS molecule across the periplasm to the outer membrane. The LOS is then flipped across the outer membrane by Imp. B: The completed LOS molecule is transported across the inner membrane by MsbA. The LOS molecule then moves via a point of direct contact between the the inner and outer membranes (Bayer junction, indicated in orange). Imp then flips the LOS molecule across the outer membrane (adapted from Bos and Tommassen, 2004)

1.5.2 Capsular polysaccharide

Until 2000, the presence of a capsule structure on the surface of *C. jejuni* was unconfirmed. The usual methods of capsule detection (e.g. India ink) did not work as the capsule is very thin. In 2000, Karlyshev *et al.*, analysed the genome sequence of *C. jejuni* and demostrated with gene knockouts that *C. jejuni* possessed structures similar to the K-antigens of *E. coli* and *Salmonella* (Group II Gram-negative capsules, see below). Final confirmation was obtained when Karlyshev *et al.* (2001) was able to stain and visualise the capsule with Alcian blue, using electron microscopy.

Comparison of the capsular polysaccharide (CPS) genes across the Gram-negatives has been hampered by a non-consistent nomenclature. In 1996, Reeves *et al.*, proposed a system for naming the sugar transferases found in the capsular polysaccharide loci of *E. coli* and *Salmonella*. Subsequent comparison of the loci revealed that four arrangements of genes are found but an overlying structure to all four groups exists. In all cases, the genes responsible for the ABC-transporter system flank the sugar transferases.

CPS is composed of a lipid modified with undecaprenol and a polysaccharide chain. The synthesis pathway and locus structure is conserved throughout the Gram negative bacteria. Synthesis of the modified lipid and the polysaccharide chain occurs separately within the cytoplasm (see Figure 1.10).

KpsU carries undecaprenol-CMP to the KpsC which uses it for modification of a lipid in the inner leaflet of the cytoplasmic membrane. The modified lipid is then transported across by the Kps ABC transporter system. The polysaccharide is

synthesised by the sequential addition of sugar residues to a Kdo-modified lipid in the cytoplasmic leaflet. The composition and structure of the chain is determined by gene expression and complement. On completion the polysaccharide chain is transported across by the Kps ABC transporter system. It is not yet known when the lipid and polysaccharide chain are attached to each other.

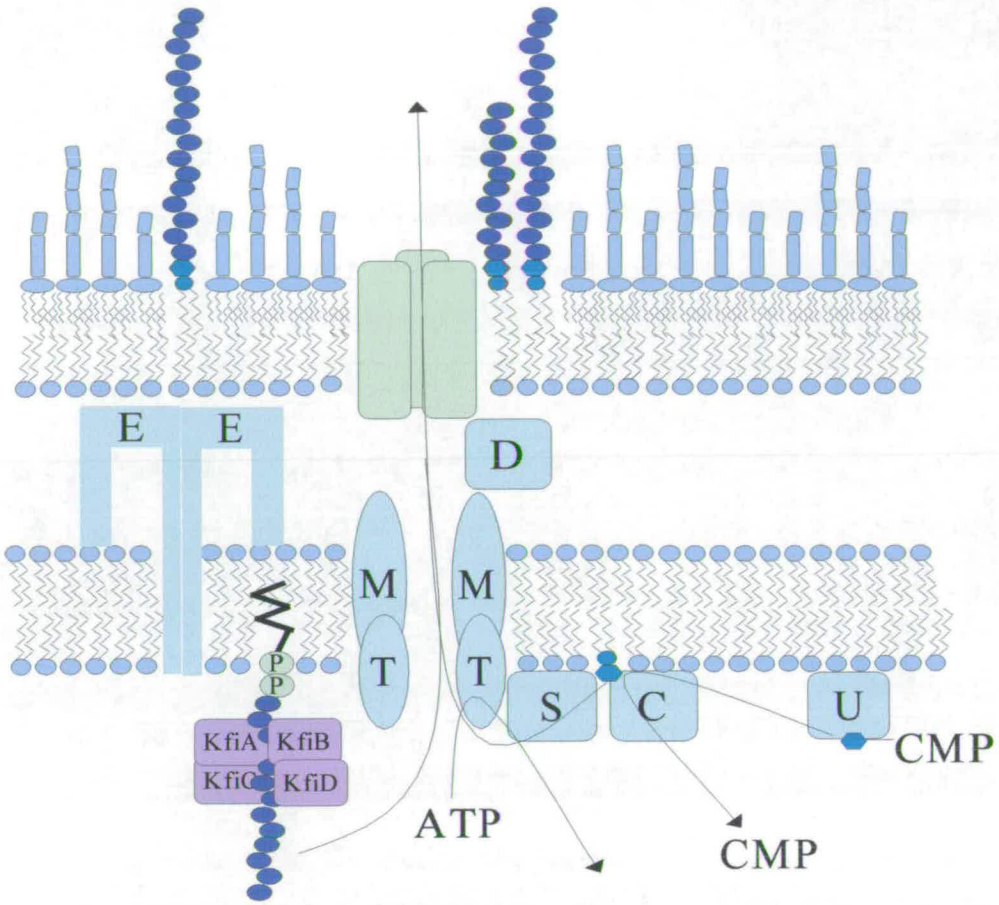


Figure 1.10 Schematic of the capsular biosynthesis pathway in *E. coli*. Kdo-CMP is transported to KpsC by KpsU; where the Kdo residue is attached to a lipid in the inner membrane. The polysaccharide chain is synthesised separately on a modified lipid within the inner membrane by KfiA, B, C and D. Both the modified lipid and the polysaccharide chain are transported across the inner membrane by the ABC-transporter KpsS,T and M. It is not known whether the polysaccharide chain is attached to the modified lipid before, during or after transport. Transport across the outer membrane is achieved via porin.

1.5.3 Post-translational modification of protein

Post-translational modification of proteins with glycans is the most common form of modification seen in eukaryotic organisms (Nita-Lazar *et al.*, 2005). Until recently, this form of modification was believed to be exclusive to eukaryotes and archaea. An increasing number of pathogenic bacteria have been found to glycosylate surface exposed proteins (Power and Jennings, 2003).

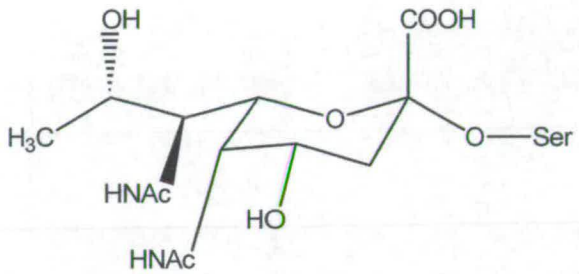
1.5.3.1 O-linked glycosylation

O-linked glycosylation has been found in a number of bacterial species: *Pseudomonas aeruginosa*, *Caulobacter crecentus*, *Aeromonas caviae*, *H. pylori* and *Neisseria* spp. Glycosylation of *Campylobacter* flagellum was first reported in 1989 by Logan *et al.* Non-glycosylated flagella have been shown to cause an apparent decrease in motility, adherence and invasive capability (Szymanski *et al.*, 2002).

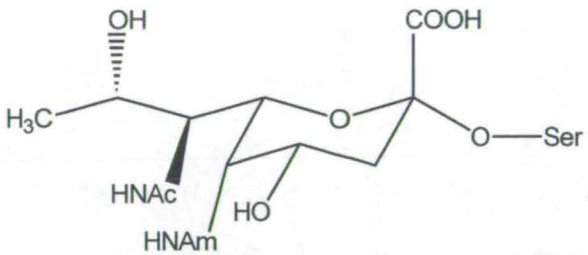
Analysis of the glycan moieties present on flagella have shown the presence of pseudaminic acid or an acetamindino subsituted analogue of pseudaminic acid (see Figure 1.11) (Thibault *et al.*, 2001). The glycans are attached within a region of 19 serine and theronine residues within the central hydrophobic region of FlaA or FlaB. Attachment is not dependent on a recognition sequence (c.f. N-linked glycosylation (see 1.5.3.2)) but is dependent on the tertiary structure of the protein (Karlyshev *et al.*, 2005). The number and type of residues attached to the flagella subunits is thought to be both species and phase variable (Karylshev *et al.*, 2005).

The genes associated with O-linked glycosylation are co-located with the flagella biosynthesis locus. The glycosylation locus contains ~ 50 genes and shows a

high degree of variability between species (Karlyshev *et al.*, 2005). Mutation of the genes with the glycosylation locus affects expression and assembly of the flagellar apparatus. Importantly, mutations that prevent glycosylation cause the accumulation of unmodified subunits in the cytoplasm (Goon *et al.*, 2003); suggesting that glycosylation of the subunits is require for recognition by the flagella secretion/assembly apparatus (Karlyshev *et al.*, 2005).



5,7-Diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulsonic acid



7-Acetamido-5-acetimidoyl-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-nonulosonic acid

Figure 1.11 Structure of two pseudaminic acid derivatives found attached by the O-linked glycosylation systems of Gram negative bacteria. Ac – acetyl Am – acetimidoyl (adapted from Schmidt *et al.*, 2003)

1.5.3.2 N-linked glycosylation

The N-linked protein glycosylation pathway is highly conserved in eukaryotes (Nita-Lazar *et al.*, 2005); comparison of putative genes from *C. jejuni* revealed a homologue of *stt3* the most highly conserved protein in the pathway (Wacker *et al.*, 2002). So far 21 proteins have been isolated from *C. jejuni* in glycosylated forms (Szymanski *et al.*, 2003).

The glycosylation pathway can be split into two events: 1. the assembly of a lipid-linked polysaccharide in the inner membrane, and 2. the transfer of the preassembled oligosaccharide to the protein and attachment at the N-X-S/T motif. The conservation of the pathway between *C. jejuni* and eukaryotes is extremely high with only the complexity of the polysaccharide being different. The assembly of the polysaccharide occurs in a stepwise fashion by the addition of single sugar units to a lipid carrier in the inner membrane (see Figure 1.12 and Figure 1.13) by the enzymes PglA, C, H, I and J, plus two unidentified enzymes; resulting in a branched heptasaccharide, GalNAc2(Glc)GalNAc3Bac. The completed glycan is then flipped across the membrane and attached to an asparagine residue in the motif N-X-S/T (where X is any amino acid except proline) by the Stt3 homologue – PglB (see Figure 1.13). Recent experiments with *E. coli* transformed with the *C. jejuni* glycosylation apparatus have shown that the N-X-S/T motif is not the only requirement for glycosylation. Nita-Lazar *et al.* (2005), showed that there is also an (as yet unidentified) structural requirement for glycosylation and this likely to be associated with the way in which the target protein is transported across the inner membrane.

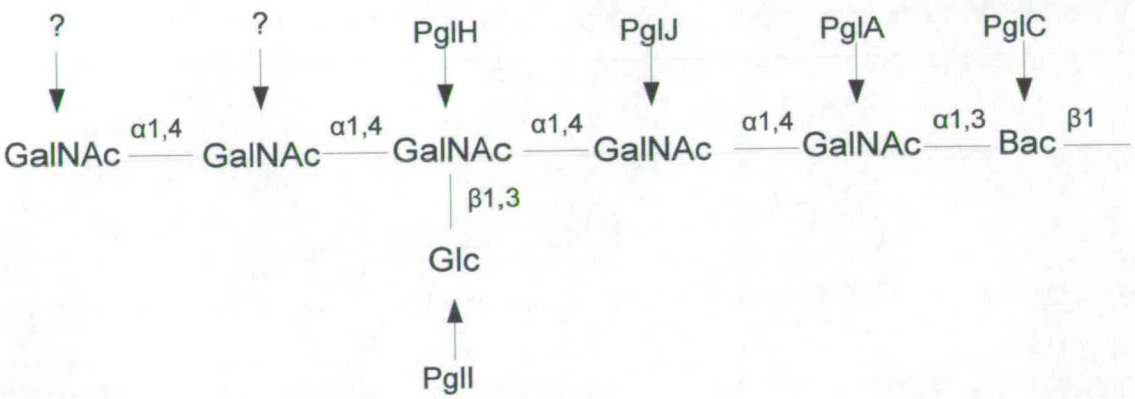


Figure 1.12 Schematic of the biosynthesis of the N-linked glycan in *C. jejuni*. Arrows indicate the residue added by the indicated glycosyltransferase (adapted from Linton *et al.*, 2005)

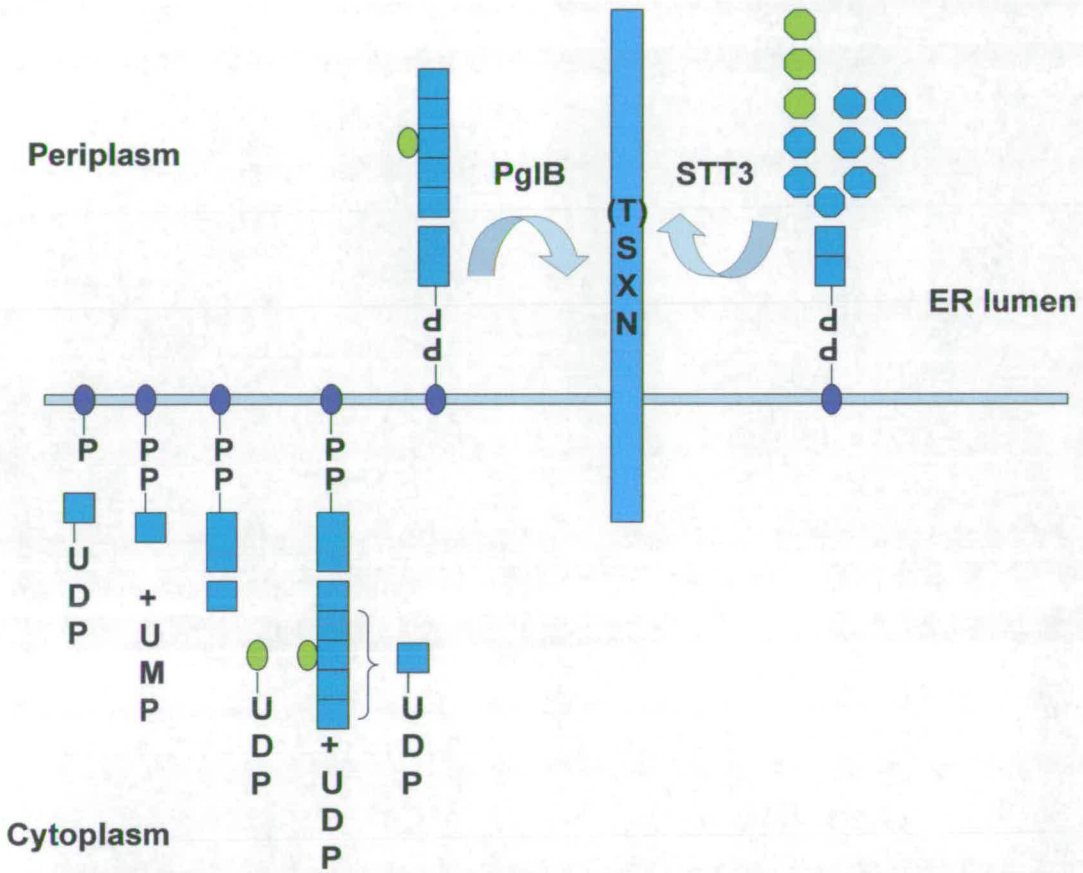


Figure 1.13 Model of N-glycan synthesis in *C. jejuni* and a comparison of the N-glycan structure from *Saccharomyces cerevisiae*. Sugar resdiues are added sequentially to a lipid carrier by the Pgl glycosyltransferases. PglB then flips the N-glycan across the membrane into the periplasm and attaches the glycan to the protein at the N-X-S/T motif. In *S. cerevisiae*, the glycan is synthesised in the same fashion but after being flipped into the ER lumen is further modified. The PglB homologue, Stt3 then attaches the N-glycan to the nascent protein (adapted from Szymanski *et al.*, 2003)

1.6 Typing methods used with *Campylobacter* spp.

1.6.1 Serotyping

1.6.1.1 Penner

The Penner serotyping systen was first described in 1980 (Penner and Hennessy, 1980). The system uses sensitised erythrocytes and antisera, raised against heat-stable antigens, to type isolates by passive agglutination. The exact nature of the serodeterminant was unknown until 1996 when Chart *et al.* showed that Penner antisera did not recognise LOS (via immunoblotting) but did recognise antigenic material extracted from the cell envelope at 50 °C. Previous reports had shown that CPS could be extracted from *E. coli* at low temperatures (Orskov and Orskov, 1989). CPS was confirmed as the serodeterminant by Karlyshev *et al.*, using mutants in *kpsM, S* and *C* which were deficient in CPS production (Karlyshev *et al.*, 2000).

Penner serotyping is the most commonly used non-genomic based typing scheme; due to its high level of simplicity and repeatability between typing labs (c.f. Lior serotyping (see 1.6.1.2) and *fla*-RFLP (see 1.6.4)). Initially, 24 Penner serotypes were described including both *C. jejuni* and *C. coli* (Penner and Hennessy, 1980), and currently >60 Penner serotypes (for *C. jejuni* alone) are included in the typing scheme.

1.6.1.2 Lior

The Lior serotyping system is a rapid slide agglutination protocol that uses heat-labile antigens to raise antisera (Lior *et al.*, 1982). The exact nature of the serodeterminants in this system is not known, but there is some evidence that the flagella may be important (Alm *et al.*, 1991). The heat-labile nature of the antigens has made standardisation difficult between different laboratories; as a slight variation in protocol can produce major variations in serotype detected.

1.6.2 Multi-locus Sequence Typing

Multi-locus sequence typing (MLST) is a similar technique to multi-locus enzyme electrophoresis (MLEE) where the mobility of conserved 'housekeeping' enzymes is compared. In MLST, it is the DNA sequence of housekeeping genes rather than enzymes being compared, making it highly discriminatory and reproducible. In *C. jejuni*, MLST has been performed using the genes *adk,* ***aspA***, *Cj1585c*, ***glnA***, ***gltA***, ***glyA***, *ilvD*, *mdh*, *pgi,* ***pgm***, ***tkt*** and ***uncA*** (Manning *et al.*, 2003). Two schemes exist for MLST of *C. jejuni*, scheme A, those genes indicated in bold, was selected after the completion of the *C. jejuni* NCTC 11168 genome in 2000 (Dingle *et al.*, 2002). Once each gene has been sequenced comparisons of each gene between strains is used to assign sequence types (ST).

MLST is highly reproducible and is widely used on a number of organisms. Primers and genes used with each organism has been standardised internationally and an MLST profile database set up (http:\\www.mlst.net).

1.6.3 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is considered to be the 'gold standard' for molecular techniques (Olive and Bean, 1999). For PFGE whole bacterial cells are combined with molten agar and allowed to set in plastic molds. The resultant plugs are treated to lyse the cells and destroy any proteineous material. Extensive washing steps remove any debris before restriction with an infrequently cutting restriction enzyme. The plugs are then inserted into an agarose gel and subjected to electrophoresis in a changing electric field. The electric field is manipulated so that both the polarity, the angle of the field relative to the gel (for some PFGE subtypes only) and the time for each change (switch time) maximises the separation between fragments. Restriction patterns can then be visualised using ethidium bromide staining.

The restriction fragments obtained in this method are very large, typically 30 kb to 2 Mb. Restricting whole genomic DNA in an agarose plug, stabilises the fragments and prevents shearing due to mechanical stress. The large fragment size also affects the mobility of the DNA within an agarose gel. The changing electric field causes DNA to reorientate, small fragments reorientating and migrating faster than larger, exaggerating the difference in size (see Figure 1.14). As switch time increases the larger fragments have more time to reorientate and migrate through the gel.

PFGE has been used successfully on many bacterial species including *E. coli* (Arbeit *et al.*, 1990), *Stapylococcus aureus* (Saulnier *et al.*, 1993), *Ps. aeruginosa* (Grundmann *et al.*, 1995) and *C. concisus* (Matsheka *et al.*, 2002).

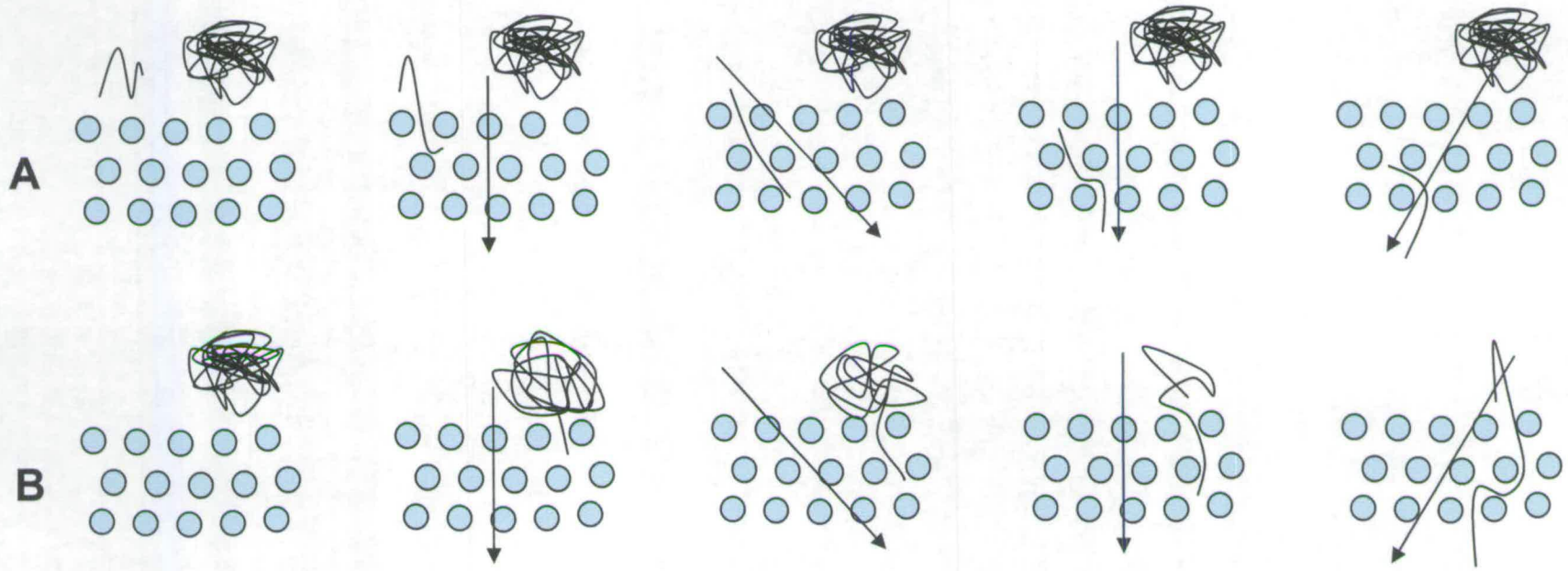


Figure 1.14 Diagrammatic representation of the movement of DNA molecules through the agarose matrix of a PFGE gel. Arrows indicate the direction of the electric field. A - When the electric field direction is changing quickly (short switch time) large fragments are unable to reorient themselves before the electric field changes direction. Short fragments are able to reorient quickly and move through the gel matrix. B – As the time spent at each field direction increases (long switch time) the large fragments are able to reorient and move through the gel matrix.

1.6.4 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) involves visualisation of whole genome restriction fragments by electrophoresis, Southern blotting and probing for specific gene sequences. This technique has been successfully used to type *Ps. aeruginosa* (Loutit and Tompkins, 1991) and *Legionella pneumophila* (Tram *et al.*, 1990). The complexity of RFLP patterns is dependent on the restriction enzyme and probe combination. Ribotyping, a variation of RFLP, uses probes against 16S and 23S rRNA genes and has been used extensively for typing *C. jejuni* and *Helicobacter* spp. (Moore *et al.*, 2002, and Vos *et al.*, 1999).

More recently the use of PCR in place of Southern blotting and probing has become the preferred method. PCR-RFLP of the 16S rRNA gene in *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. has been shown as discriminatory as RFLP (Marshall *et al.*, 1999). Other PCR-RFLP techniques for typing *C. jejuni* have been studied with particular emphasis on *fla*-RFLP. *Fla*-RFLP uses primers for the flanking conserved regions of the *flaA* gene and typically the restriction enzyme *Dde*I (Heitt *et al.*, 2002, Mohran *et al.*, 1996, Nishimura *et al.*, 1996). Several variations on this technique including amplification of both *flaA* and *flaB* (Petersen and Newell, 2001) or more discriminatory electrophoresis (Hein *et al.*, 2003) have been developed in an effort to increase the techniques discriminatory power.

1.6.5 Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) was first described in 1995 by Vos *et al.* Since then it has been used to type a large number of organisms including both eukaryotes (Rubio *et al.*, 2000) and prokaryotes (Peters and Threlfall,

2001 and Valsangiacomo *et al.*, 1995). The technique is similar to RFLP, whole genomic DNA is digested with one or two restriction enzymes; restriction site specific adaptors are then ligated to the restriction fragments. Restriction patterns are visualised by amplifying the restriction fragments with primers specific for the adaptors and separation of the fragments by electrophoresis. A major advantage of AFLP over other techniques is the use of the restriction site specific adaptors. The use of adaptors as priming sites means that no previous knowledge of the genomic sequence is required and the same adaptors can be used with multiple organisms.

Visualisation of restriction patterns is primarily achieved by the use of fluorescently labelled primers and sequencing machines. Changes in the primer design are used to decrease the number of restriction fragments visualised; typically the addition of selective bases to the 3' end of the primer or only labelling one primer in multi-restriction enzyme protocols (Duim *et al.*, 2001).

Typing of *C. jejuni* with the protocol first described by Vos (1995) has been extensively carried out on large sets of *Campylobacter* spp. isolates; both clinical and non-clinical in nature (Broman *et al.*, 2000, Desai *et al.*, 2001, Duim *et al.*, 1999, 2000 & 2001, On and Harrington, 2000). However, optimisation of the restriction enzyme set used for *C. jejuni* typing has not been carried out.

1.7 Monoclonal antibodies and their uses

Polyclonal antibodies are obtained after challenge of an animal (usually sheep, horse or rabbit) with the target antigen. After booster challenges the animal is bled and serum extracted from the bleed. This gives a small amount of an undefined antibody mixture. Repeated bleeds can be taken but animal welfare considerations must be taken into account, as well as, the changing nature of the antibody mixture obtained.

Monoclonal antibody (mAb) preparation allows continuous production of one Ab with defined specificity and affinity. mAbs are of most use where repeatability is important; consequently they are used in a variety of diagnostic techniques such as, Enzyme linked immuno-sorbent assay (ELISA), Immuno-fluorescent assays (IFA), Western blotting and protein arrays. The use of mAbs as therapeutic agents has met with some success, problems with toxicity and immune-suppression of the patient, however, prevented widescale production (Glennie and Johnson, 2000). Recent advances in production and a new understanding of human genomic sequences now allow for the production of human mAbs from engineered mice or phage display (Vaughan *et al.*, 1998).

1.7.1 Systems for producing monoclonal antibodies

1.7.1.1 Hybridoma-based systems

The first monoclonal antibodies were created by Kohler and Milstein, 1975. They fused spleenocytes and myelomas using Sendai virus. Since these first experiments the fusion methods have been refined to include electroshock (Lo *et al.*, 1984) and polyethylene glycol (PEG) mediated fusion, with PEG mediated fusion being the most commonly used. However, the exact method by which cells fuse when PEG is used is currently unknown and it is also not known if it is PEG or a molecule that co-purifies that mediates fusion.

There are several different mouse myeloma cell lines avilable including P3-X53-Ag8, SP2/0' and their derivatives. It is possible to fuse myeloma cells from a species different to the species that the splenocytecs are harvested from but the resultant hybridomas are less stable. The basic myeloma cell lines are usually 'producing' cell lines. 'Producing' cell lines contain the loci that encode antibodies from the species in which the myeloma was created. Hybridomas created using these cell lines may therefore produce non-functional or non-specific Ab.

Derivatives of the basic cell lines e.g. SP2/O'-Ag14, have been produced by selecting for cells that show beneficial characteristics such as increased growth rate, robustness and commonly 'non-producing'. 'Non-producing' cell lines lack the loci encoding the antibodies; thereby increasing the efficiency of fusion as all Ab producing hybridomas will produce Ab specific for the target Ag (Fazekas de St Groth and Scheidegger, 1980).

Hybridoma production can be split into four stages (see Figure 1.15): 1. production of splenocytes and myelomas 2. fusion 3. selection and 4. propagation. The first two stages depend upon the myeloma cell line, the antigen and the species used for splenocyte production. Selection and propagation are common to all hybridoma protocols. Selection is achieved by the use of the hypoxanthine guanine phosphoribosyl transferase (HGPRT) salvage pathway and the alternative thymidine salvage pathway. Growth media is supplemented with hypoxanthine, thymidine and aminopterin. Aminopterin blocks the manufacture of GTP and TTP (see Figure 1.16), and consequently RNA and DNA in unfused myeloma cells; splenocytes possess the HGPRT salvage pathway but are unculturable. Fused cells possess the culturable nature of the myelomas and the HGPRT salvage pathway from the spleenocytes; consequently fused cells are able to replicate and grow. Once all of the unfused cells have died, selection is gradually relaxed so that fused cells can revert to the normal GTP and TTP synthesis pathways.

