Glycosylation of *Campylobacter* iron transport systems and the role of host stress hormones

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Campylobacter jejuni is the main cause of gastroenteritis in developed countries and the exact nature of the organism's virulence continues to be the subject of much research. Initially the main area of focus for this project was to establish whether the glycosylation of iron uptake proteins is necessary for their function. We know that protein glycosylation is essential for interaction with host cells and that bacteria also require functioning iron uptake systems in order to colonise their host. Mutants in *pglB*, *pglI* and *pglK* were utilised in growth assays and compared with mutants in all known iron uptake systems. The haem uptake system was investigated and a haem biosynthesis gene *hemE* targeted, which will be used to establish whether exogenous haem can be used for metabolism. The *C. jejuni* haem oxygenase ChuZ has been expressed, purified and its viability assessed by absorbance spectrophotometry, with a view to solving the structure through X-ray crystallography. With knowledge of the structure it is anticipated that it will be possible to clarify the exact function of the protein. The catechol noradrenaline has been shown to enhance growth in iron-restricted conditions through the receptor CfrA. This study has investigated the effects of glycosylation on iron uptake via CfrA. The gluconeogenic enzyme GAPDH has been shown to localise to the outer membrane where it interacts with host transferrins. Whilst the effects of glycosylation on the uptake of iron from lactoferrin have been investigated, work has also begun on constructing a *gapA* mutant which will be used to investigate the interaction with lactoferrin and transferrin. This study has revealed that glycosylation of iron uptake proteins was not necessary for their function. Levels of natural competence were also studied and it was found that mutants in *pglB* and *pglK* were compromised with respect to their ability to naturally transform.

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Contents

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List of abbreviations

A	ampere (mA milliamperes)
ADP	adenosine diphosphate
AI	auto-inducer
AIDP	acute inflammatory demyelinating polyneuropathy
AMAN	acute motor axonal neuropathy
ANS	autonomic nervous system
ATP	adenosine triphosphate
BA	brucella agar
bp	base pair
BSA	bovine serum albumin
CAC	citric acid cycle
CDG	congenital disorders of glycosylation
CDT	cytolethal distending toxin
CEB	*Campylobacter* electroporation buffer
cfu	colony forming units (per ml)
CPS	capsular polysaccharide
Cts	*Campylobacter* transformation system
Da	Daltons (kDa kiloDaltons)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	disodium ethylenediaminetetraacetate
EM	Embden-Meyerhof
ENS	enteric nervous system

EtBr	ethidium bromide
fOS	free oligosaccharides
FSA	Food Standards Agency
g	angular velocity as multiple of the acceleration due to gravity
g	grams
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GAPDH	glyceraldehyde-3-phosphate dehyrogenase
GBS	Guillain-Barré syndrome
h	hour
HO	haem oxygenase
HPA	Health Protection Agency
IPTG	isopropythio-β-D-galactoside
K	kilo
Kb	kilobase pair
LB	Lysogeny broth
Lf	lactoferrin
LOS	lipooligosaccharide
LPS	lipopolysaccharide
M	moles
MEZ	malic enzyme
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
min	minute

MFS	Miller Fisher syndrome
NA	noradrenaline
OAA	oxaloacetate
Ω	Ohms (electrical resistance)
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCK	phosphoenolpyruvate carboxykinase
PCR	polymerase chain reaction
PEP	phosphoenol-pyruvate
PYC	pyruvate carboxylase
PYK	pyruvate kinase
RA	rhodotorulic acid
ReA	reactive arthritis
s	second
SAP	Shrimp alkaline phosphatise
SBA	soy bean agglutinin
SDS	sodium lauryl sulphate
T4SS	type-IV secretion system
T_m	melting temperature
Tf	transferrin
TLR	toll-like receptor
UV	ultraviolet
v/v	volume per volume

V	volts
VAIN	variable atmosphere incubator
W	watts
w/v	weight per volume
WHO	World Health Organisation
X-Gal	5-Bromo-4-chloro-3-indol-β-D-galactoside

Chapter 1: Introduction

1.1 Overview

It is now generally accepted that *Campylobacter jejuni* is the main cause of gastroenteritis in developed countries. The organism is a Gram-negative, highly motile bacterium and is a commensal organism in poultry. The *C. jejuni* NCTC11168 genome was sequenced and annotated by Parkhill *et al*. in 2000 and the exact nature of the organism's virulence continues to be the subject of much research. The study of *C. jejuni* is confounded by the organism's exacting growth requirements; it is microaerophilic and therefore requires a highly specific environment for successful culture.

1.1.2 History and taxonomy

The earliest known description of *Campylobacter* spp was by Theodor Escherich in 1886 (Escherich, 1886). This original account was largely based upon morphological characteristics. The isolation of an organism in 1913 by McFadyean and Stockman led to the characterisation of the *Vibrio* species (McFadyean & Stockman, 1913). An organism which was thought only to infect cattle was implicated in human disease and thus *Vibrio fetus* was isolated (King, 1957). In addition to the animal strains identified, King also discussed the presence of a 'related *Vibrio*' (King, 1957). Eventually in 1963 *C. jejuni* and *C. coli*, which had been previously reported as *Vibrio* were re-designated as a new genus, *Campylobacter* (Sebald, 1963). In addition, this new genus included *C. fetus* and *C. bubulus*, the former having been originally isolated from spontaneously aborted bovine foetuses by McFadyean and Stockman (1913). Although various *Vibrio*-like organisms had been described, the *Campylobacter* genus was ultimately

characterised in some detail in 1973 (Veron & Chatelain, 1973). Research interest was revived following successful isolation and growth techniques developed in the 1980s (Skirrow, 1977). This led to an increase in the volume of research and resulting publications throughout the latter part of the twentieth century. There are now 17 species and sub-species detailed within the *Campylobacter* genus (Moore *et al.*, 2005).

The characteristic spiral morphology of the *Campylobacteraceae* group of organisms influences their corkscrew-like tumbling motion with movement being achieved by way of one or two polar flagella. *Campylobacter* species do not ferment sugars, relying instead on amino acids or metabolic intermediates as carbon sources. Whilst a full, detailed account of all *Campylobacter* species is beyond the scope of this chapter, several organisms should be highlighted. *C fetus* was the first of the *Campylobacter* spp to be isolated (McFadyean & Stockman, 1913). Since the initial classification, the species now consists of two subspecies, *C. fetus fetus* and *C. fetus venerealis*. *C. fetus* subsp. *fetus* is responsible for both bovine and ovine spontaneous abortion. *C. fetus* subsp. *venerealis* has been associated with bovine genital tract infections. *C lari* has been isolated from wild birds, shellfish and surface water.

Campylobacter spp responsible for the majority of human infections are *C. jejuni* and *C. coli* with *C. jejuni* making up most of the reported cases (see Figure 1.1 for annual incidence of human infection by *C. jejuni*) (Frost, 2001; Gillespie *et al.*, 2002; Skirrow & Benjamin, 1980) (Health Protection Agency, accessed 29 July 2010). Both organisms have been isolated from commercially-farmed food animals, such as chickens, pigs and sheep as well as from humans with campylobacteriosis (Frost, 2001). Infections caused by *C. jejuni* are antecedent to complications such as Guillain-Barré and Miller-Fisher syndromes. Other species which have been linked with human infection are *C. rectus* (Tanner *et al.*, 1981) and *C. fetus* (Skirrow & Benjamin, 1980). *C. rectus* has been

isolated from patients with periodontal disease and is particularly prevalent in those individuals who are immuno-compromised and patients with AIDS (Gillespie *et al.*, 1992).

1.2 Campylobacter biology

1.2.1 The Campylobacter genome

The *C. jejuni* NCTC11168 genome sequence and annotation was published in 2000 (Parkhill *et al.*, 2000). The work showed *C. jejuni* to have a circular chromosome of 1,641,481 base pairs containing some 1654 predicted genes. The average gene size is a little under 1 kb and with a probable 94.3% of the genome coding for functional proteins, the *C. jejuni* genome was the densest known at the time it was sequenced (Parkhill *et al.*, 2000). The size of the *C. jejuni* genome and number of genes are considerably smaller than say *Escherichia coli* K-12 strain MG1655 which has a genome of 4,639,221 bp with 4289 genes (Blattner *et al.*, 1997). Parkhill *et al.* (2000) also showed that the *C. jejuni* genome exhibited the most sequence similarity (55.4%) with *Helicobacter pylori* which is of comparable size (Parkhill *et al.*, 2000). The NCTC11168 genome contains few insertion, repeat or phage-associated sequences but there are hypervariable sequences which tend to be associated with the genes encoding surface structures. Of four observed repeat sequences, three are found in the ribosomal RNA operon (Parkhill *et al.*, 2000). Sequence annotation revealed an incomplete glycolysis pathway, though the gluconeogenesis pathway is complete, as is the tricarboxylic acid (TCA) cycle (Parkhill *et al.*, 2000) The apparent lack of genes for carbohydrate or amino acid degradation confirmed previous observations that *C. jejuni* is unable to utilise these as carbon sources (Parkhill *et al.*, 2000). There are several

other *Campylobacter* spp genomes now available. These include *C. jejuni* RM1221 a similar strain to NCTC11168 (Fouts *et al.*, 2005) and 81-176 (Hofreuter *et al.*, 2006). The strains NCTC11168 and 81-176 differ from each other in a number of ways namely, 81-176 has a slightly smaller genome. The gene clusters for capsule synthesis and flagellar modification are variable and in 81-176 the clusters are smaller (Hofreuter *et al.*, 2006). *C. jejuni* 81-176 has two plasmids, unlike other well-documented strains NCTC11168, 81116 and RM1221 (Bacon *et al.*, 2002; Batchelor *et al.*, 2003; Fouts *et al.*, 2005; Parkhill *et al.*, 2000; Pearson *et al.*, 2007).

1.2.2 Plasmids

The *C. jejuni* strain 81-176 harbours two plasmids, pVir and pTet (Bacon *et al.*, 2002; Batchelor *et al.*, 2003). Some bacterial plasmids are able to confer antibiotic resistance and *Campylobacter* plasmids are no exception to this; the conjugative pTet plasmid having a gene, *tet*(O), encoding tetracycline resistance (Batchelor *et al.*, 2003). It is thought that *tet*(O) may have been acquired from another species of bacteria, namely *Streptococcus mutans* because of the relatively high GC (40%) content and the fact that there is a 99.4% homology to the *S. mutans tet*(O) (Sougakoff *et al.*, 1987). The plasmid is large at 45.2 kb and has a high level of sequence similarity (94.3%) to the pCC31 plasmid found in the *C. coli* strain CC31 (Batchelor *et al.*, 2003)..

The pVir plasmid is relatively large at approximately 37 kb (Bacon *et al.*, 2002). Interestingly, unlike the pTet plasmid, the GC content of pVir is low at 26%, which implies that the plasmid may have been attained through horizontal transfer from other *Campylobacter* species. A total of 54 genes have been identified, although not all are thought to encode functional proteins, with a possible 83% of the plasmid being coding sequence (Luo & Zhang, 2001). Possession of pVir alone would seem not to determine

virulence, as electroporation of pVir into NCTC11168 did not increase the invasion potential of that strain (Bacon *et al.*, 2002).

1.3 The Campylobacter glycome

A significant amount of research into the biosynthesis of carbohydrate surface structures has taken place since the sequencing of the NCTC11168 genome. The sequence revealed that a hitherto unexpected amount of the genome was devoted to the *Campylobacter* glycome (Parkhill *et al.*, 2000). During the last 20 years or so, industry research into glycomics has become therapeutically important with the potential development of vaccination targets and treatment of congenital disorders of glycosylation.

C. jejuni synthesises lipooligosaccharide (LOS), the structure of which varies greatly between strains. LOS differs from lipopolysaccharide (LPS) in that it lacks the chain of multiple oligosaccharide units seen in LPS (Rietschel *et al.*, 1994). LOS forms an essential component of the cell being part of the lipid bilayer (Karlyshev *et al.*, 2005). It is of low molecular weight and consists of lipid A and a core of oligosaccharide which possesses inner and outer regions (Moran, 1997). Within lipid A is a disaccharide backbone (2,3-diamino-2,3-dideoxy-D-glucose and D-glucosamine) the composition of which varies from strain to strain (Karlyshev *et al.*, 2005). Lipid A is modified as a response to environmental changes and in order to avoid detection by the host's immune system (van Mourik *et al.*, 2010). It is thought that *C. jejuni* synthesises LOS but not LPS. This is because the O-chain, once thought to be associated with LPS, is in fact part of the capsule (Oldfield *et al.*, 2002). The structure of the LOS core mimics the surface carbohydrates of the nerve cells and in doing so initiates the autoimmune response that

leads to GBS (Gilbert *et al.*, 2004). The LOS genes also exhibit phase variation, with the existence of homopolymeric tracts within a number of genes in the locus (Linton *et al.*, 2000). As *C. jejuni* is naturally competent (Wang & Taylor, 1990), numerous crossover events are likely to secure the acquisition of genetic variation between strains (Gilbert *et al.*, 2004).

C. jejuni CPS is also likely to exhibit variation. The capsule is essential for survival of the cell particularly within a challenging environment. The CPS locus itself has three regions consisting of two outer flanks, which are necessary for, amongst other things, the export of the CPS to the cell surface. In addition there is a central region which contains the genes for the CPS repeat units. This region has been shown to be highly variable (Karlyshev *et al.*, 2005). The first evidence for the existence of a capsule in *C. jejuni* was in 1999 during sequencing of a random NCTC11168 library (Karlyshev *et al.*, 1999). These findings were later supported by the publication of the genome sequence and expanded upon by the publication of data on different strains (Karlyshev *et al.*, 2000; Parkhill *et al.*, 2000).

1.3.1 O-linked glycosylation

The post-translational modification of the *Campylobacter* flagellum was first described in 1989 (Logan *et al.*, 1989), with structural analysis and confirmation of flagellin glycosylation following in 1996 and characterisation of the FlaA protein in 2001 (Doig *et al.*, 1996a; Thibault *et al.*, 2001). *O*-linked glycosylation is restricted to flagellar subunits (Doig *et al.*, 1996a; Szymanski *et al.*, 2003). Later studies revealed the precise nature of *O*-linked glycosylation with the finding that as many as 19 sites are glycosylated (Logan *et al.*, 2002; Logan, 2006; Samatey *et al.*, 2001; Thibault *et al.*, 2001). It has been revealed that although most polar flagella are glycosylated (Logan, 2006), in *Campylobacter* flagellar assembly is dependent upon the glycosylation of

FlaA and FlaB (Guerry & Szymanski, 2008). There is some evidence that glycosylation may be necessary in the secretion of flagellar subunits (Logan *et al.*, 2002), although no precise role has been assigned to flagellar glycosylation in protein secretion. The processes of *N*- and *O*-linked glycosylation also differ in that in *N*-linked glycosylation, glycan attachment depends upon the presence of the exact recognition sequence, whereas flagellar glycosylation would appear to be determined by the overall protein structure (Thibault *et al.*, 2001). However, it has been shown that the glycosylation site is in part associated with areas of hydrophobicity around serine or threonine residues which may be within easy reach of the transferases (Thibault *et al.*, 2001).