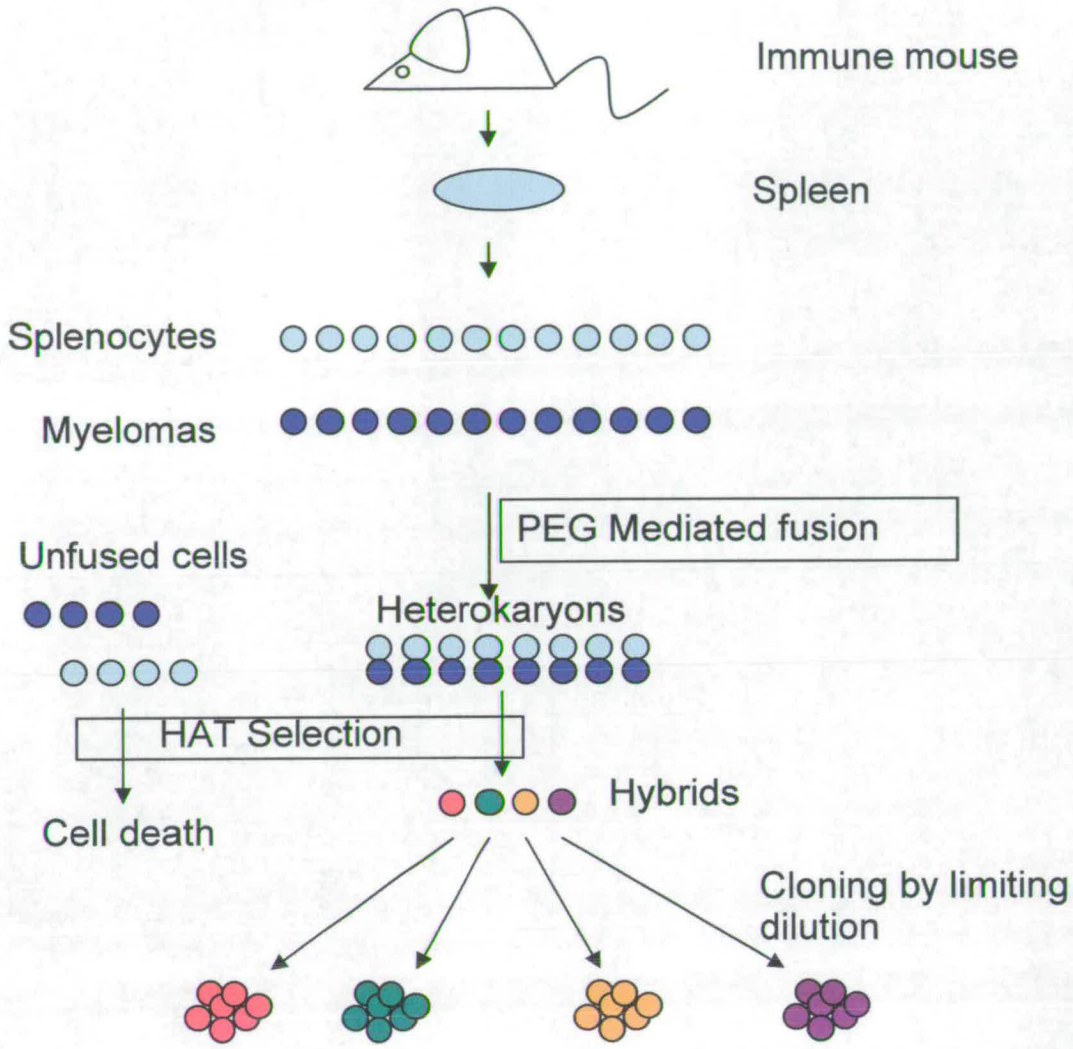


Figure 1.15 Schematic of the hybridoma formation process. Splenocytes are harvested from a challenged mouse and fused with myeloma cells. Unfused cells are killed by the addition of HAT to the culture medium. Fused cells are tested for the production of antibody against the target and if positive propagated by limiting dilution. After three rounds of limiting dilution the resultant colonies are clonal and can be used for mAb production.

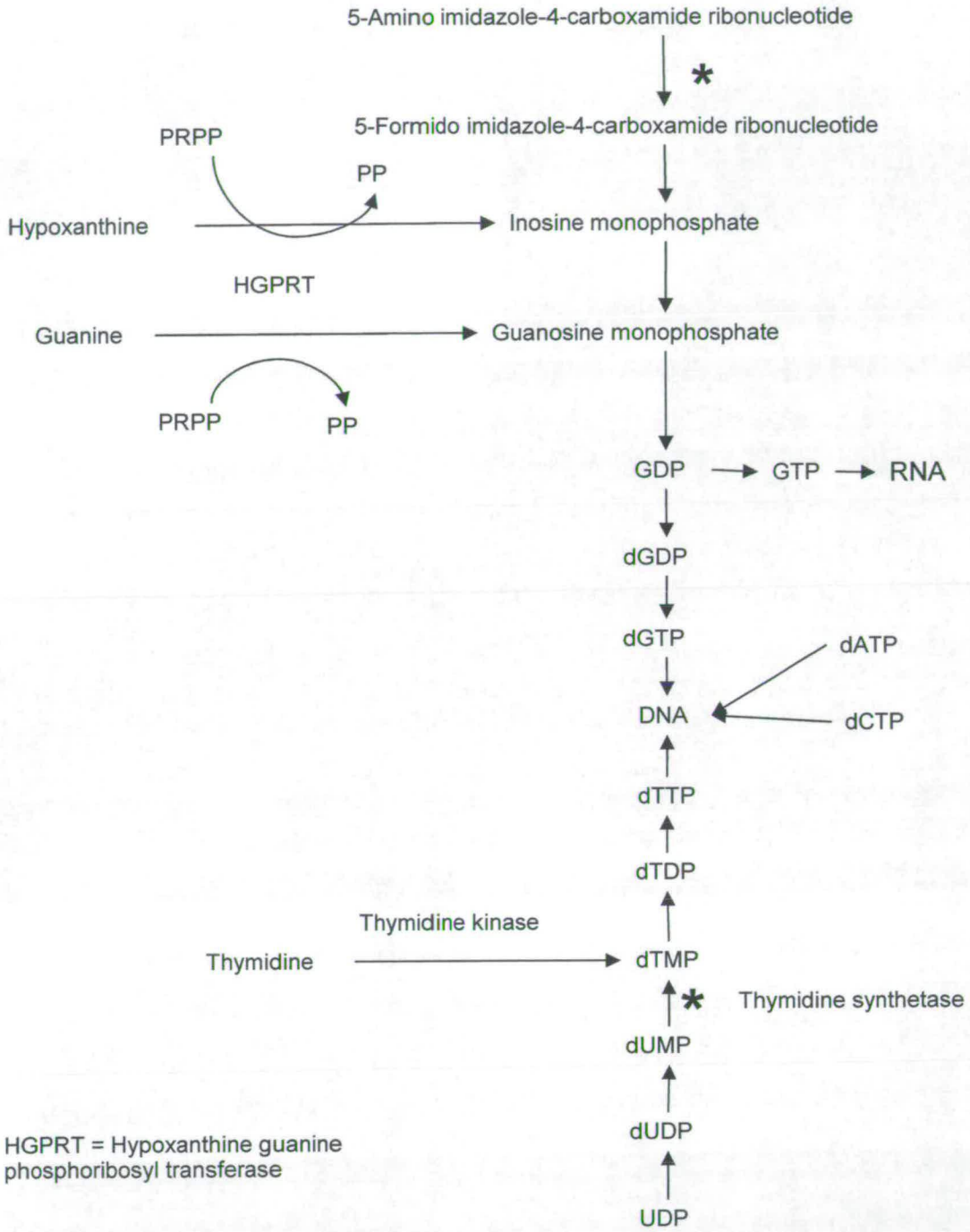


Figure 1.16 The dTTP and dGTP biosynthetic pathways. HAT selection supplements the culture media with hypoxanthine, aminopterin and thymidine. Aminopterin prevents the synthesis of dTMP and 5-formido imidazole-4-carboxamide ribonucleotide (*). Consequently preventing formation of dTTP and dGTP. Fused cells posses HGPRT and so are able to use the alternative pathway for the synthesis of dGTP. The supplement of thymidine in the media allows dTTP synthesis. Unfused cells are unable to replicate and die.

Propagation is essential to hybridoma production as they are inherently unstable. After fusion, the hybridomas possess double the normal amount of genetic material for a cell. In this situation the cell stabilises by allowing chromosomes to be lost. The order in which chromosomes are lost is random for each culture. After three rounds of propagation hybridomas become more stable and the likelihood of losing antibody production lessens. Propagation is also important as it is the means of splitting a possible mixture of Abs into mAbs. As soon as a positive culture is identified it is diluted and split (limiting dilution). After a further two dilutions/splits if 99% of the resultant cultures are negative for antibody production, statistically, the 1% producing antibody will represent single clones.

1.7.1.2 Phage display

Phage display is a highly versatile system for the screening of proteins that possess the ability to bind to a target protein. Phage display works by fusing a protein of interest or more commonly a library of proteins to the phage coat protein pIII. Phage containing this fusion are then used to infect *E. coli* where the protein fusion can be expressed. mAbs were first expressed as phage fusion by Breitling *et al.* in 1991. A single chain variable fragment was cloned into a phagemid based upon the genome sequence of fdtet. Since this initial experiment phagemid vectors have been designed to increase the efficiency of cloning, display and expression of Ab-pIII fusions (McCafferty *et al.*, 1990 and Smith, 1985). An example of such a phagemid is shown in Figure 1.17 and some of the antibody display formats in Figure 1.18.

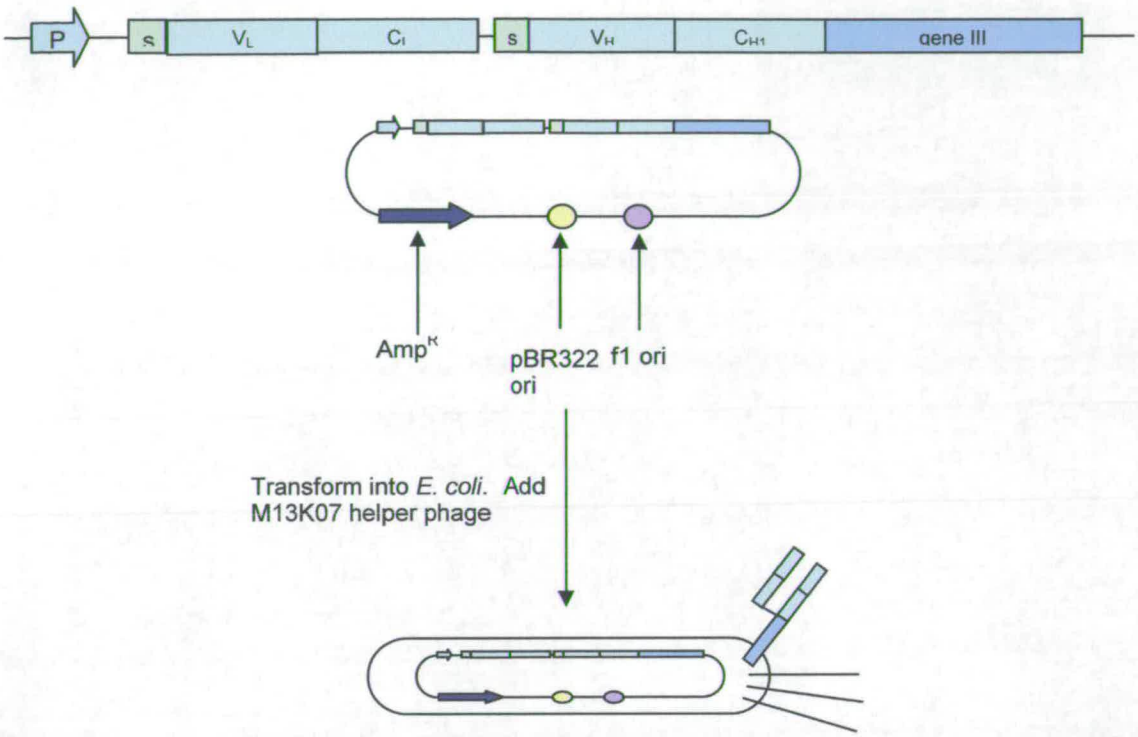


Figure 1.17 Map of a phagemid carrying a co-expressed Ab light chain and heavy chain-gene III fusion. V_L = Variable region of light chain C_L = Constant region of light chain V_H = Variable region of heavy chain C_{H1} = First constant region of heavy chain P = Promoter region S = Start of transcribed region.

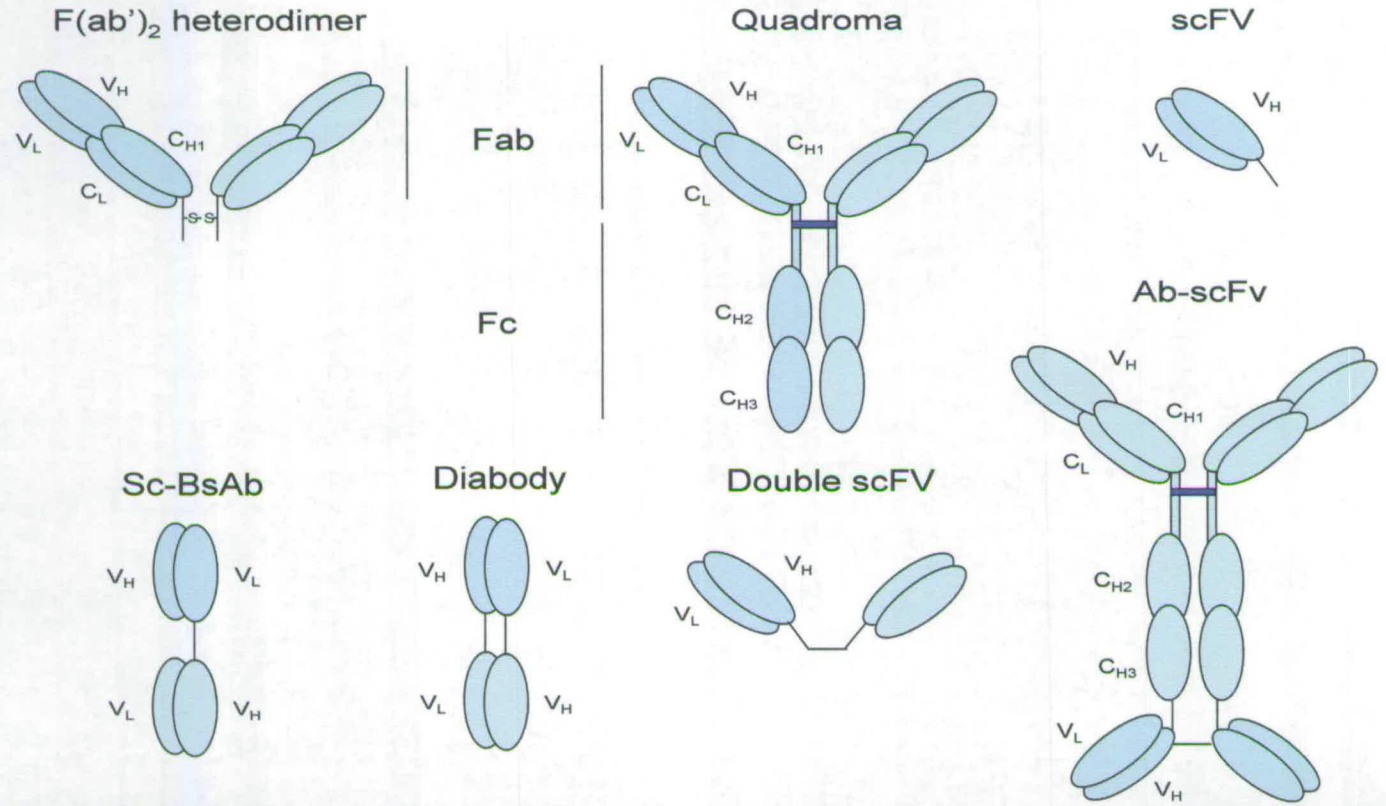


Figure 1.18 Representations of the antibody formats that are currently possible using phage display as a production method. The bottom row represents bispecific antibodies i.e. target more than one antigen. V_H – Variable heavy domain C_H –Constant heavy domain V_L – Variable light domain C_L – Constant light domain scFV – Single chain variable fragment Sc-BsAb – Single chain bispecific antibody

1.8 Aims

- To optimise the AFLP typing system using the published sequence of *C. jejuni* NCTC 11168.
- To devise a novel typing system based upon the CPS locus that will: 1. combine the simplicity and speed of RFLP and 2. harmonise DNA-DNA based typing systems with Penner serotyping.
- Clone the suspected outer membrane protein PEB3, raise monoclonal and polyclonal antibodies against it for use in an immunomagnetic separation system.

Materials and Methods

2.1 Health and Safety

Throughout the project each protocol was assessed before it was carried out according to the University of Edinburgh guidelines, which conform to the Health and Safety at Work Act. At all times care was taken to use reduce the risk to everyone who would come into contact with material used in each experiment. Where potentially hazardous material was used personal protective equipment appropriate to the risk involved was worn. The used of genetically modified organisms was approved, before being performed, by the departmental board according to the relevant legislation. At all times care was taken to ensure that genetically modified organisms were not released into the environment. In all the experiments involving the use of animals, Home Office permission was obtained and the work carried out according to the specifications of each project license. In all cases material was disposed of in an appropriate manner.

2.2 Materials used

2.2.1 Primers and plasmids

The primers used in this work are detailed in Table 2.1 and the plasmids in Figure 2.1 and Figure 2.2.

Name	Target	Sequence	Source
Cj1430Fwd	*cj1430*	ATGGCAATAGAATTTGATATACAAG	Karlyshev *et al.*, 2005
Cj1430Rev	*cj1430*	GCAAGAATATCGCGATCGGAAAGAATAGGCG	Karlyshev *et al.*, 2005
hddCFwdc	*hddC*	GCCGCCACAAAGTATTATTGCTTGCAA	Karlyshev *et al.*, 2005
gmhA2Fwd	*gmhA2*	ATGGAGAATTTAAATTCCTATATAAAAGG	Karlyshev *et al.*, 2005
ak186	*kpsF*	GAAAAGGAAGCTTGTCCTTTGCAGCTTGC	Karlyshev *et al.*, 2005
KpsD-SFwd	*kpsD*	CGTTAATGCTCCAGGACTTTATCAAGGCAT	This project
KpsD-SRev	*kpsS*	CCTTAGTCGGACAGCCTTCTAAAATAGCACT	This project
FclFwd	*Fcl*	TAGCGGGGCACAAAGGAAC	This project
FclRev	*Fcl*	CAATCCATAAGTCTATCCATTGTGC	This project
KpsSFwd	*kpsS*	CCTAGCGGAACGAGGTGTAAGTGTG	This project
KpsSRev	*kpsS*	GTCGGACAGCCTTCTAAAATAGCAC	This project
PEB3Fwd	*peb3*	CATATGACGGCCTTAAAAGATATAG	This project
PEB3Rev	*peb3*	GGATCCAAATCTTGTGTTTCTTTGCTCG	This project
EcoRIAdap1		CTCGTAGACTGCGTACC	Vos *et al.*, 1995
EcoRIAdap2		ATTGGTACGCAGTCTAC	Vos *et al.*, 1995
EcoRIPr	EcoRIAdap	GACTGCGTACCAATT	Vos *et al.*, 1995

Table 2.1 Table of the primers/oligonucleotides and their sequence sources; used throughout this project. All primers were diluted to 100 µM for stock solutions and to 10 µM for 10× working solutions in 0.5 M Tris pH 8.0 and stored at -20 °C.

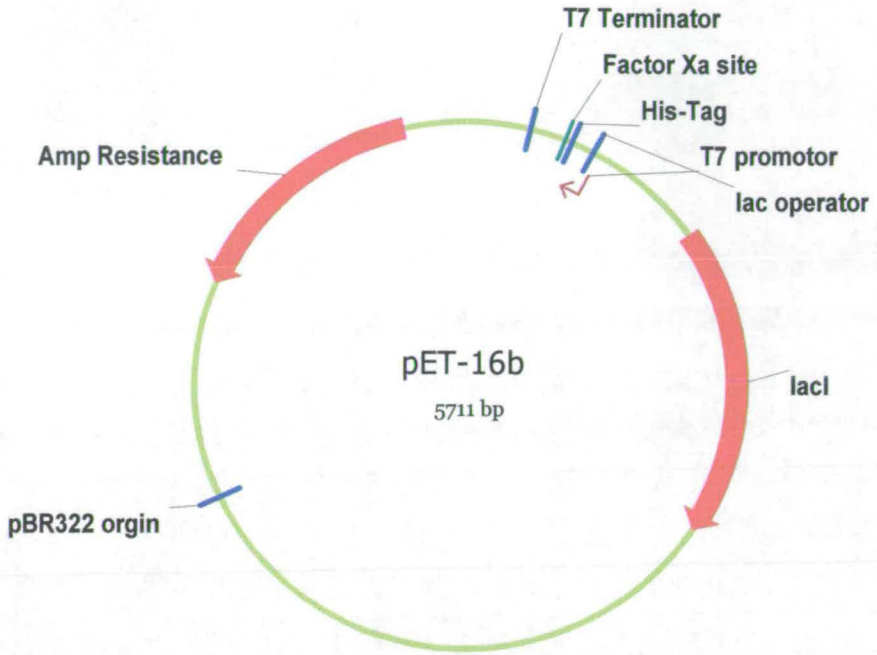


Figure 2.1 Schematic of the plasmid pET-16b (Novagen) showing the location of the His.Tag sequence. The multiple cloning region is situated between the His-tag and Factor Xa site.

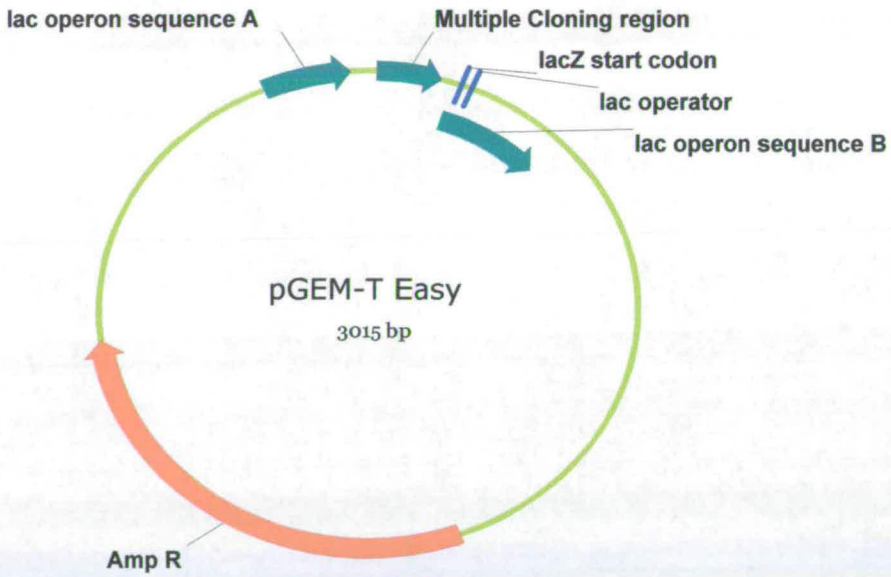


Figure 2.2 Schematic of the plasmid pGEM-T/-Easy (Promega) showing the location of the multiple cloning site and its position in relation to the *lac* operon. The two vector version, -T and –TEasy, are identical except for the number of restriction sites in the multiple cloning region.

2.2.2 Bacterial strains

The bacterial strains used in this work are detailed in Table 2.2 and Table2.3.

Species	Clonal Complex	Strain	Year first isolated	Source	Penner	Donor
C. jejuni	ST22	3752	1991	Human	19	M. Maiden
C. jejuni	ST42	3744	1991	Human	23	M. Maiden
C. jejuni	ST45	409266	1999	Human	57	M. Maiden
C. jejuni	ST49	3060	1991	Human	18	M. Maiden
C. jejuni	ST61	450964	1998	Beef Ofal	50	M. Maiden
C. jejuni	ST177	79196	1994	Sand	NT	M. Maiden
C. jejuni	ST206	2666	1991	Human	NT	M. Maiden
C. jejuni	ST362	C649/94	1994	Human	41	M. Maiden
C. jejuni	ST43	NCTC 11168	1977	Human	2	T. Humphries
C. jejuni	ST267	NCTC 81116	1981	Human	-	T. Humphries
C. coli		NCTC 11366	1980	Pig	4	T. Humphries
C. lari		NCTC 11352	1980	Herring Gull	-	T. Humphries

Table 2.2 Table of *Campylobacter* spp. used throughout this project. Dr M. Maiden - Dept. of Zoology, University of Cambridge. Prof. T. Humphries - University of Bristol

Species	Strain	Genotype	Source
E. coli	TG1		G. Reid
E. coli	BL21(DE3)	F- *ompT hsdSB(rB- mB-) gal dcm* (DE3)	M. Gallagher
E. coli	Tuner™ (DE3)	F- *ompT hsdSB (rB- mB-) gal dcm lacY1* (DE3)	Novagen

Table 2.3 Table of *E. coli* strains used in cloning experiments. Prof. G. Reid - University of Edinburgh. Dr. M. Gallagher - University of Edinburgh

2.2.3 Myeloma cell lines

The myeloma cell lines used in this work are detailed in Table 2.4.

Cell line	Description	Source
P3-X63-Ag8	Derived from the P3X27 cell line, itself a derivative of mineral oil-induced plasmacytoma MOPC-21 in BALB/c mice. Resistant to 20µg/ml azaguanine, does not grow in selective medium. HGPRT neg. dies in HAT. Doubling time 16-26 h.	A. Merdes
Sp2/0'-Ag14	Sp2/0-Ag14 is a non-Ig-secreting or synthesising line derived from a cell line created by fusing a BALB/c mouse spleen cell and the mouse myeloma P3X63Ag8. Resistant to 8-azaguanine at 20ug/ml and does not survive in HAT containing media.	J. Ross

Table 2.4 Myeloma cell lines used in hybridoma experiments, a short description of the cell line and their source. Dr A Merdes - University of Edinburgh. Dr J Ross - University of Edinburgh.

2.2.4 Stock solutions

All stock solutions were stored at room temperature and sterilised at 121 °C for 20 min unless otherwise stated. Where more than one grade of reagent was available, the highest quality was always used.

2.2.4.1 0.2 M Calcium chloride

A solution of 43.8 g $CaCl_2 \cdot 6H_2O$ in 800 ml distilled water was prepared. Once the components were dissolved completely the volume was adjusted to 1 l before sterilisation.

2.2.4.2 O.5 M EDTA

A solution of 186.12 g disodium ethylenediaminetetraacetate (Na_2EDTA) in 800 ml distilled water was prepared. The pH of the media was adjusted to ~8.0 using

NaOH pellets and then accurately adjusted to 8.0 using 10 M NaOH. The final volume of the medium was adjusted to 1 l before sterilisation.

2.2.4.3 1 M Tris

A solution of 121.14 g tris(hydroxymethyl)aminomethane (Tris) in 800 ml distilled water was prepared. The pH of the solution was adjusted to 7.4, 7.6 or 8.0 using concentrated HCl. The final volume was adjusted to 1 l with distilled water before sterilisation.

2.2.4.4 20% (w/v) SDS

A solution of 200 g sodium dodecyl sulphate crystals (SDS) in 900 ml distilled water was prepared. The solution was heated, with stirring, until all the crystals had dissolved. Once the components were dissolved completely, the pH was adjusted to 7.2 with HCl and the final volume adjusted to 1 l with distilled water. This solution was not sterilised.

2.2.4.5 0.1 M IPTG

A solution of 1.19 g isopropyl β-D-thiogalactopyranoside (IPTG) in 40 ml distilled water was prepared. Once the components were dissolved completely the final volume was adjusted to 50 ml and filter sterilised with a 0.22 μm filter. Aliquots (1 ml) were made and stored at -20 °C until needed; working stocks were stored at 5 °C in the dark for a maximum of 2 weeks.

2.2.4.6 X-gal (20 mgml^{-1})

A solution of 400 mg 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) in 20 ml N,N'-dimethyl formamide was prepared. Aliquots were made and stored at -20 °C protected from light; working stocks were stored at 5 °C in the dark for a maximum of 2 weeks. This solution was not sterilised.

2.2.4.7 10× PBS

A solution of 80 gNaCl, 2 g KCl, 26.8 g $Na_2HPO_4 \cdot 7H_2O$ and 2.4 g KH_2PO_4 in 800 ml distilled water was prepared. The pH of the solution was then adjusted to 7.4 using HCl and then the final volume adjusted to 1 l with distilled water.

2.2.4.8 TE

To make TE solution, 10 ml 1 M Tris (see 2.2.4.3) at the appropriate pH and 2 ml 0.5 M EDTA (see 2.2.4.2) was added to 800 ml distilled water. Once mixed thoroughly, the volume was adjusted to 1 l using distilled water before sterilisation.

2.2.4.9 0.2 M Sodium phosphate, mono-sodium salt

A solution of 27.6 g $NaH_2PO_4 \cdot H_2O$ in 500 ml distilled water was prepared. Once dissolved the final volume was adjusted to 1 l with distilled water before sterilisation.

2.2.4.10 0.2 M Sodium phosphate, di-sodium salt

A solution of 53.62 g $Na_2HPO_4 \cdot 7H_2O$ in 500 ml distilled water was prepared. Once dissolved the final volume was adjusted to 1 l with distilled water before sterilisation.

2.2.4.11 0.1 M Sodium phosphate

To make 0.1 M sodium phosphate of a specific pH, 0.1 M sodium phosphate mono sodium salt (see 2.2.4.9) and 0.2 M sodium phosphate di-sodium salt (see 2.2.4.10) was mixed according to the Table in appendix A to obtain a 200 ml solution of 0.1 M sodium phosphate with a specific pH.

2.2.4.12 10× agarose gel sample buffer

A solution of 250 mg bromophenol blue and 250 mg xylene cyanol in 33 ml 150 mM Tris pH 7.6 (see 2.2.4.3) was prepared. Once dissolved, 60 ml sterile glycerol and 7 ml distilled water were added. This solution was not sterilised.

2.2.4.13 10× TBE

A solution of 108 g Tris and 55 g Boric acid in 900 ml distilled water was prepared. Once dissolved, 40 ml 0.5 M EDTA pH 8.0 (see 2.2.4.2) was added. The pH was checked and adjusted to 8.0. The final volume was then brought up to 1 l with distilled water.