The *O*-linked glycosylation locus contains in the order of 50 genes (Parkhill *et al.*, 2000) and shows considerable variation between strains (Karlyshev *et al.*, 2005). The presence of homopolymeric tracts within some of the genes suggests there may be phase variation of those genes (Karlyshev *et al.*, 2002; Karlyshev *et al.*, 2005). This variation and the ability to vary the flagellar structure could assist in evasion of the host's immune system as well as allowing adaptation to a changing environment (Karlyshev *et al.*, 2005).

1.3.2 N-linked glycosylation

Protein glycosylation is a post- or co-translational modification that was originally believed to be confined to eukaryotes. Extensive studies have been carried out on the glycosylation of human proteins, partly driven by the need to investigate a group of genetic disorders of glycosylation, known as CDG (Congenital Disorders of Glycosylation). There are now a total of twenty eight known conditions, 16 of which are associated with the *N*-linked protein glycosylation process (Jaeken & Matthijs, 2007). The effects of CDG are broad and varied, ranging from very mild (affecting one organ)

to severe, affecting all systems within the body and causing a plethora of disease phenotypes (Jaeken & Matthijs, 2007). A prokaryotic system was initially described in the 1970s when Mescher and Strominger reported the presence of *N*-linked glycoproteins in the cell envelope of *Halobacterium salinarium* (Mescher *et al.*, 1974; Mescher & Strominger, 1976). Subsequent research by Sleytr and Thorne described glycoproteins in *Clostridia* (Sleytr & Thorne, 1976). The sequencing of *C. jejuni* NCTC11168 revealed that a large part of the genome was dedicated to the biosynthesis of cell carbohydrates (Parkhill *et al.*, 2000). Amongst these were two distinct glycosylation systems, the possession of which made *C. jejuni* unique amongst bacteria at the time the genome was sequenced. The system of *Campylobacter O*-linked, flagellar glycosylation was first described by Logan *et al.* in 1989. Evidence to date shows that *O*-linked glycosylation is limited to flagellar subunits (Szymanski *et al.*, 2003) and is required for correct flagellar assembly and secretion (Logan *et al.*, 2002). The genes involved in *N*-linked protein glycosylation (*pgl*) in *Campylobacter* are adjacent to those involved in lipopolysaccharide biosynthesis and this initially led to them being assigned an incorrect function (Fry *et al.*, 1998). Observations were made that the genes were not variable however the correct function was still not known (Wood *et al.*, 1999). *N*-linked glycosylation in *Campylobacter* was later described in more detail by Szymanski *et al*. (1999). That group concentrated on making site-specific mutations in order to investigate the function of each gene within lipopolysaccharide (LPS) biosynthesis, only to find that there was no link between the *pgl* genes and LPS. Instead, the evidence pointed to the existence of a system of protein glycosylation in *Campylobacter*. The protein glycosylation genes were termed *pgl* following the earlier establishment of a system of nomenclature in *Neisseria meningitidis* as published by Jennings and co-workers (1998). It later became apparent that glycosylation was widespread throughout many prokaryotic species, however *C.*

jejuni was the first Gram-negative bacterium in which the process of *N*-linked glycosylation was described in detail (Szymanski *et al.*, 1999).

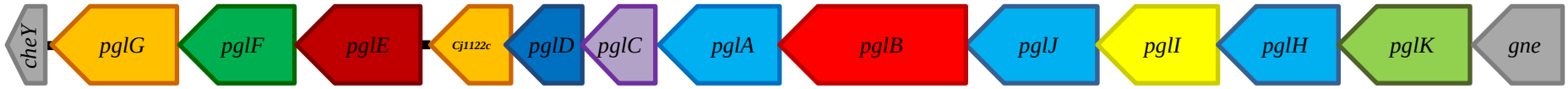


Figure 1.1. The protein glycosylation locus in NCTC11168. The colours represent the different enzyme functions. Light green = flippase, turquoise = GalNAc transferase, yellow = glucosyl transferase, red = oligosaccharyltransferase, lilac = galactosyltransferase, dark blue = acetyltransferase, orange = unknown, dark red = UDP-4-keto-6-deoxy-GlcNAc C4 aminotransferase and dark green = UDP-GlcNAc C4,6 dehydratase. The two genes that flank the *pgl* cluster, *cheY* and *gne* are shown in grey. The arrow direction indicates the direction of transcription.

1.3.2.1 An overview of the process of N-linked glycosylation

N-linked glycosylation is the process by which a heptasaccharide glycan is assembled and attached to a nascent polypeptide. The exact structure of the *N*-linked glycan in *C. jejuni* was first described by Young *et al.* (2002), who utilised mass-spectrometry techniques to investigate an antigenic peptide, PEB3 (Cj0289c). In addition, their research identified a total of 38 proteins which were glycosylated (Young *et al.*, 2002)(Appendix). Further examination using nano-NMR revealed a glycan structure of GalNAc-α1,4-GalNAc-α1,4-(Glcβ1,3)- GalNAc-α1,4- GalNAc-α1,4- GalNAc-α1,3-Bac. In *C. jejuni* NCTC11168 there are 11 known *pgl* genes, *pglA – pglK*, which make up a ~16 kb locus (Szymanski *et al.*, 1999). Gene sizes range from 2142 bp (*pglB*) down to 588 bp (*pglD*); there are four classes of enzymes encoded by the genes, namely those enzymes which synthesise or modify bacillosamine (PglD,E and F), glycosyltransferases (PglA, C, H, I and J), an oligosaccharyl-transferase (OTase), PglB and a flippase PglK (Figure 1.1). PglK was formerly called WlaB and is an ABC transporter which is responsible for the transposition of the glycan across the inner membrane and into the periplasm. The function of PglG is not yet known. The locus is largely conserved throughout *Campylobacter* species. During glycan assembly sugars are either added sequentially or modified on a lipid carrier (polyisoprenyl diphosphate) on the inner membrane as shown in Figure 1.2. Each enzyme is encoded by a *pgl* gene and is responsible for at least one sequential step in the heptasaccharide's biosynthesis or modification. Initially PglF, E and D convert UDP-GlcNAc to UDP-4-keto-6-deoxy-D-GlcNAc and then to UDP-2,4-diacetamido-2,4,6-trideoxyglucopyranose (UDP-diNAcBac)(Guerry & Szymanski, 2008). PglC transfers the UDP-diNAcBac onto the membrane-bound UDP-pyrophosphate (Guerry & Szymanski, 2008).