2.2.4.14 Ethidium Bromide

A solution of 1 g ethidium bromide in 100 ml distilled water was prepared. The solution was not sterilised and stored at room temperature in the dark.

2.2.4.15 10× SDS PAGE running buffer

A solution of 10 g SDS, 30.3 g Tris and 144.1 g glycine in 800 ml distilled water was prepared. Once dissolved, the final volume was adjusted to 1 l with distilled water. This solution was not sterilised.

2.2.4.16 2× SDS PAGE sample buffer

To make 2× SDS PAGE buffer, 10 ml 1.5 M Tris pH 6.8 was mixed with 6 ml 20% (w/v) SDS (see 2.2.4.4), 30 ml glycerol and 15 ml β-mercaptoethanol. Once mixed thoroughly, 3.6 mg bromophenol blue was dissolved in the mixture and the final volume adjusted to 100 ml. The solution was divided into aliquots (5 ml) and stored at -20 °C. This solution was not sterilised.

2.2.4.17 Coomassie Blue staining solution

A solution of 2.5 g 'Coomassie Brilliant Blue R-250' in a mixture of 450 ml methanol, 100 ml acetic acid and 400 ml distilled water was prepared. The final volume was then adjusted to 1 l with distilled water and filter through Whatman No. 1 filter paper before use. This solution was not sterilised.

2.2.4.18 Coomassie Blue destaining solution

To make Coomassie blue destaining solution, 450 ml methanol, 100 ml acetic acid and 400 ml distilled water were mixed. Once mixed thoroughly, the final volume was adjusted to 1 l with distilled water. This solution was not sterilised.

2.2.4.19 1000× Ampicillin

A solution of 1 g ampicillin (amp) in 10 ml distilled water was prepared. Once dissolved, the solution was sterilised with a 0.22 μm filter and stored at -20 °C.

2.2.4.20 1000× Chloramphenicol

A solution of 3.5 g chloramphenicol (cmr) in 10 ml absolute ethanol was prepared. Once dissolved, the solution was sterilised with a 0.22 μm filter and stored at -20 °C.

2.2.4.21 LB

A solution of 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 10 g NaCl in 900 ml distilled water was prepared. The pH of the solution was adjusted to 7.0 with 10 M NaOH and the volume adjusted to 1 l with distilled water before sterilisation.

2.2.4.22 SOC

A solution 20 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 0.5 g NaCl, and 2.5 ml 1 M KCl was mixed with 900 ml distilled water. The pH was adjusted to 7.0 with 10 M NaOH and the volume adjusted to 990 ml with distilled water sterilisation. Before use 10 ml sterile 1 M $MgCl_2$ and 20 ml of 1 M glucose were added. After addition of $MgCl_2$ and glucose the SOC was stored at 5 °C.

2.2.4.23 Brucella broth

To make Brucella broth, 28 g dehydrated brucella broth was added to 1 l distilled water. The broth was heated with stirring until completely dissolved.

2.2.4.24 Denaturing lysis buffer

A solution of 13.8 g $NaH_2PO_4 \cdot H_2O$, 1.2 g Tris and 480.5 g urea in 600 ml distilled water was prepared. Once dissolved, the pH was adjusted to 8.0 with NaOH and the final volume to 1 l with distilled water. This buffer was not sterilised. Before use the pH of the buffer was checked and readjusted as necessary.

2.2.4.25 Wash buffer

Composition of wash buffer is identical to denaturing lysis buffer (see 2.2.4.24). Immediately before use the pH of an appropriate amount of denaturing lysis buffer was adjusted to 6.3.

2.2.4.26 Elution buffer A

Composition of elution buffer A is identical to denaturing lysis buffer (see 2.2.4.24). Immediately before use the pH of an appropriate amount of denaturing lysis buffer was adjusted to 5.9.

2.2.4.27 Elution buffer B

Composition of elution buffer B is identical to denaturing lysis buffer (see 2.2.4.24). Immediately before use the pH of an appropriate amount of denaturing lysis buffer was adjusted to 4.5.

2.2.4.28 ELISA coating buffer

A solution of 794 mg Na_2CO_3, 1.47 g $NaHCO_3$ in 800 ml of distilled water was prepared. Once the components were dissolved completely, 100 µl 0.01 % (w/v) NaN_3 was added; the pH adjusted to 9.5 with NaOH and the final volume brought up to 1 l with distilled water. This buffer was not sterilised.

2.2.4.29 0.1 M Citric acid

A solution of 19.2 g citric acid in 1 l of distilled water was prepared; filter sterilised with a 0.22 µm filter and stored at 5 °C in the dark.

2.2.4.30 ELISA substrate buffer

Substrate buffer was made immediately before use by mixing 6 ml 0.1 M citric acid (see 2.2.4.29), 6.4 ml 0.2 Na_2HPO_4 (see 2.2.4.10), 12.5 ml distilled water and dissolving 20 mg O-phenylenediamine in the mixture. Once the components were dissolved completely and mixed, 10 µl 30% (w/v) H_2O_2 was added. This solution was not sterilised.

2.2.5 Tissue culture related media and stock solutions

All tissue culture media was filter sterilised (0.22 μm filter) or obtained sterile from the supplier (Gibco, unless otherwise indicated) and tissue culture grade. All subsequent mixing of media was carried out in a Class II air flow cabinet.

2.2.5.1 Freund's complete adjuvant

Freund's complete adjuvant (FCA) is a suspension of either *Mycobacterium tuberculosis* or *M. butryicum* in a mixture of paraffin oil and mannide monooleate. Before injection FCA is mixed with the antigen to form a water-oil emulsion.

2.2.5.2 Freund's incomplete adjuvant

Freund's incomplete adjuvant (FIA) has the same composition as FCA (see 2.2.5.1) but does not contain any *Mycobacterium* spp.

2.2.5.3 100 mM sodium pyruvate

A solution of 550 mg sodium pyruvate (Sigma) in 50 ml distilled water was prepared. After sterilisation sodium pyruvate was stored at 5 °C.

2.2.5.4 Myeloma culture media

To 500 ml of RPMI-1640 with glutamine, 50 ml foetal bovine serum (USDA Approved), 10 ml 200 mM glutamine, 5 ml Pen-strep, 3 ml 100× non-essential amino acids and 5 ml 100 mM sodium pyruvate (see 2.2.5.3) was added. The complete myeloma culture media (MCM) was stored at 5 °C.

2.2.5.5 Serum free medium

To 500 ml of RPMI-1640 with glutamine, 10 ml 200 mM glutamine, 5 ml Pen-strep, 3 ml 100× non-essential amino acids and 5 ml 100 mM sodium pyruvate (see 2.2.5.3) was added. The serum free media (SFM) was stored at 5 °C.

2.2.5.6 Complete hybridoma medium

To 500 ml of RPMI-1640 with glutamine, 50 ml foetal bovine serum (Fusion tested, USDA Approved), 10 ml 200 mM glutamine, 5 ml Pen-strep, 3 ml 100× non-essential amino acids, 5 ml 100 mM sodium pyruvate (see 2.2.5.3) and 50 ml PH-Comdimed (Roche). The complete hybridoma medium (CHM) was stored at 5 °C.

2.2.5.7 HAT medium

To make hypoxyanthine-aminopterin-thymidine medium (HAT), one vial each of HT Hybri-Max (Sigma) and aminopterin Hybri-Max (Sigma) were added to 500 ml CHM (see 2.2.5.6) according to manufacturer's instructions. HAT was stored at 5 °C.

2.2.5.8 HT medium

One vial of HT Hybrid-Max (Sigma) was added to 500 ml of CHM (see 2.2.5.6) according to the manufacturer's instructions. HT was stored at 5 °C.

2.3 Growth of bacterial species

2.3.1 Growth of *Campylobacter* spp.

Campylobacter spp. cultures were incubated at 42 °C in Brucella broth for 48 h under micro-aerophilic conditions in gas jars (5% O_2, 10% CO_2, 80% N_2 (BOC)). When necessary Skirrow antibiotics (see Table 2.6) were used to inhibit any contaminating bacteria.

Component	Amount
Vancomycin	5 mg
Trimethoprim	2.5 mg
Polymixin B	1250 IU
Distilled water	2 ml

Table 2.5 Composition of 250× Skirrow antibiotics. IU= inhibitory units

2.3.2 Growth of *E. coli* strains

All *E. coli* strains were grown at 37 °C in LB (see 2.2.4.21) or on LB-agar. Broth cultures were grown in a vessel approximately five times the size of the culture volume and shaken at 200 rpm for the duration of the incubation. Where necessary antibiotics were added from the 1000× stock solutions (LB-amp, LB-cmr or LB-amp-cmr) (see 2.2.4.19 and 2.2.4.20).

2.4 DNA Purification

2.4.1 Genomic DNA purification

Genomic DNA was isolated using either the Wizard Genomic Kit (Promega) or the AquaPure Genomic Kit (BioRad). In both cases the manufacturers' instructions were followed except that 10 ml of *Campylobacter* spp. was substituted for 1 ml of *E. coli* culture.

2.4.2 Plasmid purification

Plasmids were purified from 1 ml of overnight culture using the Wizard Miniprep kit (Promega).

2.4.3 Gel purification of PCR products and plasmids

After electrophoresis (see 2.5.1) the band of interest was excised from the agarose gel using a clean scalpel. DNA was then extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen).

2.4.4 Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit (Qiagen) and long template PCR (LT-PCR) products were purified using the Chargeswitch PCR purification kit (Invitrogen).

2.5 DNA detection and analysis

2.5.1 Electrophoresis of DNA

All agarose gels were made by weighing an appropriate amount of electrophoresis grade agarose (Invitrogen) and dissolving, using a microwave oven, in 100 ml 0.5× TBE (see 2.2.4.13). Once the agarose had cooled slightly the solution was poured into a gel tray, a 16 well comb attached and the gel allowed to set. DNA samples were prepared by adding 5 µl agarose gel loading buffer (see 2.2.4.12) into the sample. Either a 100 bp or 1 kb (NEB) DNA ladder was loaded at either side of the gel as appropriate for the size of bands expected. A maximum of 30 µl of sample was then loaded in the intermediate lanes. All gels were run in 0.5× TBE (see 2.2.4.13) and at 100 V. Once complete the gels were stained with ethidium bromide as described in 2.5.3.

2.5.2 Pulsed Field Gel Electrophoresis of LT-PCR products

PFGE gels was made by dissolving 1.2 g of PFGE certified agarose (BioRad) in 120 ml 0.5× TBE (see 2.2.4.13), using a microwave oven. After allowing the solution to cool slightly the gel was poured into the casting tray, a 20 well comb attached and the gel allowed to set. While the gel was setting 2 l 0.5× TBE (see 2.2.4.13) was put into the PFGE tank and cooled to 14.0 °C.

PCR products were purified as described in 2.4.4, then 5 µl agarose gel loading buffer was added to 50 µl purified PCR product. Care was taken to mix the sample carefully to prevent undue DNA shearing. Mono-cut PFGE marker (1 µg) (NEB) was

loaded at either side of the gel and the samples loaded in the intermediate lanes. Gels were run with the following conditions using the CHEF-DR II PFGE system (BioRad):

- 6 Vcm^{-1}
- Run time = 22 h
- Switch times = 0.5 – 1.5 sec (linear gradient)

Once the run was complete, gels were stained using ethidium bromide as described in 2.5.3.

2.5.3 Staining of agarose gels

Stock ethidium bromide solution (100 µl) (see 2.2.4.14) was added to 500 ml distilled water. Gels were immersed in the solution for 15 min and photographed under UV light in an Epichem darkroom II (UVP). If destaining was required, gels were immersed in 500 ml distilled water for 15 min before photographing.

2.5.4 Sequencing of DNA

Sequencing reactions were set up as detailed in Table 2.7, using the BigDye sequencing kit (version 3.1, Applied Biosystems). The sequencing reaction was carried out on a MBS 0.2 G thermal cycler and the cycling conditions used were:

- 25 times
    - 30 sec at 95 °C
    - 20 sec at 50 °C
    - 4 min at 60 °C

Completed reactions were made up to a final volume of 20 µl with distilled water before analysis on an ABI 3730 genetic analyser (Applied Biosystems). The raw

sequence data was analysed using BioEdit (Department of Microbiology, North Carolina University) and aligned to known sequences using Vector NTI (version 10, Invitrogen).

Component	Amount
Template DNA	~ 400 ng
Primer	1.6 pmol
BigDye Mix	4 µl
Distilled water	To 10 µl

Table 2.6 Composition of sequencing reactions

2.6 Protein detection and analysis

2.6.1 SDS-PAGE

Stock 30% (w/v) acrylamide (Severn Biotech Ltd) was mixed with separating (1.5 M Tris pH 8.8) and stacking (0.5 M Tris pH 6.8) gel buffers according to Table 2.8. The separating gel was mixed with 200 µl of 20% (w/v) ammonium persulphate (made immediately before use) and 10 µl of TEMED. After pouring, the separating gel was covered with a layer of distilled water and left to polymerise at room temperature. Once the separating gel had polymerised, the stacking gel was mixed with 200 µl 20% (w/v) ammonium persulphate and 10 µl TEMED. A 10 well comb was inserted into the stacking gel and the gel left to polymerise at room temperature. Before running the wells were washed with distilled water to remove any traces of unpolymerised acrylamide.

Protein samples were prepared by mixing with an equal volume of 2× SDS PAGE sample buffer (see 2.2.4.16) and incubated in a boiling water bath for 5 min. The amount of each sample loaded was adjusted so that total protein amounts per lane were equal (see 2.6.5). The first well in all gels was loaded with 1 µl of the Broadrange 3

Protein ladder (NEB). Gels were run at 100 V until the dye front had run the length of the gel.

	Amount (ml)	
Final%	**4**	**12.5**
Component		
Acrylamide 30%	4	10
Separating gel buffer	-	6
Stacking gel buffer	6	-
Distilled water	14	8

Table 2.7 Composition of the Stacking (4% w/v) and separating (12.5% w/v) polyacrylamide gels used for electrophoresis of proteins.

2.6.2 Coomassie blue staining of SDS-PAGE gels

Gels were incubated with 100 ml of Coomassie blue staining solution (see 2.2.4.17) at room temperature for 15 – 20 min. After incubation the staining solution was removed and the gel incubated with Coomassie blue destaining solution until bands were visible. Destained gels were photographed backlit with white light and a Epichem II darkroom.

2.6.3 Gelcode blue staining of SDS-PAGE gels

Gels were fixed in 50 ml 5% (v/v) methanol and 7% (v/v) acetic acid for 15 min. After fixing, the gels were washed with 50 ml distilled water. The distilled water was discarded after 5 min and the gel covered in Gelcode blue (Perkin-Elmer). The gel was incubated at room temperature for 30 min to 1 h with shaking. Gels were destained with 100 ml distilled water overnight before photographing as a Coomassie stained gel (see 2.6.2).

2.6.4 Determination of total protein concentration

Total protein concentration was determined using the BioRad protein assay kit with bovine serum albumin (NEB) as a standard.

2.6.5 Equalisation of loading amounts

When it was not possible to directly measure total protein, the amount of sample to be loaded was determined by the following formula:

$$\text{Volume to load } (\mu l) = \frac{270}{16.6 x A600nm}$$

2.7 Amplified fragment length polymorphism (AFLP) analysis of *Campylobacter* spp.

2.7.1 Digestion of genomic DNA and ligation of adaptors

Approximately, 400 ng of purified genomic DNA (see 2.4.1) was incubated with 10 U *Eco*RI (NEB), 3 U T4 DNA ligase (Promega) and 50 pmol each adaptor strand (see Table 2.1). The reaction was carried out at 37 °C for 1 h. The restriction/ligation mix was made using the supplied 10× ligase reaction buffer and the final volume of the reaction was 50 µl.

2.7.2 Amplification of ligated fragments

Amplification reactions were set up according to Table 2.8 and carried out either in a MBS 0.2 G thermal cycler (ThermoElectron) or a Progene thermal cycler (Techne). The cycling conditions used were:

- 5 min at 94 °C
- 30 times
    - 30 sec at 94 °C
    - 30 sec at 55.5 °C
    - 1 min at 72 °C
- 5 min at 72 °C

Once cycling was complete the reaction was run on a 2.5% (w/v) agarose gel at 100 V for 120 min.

Component	Amount
Restriction/ligation reaction	5 µl
Primer	100 pmol
Taq DNA polymerase	1 U
10× Taq DNA polymerase buffer	5 µl
dNTPs 1 mM	1 µl
Distilled water	To 50 µl

Table 2.8 Composition of *Eco*RI-AFLP amplification reactions.

2.7.3 Amplification of AFLP controls

Each control was amplified in separate reactions from purified *C. jejuni* NCTC 11168 genomic DNA. The PCR reactions were set up according to Table 2.10 and carried out in a MBS 0.2 G thermal cycler. The cycling conditions used were:

- 5 min at 94 °C
- 30 times
    - 30 sec at 94 °C
    - 30 sec at 50 °C
    - 1 min at 72 °C
- 10 min at 72 °C

Component	Amount
Template DNA	~ 100 ng
10× Fwd primer	5 µl
10× Rev primer	5 µl
Taq DNA polymerase	0.5 µl
10× Taq DNA polymerase buffer	5 µl
dNTPs 1 mM	1 µl
Distilled water	To 50 µl

Table 2.9 Composition of PCR reactions for amplification of each AFLP control from genomic DNA.

2.8 CPS-RFLP of *C. jejuni*

2.8.1 Amplification of single genes from the CPS locus

Primers for the *kps* genes, *cj1430*, *hddC* and *gmhA2* (see Table 2.1) were designed based the genomic sequences of *C. jejuni* NCTC 11168, RM1221 and the published CPS loci sequences of Penner serotypes HS:23/36 and HS:41, using Vector NTI (version 10, Invitrogen). Individual PCR reactions were set up according Table 2.10 and amplification was carried out in a MBS 0.2 G thermal cycler (ThermoElectron). The following cycling conditions were used:

- 5 min at 94 °C
- 30 times
    - 30 sec at 94 °C
    - 30 sec at 55 °C
    - 60 sec at 72 °C
- 5 min at 72 °C

Component	Amount
Template DNA	~100 ng
Working stock forward primer	5 µl
Working stock reverse primer	5 µl
Taq DNA polymerase	0.5 µl
10× Taq DNA polymerase buffer	5 µl
dNTPs 1 mM	1 µl
Distilled water	To 50 µl

Table 2.10 Composition of PCR reactions used to amplify single genes from the CPS locus of *C. jejuni*

2.8.2 Long template PCR of the *C. jejuni* CPS locus

An aliquot of 1 ml from a two day old culture, was spun at ~ 16 000 g for five minutes in a microcentrifuge. The supernatant was discarded and the pellet was resuspended in 10 µl of distilled water. Before the reactions were set up, the 10× Expand™ DNA polymerase buffer was heated to 42 °C for 2 h. Reactions were set up according to Table 2.11 using the Expand™ 20 kb plus kit (Roche) and amplification carried out in a MBS 0.2 G thermal cycler (ThermoElectron). The following cycling conditions were used:

- 5 min at 94 °C
- 10 times
    - 30 sec at 94 °C
    - 30 sec at 50 °C
    - 10 min at 64 °C
- 20 times
    - 30 sec at 94 °C
    - 30 sec at 50 °C
    - 10 min at 64 °C plus 20 sec per cycle
- 10 min at 64°C

Component	Amount
Resuspended cells	2 µl
Working stock gmhA2Fwd	2.4 µl
Working stock KpsD-SRev2	2.4 µl
Expand™ DNA polymerase mix	1 µl
10× Expand™ DNA polymerase buffer	5 µl
dNTPs 1 mM	2.5 µl
Distilled water	To 50 µl

Table 2.11 Compositon of the long template PCR reactions used to amplify the section of the *C. jejuni* CPS locus from *gmhA2* to *KpsS* using the Expand(tm) 20 kb plus kit (Roche).

2.8.3 RFLP of long template PCR products

After purification as in 2.4.4, long template PCR products were digested with either *Eco*RI, *Bcl*I, *Bgl*II or *Nde*I. All reactions were carried out at 37 °C for 1 h and setup according to Table 2.12.

Component	Amount
Purified LT-PCR product	20 µl
Restriction enzyme	1 µl
Restriction enzyme buffer	5 µl
BSA (10 mg/ml)	1 µl
Distilled water	To 30 µl

Table 2.12 Composition of the restriction digest reaction used for CPS-RFLP.

2.9 Construction of His-tagged PEB3

2.9.1 Cloning of *peb3*

Primers for *peb3* (see Table 2.1) were designed based on the published sequence of *C. jejuni* NCTC 11168 using VectorNTI (version 10, Invitrogen). PCR reactions were set up according to Table 2.14 and amplification was carried out in a MBS 0.2 G thermal cycler (ThermoElectron). The following cycling conditions were used:

- 5 min at 94 °C
- 30 times
    - 30 sec at 94 °C
    - 30 sec at 55.5 °C
    - 60 sec at 72 °C
- 5 min at 72 °C

Component	Amount
Template DNA	~100 ng
Working stock PEB3Fwd	5 µl
Working stock PEB3Rev	5 µl
Taq DNA polymerase	0.5 µl
10× Taq DNA polymerase buffer	5 µl
dNTPs 1 mM	1 µl
Distilled water	To 50 µl

Table 2.13 Composition of the *peb3* amplification reactions

2.9.2 Preparation of chemically competent *E. coli*

LB (5 ml) (see 2.2.4.21) was inoculated with the appropriate strain of *E. coli* and incubated overnight at 37 °C with shaking at 200 rpm. LB (5 ml) was then inoculated with 200 µl overnight culture and incubated at 37 °C with shaking at 200 rpm until the A_{600nm} ~ 0.8. All 5 ml of the culture was spun at ~ 3 500 g in a microcentrifuge at 5 °C for 2 min. The supernatant was discarded and the cells were re-suspended in 2 ml ice cold 0.2 M $CaCl_2$ (see 2.2.4.1). The cells were incubated for ~ 1 h on ice and then spun at ~ 3 500 g in a microcentrifuge at 5 °C for 2 min. The supernatant was discarded and the cells were re-suspended in 1 ml ice cold 0.2 M $CaCl_2$. The cells were then incubated on ice for at least 45 min before use.

2.9.3 Ligation of insert into pGEM-T

The plasmid pGEM-T is available ready-prepared with an optimised DNA ligase from Promega. The manufacturer's instructions were followed and the ligations carried out at 5 °C overnight to maximise the number of transformants. Once complete the ligation reaction was used to transform *E. coli* TG1 cells (see 2.9.4).

2.9.4 Transformation of competent *E. coli*

An entire ligation reaction or 5 µl of purified plasmid preparation (see 2.4.2) were placed on ice with 200 µl of competent cells for 5 min. The cells were then heat shocked by incubating at 42 °C in a water bath for 1 min 45 sec. The cells were then immediately placed on ice and 800 µl of room temperature SOC (see 2.2.4.22) was added. The cells were then incubated at 37 °C for 30 min with shaking at 200 rpm.

2.9.5 Screening for pGEM-T clones

LB-amp plates were dried and had 100 µl IPTG (see 2.2.4.5) and 20 µl X-Gal (see 2.2.4.6) added and spread thoroughly across the surface of the plates. After preparation of transformed cells (see 2.9.4), the cells were spun down at ~ 16 000 g in a microcentrifuge for 5 min. The cells were resuspended in 100 µl of supernatant and spread across the surface of a plate before being incubated at 37 °C overnight. Clones positive for insert appeared as white colonies and were subcultured onto LB-amp plates. Five positive colonies were also used to inoculate 5 ml of LB-amp and incubated at 37 °C overnight with shaking at 200 rpm.

Plasmid was purified from the overnight cultures (see 2.4.2) and sequentially digested with 2 000 U of *Nde*I (NEB) for 1 h at 37 °C and then 2 000 U of *Bam*HI (NEB) for a further 1 h. Digestions were set up according to Table 2.15. Agarose gel sample buffer (5 µl) (see 2.2.4.12) was added to the digestion and 30 µl run on a 1% (w/v) agarose gel (see 2.5). Plasmids that showed an insert equal to the size of the expected insert were then sequenced (see 2.5.4). This screen was repeated until a clone containing the correct insert was identified.

Component	Amount
Purified plasmid	5 µl
*Nde*I	2 000 U
*Bam*HI	2 000 U
NEB restriction enzyme buffer 4	3 µl
BSA 10µgµl^{-1}	0.5 µl
Distilled water	To 30 µl

Table 2.14 Composition of the digestion reaction for the screening of pGEM-T-PEB3 clones.

2.9.6 Preparation of pET-16b vector

Purified pET-16b (1 µg) (Novagen) was digested sequentially with 2 000 U of *Nde*I for 1 h at 37 °C and 2 000 U of *Bam*HI for a further hour. The digestions were set up according to Table 2.16. Upon completion of digestion 1 000 U calf intestinal phosphatase (NEB) was added to the restriction reaction and the reaction was incubated at 37 °C for 1 h. Agarose gel sample buffer (5 µl) (see 2.2.4.12) was added to the dephosphorylated plasmid and run on a 1% (w/v) agarose gel (see 2.5). The plasmid was then purified from the agarose (see 2.4.3) for use in ligation reactions.

Component	Amount
Purified plasmid	5 µl
*Nde*I	2 000 U
*Bam*HI	2 000 U
NEB restriction enzyme buffer 4	3 µl
BSA 10µgµl^{-1}	0.5 µl
Distilled water	To 30 µl

Table 2.15 Composition of restriction reactions for the preparation of the pET-16b vector

2.9.7 Ligation of insert into pET-16b clones

Insert from pGEM-T-PEB3 was gel purified (see 2.4.3) and ligated into purified pET-16b (see 2.9.6) according to Table 2.1. All ligation reactions were incubated overnight at 5 °C. Once complete, the ligation reaction was used to transform *E. coli* TG1 cells (see 2.9.4).

	Ratio (vector:insert)			
Component	**2:1**	**1:1**	**1:2**	**1:3**
pET-16b	2 µl	1 µl	1 µl	1 µl
Insert	1 µl	1 µl	2 µl	3 µl
T4 DNA ligase	1 µl	1 µl	1 µl	1 µl
10× T4 DNA ligase buffer	1 µl	1 µl	1 µl	1 µl
Distilled water	To 10 µl	To 10 µl	To 10 µl	To 10 µl

Table 2.16 Composition of ligation reactions for the ligation of *peb3* into pET-16b.

2.9.8 Screening of pSAN3 transformants

After incubation (see 2.9.4 and 2.9.7), pET-16b-PEB3 (renamed pSAN3) transformed cells were spun at ~ 16 000 g in a microcentrifuge for 5 min re-suspended in 100 µl of supernatant; then spread thoroughly across the surface of 1 LB-amp-cmr plate and incubated at 37 °C overnight. After incubation, colonies were subcultured onto LB-amp-cmr plates and five colonies selected for screening. The screen was performed in the same way as for the pGEM-T-PEB3 screen (see 2.9.5).

The plasmid from one positive clone was purified (see 2.4.2) and transformed into *E. coli* Tuner™ cells (see 2.9.4). After incubation, transformed cells were spun down at ~ 16 000 g in a microcentrifuge and then re-suspended in 100 µl of supernatant. The re-suspended cells were then spread on a LB-amp-cmr plate and the plates were incubated at 37 °C overnight. To screen for protein expression, five colonies were used to inoculate 5 ml of LB-amp-cmr and incubated overnight at 37 °C with shaking at 200 rpm. From each overnight culture, 200 µl was used to inoculate 5 ml LB-amp-cmr, which was the incubated at 37 °C with shaking at 200 rpm until the A_{600nm} ~0.8. Cultures were then induced with 0.1 mM IPTG (see 2.2.4.5) and incubated for 3 h at

37 °C with shaking at 200 rpm. An extra uninduced culture was set up as a negative induction control. After induction, 1 ml culture was harvested and the cell density measured at A_{600nm} and a 1 ml aliquot was spun down at ~ 16 000 g in a microcentrifuge and resupended in 15 µl of distilled water and 15 µl of SDS-PAGE sample buffer (see 2.2.4.16).

The A_{600nm} of each culture was used to standardise the loading amounts for each culture (see 2.6.5). All samples were analysed by SDS-PAGE (see 2.6). This process was repeated until a clone over-expressing a protein at the expected Mr of the cloned protein was obtained.

2.10 Expression of His.PEB3

Fifty millilitres of LB-amp-cmr was inoculated with the pSAN3 clone and incubated overnight at 37 °C with shaking at 200 rpm. After incubation, the 50 ml overnight culture was split and used to inoculate 3× 1 l of LB-amp-cmr and incubated at 37 °C with shaking at 200 rpm until the A_{600nm} was 0.8. Once the $A_{600n\ m}$ was reached the cultures were induced with 0.1 mM IPTG (see 2.2.4.5) for 3 h at 37 °C with shaking at 200 rpm. After induction, the cells were spun down at 10 000 g for 15 min.

2.11 Denaturing purification of His.PEB3

Cell pellets from His.PEB3 inductions (see 2.10) were re-suspended in 25 ml of denaturing lysis buffer (see 2.2.4.24). The cells were lysed by mixing gently at room temperature for 60 min. The lysate was then centrifuge at 10 000 g for 20 min to pellet cell debris.

The Ni-ATA agarose (Qiagen) was shaken well before use to ensure an even suspension and half a millilitre of agarose was added to a gravity flow column and allowed to settle. The agarose was equilibrated with 4 washes of 5 ml of denaturing lysis buffer. The cleared lysate was then added to the column and mixed with the agarose at room temperature for 60 min. After mixing, the agarose was allowed to settle and drain. The column was then washed twice with 4 ml of wash buffer (see 2.2.4.25) and twice with 4ml of elution buffer A (see 2.2.4.26). The purified protein was then eluted from the column in 2 ml of elution buffer B (see 2.2.4.27). The composition of the washes and elutents was analysed by SDS PAGE (see 2.6).

2.12 Enzyme Linked Immunosorbent Assay (ELISA)

Purified His.PEB3 (see 2.11) was diluted to a final concentration of 10 $\mu g ml^{-1}$ in ELISA coating buffer (see 2.2.4.28). One hundred microlitres of diluted protein was added to each well of a 96-well flat bottomed microtitre plate and incubated at 5 °C overnight. After incubation, the plate was washed in PBS + 0.05% (v/v) Tween 20 with a Dynex M.R.W. V 2 plate washer. The washing cycle was as follows:

- 4 times
    - Remove liquid
    - Add 300 µl wash solution

After washing, any residual liquid was removed by inversion and tapping of the plate on paper towel. The plate was then blocked using 200 µl of 1% (w/v) skimmed milk in coating buffer per well and incubating at 37 °C for 1 h in a humidified incubator. During blocking primary antibody dilutions were prepared in 0.25% (w/v) skimmed

milk PBS + 0.05% (v/v) Tween 20. After blocking, plates were washed as above and residual wash solution removed. One hundred microlitres of each antibody dilution was then added to the appropriate wells on the plate and incubated at room temperature for 4 h. After incubation, the plate was washed as above and any residual buffer removed.

Secondary antibody was diluted in 0.25% (w/v) skimmed milk PBS + 0.05% (v/v) Tween 20 according to the manufacture's instructions. One hundred microlitres of diluted secondary antibody was added to each well of the plate and incubated at 37 °C for 30 min. After incubation, the plate was washed and any residual buffer removed as above.

One hundred microlitres of freshly prepared substrate buffer (see 2.2.4.30) was added to each well and the plate incubated at room temperature for 15 min. The reaction was stopped by the addition of 50 µl of 1 M H_2SO_4 and the A_{492nm} of each well read in a Ronsys anthos 2010 plate reader.

2.12.1 ELISA end point titre determination

End point titres were calculated according to the equation:

End point titre = Mean of 6 Secondary antibody controls + 0.2 OD units

(Kemeny, 1991)

2.13 Production of monoclonal antibodies

2.13.1 Challenge of BALB/C mice with His.PEB3

Two separate challenges were carried out according to Table 2.18 and Table 2.19. In both cases antigen was prepared by mixing an equal volume of antigen and adjuvant through a 19 gauge needle until a thick emulsion was formed. Once an emulsion was formed, it was injected subcutaneously into each animal. All animal work for these challenges was carried out by National Diagnostics (Scottish National Blood Transfusion Service, Bush, Scotland).

Procedure	Day	Antigen amount (µg)	Adjuvant	Date
Preimmune bleed	0			26/05/04
Challenge 1	0	15	FCA	26/05/04
Challenge 2	28	15	FIA	23/06/04
Challenge 3	56	15	FIA	21/07/04
Test bleed	61			28/07/04
Challenge 4	96	15	FIA	1/10/04
Exsanguination and Splenctomy	99			4/10/04

Table 2.17 Schedule A used to challenge two BALB/C mice with 15 µg of His.PEB3 for the production of monoclonal antibodies.

Procedure	Day	Antigen amount (µg)	Adjuvant	Date
Preimmune bleed	0			10/01/05
Challenge 1	0	100	FCA	10/01/05
Challenge 2	28	100	FIA	7/02/05
Test Bleed	35			14/02/05
Challenge 3	42	100	FIA	21/02/05
Exsangiunation and Splenctomy	46			25/02/05

Table 2.18 Schedule B used to challenge two BALB/C mice with 100 µg of His.PEB3 for the production of monoclonal antibodies.

2.13.2 Preparation of serum

Serum was prepared by allowing bleeds to clot at 4 °C overnight and then centrifugation at 10 000 rpm in a microcentrifuge for 2 min. Serum was then extracted taking care to not to disturb the clot. If any red blood cells were present in the serum, it was re-spun at 10 000 rpm in a microcentrifuge for 2 min to pellet the cells before decanting into another tube. Serum was stored at -20 °C until use.

2.13.3 Growth of myeloma cells

A vial of cells (see 2.2.3) was removed from liquid nitrogen storage and rapidly defrosted at 37 °C. The cells were then washed 3 times in 30 ml of SFM (see 2.2.5.5) and harvested by centrifugation at 2 000 rpm for 5 min. After the final wash the cell pellet was re-suspended in 10 ml of MCM (see 2.2.5.4), placed in a 55 cm^2 tissue culture Petri dish and incubated at 37 °C under 5% CO_2 for two days in a humidified incubator. After incubation, spent media was replaced by an equal volume of fresh media every 2 d until the cells reached a density of $1 - 5 \times 10^5$ cell ml^{-1}.

The cells were then harvested by gentle washing with the spent media until all cells had detached from the surface of the dish. If necessary, cells were detached by washing with 5 ml of PBS (see 2.2.4.7) and then incubating with 1 ml of trypsin-EDTA for 10 min at 37 °C. The suspension was then divided equally between 3 dishes and fresh MCM used to bring the final volume to 10 ml. This process was repeated so that cell density reached but never exceeded $1 - 5 \times 10^5$ cells ml^{-1} until the cells were needed. On the day of fusion, 1×10^8 cells were harvested and washed in 30 ml SFM.

2.13.4 Harvest of splenocytes

The spleen was placed into a Petri dish on top of a square of sterile gauze and 10 ml SFM (see 2.2.5.5) added. The sterile gauze was folded over the spleen several times to create a pouch. A cell scraper was then used to burst the spleen and squeeze out the splenocytes until only the tough spleen capsule remained. Splenocytes were washed out the gauze using the SFM in the Petri dish. The suspension of SFM and splenocytes was removed and placed to one side. The Petri dish and gauze were then thoroughly washed with SFM to ensure all splenocytes were harvested. The cells were then pelleted at 1 000 rpm for 1 min in a benchtop centrifuge. The supernatant was removed and the cells washed in 30 ml fresh SFM and pelleted as above. The cells were washed a further 4 times in 30 ml SFM.

2.13.5 Fusion of myelomas and splenocytes

Washed splenocytes (see 2.13.4) and washed myelomas (see 2.13.3) were combined in one tube and washed twice in 30 ml SFM (see 2.2.5.5). After washing, the tube was flicked to ensure an even mixture of cells within the pellet. Over the course of 30 sec, 1 ml PEG 1500 (Roche) was added to the pellet with gentle flicking; the tube was flicked gently for a further 1 min 30 sec. Fresh SFM (5ml) was added over the course of 30 sec; from approximately 8'' above the pellet, without flicking. A further 5 ml SFM was added over 30 sec with flicking; before the addition of a final 10 ml SFM.

The cells were harvested by centrifugation at 1 000 rpm for 1 min and then re-suspended in 10 ml fresh SFM. The suspension was then placed in a Petri dish and incubated at 37 °C under 5% CO_2 in a humidified incubator for 30 min. After incubation, the cells were removed from the dish and the dish washed with SFM. The

cells were harvested by centrifugation at 1 000 rpm for 1 min and then re-suspended in 30 ml CHM (see 2.2.5.6). The suspension was pipetted into 96 well tissue culture plates (100 µl per well) and then incubated for 1 day at 37 °C under 5% CO_2 in a humidified incubator.

2.13.6 Selection and screening of hybriodmas

Fused cells (see 2.13.5) were subjected to HAT selection one day after fusion. Fused cells were fed with 200 µl 2× HAT (see 2.2.5.7) per well. The plates were then incubated for 2 days at 37 °C under 5% CO_2 in a humidified incubator. Cells were fed by removing 100 µl of media from each well and replacing it with 100 µl of fresh HAT before incubating as before. After 3 feedings, the cells were then fed with HT (see 2.2.5.8) instead of HAT. This process was then repeated until all wells of a plate tested negative for Anti-His.PEB3 production (see 2.12).

2.14 Production of polyclonal antibodies

2.14.1 Challenge of New Zealand White rabbits

Three New Zealand white rabbits were challenged with 0.95 mg of His.PEB3 per injection according to the schedule in Table 2.20 (Harlan Sera-lab, Hillcrest, UK). Serum was prepared as in 2.13.2 and tested for the presence of Anti-His.PEB3 (see 2.12).

Procedure	Day	Antigen Amount (µg)	Adjuvant	Date
Preimmune bleed	0			25/07/05
Challenge 1	0	950	FCA	25/07/05
Challenge 2	14	950	FIA	8/08/05
Challenge 3	28	950	FIA	22/08/05
Test Bleed 1	35			29/08/05
Challenge 4	42	950	FIA	5/09/05
Test Bleed 2	49			12/09/05
Challenge 5	56	950	FIA	19/09/05
Test Bleed 3	63			26/09/05
Challenge 6	70	950	FIA	3/10/05
Exsanguination	77			10/10/05

Table 2.19 Schedule for the challenge of 3 New Zealand white rabbits, against His.PEB3

Results and Discussion – Typing systems

3.1 Introduction to typing systems currently in use

Epidemiological analysis relies on the use of typing systems. Without the power to discriminate between different isolates, it would be impossible to determine the relationship between separate outbreaks. Unlike *Salmonella* and *E. coli*, cases of *C. jejuni* are sporadic; often with considerable time periods between cases. Determining commonality between seemingly unrelated cases is therefore only possible by accurate identification of isolates.

A wide variety of typing systems have been developed for use with *C. jejuni* (see 1.6). The majority of these rely on DNA based comparisons to distinguish between strains e.g. 16S rRNA, *fla*-RFLP, PFGE, AFLP and MLST. DNA-DNA based comparisons are highly sensitive as often single base pair differences can influence the result. This however presents its own problems as most rely on PCR to amplify sequences for comparison. A sequence that is both stable and conserved across strains is required for primer binding.

The most popular typing scheme currently in use is Penner serotyping. This scheme uses antibodies against CPS to agglutinate sensitised erythrocytes in the presence of each *C. jejuni* serotype. The level of discriminatory power that this achieves is low when compared to the DNA based techniques. Analysis of the sequenced genome (*C. jejuni* NCTC 11168) shows that the CPS is probably phase variable, which could cause variable serotyping results for each isolate. Despite this Penner serotyping has remained widely used. The Penner scheme has survived as the primary typing scheme because it requires minimal technical skill when compared to DNA based techniques. It is also relatively difficult to translate results from one

scheme to another, making comparison to pre-DNA-era isolates difficult.

AFLP has been widely studied and used to type large numbers of *C .jejuni* isolates. Briefly, whole genomic DNA is subjected to restriction digest. Restriction site specific adaptors are then ligated to the restriction fragments. Primers specific for the adaptor sequences are then used to amplify the fragments within a specified size range (commonly 50 – 500 bp). The amplified fragments are then size separated and visualised. The technique has changed little since its first publication (Vos *et al.*, 1995), despite it being stated that the technique would require optimisation for each organism it is used with.

In this chapter an attempt to optimise the AFLP technique for use with *C. jejuni* is described. In addition a novel technique based on PCR-RFLP, that will allow straightforward comparison to Penner serotyping is also described.

3.2 Optimisation of AFLP

3.2.1 Using *in silico* analysis of the published *C. jejuni* NCTC 11168 genome to predict AFLP profiles

Previous attempts to improve the AFLP typing scheme have been based upon trial and error of different restriction enzymes. It was decided to use the published genomic DNA sequence and *in silico* analysis to select restriction enzymes for practical analysis.

The selection of restriction enzymes for *in silico* analysis was made using three criteria: A. the enzyme must cut at the restriction site B. the enzyme must produce an overhang on restriction and C. the cost of the enzyme should not prevent routine use (defined as any enzyme costing < £ 20 per 50 U). Fourteen enzymes

were selected for analysis (see Table 3.1). The published sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000) was analysed using two programs, the Comprehensive microbial resource (TIGR) and a custom built program (Needham, unpublished 2002). Results for each enzyme singly and in combination were produced from both programs simultaneously and compared. Differences were only observed in the position used for the start of each fragment but this had no effect on the calculated sizes as it was consistent throughout all calculations. Once all the data was collated the number of fragments within the range 50 – 500 bp was calculated, as this is the size range used for fluorescent AFLP analysis (see Table 3.2).

Enzyme	Recognition site
*Afl*II	C▼TTAAG
*Apa*I	GGGCC▼C
*Bam*HI	G▼GATCC
*Bcl*I	T▼GATCA
*Eco*RI	G▼AATTC
*Hpa*II	C▼CGG
*Kpn*I	GGTAC▼C
*Mlu*I	A▼CGCGT
*Pst*I	CTGCA▼G
*Xba*I	T▼CTAGA

Table 3.1 Restriction enzymes and their recognition sites picked for use in prediction of *C. jejuni* NCTC 11168 AFLP profiles (the position of the cut in the restriction site is indicated by ▼)

Enzyme	*Afl*II	*Apa*I	*Bam*HI	*Bcl*I	*Eco*RI	*Hpa*II	*Kpn*I	*Mlu*I	*Pst*I	*Xba*I
*Afl*II	50	22	30	149	92	73	25	28	79	61
*Apa*I		0	1	71	29	29	1	3	33	14
*Bam*HI			1	84	41	41	6	6	36	23
*Bcl*I				71	162	146	84	100	153	135
*Eco*RI					29	85	34	56	82	74
*Hpa*II						29	32	39	87	73
*Kpn*I							1	5	39	23
*Mlu*I								3	51	28
*Pst*I									33	66
*Xba*I										14

Table 3.2 Predicted number of fragments within the range 50 - 500 bp produced by the *in silico* restriction of *C. jejuni* NCTC 11168 by stated enzyme combinations.

The optimal restriction enzyme combination is dependent on the method used to visualise the AFLP profiles. Visualisation methods can be split into two groups based upon the amount of DNA needed for detection. High sensitivity techniques such as radio-labelling or fluorescently labelled primers require fg of DNA per fragment for detection; whereas low sensitivity techniques such as SYBR Green or EtBr staining require ng of DNA per fragment for detection. Of significance when determining the optimum restriction enzyme combination, different separation matrices are used for each sensitivity group. High sensitivity techniques use polyacrylamide or polyacrylamide mixes and usually longer gel lengths are utilised with than low sensitivity techniques. This means that fragments with 1 bp differences in size can be resolved but the size ranges that can be analysed are smaller and less flexible. Most fluorescent-primer detection methods rely on the use of automated sequencers for separation of fragments. In theory such machines should be able to resolve 450 fragments in the 50 – 500 bp range (assuming there is a fragment for each size). However, limits in signal separation mean this is rarely

achieved. The practical detection limit is approximately 300 fragments, depending on the conditions used. Low sensitivity techniques generally use agarose as the separation matrix. In comparison to polyacrylamide, a 15 cm long agarose gel has a theoretical detection limit of 30 bands. Therefore, enzyme combinations suitable for a high sensitivity technique will not necessarily be useable in low sensitivity techniques.

The optimum restriction enzyme combination can therefore be defined as: 'the enzyme combination that produces enough bands to allow the use of statistical techniques in distinguishing strains and fewer bands than the theoretical resolution limit of the detection method.'

Using the definition of the optimum enzyme combination all of the enzyme combinations in Table 3.2 could be used for AFLP analysis of *C. jejuni*. To determine which of these best fulfils the discrimination criteria for the optimal combination, practical comparison of AFLP profiles from a set of strains is needed. It was decided to use *Eco*RI initially as these had successfully used with *C. jejuni* previously (Arnold *et al.*, 1999), as well as *Hpa*II and *Xba*I. Additionally primers and adaptors were designed for use with *Xba*I. In all cases single digests were predicted to produce profiles that are suitable for EtBr visualisation and double digests suitable for fluorescent analysis.

3.2.2 *In vitro* AFLP analysis of *C. jejuni* NCTC 11168 genomic DNA

The AFLP procedure was first described by Vos *et al.* in 1995. A slight modification was made (Peters and Threlfall, 2001) to change the system into a single restriction enzyme technique. Initial experiments with *Eco*RI were not

successful, as they did not provide any visible bands after staining with EtBr. The procedure was then further modified to take into account the sensitivity difference between EtBr staining and fluorescent detection. After modification the reactions produced a ladder-like profile with bands at 30 bp and then every 30 bp thereafter. This is probably due to the formation of primer/adaptor dimers during the amplification. A series of dilutions of primers and adaptors was prepared to minimise dimer formation but had no effect.

Both primers and adaptors were re-synthesised and used in parallel with older oligos. When the re-synthesised oligos were used no ladder-like profile was seen, this suggests that the adaptor EcoRIAdap1 had been incorrectly synthesised preventing ligation of the adaptor to the restriction fragments. A gradient PCR was then used to maximise the number of bands seen (see Figure 3.1). The profile was then compared to the expected profile obtained from *in silico* analysis. Not all the expected bands were observed and all of the observed bands were sized to within 2 – 3 bp of expected bands (see Figure 3.2).

AFLP reactions were also performed with *Hpa*II and *Xba*I at the same time using the same conditions as the *Eco*RI reactions. With all enzymes the profiles obtained were consistent for 4 – 5 reactions but then became less distinct and less reproducible, particularly within the size range 50 – 500 bp. Bands observed in the 50 – 1000 bp range appeared more stable but the profile did eventually degrade.

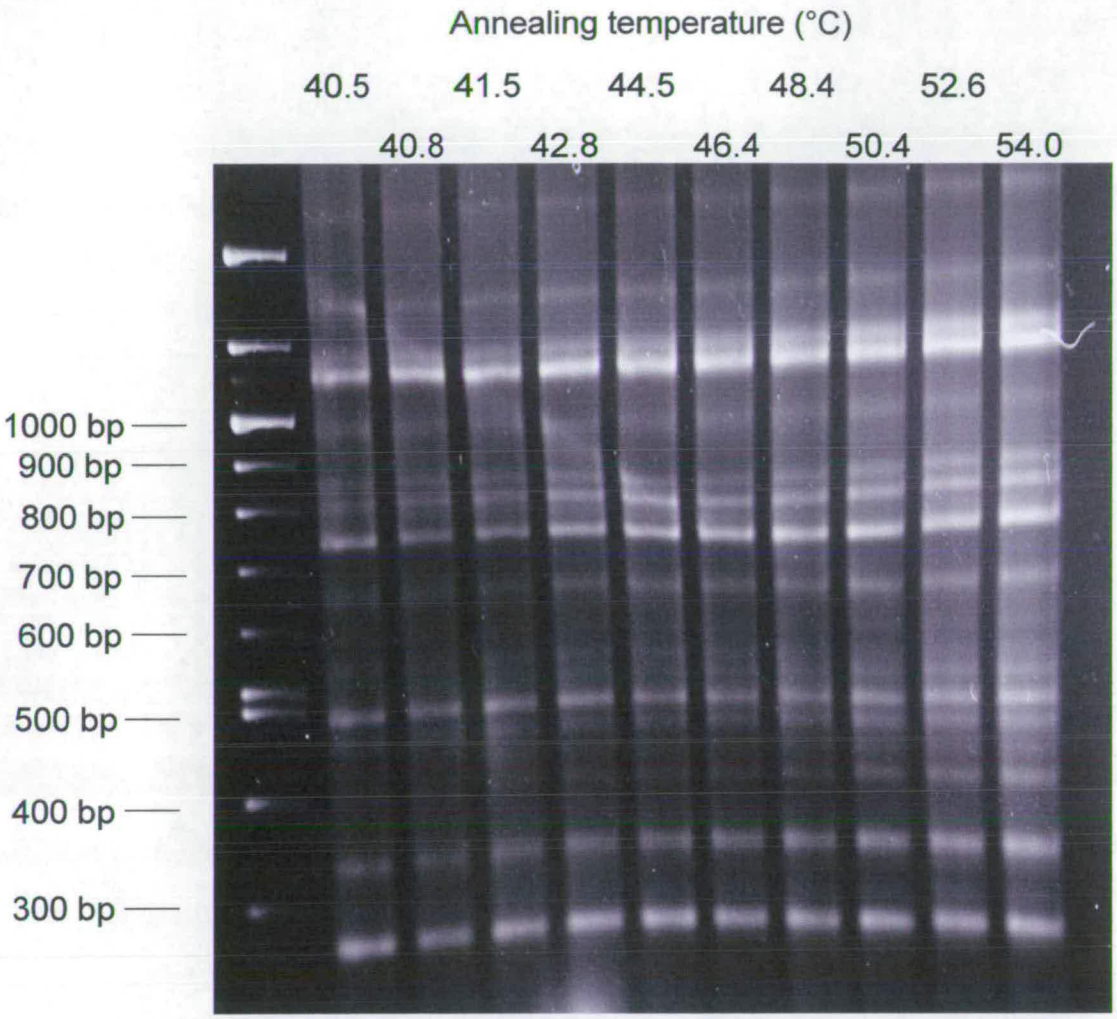


Figure 3.1 2.5 % (w/v) agarose gel showing the *Eco*RI AFLP profile of *C. jejuni* NCTC 11168 and the effect of annealing temperature on the AFLP profile. Whole genomic DNA (400 ng) from *C. jejuni* NCTC 11168 was digested with *Eco*RI. Adaptors specific to the restriction site were then ligated to the restriction fragments before amplification with primers specific to the adaptor sequence. Each represents a different annealing temperature (stated at the top of each lane) used during amplification. As the annealing temperature increases the number of fragments increases and the yield for each fragment increases.

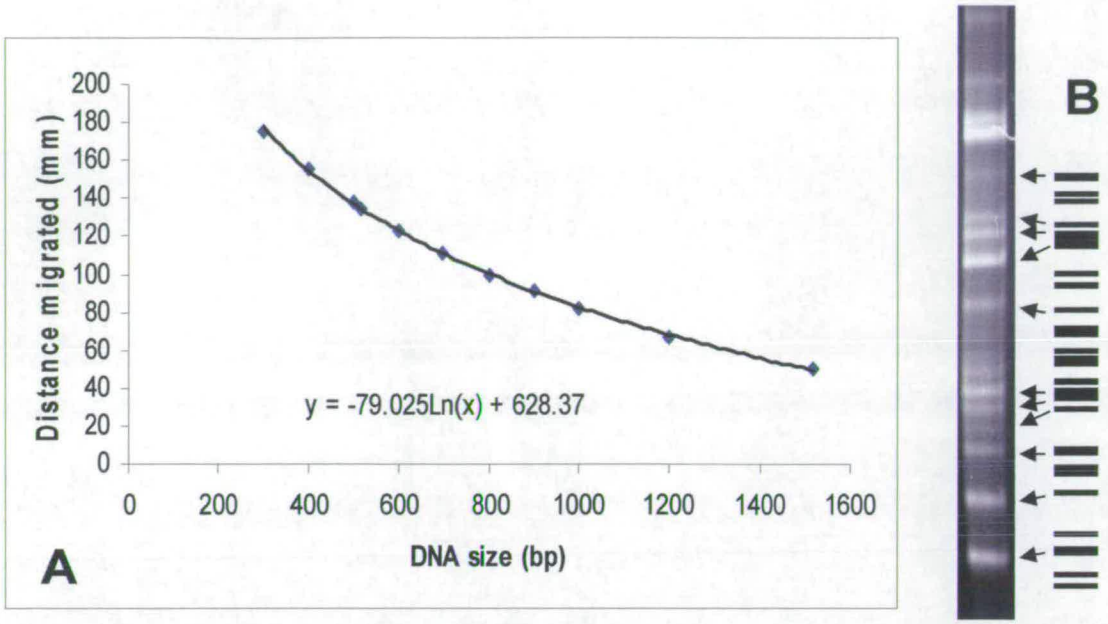


Figure 3.2 Comparison of expected fragments to observed fragments for the *Eco*RI AFLP profile of *C. jejuni* NCTC 11168. Known fragment sizes were used to calculate a standard curve (A). The equation for the standard curve (y= -79.025ln(x) + 628.37) was used to position the expected fragments against the photograph (B). Arrows indicate the position of corresponding bands between expected and observed.

A series of control fragments were designed to investigate possible variables in the AFLP protocol. Using the *in silico* analysis, primers were designed that would amplify four of the expected *Eco*RI fragments. AFLP reactions with each control fragment separately and in combination were performed. The control fragments showed that restriction and ligation were not affected by size as all fragments appeared when processed separately (see Figure 3.3). However, control fragment 1 (153 bp) was never observed when processed in combination with other fragments. Control fragment 2 (255 bp) was not observed when processed with control fragment 4 (555 bp) but was present in all other combinations. Both control fragments 3 (306 bp) and 4 were observed in all combinations. Control fragment 4 was always present in greater abundance than any other fragment. These results show clearly that larger fragments were preferentially amplified. It would be expected that

fragment size would influence amplification efficiency as a natural bias towards small fragments is normally seen in PCR reactions.

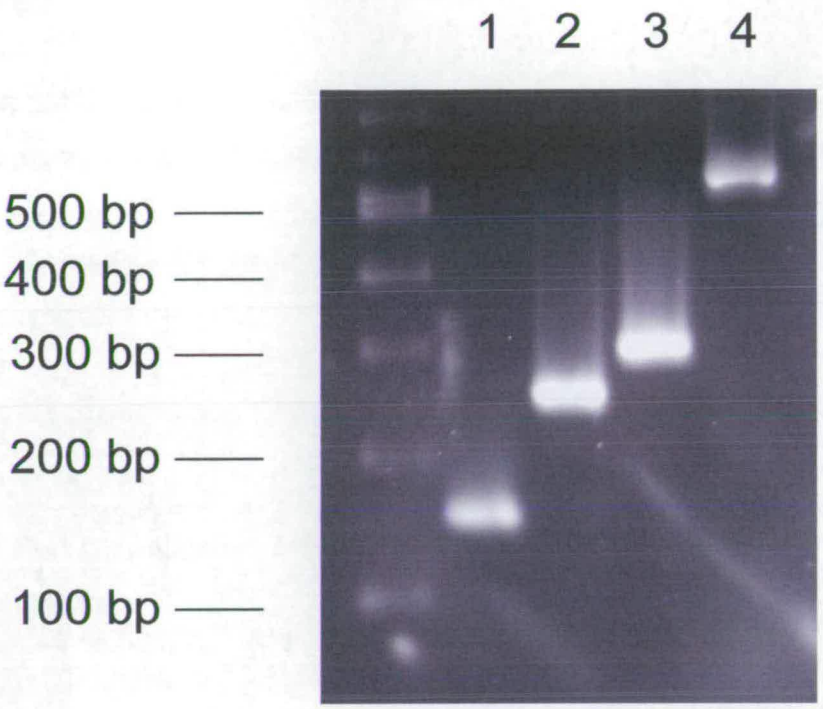


Figure 3.3 2.5 % (w/v) agarose gel showing the amplification of each *Eco*RI AFLP control after being processed using the AFLP protocol. Each control fragment was amplified from *C. jejuni* NCTC 11168 genomic DNA and then digested with *Eco*RI. Adaptors specific to the *Eco*RI restriction site were ligated to each fragment and primers specific to the adaptors used to amplify each fragment. Lane 1: Control fragment 1 (153 bp) Lane 2: Control fragment 2 (255 bp) Lane 3: Control fragment 3 (306 bp) Lane 4: Control fragment 4 (555 bp)

The efficiency of any PCR reaction is dependent on a number of factors: template quality, template amount, primer concentration, dNTP concentration, Mg^{2+} concentration, KCl concentration and enzyme amount. For most PCR reactions little or no optimisation is required. However, in more complex PCR protocols such as multiplex PCR, extensive optimisation of each variable may be required. It was decided to use troubleshooting techniques for multiplex PCR on the AFLP protocol

as the two processes are similar in nature.

Using reactions combining control fragments 2 and 4, the effect of each factor (see above), BSA addition (to stabilise enzyme) and decrease in reaction volume, was investigated. Reducing reaction volume, adding BSA, doubling dNTP or increasing the primer concentration, all caused the yield to increase (~ 2 fold) (see Figure 3.4). A 4 fold increase in the amount of enzyme added per reaction gave an increased yield of ~ 10 fold (see Figure 3.4). Changing both Mg^{2+} and KCl concentration caused the amplifications to fail. In all cases where an increase in yield was observed, control fragment 4 was amplified preferentially to control fragment 2.

The %GC value is known to affect the efficiency of long template PCR (see 3.3.1), to test the effect of %GC on AFLP reactions, both λ (%GC = 50 (Monocut DNA ladder, NEB)) and *W. succinogens* (%GC = 48.5) were processed with both *Eco*RI and *Hpa*II protocols. No effect due to %GC was apparent (see Figure 3.5).

The standard AFLP protocol does not include any steps for removing restriction fragments not of interest i.e. those above 500 bp in size. To investigate the effect of the ratio of total fragments to fragments of interest, enzymes which produced fragments with ends compatible with *Eco*RI and *Hpa*II were used. The two additional enzymes *Mfe*I (*Eco*RI compatible) and *BstB*I (*Hpa*II compatible) had ratios of 16:1 and 9:1 repectively c.f. *Eco*RI 10:1 and *Hpa*II 7:1. λ DNA (as above), *W. succinogens* and *C. jejuni* NCTC 11168 genomic DNA were processed with all four enzymes but no effect due to the ratio of total fragments to fragments of interest was apparent (see Figure 3.6).