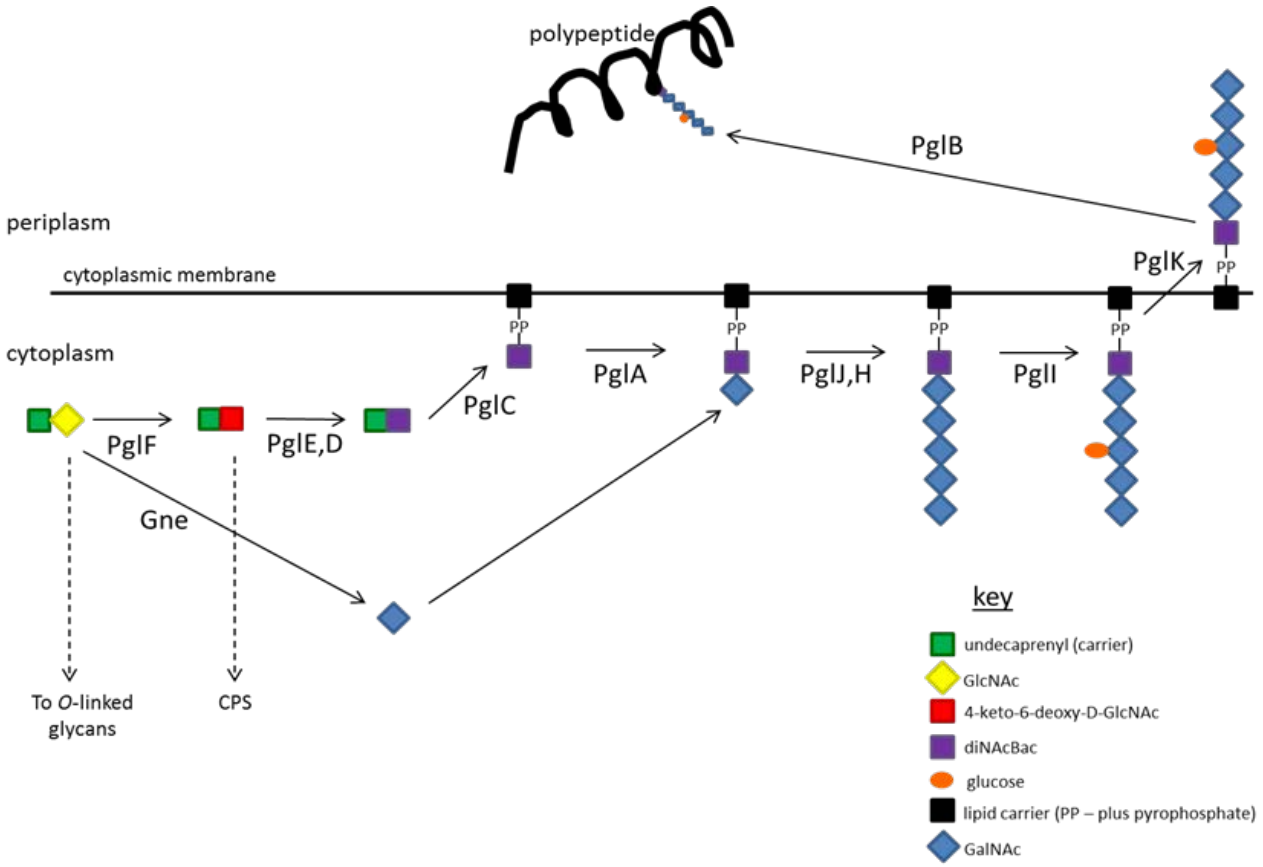


Figure 1.2 Overview of the protein glycosylation pathway. See text for explanation of individual steps of the process. PglG has been omitted because its function is as yet unknown.

The latter stages of the process are completed by PglA,J and H, with the branched glucose being added by PglI. The structure is flipped across the cytoplasmic membrane by PglK before PglB adds the heptasaccharide to a nascent polypeptide (Szymanski *et al.*, 2003).

1.3.2.2 Attachment of the glycan by PglB

All members of the family of STT3 OTases studied to date have been shown to possess a highly conserved motif, WWDYG, which is located in the *C*-terminus of PglB (Weerapana & Imperiali, 2006). Mutations in this motif abolish glycosylation which provides evidence of its significance in the process (Wacker *et al.*, 2002). The heptasaccharide, once complete is flipped across the cytoplasmic membrane by PglK and linked, by PglB, to the amide nitrogen (via a β-glycosylamide linkage) of the asparagine residue of a common consensus sequence on the nascent polypeptide (Wacker *et al.*, 2002). This sequence was until very recently believed to be identical in both eukaryotic and prokaryotic glycosylation systems; Asn – Xaa – Ser/Thr (where Xaa can be any amino acid with the exception of proline). It has been revealed however that the glycosylation recognition sequence with respect to *C. jejuni* is extended to include a negatively-charged side chain making the 'new' sequon Asp/Glu – Xaa – Asn – Xaa – Ser/Thr (where, once again Xaa can be any amino acid but proline) (Kowarik *et al.*, 2006). It has recently been suggested that PglB has 'clear preferences' for specific residues in the +2 and -2 positions of the consensus sequence. This would result in a preferred sequence of Asp-Xaa-Asn-Xaa-Thr (Chen *et al.*, 2007). Chen and co-workers also proposed that while glycosylation would occur in the presence of any residues at +1 and -1 (≠ proline), it was generally found that the favoured amino acids would be

alanine, serine and positively-charged residues at +1 and aromatic or amido residues at -1 (Chen *et al.*, 2007).

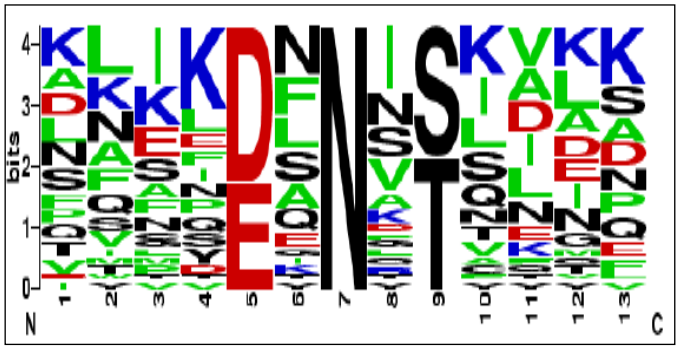


Figure 1.3. An analysis of the glycosylation site of glycoproteins in *C. jejuni*. This illustration is based on a statistical analysis of the consensus sequence of the Asn sequon. The axes shows the frequency of occurrence of the listed amino acids from the -6 to +6 positions, with the x-axis indicating the position and the y-axis shows the frequency with which a particular residue occupies that position. For example, the residue occupying position 0 will always be an asparagine. The data for this analysis was generated by Kowarick *et al.* (2006) as part of a study to identify the precise sequence for glycan attachment. The figure was created using WebLogo (http://weblogo.berkeley.edu/) – a package designed to generate sequence logos (Crooks *et al.*, 2004). Colour scheme: Black – hydrophobic, Green – polar, Blue – basic and Red – acidic.

A detailed statistical analysis of the glycosylation recognition site published by Kowarick e*t al.* (2006) is summarised in Figure 1.3. Having identified the recognition sequence for the glycosylation of the protein, it was initially assumed that all identical sequons in the peptide would be glycosylated. This, however, is not the case and it is now accepted that whilst the presence of the sequon is an absolute necessity, it is not sufficient alone for glycosylation to occur (Nita-Lazar *et al.*, 2005).

In eukaryotes, it seems that the OTase recognises an 'asparagine-turn' which is part of the secondary structure of the nascent protein; glycosylation occurring before the peptide is folded completely (Weerapana & Imperiali, 2006). PglB has been shown to have a relaxed specificity with respect to potential glycosylation targets, even to the extent where the OTase will target exogenous proteins (Wacker *et al.*, 2006).

Not every recognition site is targeted by the OTase so the question remains as to why bacteria glycosylate proteins. Research into eukaryotic glycosylation has provided the majority of the evidence as to the probable functional purposes for the process; these are likely to be further protein folding and conformational stability of the mature protein (Imperiali & O'Connor, 1999). Other possible advantages to glycosylation include protein solubility, assembly, variation and protection against cleavage by proteases (Szymanski & Wren, 2005). Eliminating protein glycosylation has been shown to alter the transcription of a number of genes (Nothaft *et al.*, 2008). A significant amount of research has been carried out relating to transcription, the existence of promoters and the effects of mutations in the *pgl* system in respect of gene expression. Mutations in the *pgl* locus also affect the expression of the *pgl* genes themselves with all *pgl* genes being down-regulated in *pglH*, *pglJ* and *pglK* mutants (Nothaft *et al.*, 2008).