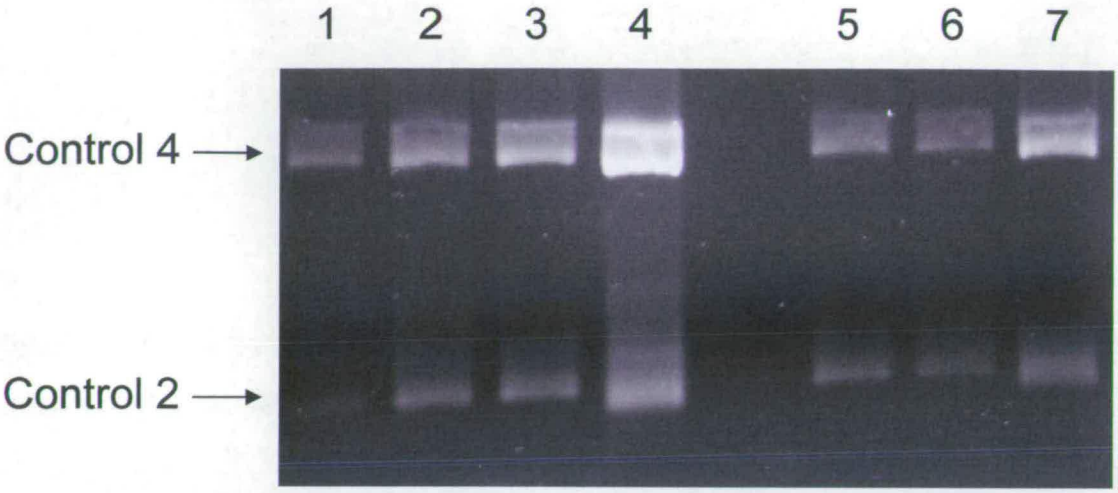


Figure 3.4 2.5 % (w/v) agarose gel showing the effects of changing reaction conditions on the amplification of *Eco*RI AFLP control fragments 2 and 4. Lane 1: Control reaction. The yield is approximately twice the control when the reaction volume is halved (2), BSA is added to a final concentration of 0.8 $\mu g \mu l^{-1}$ (3), doubling dNTP concentration (5) and increasing primer concentration (6 and 7). Increasing the amount of enzyme 4 fold in the reaction caused the yield to increase ~10 fold (4). In all cases control fragment 4 was amplified preferentially to control fragment 2.

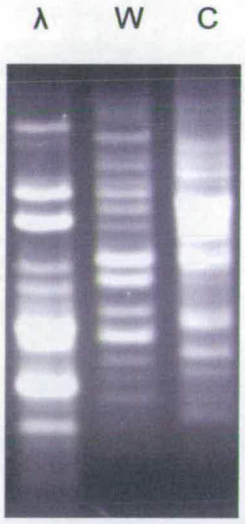
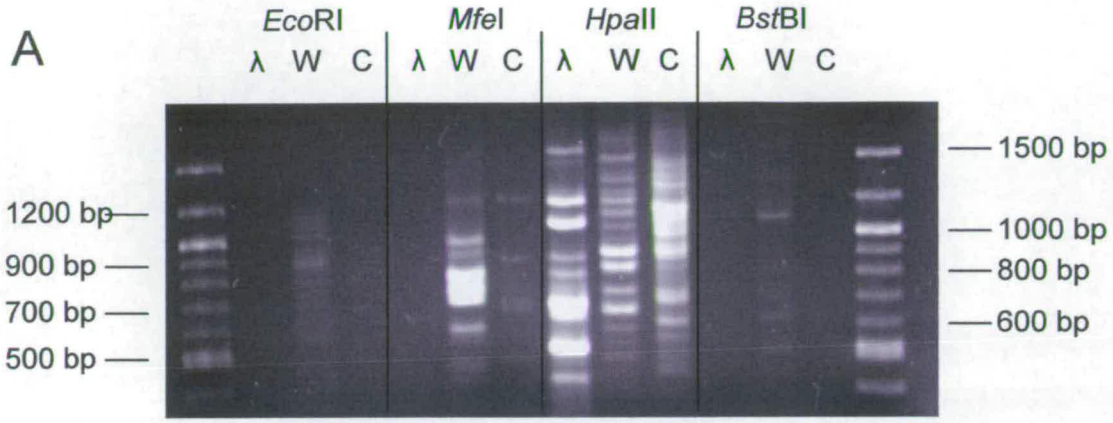


Figure 3.5 1 % (w/v) agarose gel showing that %GC does not affect the *Hpa*II AFLP profile for Lambda Mono-cut ladder (50 %) (NEB) (λ), *W. succinogens* DMSZ 1740 (48.5 %) (W) and *C. jejuni* NCTC 11168 (30.6 %) (C). Genomic DNA (400 ng) was digested with *Hpa*II and *Hpa*II restriction site specific adaptors ligated to the fragments. The fragments were then amplified with primer specific to the adaptor sequence.

A



B

Enzyme	Species	Ratio
*Eco*RI	λ	11:0
	W	11.9:1
	C	10:1
*Mfe*I	λ	14:1
	W	21:1
	C	16:1
*Hpa*II	λ	1.8:1
	W	5.5:1
	C	7:1
*Bst*BI	λ	13:0
	W	12.5:1
	C	9:1

Figure 3.6 1 % (w/v) agarose gel (A) showing the AFLP profiles for Lambda Mono-cut DNA (NEB) (λ), *W. succinogens* DMSZ 1740 (W) and *C. jejuni* NCTC 11168 (C). Genomic DNA (400 ng) from each species was digested in separate reactions with *Eco*RI, *Mfe*I, *Hpa*II and *Bst*BI. Adaptors specific to the *Eco*RI restriction site were ligated to *Eco*RI and *Mfe*I fragments. Adaptors specific to the *Hpa*II restriction site were ligated to *Hpa*II and *Bst*BI fragments. Adaptor specific primers were then used to amplify each fragment set. The ratio, total fragments:fragments in range 50 – 500 bp, was determined to see if this affected the success of the AFLP profile. The ratios are shown in B.

The original AFLP protocol uses two rounds of amplification before visualisation. The first, non-selective amplification to increase the total amount of template DNA in the range 50 – 500 bp and the second selective amplification to amplify a subset of fragments within the 50 – 500 bp range. The selective amplification uses a primer modified by the addition of between 1 and 3 extra nucleotides at the 3' end. Selective *Eco*RI primers (EcoRIPr+A and EcoRIPr+C)

were used to amplify *C. jejuni* NCTC 11168 *Eco*RI fragments after a round of non-selective amplification. In both cases no previously unobserved bands were obtained (data not shown).

3.2.3 AFLP repeatability is sensitive to a wide variety of factors both biological and technical

The experiments performed above show that the success of an individual AFLP reaction is:

- Independent of the substrate genomic DNA used
- Independent of the primers used
- Independent of the adaptors used

The variability of the AFLP reactions (even when performed in parallel) must therefore be introduced by processing differences between each individual sample. Any small differences are then magnified by the amplification step of the AFLP protocol. A known problem of porting PCR protocols between thermal cyclers is variation in the thermal profiles of different blocks, meaning a small amount of optimisation is often required to achieve equivalent results.

A key requisite of any typing system is portability and reproducibility between laboratories. The CAMPYNET project was an EU funded project to standardise typing protocols for *C. jejuni* and *C. coli*. While standardised protocols for *fla*-RFLP and PFGE were achieved (Harrington *et al.*, 2003), it proved impossible to standardise AFLP. It was concluded that differences in "separation and detection apparatus" prevented comparison of AFLP patterns produced by different laboratories and methods (http://campynet.vetinst.dk/news.htm).

3.3 The CPS locus is potentially a good target for RFLP analysis of *C. jejuni*

RFLP compares restriction pattern differences in single genes or loci between strains. Initially, RFLP was performed by restriction of whole genomic DNA, electrophoresis, Southern blotting and probing for a specific gene of interest. The advent of PCR led to a modification of the RFLP protocol where the target is amplified before restriction and the pattern visualised directly. The main advantage of PCR-RFLP is its speed when compared to RFLP.

A number of typing schemes utilising different restriction enzymes and primers against *flaA* and *flaB* in *C. jejuni* are currently in use (Harrington *et al.*, 2003). Other targets used include, 16S rRNA, 23S rRNA (Moreno *et al.*, 2002), *groEL* (Karenlampi *et al.*, 2004) and *hipO* (Slater *et al.*, 1997).

The discriminatory power of PCR-RFLP varies depending on the specific target, but is generally higher than Penner serotyping (60 serotypes) and lower than AFLP or MLST (658 sequence types). One means of increasing discriminatory power is to increase the number of target genes. Two locus based schemes have been proposed using the *wla* gene cluster (Misawa *et al.*, 2001) and the LOS locus (Nakari *et al.*, 2005). The use of the LOS locus was also an attempt to harmonise Penner serotyping with DNA based typing systems. Although a working scheme was produced, harmonisation was not achieved.

It has been shown that the serodeterminate of Penner serotyping is CPS (Karlyshev *et al.*, 2000). Described below is an attempt to create a PCR-RFLP typing scheme that uses the CPS locus as a target and harmonises Penner serotyping with RFLP.

The conserved structure of the CPS locus makes it a good choice for RFLP analysis. All of the sequenced loci conform to the Gram negative group II capsule locus structure (see Figure 3.7 and Figure 3.8); with transport genes flanking a variable region of sugar transferases. To investigate the degree of conservation within the *kps* genes (responsible for transport), primers based upon the *C. jejuni* NCTC 11168 sequences, were designed for *kpsM*, *E*, *D*, *F* and *S*. Each gene was amplified and sequenced from isolates with the following Penner serotypes (HS): 18, 23, 50 and 57. In all of the genes the sequenced sections (~ 66% of the total gene length) had between 90 % and 100 % identity (alignments for *kpsD* and *kpsS* are shown in appendix B). Primers for the conserved sequences in *kpsD* and *kpsS* were designed for amplification of the CPS locus. Because of the extreme length of the locus it was necessary to use long template (LT) PCR (see 3.3.1).

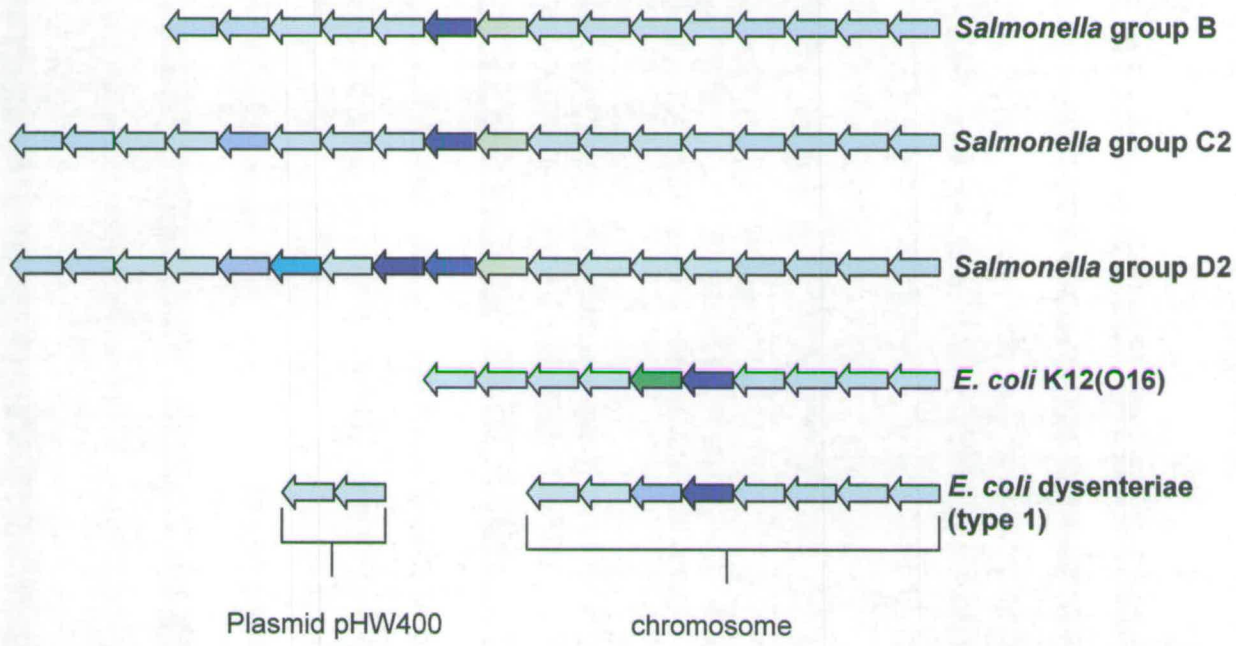


Figure 3.7 Schematic of the CPS locus in two serotype groups of *Salmonella* and two strains of *E. coli*. Each colour represents a separate branch of the CPS biosynthetic pathway. With the exception of *E. coli* dysenteriae (type1) all loci are chromosomally encoded.

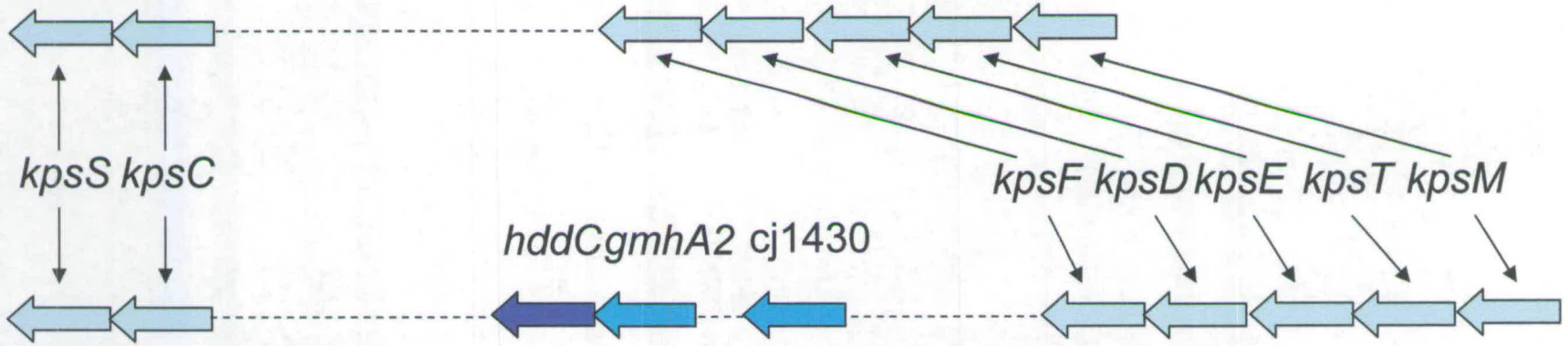


Figure 3.8 Schematic of the known CPS loci types in *C. jejuni*. Type I loci (top) follow the Gram negative Group II CPS locus structure with the *kps* genes flanking a variable region of sugar transferases. Type II loci (bottom) follow the same structure, but with have three conserved genes within the conserved region, *hddC*, *gmhA2*, and *cj1430*.

3.3.1 Long template PCR- using polymerase mixtures to amplify large (>10 kb) sequences of DNA

Standard PCR techniques utilising Taq polymerase (or other non-proofreading polymerases) have maximum amplicon lengths of ~ 5 kb. This limitation is caused by the incorporation of incorrect bases during amplification. When such a mismatch occurs, the polymerase stalls and is unable to continue replication. Proofreading enzymes, such as Pfu, do not stall on the incorporation of mismatched bases as they possess exonuclease activity and can remove the incorrect base. However, the exonuclease activity lowers the processivity of the enzyme making amplification of long sequences time consuming. In both cases the thermal stability of the polymerase is important in determining maximum amplicon length (Huang *et al.*, 1992).

One method of increasing the maximum amplicon length is to use a mixture of non-proofreading and proofreading enzyme in the amplification mixture. In such a mixture the non-proofreading enzyme provides the processivity to decrease amplification time and the proofreading enzyme to allow extension of incomplete product when mismatches occur (see Figure 3.9). Enzyme stability is increased by changes in the reaction buffer (see Table 3.3 for comparison) and using a lower extension temperature to lower thermal degradation. This technique was patented by Roche and two kits are available for the amplification of sequences up to ~28 kb in size. The maximum amplicon size is dependent on the target sequence; the largest sequences amplified from λ genomic DNA are 40 kb compared to 25 kb from human genomic DNA. The exact reason for this is unknown but has been theorised to be

caused by the %GC and base distribution of the target sequence.

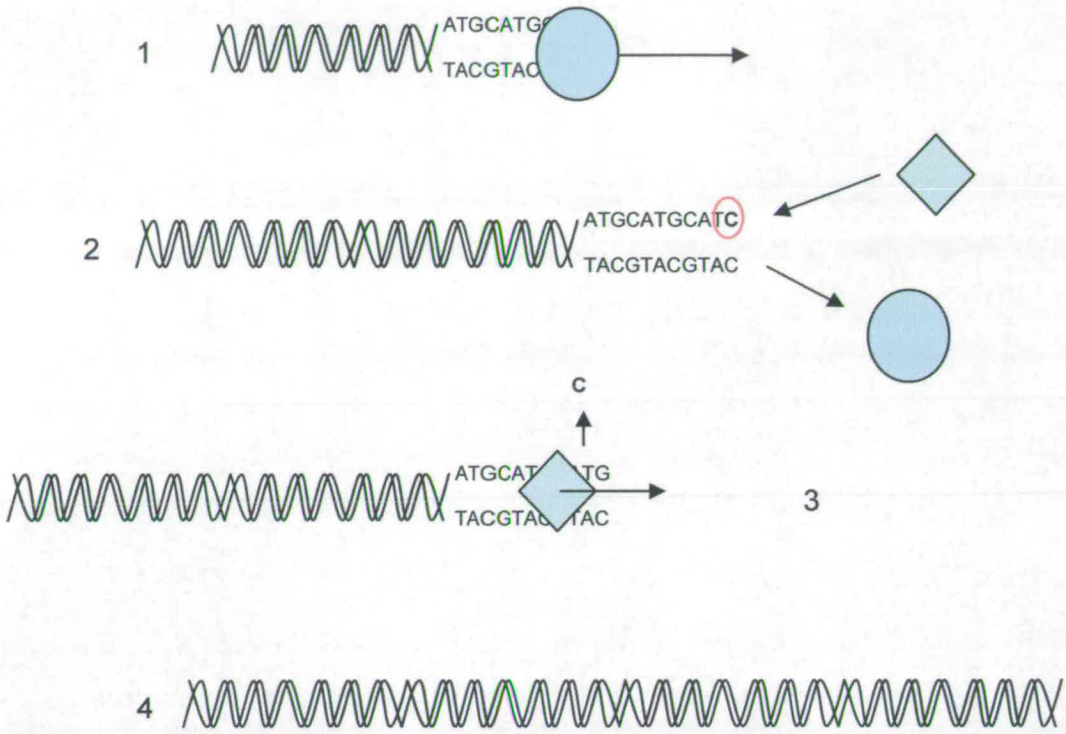


Figure 3.9 Schematic of the processes occurring during the amplification step of a Long template PCR reaction. 1. Taq polymerase (circle) extends the primer until a mismatch occurs (red circle). 2. The Taq polymerase stalls and unbinds from the DNA strands. 3. Pfu polymerase then binds and removes the mismatch and continues extension. 4. Extension is then completed by either polymerase.

3.3.2 Purification of PCR products > 10 kb

Amplification of long sequences presents problems during purification after PCR. It is desirable to purify the products before restriction digest because of the high salt content in the PCR buffer, which may inhibit restriction. Most commonly, removal of salt us achieved by EtOH/isopropanol precipitation or using silica and NaI (e.g. QIAquick PCR clean up kit (Qiagen)). Both methods have disadvantages; EtOH precipitation is not always efficient at removing salt from the DNA and silica will irreversibly bind DNA above 10 kb in size. Both methods were compared using

the Mono-cut ladder (NEB) in LT-PCR reaction buffer (data not shown). As expected no DNA was recovered when silica was used. Two protocols for EtOH precipitation were compared, 30 min at -70 °C and overnight at -20 °C. Purification at -20 °C was ~50 % efficient and no salt was co-precipitated. Purification at -70 °C was ~90 % efficient but there was significant co-precipitation of salt.

In 2005, Invitrogen introduced a new range of DNA purification products that uses a new technology called Chargeswitch™. The purification system is based on using changes in pH to influence the charge of the Chargeswitch™ material. At low pH, Chargeswitch™ is positively charged and binds nucleic acids (see Figure 3.10). A wash step at neutral pH removes any contaminants or loosely bound material. The nucleic acid is then eluted by raising the pH with Tris.Cl pH 8.0 causing the Chargeswitch™ to lose charge. In practice, paramagnetic beads are coated with Chargeswitch™ to facilitate separation of the DNA at each step. When compared to EtOH precipitation, efficiency was >90 % and there was no co-purification of salt.

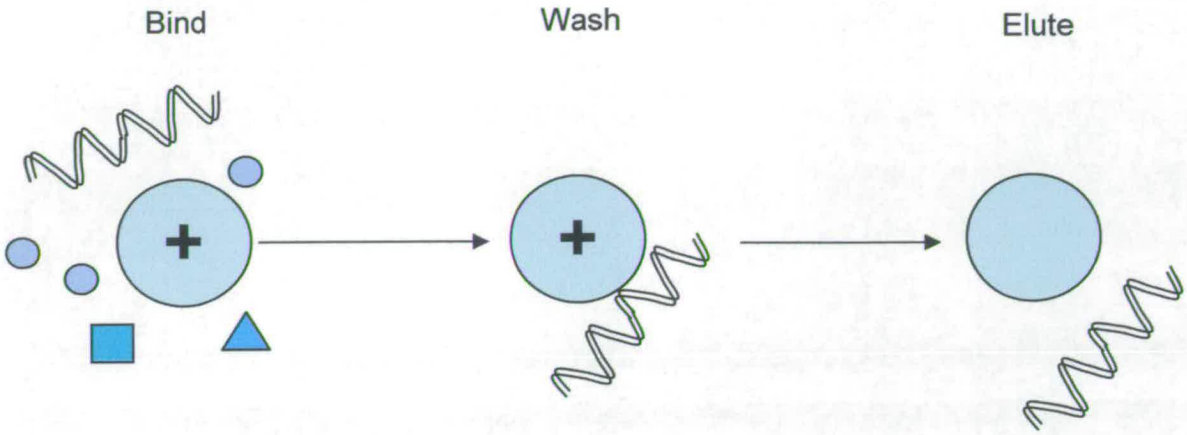


Figure 3.10 Schematic of the Chargeswitch™ purification system. DNA is electrostatically bound by mixing with Chargeswitch™ coated paramagnetic beads in an acidic solution. Loosly bound material and contaminants are then washed off with a pH neutral solution. DNA is eluted by adding and mildly alkaline solution, causing the Chargeswitch™ to lose charge and release the DNA.

3.3.3 Sequence comparison of the known CPS loci to determine likely targets for primer binding

An attempt to amplify the entire CPS locus (~40 kb) from *C. jejuni* NCTC 11168 was made but failed. It was therefore necessary to design primers that binded within the variable region of the CPS locus (see Figure 3.8). The CPS loci of six Penner serotypes, HS:1, HS:19, HS:41, HS:23/36 (HS:23 and HS:36 have identical loci) and RM1221 (serotype unknown) have been sequenced (Fouts *et al.*, 2005, Karylshev *et al.*, 2005 and Parkhill *et al.*, 2000). Comparison of the sequenced loci showed that two subgroups of locus structure exist. The first group is identical to the Gram negative group II capsule locus structure. The second group consists of loci with a small island of partially conserved genes within the variable region of the locus and the locus size is ~2 fold that of the first group. The conserved genes within this island are *hddC*, *gmhA2* and *cj1430*. Primers for *cj1430* and *gmhA2/hddC* (the primer gmhA2Rev binds internally to *hddC*) were designed and used to detect their

presence in 10 *C. jejuni* strains. *GmhA2* was detected in 8 strains and *cj1430* in 5 strains (see Figure 3.11). To amplify *cj1430* in the strain set, it was necessary to lower the annealing temperature used by 5 °C; suggesting that the sequence similarity at the primer binding sites is low. It was decided therefore, to use *gmhA2* as an internal priming site.

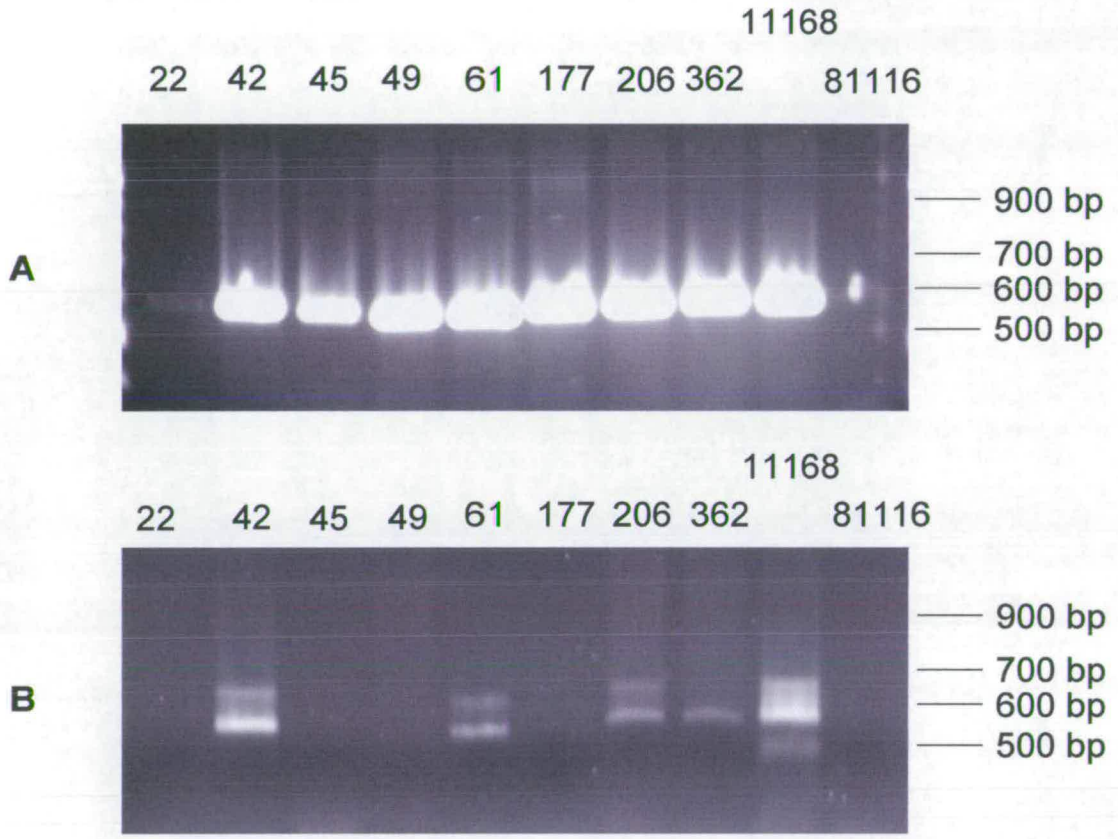


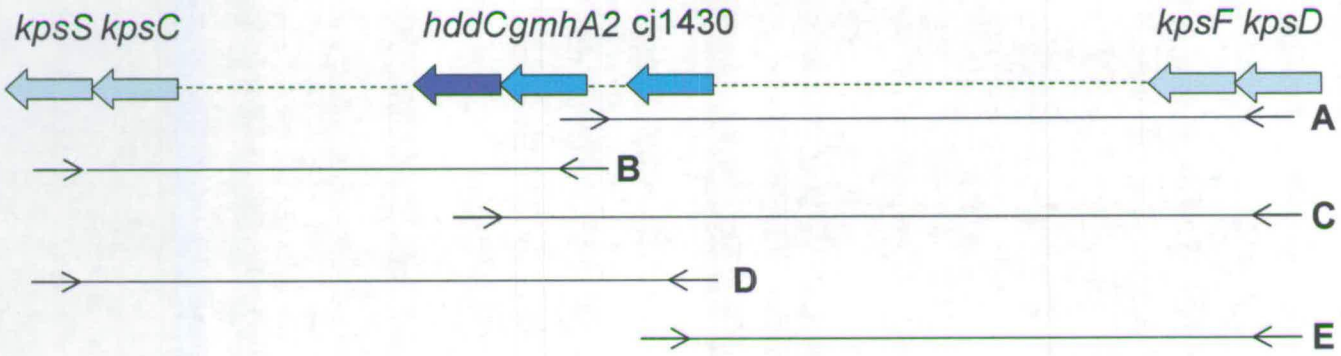
Figure 3.11 1 % (w/v) agarose gel showing the presence of *gmhA2* and *cj1430* in set of 10 *C. jejuni* strains. *GmhA2* (A) was detected in 8 strains and *cj1430* (B) in 5 strains.

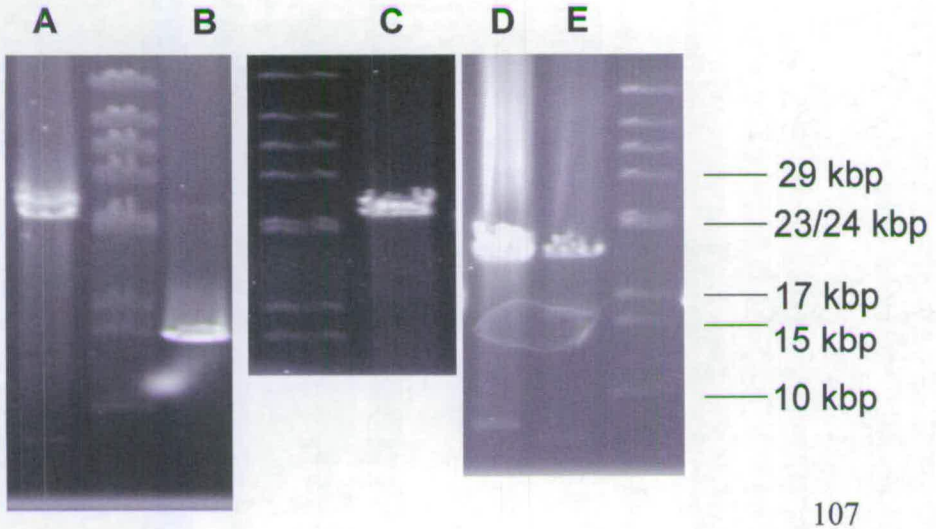
3.3.4 Amplification of the *C. jejuni* NCTC 11168 locus using multiple PCR reactions

As previously stated (see 3.3.1) the maximum possible amplicon length is sequence dependent. Using the primers designed against the internal conserved

genes (see 3.3.3), LT-PCR reactions with differing combinations of primers were setup to determine the maximum possible amplicon length. Each of the reactions was successful with amplicons up to and including 25.9 kb amplified (see Figure 3.12). During the course of these experiments the LT-PCR kit was modified by the manufacturer, with a change in the amount of proofreading enzyme and working Mg^{2+} concentration. This change caused the amplification to cease working until the extension temperature was lowered to 64 °C to counteract the increased exonuclease activity. This caused a decrease in the maximum amplicon length and a decrease in amplification yield. Subsequently, amplification of products longer than 14 kb was not achieved.

Figure 3.12 (Following page) Schematic showing the approximate binding sites of the primers used to amplify sections of the *C. jejuni* CPS locus. Each product is shown amplified after running on a 1 % (w/v) PFGE gel under the following conditions: 6 Vcm^{-1}, 0.5 – 1.5 s linear ramped switch time for 22 h, the λ Mono-cut DNA ladder included on each gel for reference. A: *gmhA2-kpsD* 25.3 kb B: *kpsS-gmhA2* 14.0 kb C: *hddC-kpsD* 25.9 kb D: *kpsS-cj1430* 19.4 kb E: *cj1430-kpsD* 20.5 kb.


kpsS kpsC
hddCgmhA2 cj1430
kpsF kpsD
A
B
C
D
E


A
B
C
D
E
29 kbp
23/24 kbp
17 kbp
15 kbp
10 kbp

3.3.5 *In silico* analysis of the sequenced CPS loci to determine appropriate restriction enzymes for experimentation

The expected amplification product from all sequenced CPS loci, using the primer pair kpsD-SRev2 and gmhA2Fwd, was generated using VectorNTI (version 10, Invitrogen). The LT-PCR products were then digested *in silico* using NEBcutter (NEB), limiting output to enzymes available commercially and with fewer than 15 restriction sites. The size distribution of the restriction fragments for each enzyme was then analysed and enzymes that produced fragments spaced across the size range 1 – 10 kb selected. The restriction profiles for each strain were then compared and enzymes that could not distinguish each strain were discarded. The enzymes *Bcl*I, *Bgl*II, *Eco*RI and *Nde*I were selected for initial experimentation. The generated restriction profile for each strain and enzyme is shown in Figure 3.13.

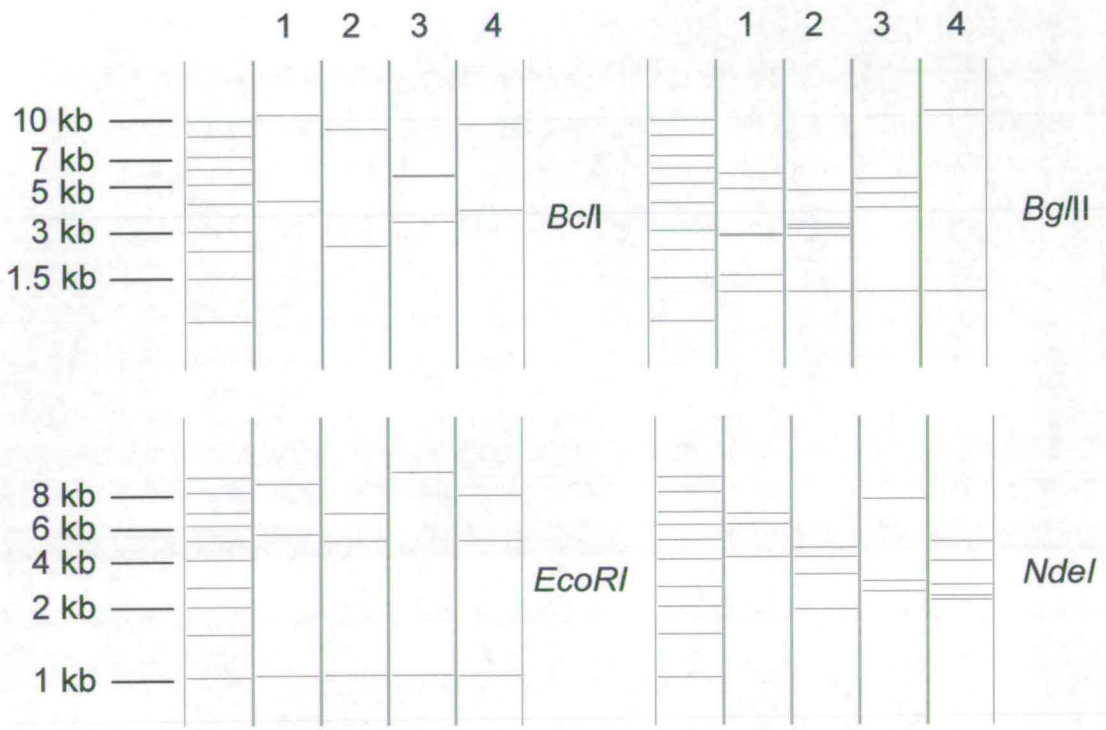


Figure 3.13 Schematics showing the theoretical restriction patterns, as would be seen on a 1 % (w/v) agarose gel, for digests of the LT-PCR product KpsS-gmhA2 from 4 *C. jejuni* strains with the stated restriction enzymes. 1: NCTC 11168 (HS2) 2: HS23/36 3: HS41 4: RM1221 (HS unknown)

3.3.6 CPS-RFLP of *C. jejuni*

Digests were initially performed on the LT-PCR product produced from *C. jejuni* NCTC 11168, to ensure the accuracy of the *in silico* prediction allow optimisation of the restriction reaction. All the expected fragments were observed and no unexpected bands were observed for each enzyme (see Figure 3.14).

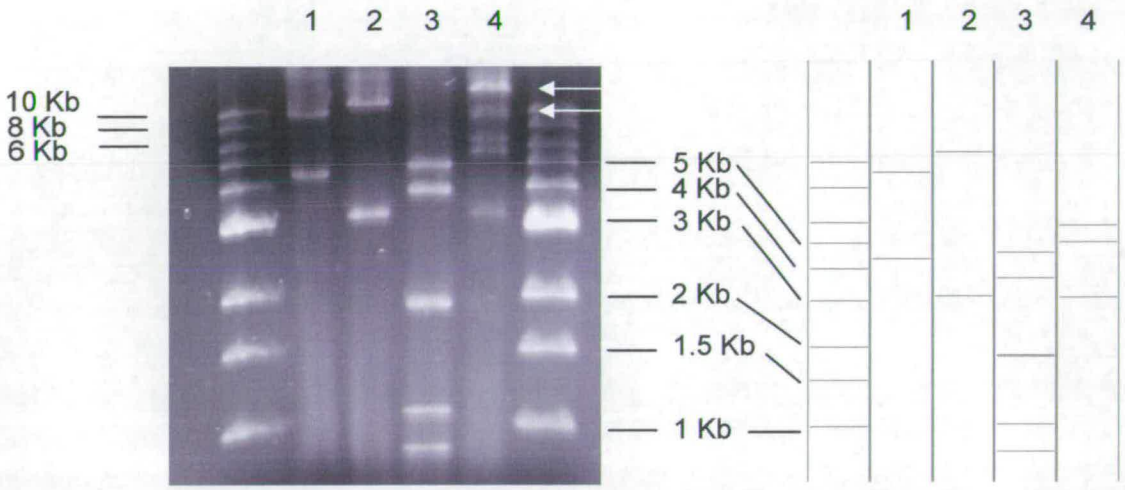


Figure 3.14 1 % (w/v) agarose gel showing the restriction profiles for the LT-PCR product KpsS-gmhA2 from *C. jejuni* NCTC 11168 and a schematic showing the theoretical restriction profile. 1: *Eco*RI 2: *Bcl*I 3: *Bgl*II 4: *Nde*I. Products of incomplete digestion are shown by white arrows.

3.3.7 LT-PCR is unsuitable for use as a typing system

Amplifications of the CPS loci in the reference strain set failed. This was unexpected as: 1. the primers had been used to amplify individual genes previously (see 3.3.3) and 2. the primers were designed based upon sequences obtained from the reference strain. Of the strains tested, only ST362's entire CPS locus is sequenced (HS:41). When compared to the locus from NCTC 11168 there is little (apart from gene complement) to distinguish it. In particular the known problems of %GC should be the same for both strains; as the %GC of each locus is almost identical (27 % and 26 %).

There are significant differences between the buffers used for LT-PCR and normal PCR (see Table 3.3). The substitution of tricine for tris stabilises the buffer pH more effectively at high temperatures, in turn increasing DNA stability. The addition of glycerol serves to increase the thermal stability of the polymerases as well as lowering the effective melting temperatures of the DNA complexes. Of most importance is the addition of DMSO, the increase in Mg^{2+} and the substitution of KCl for KOAc. DMSO lowers the effective melting temperature of the DNA strands (~5 °C for each 10 % v/v DMSO added) but lowers the thermal stability of the polymerases. The increase in Mg^{2+} is to balance the required increase in dNTP concentration and maximise polymerase efficiency. The substitution of KCl for KOAc, lowers the ionic strength of the buffer. In high %GC sequences this is thought to help destabilise any 2° structures that may form (Cheng, 1996).

Normal PCR	LT-PCR
10 mM tris (pH 8.3)	20 mM tricine (pH 8.7)
None	10 % (w/v) glycerol
None	2 % (v/v) DMSO
1.5 mM $MgCl_2$	1.1 mM $Mg(OAc)_2$
50 mM KCl	80 mM KOAc

Table 3.3 Buffer compositions use in normal PCR with Taq DNA polymerase and the LT-PCR Expand 20 kb plus kit from Roche. Detailed information regarding the composition of the buffer in LT-PCR is unknown as it is proprietry. The buffer composition presented in the table was taken from US patent 5512462 which covers the LT-PCR process.

Experimentally the most noticeable effect was the lowering of melting temperatures. To amplify any part of the *C. jejuni* NCTC 11168 CPS locus it was necessary to lower the annealing temperature of the PCR reaction by 5 °C to 50 °C. It is possible that the buffer, designed to achieve maximum efficiency with high %GC templates, is destabilising the target DNA and primers too much. Thereby preventing annealing of primers to the template or partially completed amplicons to the template and consequently lowering the efficiency of amplification.

At this time and with the Expand™ 20kb plus LT-PCR kit, CPS-RFLP as described above is impossible. The theoretical comparisons of the CPS loci show them to be good targets for RFLP possessing both conserved priming sites (see 3.3.3) and enough variable material for identification (see 3.3.5). Investigation of other LT systems is therefore needed before CPS-RFLP is dismissed.

3.4 Conclusions

- Small variations in sample processing can cause large variations in the AFLP profile observed.
- The emergent technology of LT-PCR is currently too unreliable for typing use.
- Sequence comparison of the known CPS loci, shows the potential for development of a DNA-DNA based typing scheme with the locus as a target.

Results and Discussion – Immunomagnetic separation

4.1 Immunomagnetic separation

Diagnostic laboratories can be presented with a wide variety of samples for investigation, e.g. faeces, food, swabs. Before the samples can be investigated, they must be processed so that the organisms of interest can be detected. The degree and type of processing is dependent on both the organism of interest and the final detection system. Traditionally, enrichment media was used to favour the growth of particular groups of organisms before separation into pure cultures and biochemical testing. A problem with such techniques is that the number of bacteria within an enrichment culture does not directly relate to the original number of bacteria within the sample. The traditional enrichment methods can take several days before testing can begin which is not ideal.

As technology has progressed the use of PCR based detection techniques has become more common. In many cases detection of a target bacterium directly from a sample is possible. However, many of the samples will contain both compounds that interfere with the PCR reaction and other bacteria that may cross react. Immunomagnetic separation (IMS) provides a means both of enriching a target bacterium and removing it from contaminants.

IMS works by mixing antibody coated paramagnetic beads with a sample and separation from the sample with a magnet (see Figure 4.1). Paramagnetic beads are used because they are only magnetic when a magnetic field is present, thus ensuring even mixing of the beads with the sample. The antibody which is attached to the beads is variable. Either an antibody recognising the target is directly conjugated to the beads or an anti-species antibody is conjugated to the beads; allowing the system

to be used against one or many targets. Other advantages of IMS over traditional enrichment techniques are: quick, technically simple, number of bacteria recovered relates directly to the number of bacteria within the original sample and recovered bacteria remain viable. Table 4.1 lists some of the bacteria that have been successfully recovered using IMS.

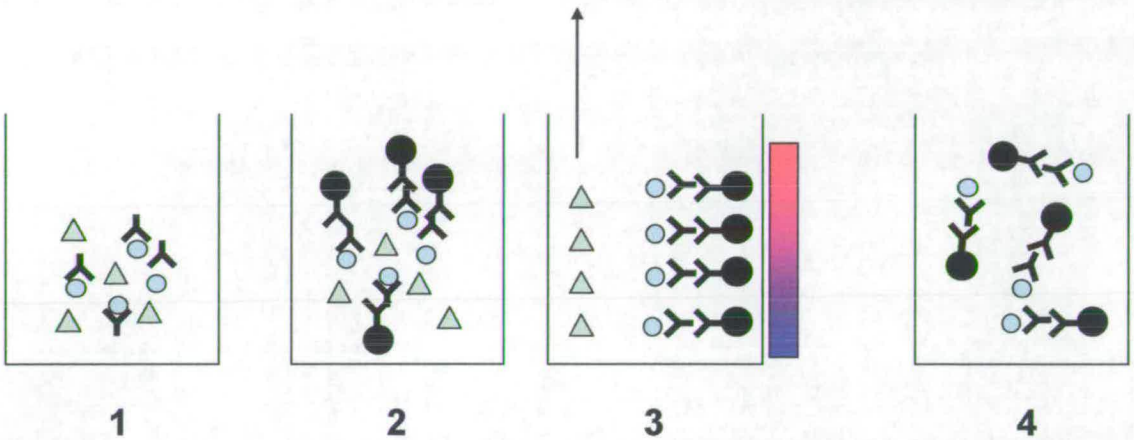


Figure 4.1 Schematic of the immunomagnetic separation procedure. 1: Antibody against the target bacterium is incubated with the contaminated sample. 2: Anti-species coated paramagnetic beads are incubated with the contaminated sample to capture the bound target. 3: A magnetic field is applied and the contaminants removed. 4: Bound target is then washed and resuspended after removal of the magnetic field. Target can then be eluted from the beads if necessary.

Target	Sample	Detection method	Reference
E. coli O157	Faeces	Conventional culture	Cubbon *et al.*, 1996
	Raw milk/Ice cream	PCR	Gooding and Choudary, 1997
Salmonella spp.	Pork sausages/chicken meat/beef	Conventional culture	Ripabelli *et al.*, 1997 Ripabelli *et al.*, 1999
Listeria spp.	Various	Conventional culture	Avoyne *et al.*, 1997
Salmonella and *Listeria* spp.	Various	Conventional culture	Karpiskova and Holasova, 1999
Campylobacter spp	Various chicken samples	Microtitre hybridisation	Lamoureux *et al.*, 1997
	Chicken and milk	PCR	Docherty *et al.*, 1996

Table 4.1 Examples of bacteria that have been isolated using immunomagnetic separation, and the method used in their detection (Adapted from Clark, 2000)

4.2 Using the cell membrane protein PEB3 as a target for IMS

4.2.1 Design of primers for the detection and cloning of *peb3*

Primers for the amplification of *peb3* were designed using the published sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000). The primers included an *Nde*I or *Bam*HI recognition site to allow for use in directional cloning. The *peb3* sequence includes an N-terminal signal sequence with the predicted cleavage point between amino acid residue 20 and 21 (see Figure 4.2).

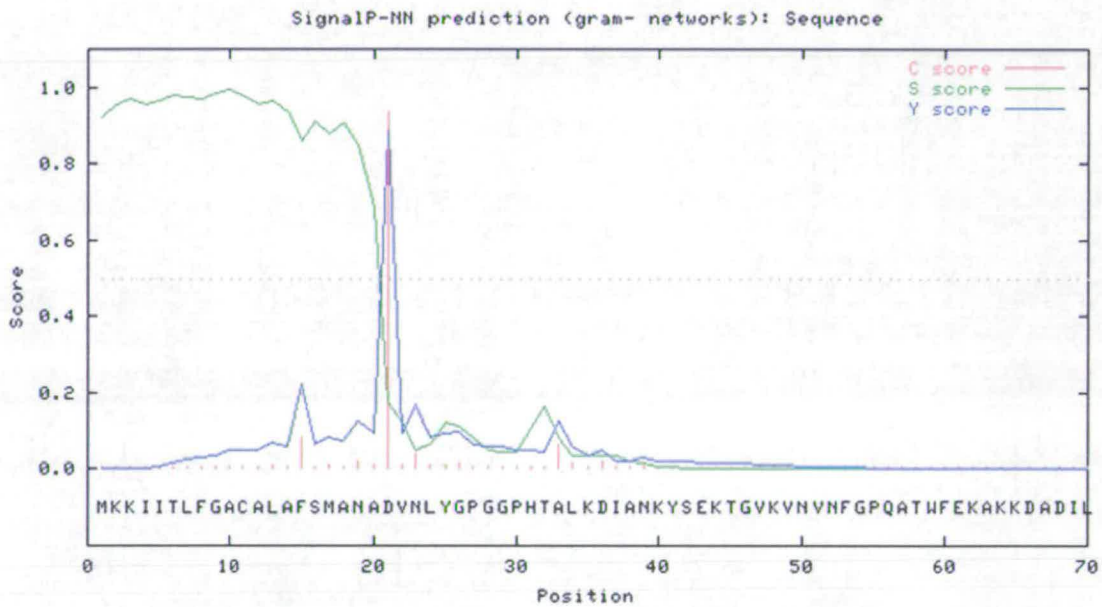


Figure 4.2 Graph of the signal sequence (S), Cleavage site (C) and a derivative of both scores (Y) for *C. jejuni* NCTC 11168 PEB3. A cleavage site is predicted where the slope of Y is steep and C is significant (> 0.5). Cleavage site predicted using Signal-P v 3.0 (Bendsten, Neilsen *et al.*, 2004)

As the signal sequence would be cleaved in the mature protein the primer PEB3-FWD was redesigned so the first 20 amino acids would not be included in the cloned gene.

4.2.2 Detection and comparison of *peb3* in *C. jejuni*

PCR was used to amplify *peb3* in the *C. jejuni* reference strains. The results are shown in Figure 4.3.

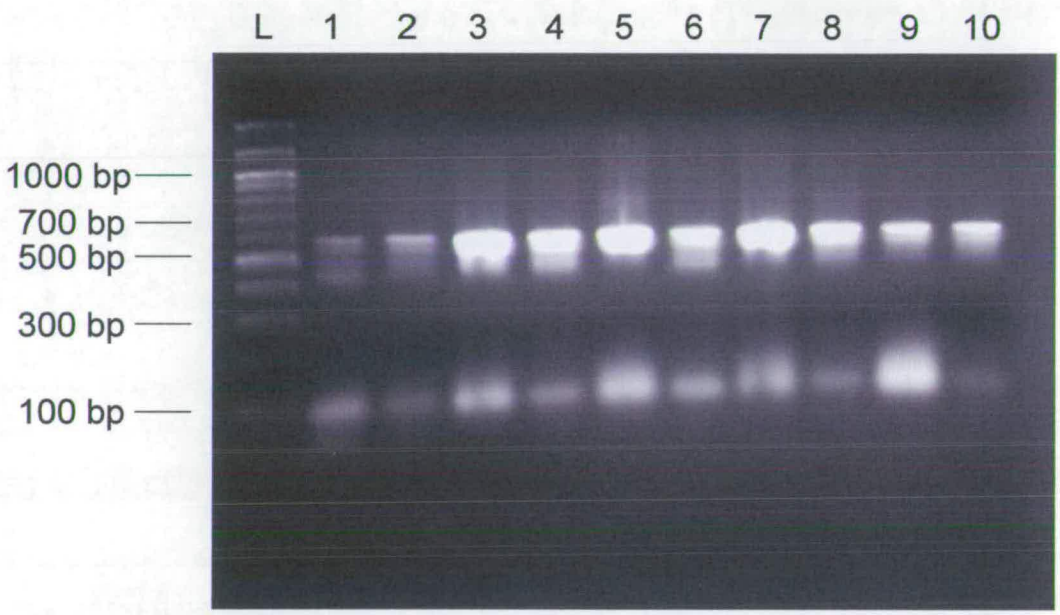


Figure 4.3 1 % (w/v) agarose gel showing the results of a PCR reaction using the primers PEB3-FWD and PEB3-REV on *C. jejuni* strains 1: NCTC 11168 2: NCTC 81116 3: ST22 4: ST42 5: ST45 6: ST49 7: ST61 8: ST177 9: ST206 10: ST 362

The results show that *peb3* is present in all of the reference *C. jejuni* strains. To determine the extent to which *peb3* varies between the strains, sequencing of *peb3* in the strains NCTC 81116, ST22 and ST42 was carried out. The sequences were aligned to the sequence strain *C. jejuni* NCTC 11168 *peb3* and no significant differences were found.

4.2.3 Directional cloning of *peb3*

The *peb3* gene was amplified as stated in 2.8.1. After amplification the PCR product was gel purified and ligated into pGEM-T Easy™ (Promega) as in 2.8.3. The ligation reaction was transformed into *E. coli* TG1 and screened using *lacZ* blue/white screening. Five of the positive clones were selected for further analysis (pGEM-TPEB31 to 35). The plasmid from each clone was purified and was checked for the presence of the insert using the primers PEB3-FWD and PEB3-REV. All of the clones tested positive for the insert in this way (data not shown). Each clone was then sequenced to check for base substitutions as Taq DNA polymerase was used in the initial construction of the insert (see Figure 4.4 for alignments).

The sequencing results from pGEM-TPEB31 showed several differences between the clone and the expected sequence. However, all of these mismatches are at the very beginning of each sequencing reaction and the corresponding bases on the complementary strand are identical to the expected sequence. Analysis of the peaks produced in the sequencing run shows that these mismatches are mostly caused by poor resolution of peaks at the beginning of each sequence (data not shown). pGEM-TPEB31 was selected as the source of the insert for creation of an expression clone. Sequential digestion of the purified plasmid with the restriction enzymes *Nde*I and *Bam*HI was used to release the insert. The expression vector pET-16b (Novagen) was prepared by sequential digestion with *Nde*I and *Bam*HI and dephosphorylation with calf intestinal phophatase to prevent recircularization. This vector was chosen because it enabled the addition of a His_{10} tag. The His_{10} tag was chosen as it does not affect the natural properties of the expressed protein significantly and allows easy purification. The gel-purified insert was ligated into the dephosphorylated pET-16b

vector as described in 2.8.7. Five clones (pSAN31-35) were screened for the presence of the insert by excision of the insert using *Nde*I and *Bam*HI (data not shown) and the results were as predicted. The clone pSAN31 was selected for use and renamed pSAN3 (Figure 4.5).

```
                1                                                   36
                |                                                   |
PCR product     CAT ATG ACG GCC TTA AAA GAT ATA GCA AAC AAA TAT
pGEM-TPEB31-FWD --- --- --- --- --- --- --- --- --- --- AAA TAT
pGEM-TPEB31-REV CAT ATG ACG GCC TTA AAA GAT ATA GCA AAC AAA TAT

37                                                                  85
|                                                                   |
AGC GAA AAA ACA GGC GTT AAA GTA AAT GTA AAT TTT GGC CCT CAA GCG
AGC GGA AAA ACA GGC GTT AAC CTA AAT GTA AAT TTT GGC CCT CAA GCG
AGC GAA AAA ACA GGC GTT AAA GTA AAT GTA AAT TTT GGC CCT CAA GCG

86                                                                  134
|                                                                   |
ACT TGG TTT GAA AAG GCT AAA AAA GAT GCA GAT ATT TTA TTT GGC GCT
ACT TGG TTT GAA AAG GCT AAA AAA GAT GCA GAT ATT TTA TTT GGC GCT
ACT TGG TTT GAA AAG GCT AAA AAA GAT GCA GAT ATT TTA TTT GGC GCT

135                                                                 183
|                                                                   |
TCA GAT CAA TCG GCT TTA GCT ATA GCG AGT GAT TTT GGA AAA GAT TTT
TCA GAT CAA TCG GCT TTA GCT ATA GCG AGT GAT TTT GGA AAA GAT TTT
TCA GAT CAA TCG GCT TTA GCT ATA GCG AGT GAT TTT GGA AAA GAT TTT

184                                                                 232
|                                                                   |
AAT GTG AGT AAA ATC AAG CCT TTA TAT TTT AGA GAA GCC ATC ATA CTT
AAT GTG AGT AAA ATC AAG CCT TTA TAT TTT AGA GAA GCC ATC ATA CTT
AAT GTG AGT AAA ATC AAG CCT TTA TAT TTT AGA GAA GCC ATC ATA CTT

233                                                                 281
|                                                                   |
ACT CAA AAA GGC AAT CCT TTA AAA ATC AAA GGT TTA AAA GAT TTG GCT
ACT CAA AAA GGC AAT CCT TTA AAA ATC AAA GGT TTA AAA GAT TTG GCT
ACT CAA AAA GGC AAT CCT TTA AAA ATC AAA GGT TTA AAA GAT TTG GCT

282                                                                 330
|                                                                   |
AAT AAA AAA GTA AGA ATC GTT GTG CCT GAA GGT GCT GGA AAG AGC AAT
AAT AAA AAA GTA AGA ATC GTT GTG CCT GAA GGT GCT GGA AAG AGC AAT
AAT AAA AAA GTA AGA ATC GTT GTG CCT GAA GGT GCT GGA AAG AGC AAT
```

Figure 4.4 Sequence alignment of pGEM-TPEB31 with the expected PEB3 clone sequence. Mismatches are highlighted in blue.

```
331                                                                 379
|                                                                   |
ACT TCT GGA ACT GGA GTT TGG GAA GAT ATG ATA GGT AGA ACT CAA GAT
ACT TCT GGA ACT GGA GTT TGG GAA GAT ATG ATA GGT AGA ACT CAA GAT
ACT TCT GGA ACT GGA GTT TGG GAA GAT ATG ATA GGT AGA ACT CAA GAT

 380                                                                428
|                                                                   |
ATA AAA ACC ATA CAA AAT TTT AGA AAC AAT ATC GTG GCC TTT GTT CCA
ATA AAA ACC ATA CAA AAT TTT AGA AAC AAT ATC GTG GCC TTT GTT CCA
ATA AAA ACC ATA CAA AAT TTT AGA AAC AAT ATC GTG GCC TTT GTT CCA


429                                                                 477
|                                                                   |
AAT AGT GGA AGT GCA AGA AAG CTT TTC GCA CAA GAT CAA GCC GAT GCT
AAT AGT GGA AGT GCA AGA AAG CTT TTC GCA CAA GAT CAA GCC GAT GCT
AAT AGT GGA AGT GCA AGA AAG CTT TTC GCA CAA GAT CAA GCC GAT GCT

478                                                                 526
|                                                                   |
TGG ATC ACT TGG ATT GAC TGG TCA AAA AGC AAT CCT GAC ATA GGA ACT
TGG ATC ACT TGG ATT GAC TGG TCA AAA AGC AAT CCT GAC ATA GGA ACT
TGG ATC ACT TGG ATT GAC TGG TCA AAA AGC AAT CCT GAC ATA GGA ACT

527                                                                 575
|                                                                   |
GCC GTA GCT ATA GAA AAA GAT TTG GTT GTT TAT AGA ACT TTT AAT GTG
GCC GTA GCT ATA GAA AAA GAT TTG GTT GTT TAT AGA ACT TTT AAT GTG
GCC GTA GCT ATA GAA AAA GAT TTG GTT GTT TAT AGA NCT TTT AAT GGA

576                                                           620
|                                                             |
ATA GCT AAA GAA GGT GCG AGC AAA GAA ACA CAA GAT TTG GAT CC
ATA GCT AAA GAA GGT GCG AGC AAA GAA ACA CAA GAT TTG GAT CC
TAG CAA AGA AGG G-- --- --- --- --- --- --- --- --- --- --
```

Figure 5.3 cont. Sequence alignment of pGEM-TPEB31 with the expected PEB3 clone sequence. Mismatches are highlighted in blue.

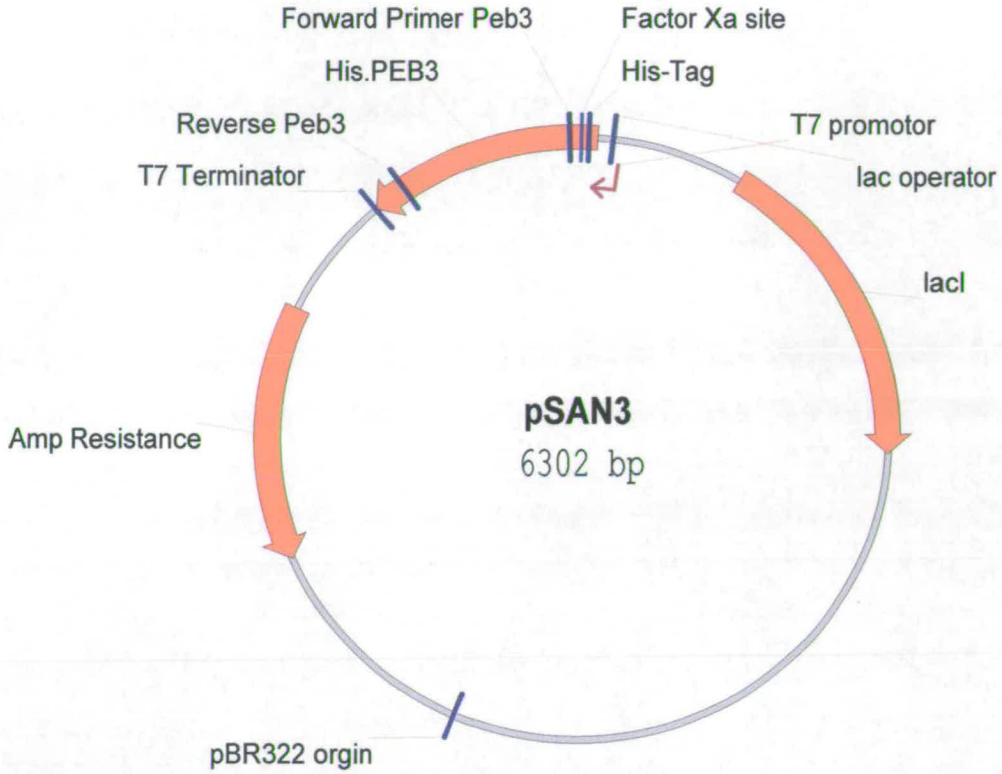


Figure 4.5 Map of pSAN3 showing important features

4.2.4 Selection of an appropriate *E. coli* strain for the expression of His.PEB3

pSAN3 was transformed into *E. coli* BL21 DE3 and induced as described in 2.9. While all the transformants tested did express PEB3 as indicated by SDS-PAGE, the level of expression was low and inconsistent (data not shown). This may have been due to either the expressed proteins insolubility (CV-CV' = 1.36 , (Wilkinson and Harrison, 1991)) or the difference in codon bias between *E. coli* (Henaut *et al.*, 1996) and *C. jejuni* (Gray and Konkel, 1999).