1.3.2.3 The effects of mutation in pgl genes

With respect to bacteria it has been shown that glycosylation mutants in *Campylobacter* show less adhesion and invasion of host cells (Hendrixson & DiRita, 2004; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002). Szymanski and co-workers showed that mutants of *pglB* and *pglE* exhibited significantly less adhesion and invasion of INT407 cells than wild-type *Campylobacter*, in addition these mutants were less able to colonise mice (Szymanski *et al.*, 2002). Subsequent research by Karlyshev *et al*. (2004) demonstrated that *pglH* mutants were less efficient in adhesion and invasion of Caco2 cells. These mutants were also less able to colonise chicks (Karlyshev *et al.*, 2004) with the most likely explanation of this being a decline in host cell interaction. This suggests a possible role for glycoproteins acting as adhesins (Karlyshev *et al.*, 2004). Any distinct phenotype for the *pgl* mutants is largely confined to the inability of the organism to effectively adhere to and invade host cells. However it has been shown that mutations in the *N*-linked glycosylation system of *C. jejuni* 81-176 affect that strain's ability to take up DNA by natural transformation (Larsen *et al.*, 2004). Work by Larsen and colleagues investigated the role of one of 81-176's plasmids (pVir) and in particular VirB10, a glycoprotein involved in natural transformation and host cell invasion. They found that mutating *pglB* and *pglE* had the same effect on the organism's natural competence as mutating the *virB10* gene (Larsen *et al.*, 2004). There is little doubt that glycosylation contributes to the overall conformation of the nascent protein; extensive studies have been carried out in eukaryotic systems using methods to deglycosylate proteins though of course this will merely give information with respect to the protein's stability (Imperiali & O'Connor, 1999). Many comparative studies have been made between eukaryotic and prokaryotic systems and the high level of homology between the two

means that the *C. jejuni* OTase PglB could be an ideal model for glycosylation systems in other organisms. There are distinct advantages in studying prokaryotic glycosylation. For example glycosylation occurs in the ER in eukaryotes and the comparative complexity of the system makes it more difficult to study. In addition, mutations in *Stt3* result in varying degrees of biosynthetic lethality thus confounding observations (Chavan *et al.*, 2003). Finally, PglB shows a relaxed specificity with respect to glycan structure which may lead ultimately to exploiting the system for glycoengineering (Weerapana & Imperiali, 2006).

1.3.2.4 Components of the protein glycosylation system

As stated earlier, proteins in the Pgl system either add or modify the components of a heptasaccharide glycan before its translocation across the cytoplasmic membrane by PglK and coupling to a nascent polypeptide by PglB. Considerably more is known about function than structure with respect to glycosylation in *Campylobacter*. The insight gained has been partly due to the study of homologous proteins in other organisms and this in turn has led to putative roles being assigned to each of the Pgl proteins. It has been established that the synthesis of the *N*-linked glycan is a step-wise process that begins with the action of PglF upon UDP-GlcNAc and ends with the flipping of the glycan across the cytoplasmic membrane (Guerry & Szymanski, 2008). Not all translocated glycans are attached to polypeptides and as a result they remain free-floating within the periplasm (Nothaft *et al.*, 2009). PglF catalyses a dehydration reaction and converts UDP-GlcNAc to UDP-4-keto-6-deoxy-D-GlcNAc (Olivier *et al.*, 2006; Schoenhofen *et al.*, 2006). This reaction is dependent upon the presence of NAD^+ (Olivier & Imperiali, 2008). PglE is the next enzyme in the pathway and through transamination forms a UDP-4-amino sugar (Schoenhofen *et al.*, 2006). PglD acetylates the amino sugar to form UDP-$BacAc_2$ by using acetyl-coenzyme A for the acetyl group

(Olivier & Imperiali, 2008)). This forms the first sugar in the structure of the heptasaccharide.

A recent study has solved the crystal structure of PglD (Olivier & Imperiali, 2008; Rangarajan *et al.*, 2008). The enzyme exists as a trimer, with each of the monomers consisting of a left-handed β helix (LβH) at the C-terminus and an α/β domain at the N-terminus. Interaction between the β-helices and the α/β-domains assist in stabilising the overall structure whilst creating an active binding site between the domains (Rangarajan *et al.*, 2008). PglC is a glycosyl-1-phosphate transferase and transfers the UDP-diNAcBac to the undecaprenylphosphate UDP-PP on the lipid membrane (Glover *et al.*, 2006; Guerry & Szymanski, 2008). PglA is the first of the glycosyltransferases and adds an *N*-acetylgalactoseamine (GalNAc). Following this step a single GalNAc molecule is added by PglJ and a further three by PglH (Glover *et al.*, 2005) before the branched glucose is added by PglI (Olivier *et al.*, 2006). The four glycosyltransferases are of similar size and PglA, H and J lack any putative transmembrane domains, however it has been suggested that there may be some interaction between the cytoplasmic membrane and these proteins (Glover *et al.*, 2005). PglA, H and J interact with each other and the indications are that all three are necessary for completion of the saccharide structure (Glover *et al.*, 2005). Although a role has yet to be assigned to PglG, homology with the Pgl proteins in other organisms point to it being another glycosyltransferase. PglI has been reported as being more hydrophobic than the other glycosylatransferases and may have a transmembrane domain in its C-terminal (Glover *et al.*, 2005). The protein adds the final single branched glucose to the heptasaccharide (Glover *et al.*, 2005). PglK was originally known as WlaB until its established role in *N*-linked glycosylation resulted in a change of name (Alaimo *et al.*, 2006). The *Campylobacter* periplasm had already been identified as the site for protein

glycosylation during research into the consensus sequence on the glycoprotein AcrA (Nita-Lazar *et al.*, 2005). This posed the question as to how the heptasaccharide was translocated from the cytoplasmic side of the inner membrane into the periplasm. Here comparisons with eukaryotic models for glycosylation led to an understanding of the system in *Campylobacter*. It was found that PglK exhibits a relaxed specificity with respect to the target oligosaccharide, even to the extent where it will transport incomplete chains (Nachamkin, 2008). The functional requirement for PglK appears to be a 2-acetamido group that is linked to UDP (Alaimo *et al.*, 2006). The process is ATP-dependent and the presence of an ATP-binding cassette classifies the PglK as an ABC-type transporter. Research also revealed that translocation of the glycan is not a spontaneous process (Nachamkin, 2008), though little is known about events which may stimulate 'flipping'. There are a large number of free oligosaccharides (fOS) within the periplasm and this is a widely-distributed feature within the Proteobacteria (Bohin, 2000). The synthesis of glucans has been shown to increase under hypoosmotic conditions (Bohin & Lacroix, 2006). It has been suggested that fOS in the periplasm are key to antibiotic resistance, pathogen-host cell interaction and biofilm formation (Mah *et al.*, 2003; Page *et al.*, 2001). The number of fOS has been reported to vary in accordance with the phase of growth and to decrease in high levels of salt and sucrose (Nothaft *et al.*, 2009). With fOS implicated in pathogenicity and in particular host cell interaction the question arises as to whether the glycosylation of certain virulence-associated proteins is really necessary for their function.

1.4 Metabolism

1.4.1 Gluconeogenesis and glycolysis

It has been known for some time that *C. jejuni* is unable to ferment hexose sugars such as glucose and fructose (Smibert, 1984). However, genes encoding enzymes in the Embden-Meyerhof (EM) pathway are present (Figure 1.4) (Parkhill *et al.*, 2000). Most forward reactions in the pathway are reversible yet *C. jejuni* does not possess a gene that could encode 6-phosphofructokinase (Parkhill *et al.*, 2000).