Apparent solubility of a protein is affected by the folding characteristics, net charge and hydrophilicity of the protein. When expressed natively (i.e. at normal levels and the native organism) these factors ensure maximum solubility. When a protein is cloned and over expressed the non-optimal conditions can lead to incorrect

folding (see Figure 4.6) and inclusion body formation. By manipulation of factors controlling the expression (speed and level) it is possible to minimize inclusion body formation. To test whether solubility of the His.PEB3 was limiting expression, pSAN3 was transformed into another *E. coli* strain, Tuner™ DE3 pLysS (Novagen) and the expression profiles between the two strains compared.

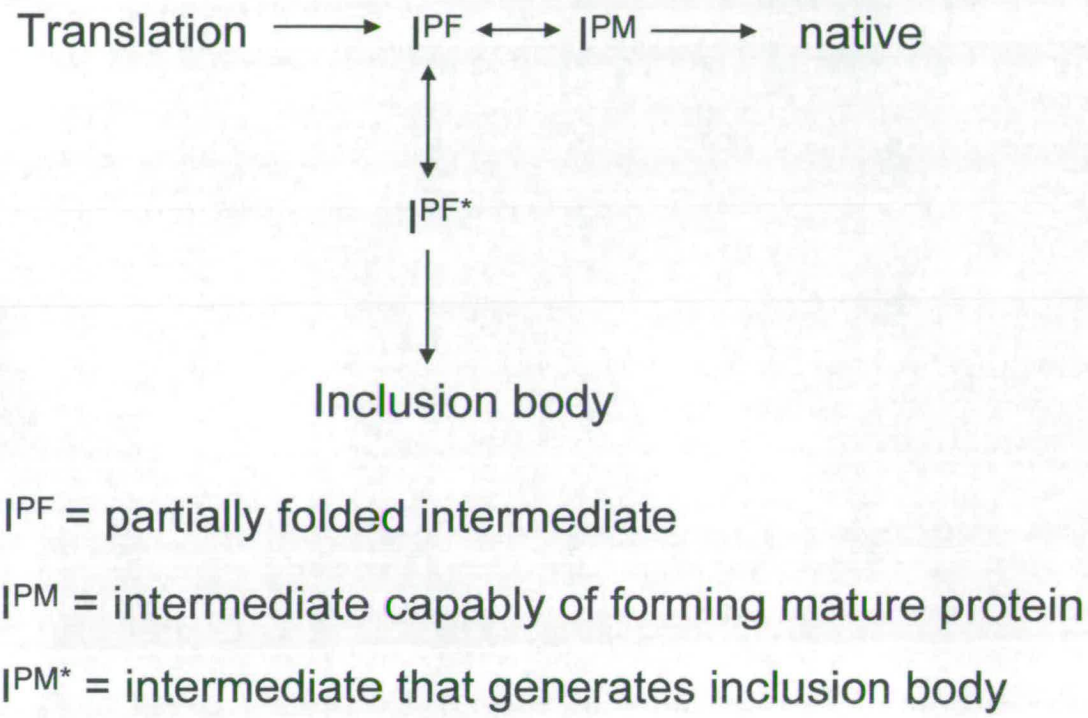


Figure 4.6 Mitraki and King model of inclusion body formation

Tuner™ DE3 pLysS cells are *lacY1* deletion mutants of *E. coli* BL21. The *lacY1* gene codes for a lactose-specific transporter. Expression of *lacY* varies between cells from no expression to high levels of expression. This means that cells within a culture will be taking up IPTG at different rates and consequently the levels of expression between cells will vary within the culture. Tuner™ DE3 pLysS cells cannot actively uptake IPTG and so all cells express at the same rate and the rate of expression is dependent on IPTG concentration. The rate at which a recombinant

protein is expressed has been shown to affect its apparent solubility. Additionally, the host strain contains the plasmid pLysS, which encodes the T7 lysozyme, and this prevents basal expression of the recombinant protein. Tuner™ DE3 pLysS cells allow fine control of the rate of expression and can enhance the apparent solubility of a recombinant protein.

Two 500ml cultures (1 BL21, 1 Tuner™) were induced with 0.1 mM IPTG for 3 hours at 37°C with shaking. Total protein samples were run on 12.5 % (w/v) polyacrylamide cells and compared (data not shown). As before little or no expression was seen in the BL21 expression strain. Expression was seen in the Tuner™ strain and so this was used in subsequent expression experiments.

4.2.5 Selection of optimal expression conditions

The conditions of protein expression were optimized to give the greatest possible yield. Two factors that have a large affect on the yield are the length of time between induction and harvest, and the concentration of IPTG used to induce. To find the optimum time between induction and harvest, one 500 ml culture was induced with 0.1 mM IPTG and time points were taken at 0.5, 1, 2, 3 hours and overnight. Total protein complement was then compared between each time point. No difference in the level of expression was seen after 1 h post-induction (data not shown).

Cultures were induced with 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM IPTG at 37°C for 3 hours with shaking. Aliquots (1 ml) of each culture were harvested and the total protein complement was compared (see Figure 4.7). Changing the IPTG concentration had no significant effect on the level of His.PEB3 expression.

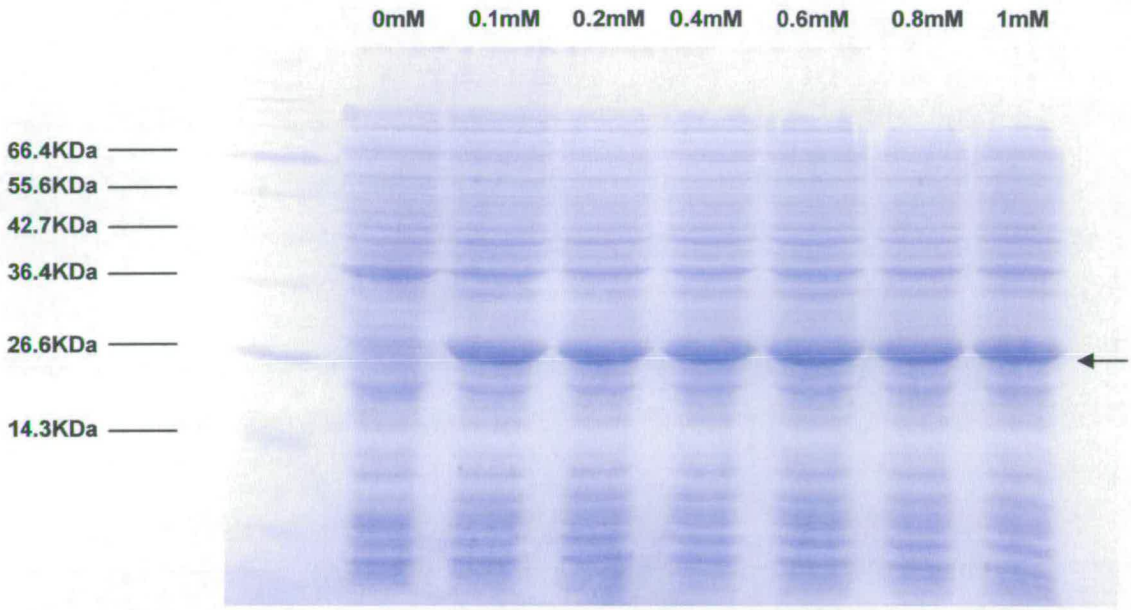


Figure 4.7 12.5 % (w/v) polyacrylamide gel stained for total protein showing the affect of different IPTG concentrations on yield of His.PEB3 (indicated by arrow) after induction for 3 hours at 37 °C.

4.2.6 Native vs. denaturing metal affinity purification of His.PEB3

Two pathways are available for purification of a His-tagged protein; native and denaturing. The native pathway is the preferable pathway as it retains the conformation of the protein. The yield however, can be significantly less than using the denaturing method as the yield is affected by the efficiency of lysis and solubilization of the target protein. The yield of denaturing purification is often higher as lysis is more efficient and solubilization more complete, but the conformation of the protein is lost.

Protein yield and purity was compared between four different purification methods: two native and two denaturing. Four 500ml cultures of Tuner™ pSAN3 were induced for 3 h at 37 °C with 1 mM IPTG. Each culture was then subjected to a different lysis method: (a) sonication (b) cell disruption (c) 8 M Urea (d) 6 M

Guanidine HCl. The first culture was exposed to 5 times 30 sec bursts of high-frequency sound (sonication) with a 30 sec rest interval between each burst. The second culture was passed 3 times through a cell disrupter (Constant cell disruption systems, Daventry). These two native purifications were then treated identically. The third culture was treated as in 2.10. The fourth culture was treated with denaturing lysis buffer that had 6 M Guanidine HCl substituted for the 8 M Urea. Additionally, the equilibration of the nickel-agarose column used Guanidine HCl containing buffer but the column was washed and the protein eluted using a Urea based buffer.

The purity of protein obtained was equivalent in each purification method (estimated at >95 % on a GelCode blue (Pierce) stained gel, see Figure 4.8). However, the yield of the native purification methods was less than half of the yield of the denaturing methods (see Table 4.2). The denaturing (urea) method was selected for purification of protein for use in raising antibodies. A final purification of 3 l of induced Tuner™ pSAN3, yielded 3.9 mg of protein. Protein from this lot was used in all further His.PEB3 experiments.

Strategy	Protein Yield ($\mu g ml^{-1}$)
Native (Sonication)	912
Native (Cell Disrupter)	853
Denaturing (Urea)	2238
Denaturing (Guanidine HCL)	2087

Table 4.2 Comparison of yields for different strategies of purification on a Nickel-agarose column.

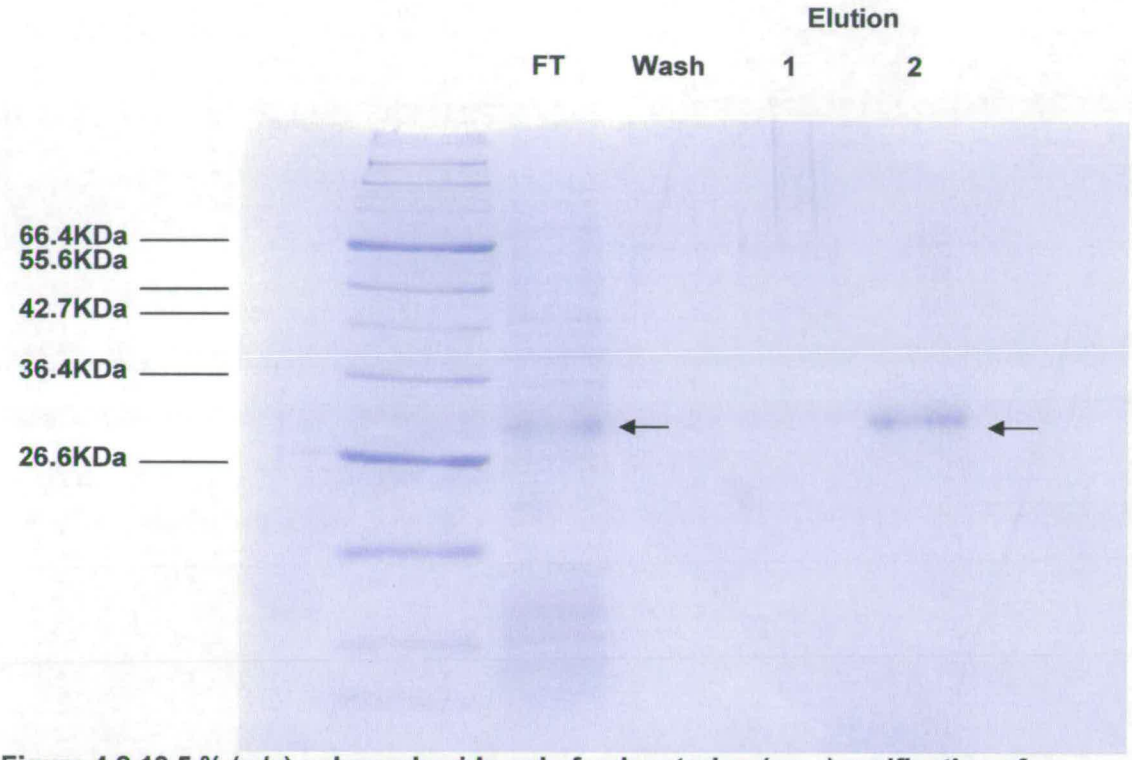


Figure 4.8 12.5 % (w/v) polyacrylamide gel of a denaturing (urea) purification of His.PEB3 (indicated by arrows) from Tuner™ pSAN3 cells. FT = Flow through, Elution 1 = eluent at pH 5.9, Elution 2 = eluent at pH 4.3

4.3 Antibody production

4.3.1 Production of monoclonal antibody against His.PEB3

Two attempts at making monoclonal antibodies against His.PEB3 were performed. Purified His.PEB3 was dialysed against PBS, ready for mixing with Freund's complete or incomplete adjuvant. During dialysis the purified protein precipitated out of solution. The precipitated protein was vigourously resuspended and divided into aliquots containing ~ 15 µg of protein. The aliquots were then sent to National Diagnostics (Bush, Scotland). The immunisation schedule is shown in 2.12.1.

Test bleeds were taken from two BALB/C mice before challenge and three

months post-challenge. Serum antibody levels were determined using ELISA and the end point titre calculated according to the equation:

End point = 1st titre > Mean of 6 secondary antibody controls + 0.2 OD units

Both mice had the same end point titre of 1:1024 (which suggest a reasonably poor response) and so it was decided to harvest the spleen from mouse 1 and keep mouse 2 for use later. As well as harvesting the spleen, exsanguinate was collected and tested for anti-His.PEB3 antibodies (End point titre 1:2). Splenocytes were harvested on the same day as the splenectomy and frozen at -80 °C for 2 days. On the day of fusion 1 x 10^8 P3-X53-Ag8 (Ag8) myeloma cells were harvested and fused with the splenocytes. Tissue culture treated 96 well were seeded with 100 µl of fused cells per well (592 wells total). The fused cells were allowed to recover and then subjected to HAT selection to select hybridoma cells from splenocytes and myelomas.

Wells were examined daily for the appearance of hybridoma colonies. When a hybridoma colony occupied approximately 1/3 to 1/2 of the well surface, the culture medium was tested for the presence of anti-His.PEB3 antibodies. A total of 54 wells (9 %) had hybridoma colonies and were tested for antibody production. None of the hybridoma colonies tested positive for anti-His.PEB3 antibody production.

Antibody amount and specificity is dependent on the processing of antigen by antigen presenting cells (APCs). Processing and presentation is known to be affected by: physical form of antigen, injection site, adjuvant used and the nature of the APC that encounters the antigen. How each factor increases or decreases the

immunogenicity of the antigen is specific to each antigen. As a result it is necessary to determine the affect of each factor empirically (Trombetta and Mellman, 2005). Table 4.3 lists factors that affect the immunogenicity of proteins and the effect seen with the majority of proteins.

Parameter	Increased immunogenicity	Decreased immunogenicity
Size	Large	Small (MW < 2 500)
Dose	Intermediate	High or Low
Route	Subcutaneous > intraperitoneal	> intravenous or intragastric
Composition	Complex	Simple
Form	Particulate	Soluble
	Denatured	Native
Similarity to self protein	Multiple differences	Few differences
Adjuvants	Slow release	Rapid release
	Bacteria	No bacteria
Interaction with host MHC	Effective	Ineffective

Table 4.3 Intrinsic protpeties and extrinsic factors that affect the immunogenicty of proteins.

To maximise the immunogenicity of the His.PEB3, it was decided to increase the dose per challenge from 15 μg to 100 μg. Two BALB/C mice were challenged according to the schedule in 2.12.1. A pre-immune bleed and test bleed were taken and tested for anti-His.PEB3 antibodies. It was not possible to calculate and end point titre as before because only a small amount of sera (~ 30 μl) was supplied. However, both mice tested positive for antibody production with A_{492nm} > 3 times the standard deviation of the negative control. As mouse 1 had responded marginally stronger, it was chosen for fusion and the splenocytes from mouse 2 harvested and frozen for later use. The exsanguinate from both mice was tested for the presence of anti-His.PEB3 antibodies and gave an end point titre of 1:2.

Splenocytes from mouse 1 were fused with SP2/0'-Ag14 meyeloma cells using the same protocol as with Ag8 myelomas. The switch between myeloma cell lines was intended to increase the number of anti-His.PEB3 producing hybridomas, as SP2/0'-Ag14 is a non-producing cell line (see 1.7.1.1). After fusion, 96 well tissue culture treated plates were seeded with 100 μl of fused cells per well (576 wells total). As soon as hybridoma colonies appeared, the culture media was tested for anti-His.PEB3 antibodies. No colonies tested positive for anti-His.PEB3 production.

In both experiments, an immune response against the His.PEB3 protein was observed, but no specific anti-His.PEB3 hybridomas produced. In the first experiment, the delay between the third challenge and the final challenge is the most likely cause. The spacing of challenges is designed to boost the immune response i.e. increase the number of B cells producing the desired antibody; as well as increasing the antibody affinity and diversity. During the time between the third challenge and

The pre-immune bleeds tested negative for all three rabbits (data not shown); the end point titre for all subsequent bleeds is shown in Figure 4.9.

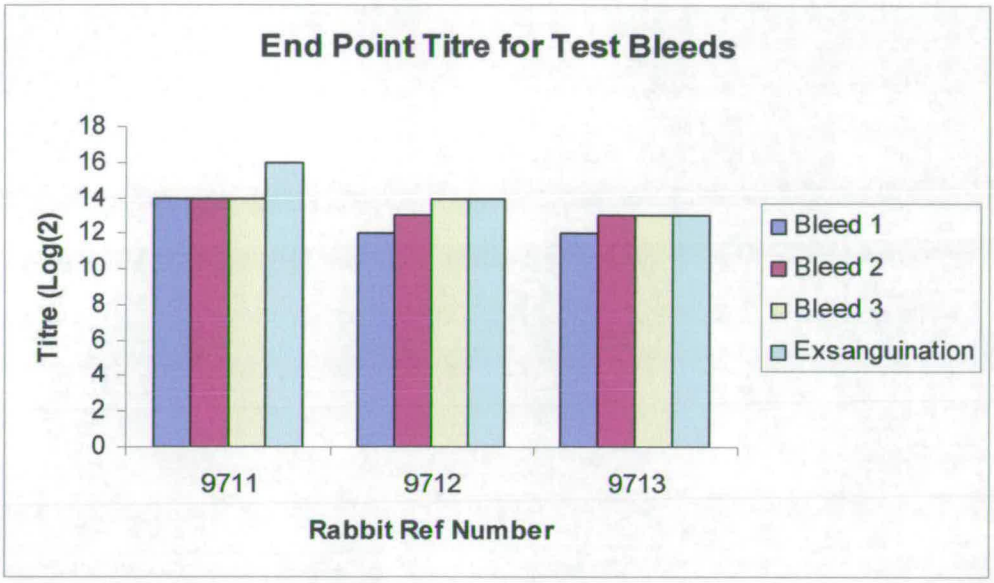


Figure 4.9 Graph showing the levels of anti-His.PEB3 antibodies in three rabbits as determined by ELISA and calculation of end point titres. End point titres were calculated according to the equation: End point = 1st titre > mean of 6 secondary antibody controls + 0.2 OD units. Bleed 1 taken at 3 days, bleed 2 taken at 49 days, bleed 3 taken at 63 days and the exsanguinate at 77 days.

4.4 Optimisation of IMS technique for maximum recovery of pure *C. jejuni*

Polyclonal serum from rabbit 9711 was used in the following experiments, as it had the highest end point titre. It has been found that the maximum number of *C. jejuni* cells on a chicken carcass is generally 10^5 cells in total (Park *et al.*, 1991). It is necessary to use saturating amounts of antibody in IMS; so that antibody binding and concentration is not a limiting factor. Excess antibody is not a problem as it will be removed by washing of captured cells. To determine the volume of sera that gave saturating amounts of antibody, varying volumes of sera (5 – 50 µl) were mixed with 1 x 10^6 *C. jejuni* cells for 2 h. Excess and unbound antibody was then washed away before incubation with anti-rabbit IgG coated paramagnetic beads. After washing to

the final challenge it would be expected that the number of circulating B cells would decrease, antibody levels will fall and the number of memory cells would increase. After the final challenge it would be anticipated that memory cells would revert to B cells and cause an expansion in the B cell population. The quality of this expansion can be assessed by measuring the serum antibody levels. The low end point titre infers that either this expansion did not occur or was very small. Consequently, the efficiency of the fusion would be corresponding low.

Similarly, in the second experiment a drop in antibody levels and therefore B cell numbers, occurred between the second challenge and exsanguinations. It would be expected that the third challenge would cause the B cell population to expand. The lack of expansion means that either the His.PEB3 was no longer immunogenic (e.g. completely degraded) or the inoculum was different from previous challenges (e.g. improper mixing, smaller dose).

4.3.2 Production of high titre polyclonal serum with rabbits

Although it is beneficial to use monoclonal antibodies in IMS, polyclonal sera can be used. The use of polyclonal sera would be expected to lower both sensitivity and specificity. Polyclonal sera were raised by challenging three New Zealand white rabbits with 1 μg of purified and dialysed His.PEB3. The challenges were carried out according to the schedule in 2.13.1 and by Harlan Seralabs (Hilcrest, UK).

A pre-immune bleed, three test bleeds and exsanguinate were tested for the presence of anti-His.PEB3 antibodies by ELISA. The end point titres for each bleed were calculated according to the equation:

End point = 1^{st} titre > Mean of 6 secondary antibody controls + 0.2 OD units

remove any non-specifically bound or weakly bound material; captured cells were eluted from the beads and DAPI stained. Stained cells were then immobilised on black polycarbonate filters before counting under a microscope with UV excitation. No cells were recovered with any of the serum amounts tested.

There are two possibilities as to why no cells were recovered using the polyclonal serum: 1. PEB3 is not surface exposed and 2. the polyclonal antibodies are not recognising native epitopes. The location of PEB3 within the cell is unknown but the evidence suggests that it is integral to the outer membrane (see Figure 4.10). PEB3 must at least be periplasmic as it is glycosylated and the attachment of the N-linked glycan occurs in the periplasm (see 1.5.3.2). The glycosylation also suggests that it is surface exposed because glycosylation is thought to be involved in attachment to host cells and immune evasion; both of which would require the glycan to be surface exposed. Additionally, PEB3 was discovered by western blotting of an acid glycine extract, which preferentially extracts periplasmic and outer membrane associated proteins. Secondary structure prediction (see Figure 4.11) shows a number of alpha helices which is common to membrane embedded proteins. However, the size of the helices is too small for any to be transmembrane (minimum number of amino acids = 20). The most likely location of PEB3 is therefore, embedded in the outer membrane on the cell surface with the glycan exposed to the extracellular medium.

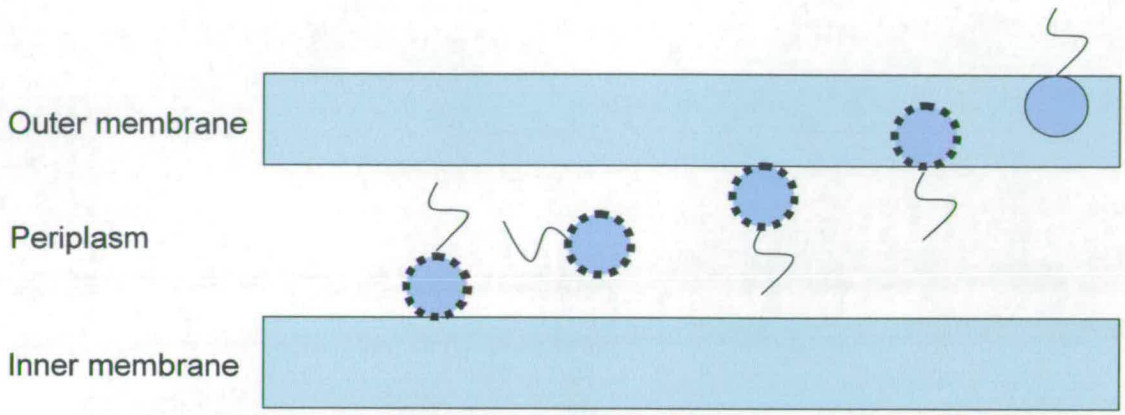


Figure 4.10 Possible locations of the protein PEB3 based upon the current evidence. The most likely location is indicated by a circle with a solid line.

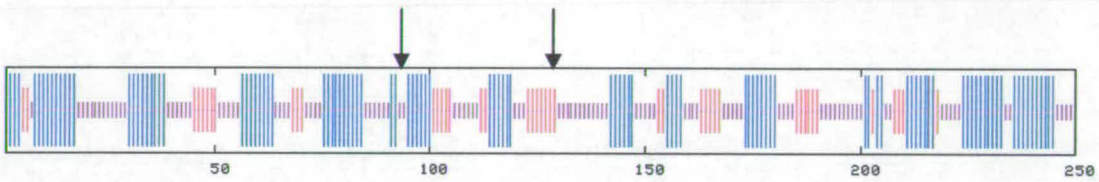


Figure 4.11 Predicted secondary structure for the protein PEB3. Blue lines indicate helices, red lines sheets and purple random coils. Arrows indicate the approximate location of the two potential glycosylation sites. Prediction performed using hierarchical neural network - (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_nn.html)

4.5 Conclusions

- The protein PEB3 is conserved across *C. jejuni* strains and as such is a possible target for IMS.
- The mice used for the production of monoclonal antibodies did not respond adequately.
- The cloned protein was immunogenic producing a good response in rabbits.
- The antibodies were not of sufficient quality or did not recognise native PEB3 epitopes.

Discussion and Further Work

5.1 *C. jejuni* typing schemes – one question, many answers!

The knowledge that *C. jejuni* is the most prevalent cause of bacterial food-poisoning has encouraged the research into typing schemes. A reliable typing scheme is critical for public health surveillance and for the comparison of data between multiple research laboratories. This need has given rise to a large number of typing schemes; some of which are extremely successful and others which have fallen into disuse. Of the typing systems currently in use, the Penner serotyping scheme, is perhaps the most important. This scheme was one of the first available and even with the advent of DNA-DNA based schemes, is still widely used. Other schemes in common use are: Lior serotyping, *fla*-RFLP, PFGE and MLST (see 1.6 for descriptions of each scheme).

It is possible to sustain multiple schemes because they fulfil differing requirements both scientific and practical (see Table 5.1). For example, *fla*-RFLP is a standardised technique that would most likely be used for following the course of an outbreak because it is easy to perform, relatively cheap and quick. Penner serotyping is useful for deciding treatment regimes as *C. jejuni* infection-associated diseases are often linked to particular serotypes (see 1.2). MLST is a good research tool as it can provide data about evolutionary relationships between strains; but is less likely to be used for public health as it is relatively expensive and slow to perform.

Typing Scheme	Approximate time required	Skill required	Cost	Level of discrimination
Penner serotyping	3 days	Low	++	+
Lior serotyping	3 days	Low	++	+
fla-RFLP	1 to 3 days	Low	+	++
PFGE	5 days	Medium	++	++
MLST	4 days	High	+++	+++

Table 5.1 Typing schemes currently used with *C. jejuni* and *C. coli* and the factors affecting the usage of each scheme.

The most important requirements that any typing system must fulfil are repeatability and portability. The portability of a scheme cannot be determined until the scheme has been used by multiple laboratories and the results compared. In the case of *Campylobacter* spp., an EU funded network (Campynet) was set up to investigate and devise standard protocols for the typing of *C. jejuni* and *C. coli*. While this project succeeded for *fla*-RFLP and PFGE, a standardised protocol for AFLP was not produced. AFLP therefore is useful for intra-laboratory use but not for inter-laboratory comparisons.

With the number of typing schemes available, the merit of developing of another is perhaps questionable. Although all the typing schemes fulfil the particular criteria for their niche, one significant problem remains. Each typing system stands alone and conversion of results from one scheme to another is difficult or impossible.

The aims of developing the CPS-RFLP typing scheme were:

- To combine the simplicity/speed of RFLP schemes and create a DNA-DNA based system that is directly comparable to Penner serotyping.
- To devise a scheme that might reveal data on the relationships between different serotypes.

These aims could also be partially filled by the use of microarrays, which have been used as epidemiological tools (Wren *et al.*, 2001). Microarrays are extremely powerful tools allowing the analysis of whole genomes and generate large amounts of data about each comparison. They therefore could easily reveal relationships at the DNA level between the different serotypes. At the same time this power makes them unsuitable for routine typing. As already stated some practical considerations that must be taken into account is the cost, technical skill required and the time taken to perform each test. Microarrays are a relatively new technology and so the cost associated with each test is very high and the technical skill required both to perform the test and analyse the data is high. CPS-RFLP would not be as discriminatory as using a microarray but would be considerably cheaper and easier to perform.

Although amplifications of the CPS locus from strains other than *C. jejuni* NCTC 11168 failed, the *in silico* analysis showed that in theory the system would have fulfilled the first aim. A possible reason for the failure was the reliance on the Expand™ 20 kb plus LT-PCR kit from Roche. There is considerable evidence to suggest that DNA polymerases are evolving to be more specific in the substrate they can process, as well as, becoming more accurate. The release of more LT-PCR systems means that the choice of enzymes available has expanded considerably since

the start of the project. Comparison of the enzymes within these kits to the *C. jejuni* DNA polymerase could determine whether any of the new kits would perform better than the Expand™ kit.

The new technique of whole genome amplification may also provide some possibilities. This technique uses the highly processive polymerase from the phage φ29 to amplify genomic DNA, using hexanucleotide primers, by rolling circle amplification (see Figure 5.1). The φ29 DNA polymerase is unusual in that is highly processive (Blanco and Salas, 1984) and possesses extremely high fidelity (Esteban, 1993). Such whole genome amplifications produce ~ 100 fold the starting amount of DNA with an average amplicon length of >10 kb. In such a system as the whole genome amplification the maximum possible amplicon length obtained is due in part to: 1. the length of time over which the amplification is performed (between 1.5 and 5 h (GE Healthcare GenomiPhi amplification kits)) and 2. enzyme stability. The stability of the φ29 DNA polymerase would need to be improved before it could be used in traditional PCR type techniques; as it is mesophilic and inactivated by incubation at 60 °C in 10 min.

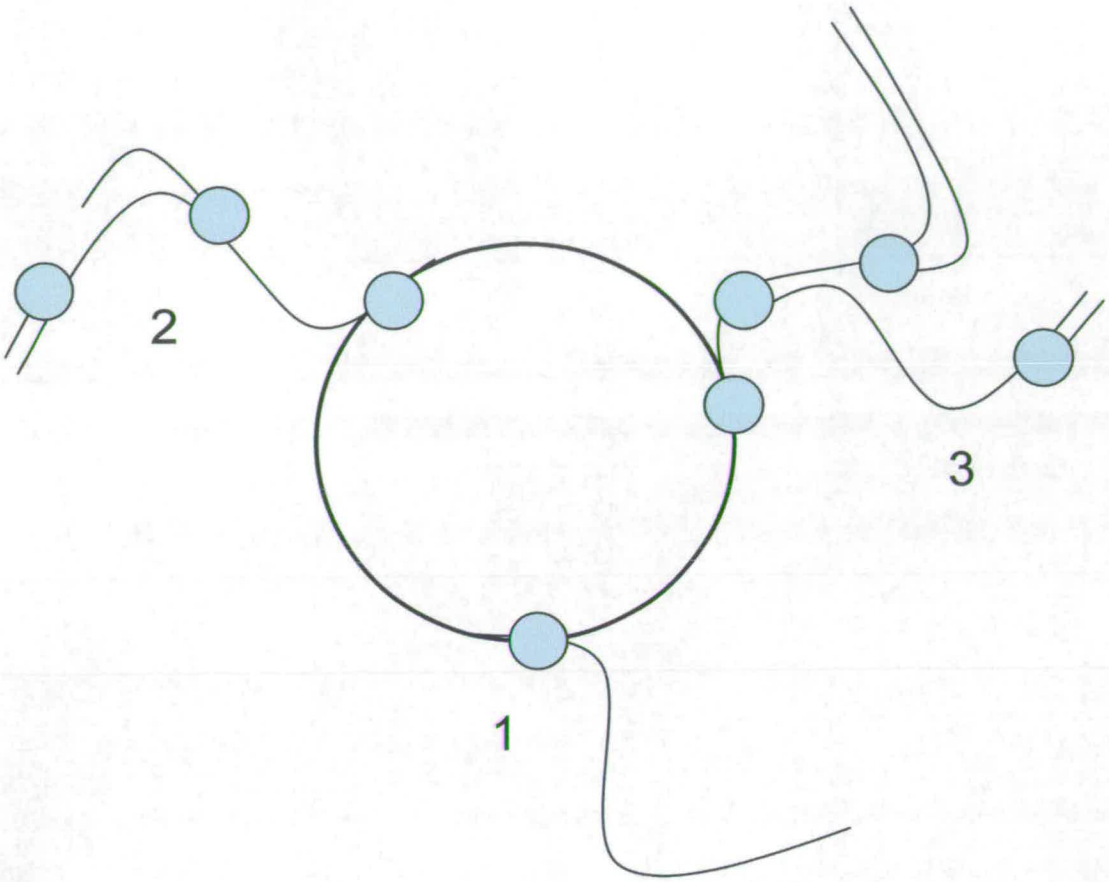


Figure 5.1 Schematic showing the process of whole genome amplification by φ29 DNA polymerase utilising hexanucleotide primers and strand displacement. 1. Replication initiates at multiple points on the chromosome by hexannucleotide primers and the DNA polymerase moves around the chromosome displacing strands of DNA synthesised by other polymerase molecules in front of it. 2. Hexanulceotide primers are then able to bind to the displaced strand. The polymerase is then able to initiate replication on the displaced strands, displacing any synthesised strands in front of it. 3. The displacement-replication cycle continues until the reaction is stopped or dNTPs are exhausted.

Another possible scheme to investigate the CPS locus as a target is multiplex PCR. This has been used successfully in *Yersinia pseudotuberculosis* to genetically 'serotype' strains. In the case of *Y. pseudotuberculosis* the LPS loci of all of the serogroups were (see Figure 5.2) known and seven targets (eight genes) allowed each of the 15 serogroups to be distinguished (Bogdanovich *et al.*, 2003). Although only a

few *C. jejuni* CPS loci are known the comparisons performed in 3.3.5 and the amplifications performed in 3.3.3 highlight some candidates for a multiplex PCR system. Such a scheme might include:

- One *kps* gene as an internal control (all loci should contain the *kps* genes)
- *hddC* and *gmhA2*. Found only in type 2 loci and was found in the majority of the reference strain set.
- *cj1430*. Found only in type 2 loci and is less common than *hddC* or *gmhA2*.
- *fcl*. The *in silico* analysis shows it to be present only in two of the sequenced loci.

As more CPS loci sequences for *C. jejuni* become available, the construction of a multiplex PCR that would distinguish between serotypes would become easier. The sequences would also allow the multiplex PCR to be related to structural differences between the CPS of each serotype (as is the case in *Y. pseudotuberculocis*). Such a system would be easily comparable to the current Penner serotyping scheme and would possibly reveal genetic relationships between the serotypes.

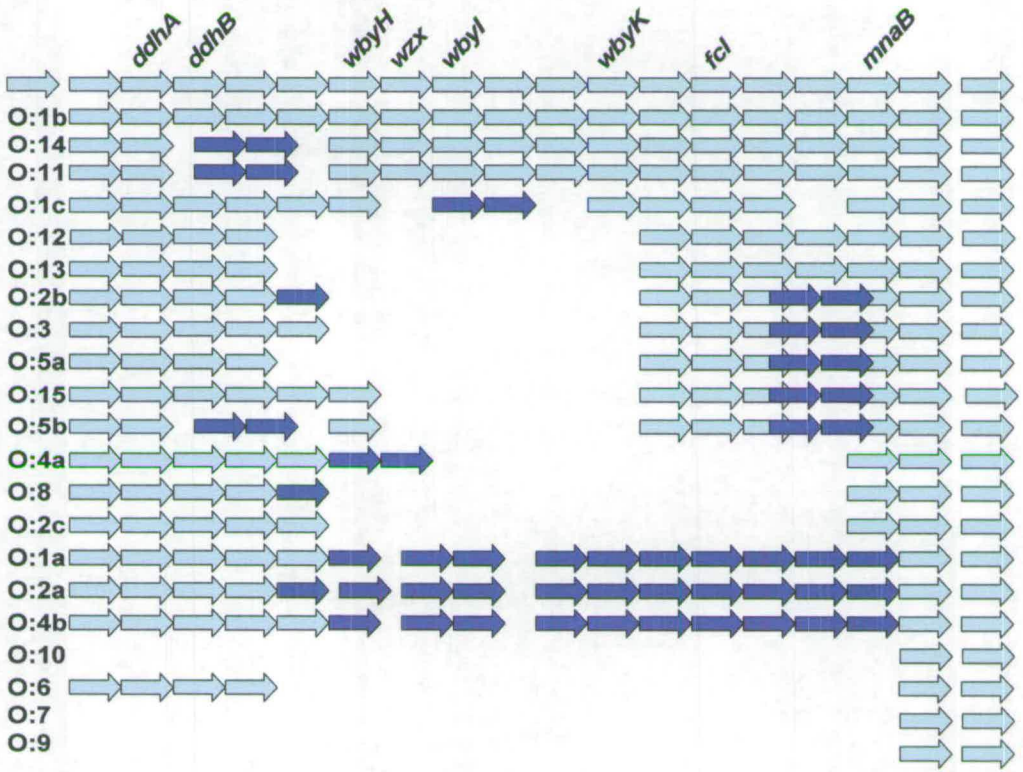


Figure 5.2 Overview of the genetic organistaion of the O-antigen gene clusters from *Yersinia pseudotuberculosis* serogroups O:1-O:15. The top locus is the O-antigen cluster from *Y. pestis* EV76. Light blue arrows indicate genes conserved between serogroups. Dark blue arrows indicate nonhomologus genes. The genes used for a genomic 'serotyping' scheme are listed above the The genes used for a genomic 'serotyping' scheme are listed above the *Y. pestis* locus. (Adapted from Skurnik, 2003)

5.2 Selection of targets for IMS

A number of IMS systems for the isolation of food borne pathogens, including *C. jejuni*, have been described (see Table 4.1). The success of each of these systems is dependent on both the target selected and quality (i.e. specificity and affinity) of the antibodies used. The results of the PEB3 based IMS detection system in Chapter 4 raise a number of questions:

- Was PEB3 a good choice of target for an IMS system?
- What is the best method for selecting targets?
- Is hybridoma-based antibody production the right choice for the production of antibodies?

The selection of PEB3 as a target was influenced heavily by the successful application of PEB1 in an IMS system (Clark, 2000). The selection of PEB1 as a target was good because of its role in adherence to host cells factors, indicating that it is surface exposed. PEB4, which has been studied along side PEB1 would not make a suitable target for IMS as it is part of an ABC-transporter system and resides in the periplasm. Despite being detected simultaneously it appears that each of the PEB proteins are unrelated to each other. The detection of PEB4 alongside the other PEB proteins is most likely due to cell lysis during infection, allowing processing of periplasmic and cytoplasmic proteins by the immune system.

The information on PEB3 suggests that it may have a similar function to PEB1. PEB3 was the first protein to be identified as glycosylated by the N-linked

glycosylation system in *C. jejuni*. The N-linked glycan is known to be involved in adherence (Karlyshev *et al.*, 2004) and cell competence (Larsen *et al.*, 2004); both of which would require PEB3 to be surface exposed. PEB3 therefore would seem to be ideal for a target of an IMS system. Other possible targets for IMS include the adherence factors that could be used as targets for IMS are CadF (Konkel *et al*, 1997), JlpA (Jin *et al.*, 2001), MOMP (Schroder and Moser, 1997) and the 21 other glycosylated proteins that have been isolated from *C. jejuni* (Szymanski *et al.*, 2003).

Although all the available evidence suggests that PEB3 is a 'good' target, the failure to produce useful antibodies suggests that the methodology used to select it as a target is not ideal. When only minimal information is available for the surface composition of the bacteria, how should targets be selected? The answer to this question is dependent on the method by which the antibodies will eventually be produced. Traditionally monoclonal antibodies are produced by hybridoma production. This technique is however unsuitable when a target cannot be identified in advance. The technique is limited by its use of animals, the length of time it takes to be performed, the level of technical skill required and its cost. The newer technology of phage display and over-expression of antibodies within *E. coli* overcomes these limitations.

Phage display (see 1.7.1.2) is a very powerful tool especially in the situation described above. Large numbers of possible targets can be screened very quickly and without any need for prior knowledge of the specific target. Phage display also provides a greater degree of control over the final antibody with fine tuning both of specificity and affinity being regulated. The only major problem with phage display is the creation of the initial antibody library. If the initial library is incorrectly

prepared then the some of the potential targets may be missed. Additionally, the creation of the initial library is relatively time consuming task and technically difficult task. The advantage being that once created the library can be used multiple times against multiple targets.

With hindsight, hybridoma-based antibody production was not a suitable choice when such a small amount of information was available on PEB3 because there is very little control over the final nature of antibodies. The use of phage display would have enabled screening of antibodies against the cloned protein and protein in its natural confirmation simultaneously; selecting for antibodies that will capture whole cells. Additionally the antibodies produced by hybridoma-based production are fixed in their properties. Once produced no further modification of affinity or specificity is possible. The properties of the antibodies are reliant on the quality of the host immune response; which for the BALB/C mice used in this experiment appeared to be of low quality. With the phage display method low quality antibodies can be artificially improved, with the use of site-directed mutagenesis or an *E. coli* host strain with a high mutation rate, to produce antibodies with the desired properties.

5.3 Further work

Although neither the CPS-RFLP typing scheme or the IMS system fulfilled their initial aims, they both are worthy of further investigation. In theory the CPS-RFLP scheme, would fill a gap in the current range of typing schemes in use. The unusually low %GC of the CPS locus will be problematic, as the majority of organisms currently being investigated are of higher %GC and consequently

protocols will be optimised as such. However, analysis of the information regarding ratios of enzymes in the LT-PCR kits and the organisms from which the enzymes are cloned should allow a selection of a kit suitable for use with *C. jejuni*. The comparison of the *C. jejuni* DNA polymerase to commercially available enzymes might help in the selection of enzymes better suited to low %GC templates.

The identification of targets for IMS based upon the current information available is difficult and therefore identification of targets would be better accomplished with a phage display antibody library. If such an approach was followed the screening of the library should be performed:

- On intact cells, thereby only producing antibodies against native epitopes.
- As many *C. jejuni* strains as are available, allowing the selection of a conserved epitope.
- Other intestinal organisms such as *Arcobacter* spp., *E. coli* and *Salmonella* spp., to prevent cross reactivity.

As already stated, phage display removes the need for prior knowledge of the target. It would therefore be necessary to identify the target post-screening by western blotting of cell surface extracts. The selection of targets in such a manner, would allow the production of an IMS system against the best target, without preconception.

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Appendix A

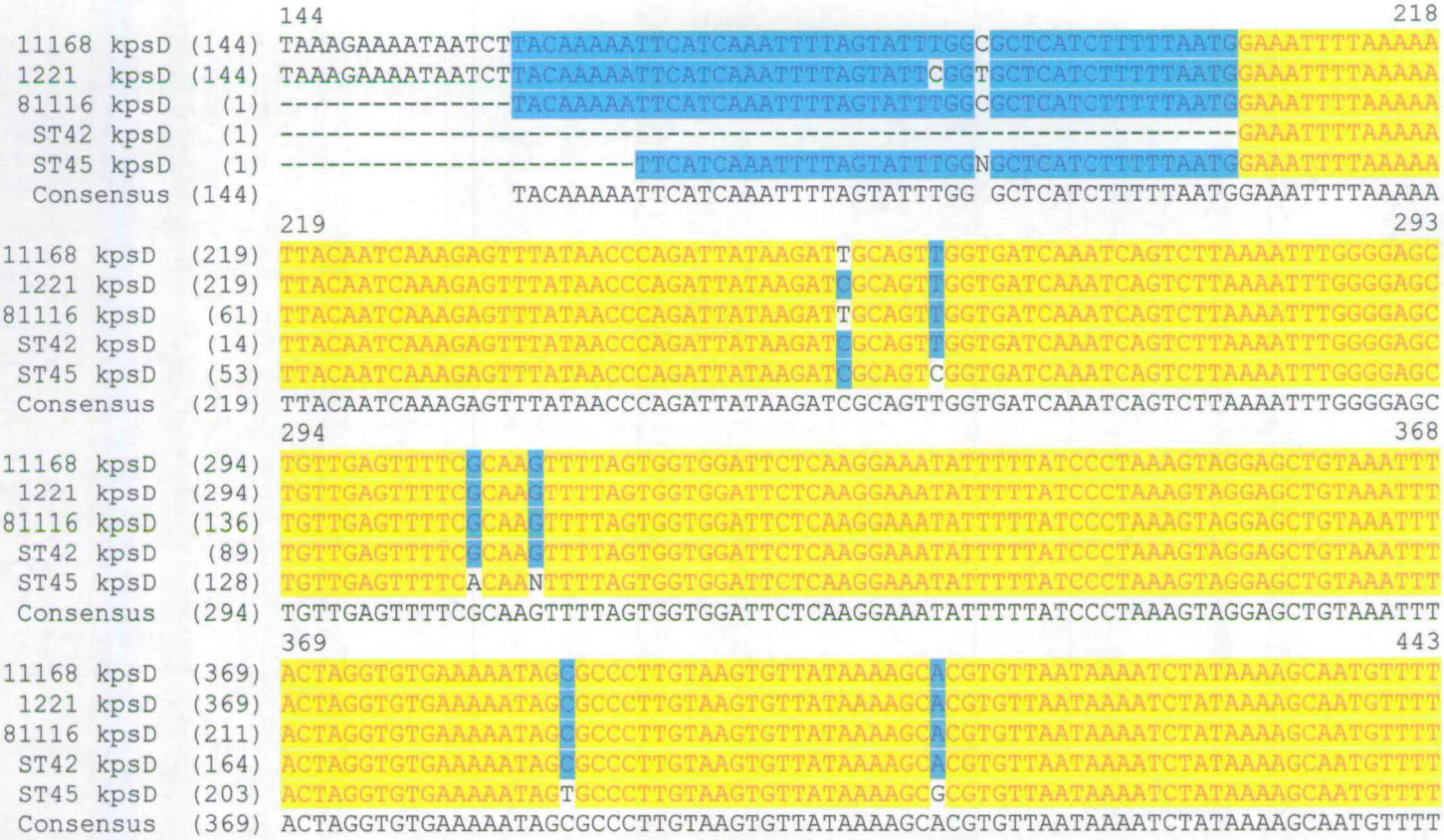
Phosphate buffer composition

Desired pH	0.2 M sodium phosphate, mono salt (ml)	0.2 M sodium phosphate, di salt (ml)	Distilled water (ml)
5.7	93.5	6.5	100
5.8	92.0	8.0	100
5.9	90.0	10.0	100
6.0	87.7	12.3	100
6.1	85.0	15.0	100
6.2	81.5	18.5	100
6.3	77.5	22.5	100
6.4	73.5	26.5	100
6.5	68.5	31.5	100
6.6	62.5	37.5	100
6.7	56.5	43.5	100
6.8	51.0	49.0	100
6.9	45.0	55.0	100
7.0	39.0	61.0	100
7.1	33.0	67.0	100
7.2	28.0	72.0	100
7.3	23.0	77.0	100
7.4	19.0	81.0	100
7.5	16.0	84.0	100
7.6	13.0	87.0	100
7.7	10.5	90.5	100
7.8	8.5	91.5	100
7.9	7.0	93.0	100
8.0	5.3	94.7	100

Appendix A Recipes for making 0.1 M sodium phosphate solution of a desired pH from 0.2 M sodium phosphate mono-salt and sodium phosphate di-salt solutions.

Appendix B

Sequence alignments



Appendix A-1 Alignments of the sequenced regions of *kpsD* from *C. jejuni* NCTC 11168, 81116, 3744 and 409266. Red on yellow residues indicate that the consesnsuu sequence was derived from a completely conserved residue. Blue on cyan residues indicate that the consensus sequence was derived from a block of conserved residues. Alignments were perfomed in VectorNTI v10 – AlignX module (Invitrogen)

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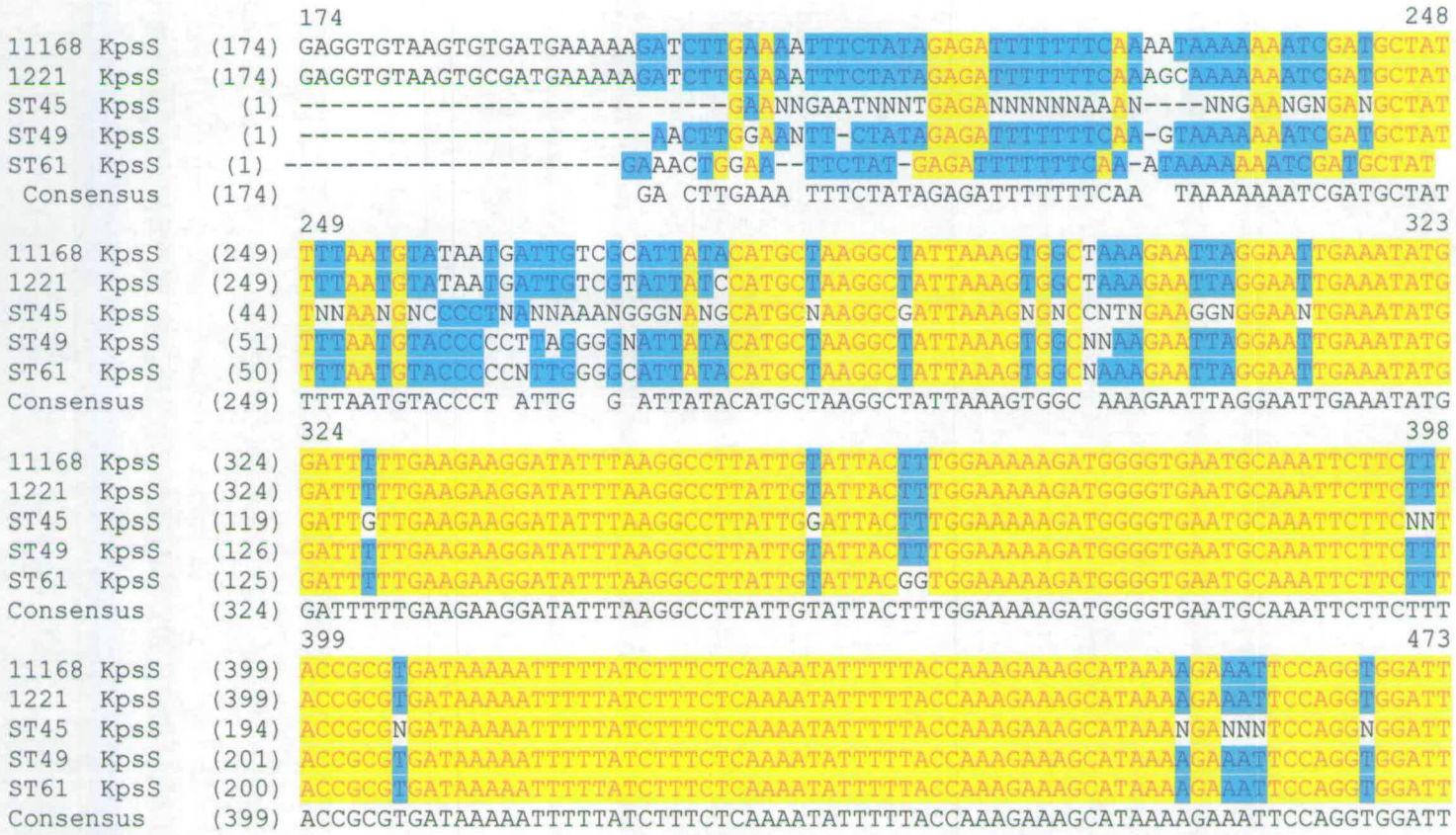
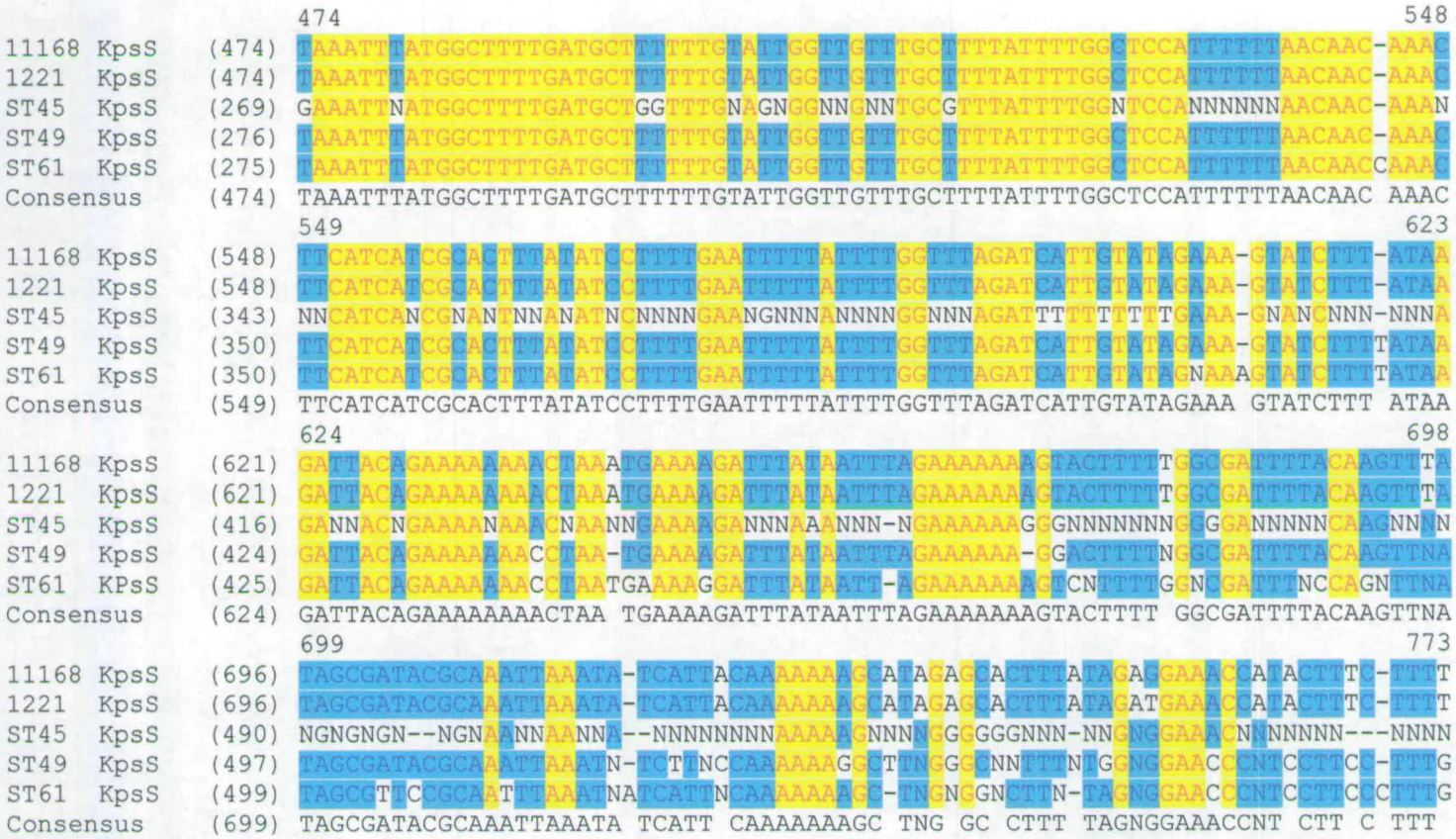
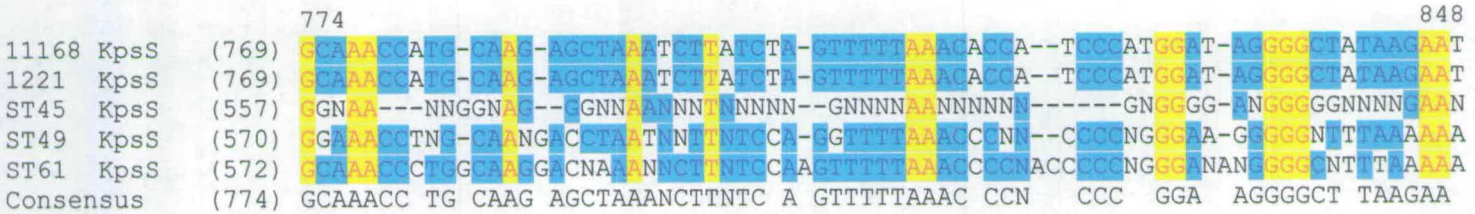


Appendix A-2 Alignments of the sequenced regions of *kpsS* from *C. jejuni* NCTC 11168, RM1221, 409266, 3060 and 450964. Red on yellow residues indicate that the consesnsuu sequence was derived from a completely conserved residue. Blue on cyan residues indicate that the consensus sequence was derived from a block of conserved residues. Alignments were perfomed in VectorNTI v10 – AlignX module (Invitrogen)



Appendix A-2 Alignments of the sequenced regions of *kpsS* from *C. jejuni* NCTC 11168, RM1221, 409266, 3060 and 450964. Red on yellow residues indicate that the consesnsuu sequence was derived from a completely conserved residue. Blue on cyan residues indicate that the consensus sequence was derived from a block of conserved residues. Alignments were perfomed in VectorNTI v10 – AlignX module (Invitrogen)



Appendix A-2 Alignments of the sequenced regions of *kpsS* from *C. jejuni* NCTC 11168, RM1221, 409266, 3060 and 450964. Red on yellow residues indicate that the consesnsuu sequence was derived from a completely conserved residue. Blue on cyan residues indicate that the consensus sequence was derived from a block of conserved residues. Alignments were perfomed in VectorNTI v10 – AlignX module (Invitrogen)