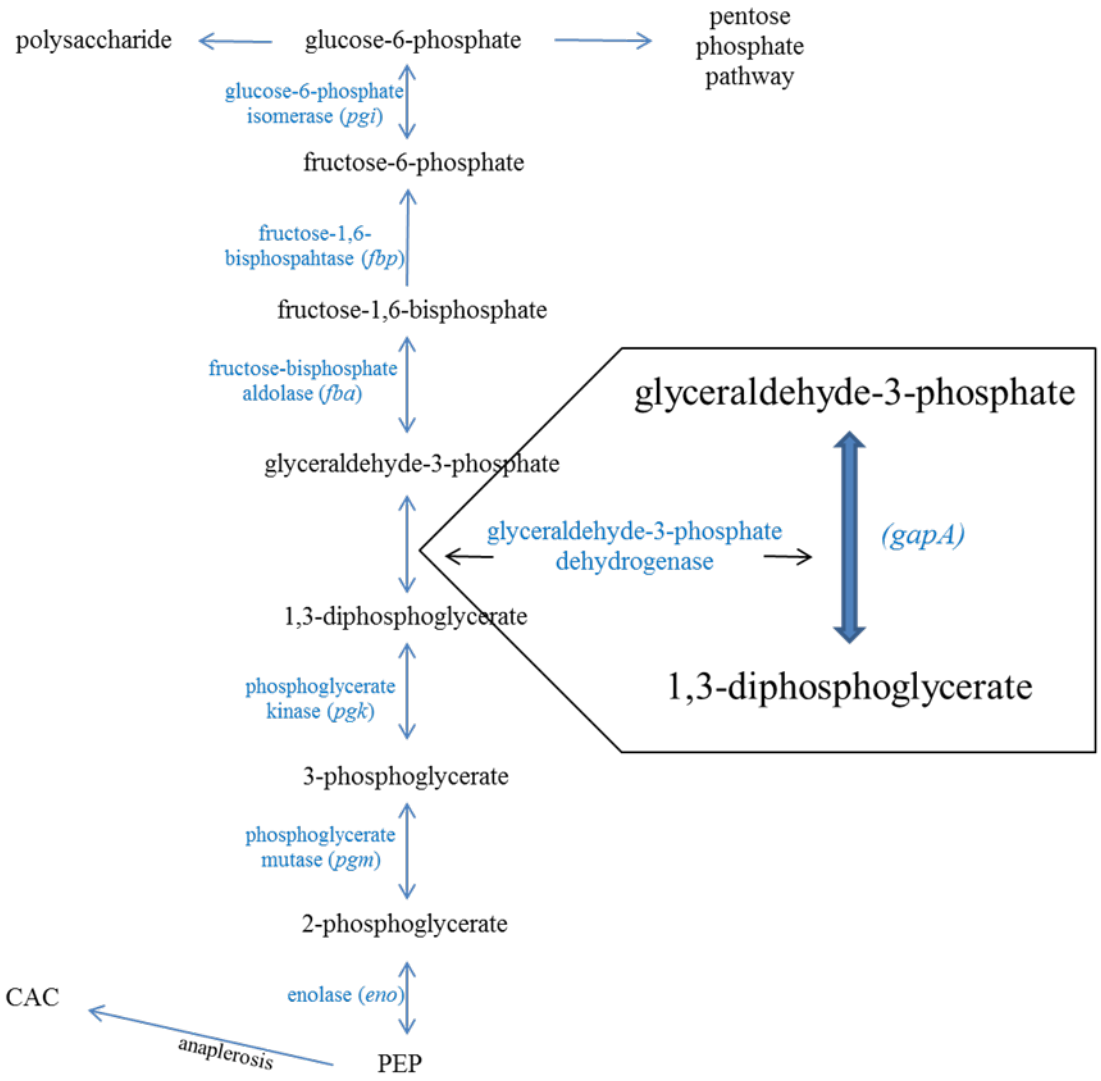


Figure 1.4. The Embden-Meyerhof pathway in *C. jejuni*. The pathway is intact with the exception of 6-phosphofructokinase which means that there is no glycolysis, although all enzymes required for gluconeogenesis are present. Enzymes are shown in blue with the respective genes named in brackets. (Parkhill *et al.*, 2000; Velayudhan & Kelly, 2002).

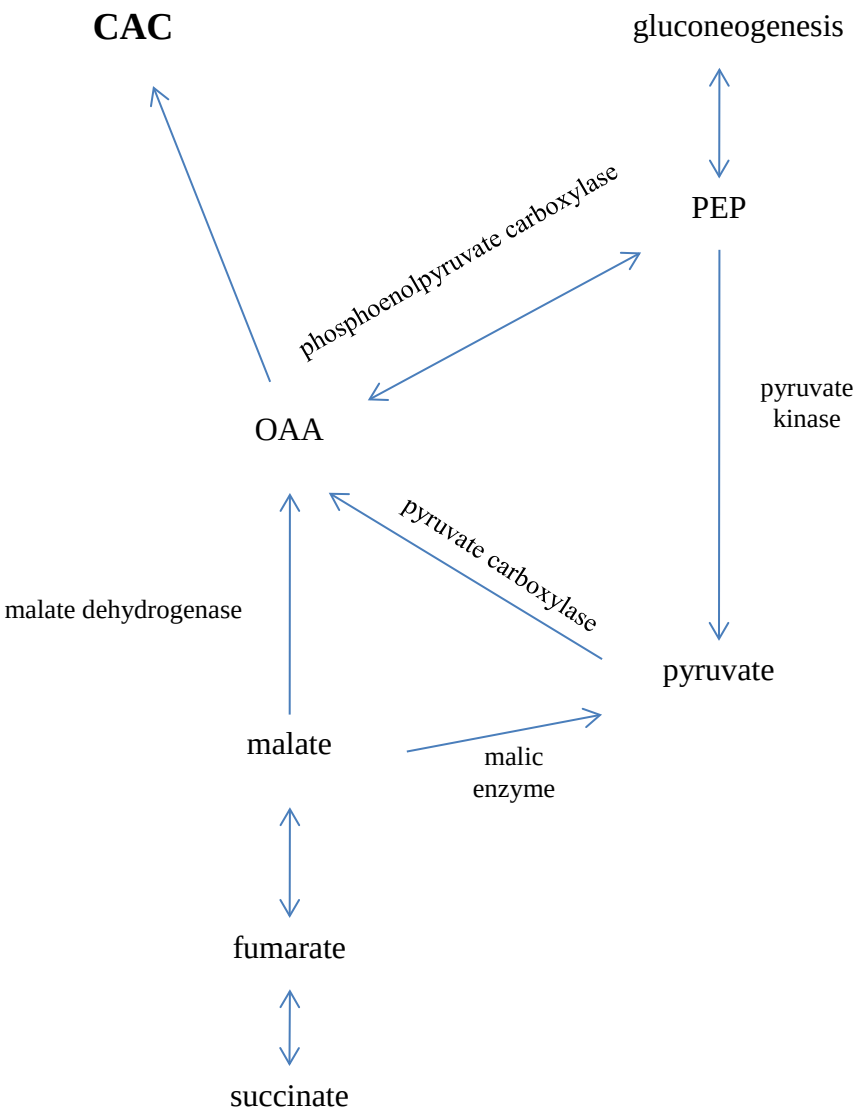


Figure 1.5. The anaplerotic reaction in *C. jejuni*. The reaction replenishes intermediates for the CAC in addition to providing PEP for gluconeogenesis. Figure adapted from (Velayudhan & Kelly, 2002)

This could explain why the bacterium is unable to utilise glucose as a carbon source (Velayudhan & Kelly, 2002). The fact that the rest of the EM pathway is intact would suggest that its purpose is in fact gluconeogenesis. Surprisingly *C. jejuni* possesses a homologue (Cj0392c) of pyruvate kinase (PYK) which is needed in the final stage of the glycolytic pathway (Parkhill *et al.*, 2000). PYK normally drives the conversion of phosphoenol-pyruvate (PEP) and ADP to pyruvate and ATP and is also necessary for the regulation of the final part of the pathway (Velayudhan & Kelly, 2002). In the absence of a role for PYK in glycolysis, it is possible that the enzyme functions in an as yet unknown part of the pathway. Pyruvate is required for conversion to acetyl-CoA, which then links into the intact citric acid cycle (CAC) (Kelly, 2001; Parkhill *et al.*, 2000). It is likely then that *C. jejuni* relies upon the catabolism of amino acids to provide a source of pyruvate (Guccione *et al.*, 2008).

1.4.2 Amino acid metabolism

A number of amino acids have been shown to be necessary for *Campylobacter* metabolism following culture in supplemented media (Guccione *et al.*, 2008; Leach *et al.*, 1997). In addition to the strain-specific L-asparagine and glutathione, other amino acids utilised were L-glutamate, L-proline, L-serine and L-aspartate (Guccione *et al.*, 2008). Pyruvate can be produced by the dehydration of serine and a transamination reaction involving alanine (Velayudhan *et al.*, 2004). Several enzymes have been identified which are involved in the catabolism of amino acids. PutA converts proline to glutamate, which is then transaminated by AspB to form α-ketoglutarate which in turn can directly enter the CAC (Guccione *et al.*, 2008). AspA deaminates aspartate to produce fumarate which is also able to enter the CAC (Guccione *et al.*, 2008). Malate is

converted directly to pyruvate by MEZ, the anaplerotic enzyme (Parkhill *et al.*, 2000; Sauer & Eikmanns, 2005). The ability of *Campylobacter* to utilise glutamate, proline and serine in particular is pertinent as these are the amino acids most commonly found in chicken faeces (Parsons *et al.*, 1982). Of the aforementioned amino acids, serine has been shown to be the most utilised (Leach *et al.*, 1997; Mendz *et al.*, 1997). This could be because serine is readily converted to pyruvate by serine dehydratase (Mendz *et al.*, 1997). A gene encoding the dehydratase (*sdaA*) has been characterised in *C. jejuni* and the gene is required for effective colonisation of chickens (Velayudhan *et al.*, 2004).

1.4.3 Electron transport

C. jejuni is able to use a number of terminal electron acceptors as well as the more commonly utilised molecular oxygen (Kelly, 2001). Studies investigating the electron transport system have highlighted the complexity of respiration in this organism. It is clear from these studies that it is the possession of a highly branched system that allows *C. jejuni* the flexibility to carry out reactions using different electron donors and acceptors (Sellars *et al.*, 2002). Donors include malate, lactate and succinate (Hoffman & Goodman, 1982). Genome analysis reveals various reductases and dehydrogenases likely to be implicated in respiration (Parkhill *et al.*, 2000). The work of Harvey and Lascelles (1980) and Lascelles and Calder (1985) enabled the identification of a number of cytochromes that function as electron carriers and terminal oxidases. Two cytochrome types, b and c, in addition to menaquinone are responsible for electron flow in *Campylobacter* (Moss *et al.*, 1984; Parkhill *et al.*, 2000).

1.4.4 The citric acid cycle

The CAC appears to be complete in *C. jejuni* (Parkhill *et al.*, 2000), with notable anaerobic adaptations such as the absence of genes for the pyruvate and 2-oxoglutarate

dehydrogenase complexes (Daucher & Kreig, 1995). The ferredoxin-dependent oxidoreductase present in *C. jejuni* catalyses the oxidative decarboxylation of pyruvate which allows entry of carbon into the CAC (Daucher & Kreig, 1995). The synthesis of oxaloacetate (OAA) from either phosphoenolpyruvate (PEP) or pyruvate is necessary for the complete function of the CAC (Velayudhan & Kelly, 2002). *C. jejuni* appears to possess all the enzymes required for anaplerotic reactions (Parkhill *et al.*, 2000; Velayudhan & Kelly, 2002).

1.4.5 The anaplerotic reactions

Anaplerosis replenishes the intermediates for the CAC and provides PEP for gluconeogenesis (Velayudhan & Kelly, 2002) (Figure 1.5). The key enzymes in the process are pyruvate carboxylase (PYC) and phosphoenolpyruvate carboxykinase (PCK) (Parkhill *et al.*, 2000; Velayudhan & Kelly, 2002). PYC contains biotin and catalyses the carboxylation of pyruvate in an ATP-dependent reaction to give OAA (Velayudhan & Kelly, 2002). PCK catalyses a decarboxylation reaction, namely the ATP-dependent conversion of OAA to PEP (Velayudhan & Kelly, 2002). A third enzyme, malate oxidoreductase or malic enzyme (MEZ) is predicted to convert malate to pyruvate by oxidative decarboxylation (Parkhill *et al.*, 2000; Velayudhan & Kelly, 2002).

1.5 Importance and epidemiology

1.5.1 Overview

Campylobacter infection often presents with gastroenteritis, diarrhoea of varying severity and occasionally vomiting. There are distinct differences in the course of the

disease between industrialised and developing countries, incidence in the latter being largely confined to children under the age of 5 (Blaser, 1997), whilst in developed countries the illness tends to be more common amongst young adults (Nachamkin, 2008). In contrast with industrialised countries, the incidence of *Campylobacter* infection in developing countries shows no apparent seasonal trends. The most common cause of infection in developed countries is the consumption of undercooked meat, particularly poultry, the carcasses of the birds having been contaminated with faecal matter during slaughter. Although less common, it is possible for infection to be acquired from an infected family pet, from contaminated milk or from surface water (Ketley, 1997).

1.5.2 The burden of illness in developed countries.

The burden of illness caused by *Campylobacter* in industrialised countries is considerable (Allos, 2001). The figures for working days lost give a better representation of the cost to these countries than figures for mortality and morbidity. In England and Wales, Health Protection Agency (HPA) data show that despite a general decrease in the reported numbers of *Campylobacter* cases between 2000 and 2004, incidences of infection have increased again since 2004 (see Figure 1.6). It is estimated that the actual number of cases may be as many as 10 times higher than those reported (Adak *et al.*, 2002). Cases are at their lowest during the winter months, with a steady increase from spring to late summer. In England, the lowest incidence of infection is around the London area, with the highest being in the South-West and South-East of the country. In October 2009, the Food Standards Agency (FSA) released the findings of a government-backed survey carried out during 2007/8, into the levels of bacterial contamination of raw poultry on sale within the UK. Of 3274 pieces of raw chicken sampled, 65% were found to contain *Campylobacter*, compared to a 6% level of

Salmonella (Health Protection Agency (HPA) website, accessed 20 August 2010). According to NHS and HPA statistics there are around 300,000 cases of *Campylobacter* infection per annum in the UK, of which approximately 10% are reported to a GP. There are on average 70 *Campylobacter*-related deaths in the UK each year (Adak *et al.*, 2002)(Hospital Episode Statistics). HPA data also show that cases of *Campylobacter* infection are higher than any other bacteria.

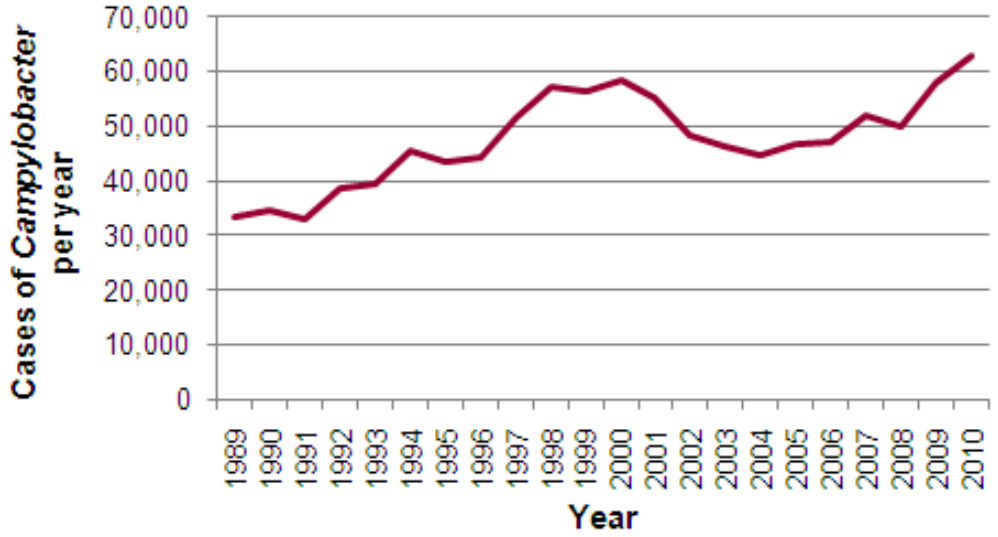


Figure 1.6. Number of reported cases of *Campylobacter* infection in England and Wales reported to the HPA from 1989 to 2010. Graph was re-drawn from data on the HPA website http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Campylobacter/EpidemiologicalData/campyDataEw/

Accessed 2 July 2012

1.5.3 The incidence of Campylobacter infection in developing countries

The true situation of food-borne illness throughout the poorer countries of the world is unknown, so much so that it has become the focus of a new initiative by the World Health Organisation (WHO) (Newell *et al.*, 2010). An estimated 1.8 million people died as a consequence of diarrhoeal disease (WHO). *Campylobacter* infection and the subsequent diarrhoea is generally milder in developing countries and, as stated above, is more prevalent in young children, with many cases being asymptomatic (van Vliet & Ketley, 2001). The difference could be accounted for by earlier immunity through frequent exposure to the organism, as few older children or adults show signs of the disease despite the presence of *Campylobacter* (Ketley, 1997). Unlike those cases in industrialised countries, there appears to be no seasonal trend in developing countries (Ketley, 1997).

1.6 Clinical disease and complications

1.6.1 Clinical presentation and disease progression

Following ingestion of *Campylobacter* the first sign of infection is often acute abdominal pain. This is generally after an incubation period of between 1 to 7 days, with 3 days being the average (van Vliet & Ketley, 2001). Infected individuals often feel generally unwell with fever and malaise (van Vliet & Ketley, 2001). The abdominal pain is frequently accompanied by copious watery, sometimes bloody diarrhoea (Ketley, 1997). Infection is self-limiting and is unlikely to cause any long-term problems to healthy individuals, however there are more serious implications for the

young, elderly or those who are immunologically compromised. In addition to the primary infection, there may also be post-infection complications including the neurological conditions Guillain-Barré and Miller-Fisher syndromes.

1.6.2 Host immune response

As mentioned previously, *Campylobacter* infection is generally self-limiting, with the majority of cases clearing without medical intervention after 7-10 days. Host antibodies are usually produced around day 5 of the infection (Black *et al.*, 1988). Unpublished data show that repeated infections may be required before the host immune response becomes effective (Nachamkin, 2008). In developing countries, it is successive infections during childhood that are responsible for the gradual decrease in the severity of the disease. It has been noted that this response is mediated by an increase in IgA antibody (Blaser *et al.*, 1986). Death from the infection is rare in all areas of the world and is generally restricted to the elderly or those with serious chronic illness (Skirrow *et al.*, 1993). Post-infection complications are uncommon but are potentially serious and include appendicitis, colitis, bacteremia (approximately 0.15%, (Skirrow *et al.*, 1993), hepatitis, pancreatitis, peritonitis and myocarditis. Longer-term complications include reactive arthritis (ReA) and Guillain-Barré syndrome (GBS).

1.6.3 Guillain-Barré syndrome

GBS has now replaced poliomyelitis as the major cause of neuromuscular paralysis in industrialised countries (Nachamkin *et al.*, 1998). The main features of the condition were first described by Landry (1859), with more detailed observations and the naming of the syndrome in 1916 (Guillain *et al.*, 1916). At that time the presence of a large amount of protein in the cerebro-spinal fluid, in association with a normal cell count

were fundamental to the diagnosis of GBS. Approximately two-thirds of GBS sufferers present as having had gastric or respiratory infections in the weeks preceding the illness (van Koningsveld *et al.*, 2000). Typically, GBS will first manifest as numbness in the extremities of the limbs, progressing to a loss of strength in the affected areas. Over the coming days (or in some cases, weeks) the feeling of numbness will progress to a widespread paralysis. Around one-third of patients will require artificial ventilation at this stage due to the involvement of nerves controlling the diaphragm and other respiratory muscles. Despite the apparent severity of this final stage of the condition, mortality rates are low at between 5 and 10% (Ropper, 1988). Some patients go on to make a full recovery, although the return to normal health can be somewhat slow. Most patients are left with some remaining dysfunction and those with severe respiratory problems are likely to have a poorer prognosis.

The association between GBS and *Campylobacter* was first documented in 1984 (Kaldor & Speed, 1984) following the isolation of *C. jejuni* from the faeces of a GBS patient. Although the majority of cases of GBS follow infections with *C. jejuni*, there are several other organisms which have also been implicated, notably *Mycoplasma pneumoniae* and *Haemophilus influenzae* (Houliston *et al.*, 2007; Ju *et al.*, 2004). Having found the main causative agent of GBS to be *C. jejuni*, it became the focus of research in that field to discover the mechanism of disease progression. Within GBS sufferers there are two subgroups that are named depending on the nature of degeneration of the peripheral nerves. These are acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN). Clinically the two present differently in that the progression of AMAN tends to be quicker and the patient's recovery is either very quick or very slow (Cosi & Versino, 2006). Localised

neuronal damage is generally caused by the activation of the patient's complement system by a number of antiganglioside antibodies. This class of antibodies has been shown to be present in GBS patients and their production may be triggered by the molecular mimicry of peripheral gangliosides by *C. jejuni* lipooligosaccharides (LOS) (Yuki *et al.*, 1993). There is still a large disparity between the number of cases of *Campylobacter* infection and the incidence of GBS. The reason for this difference remains unclear though it is thought that it may be through the involvement of other virulence factors. It is possible that the further study of molecular mimicry in *C. jejuni* might facilitate therapeutic treatment for GBS (Komagamine & Yuki, 2006).

1.7 Pathogenesis

C. jejuni and *C. coli* are responsible for the majority of food-borne *Campylobacter* infections, with *C. jejuni* being the causative agent in 90% of those cases (Ketley, 1997). Along with other campylobacters, they are able to colonise the host intestine with great success. This colonisation is dependent upon a number of virulence factors which are expressed at a time appropriate to the stage of invasion. Initially the organism has to survive the acid environment in the stomach in order to pass through to the intestine. Once adhered to the epithelial cells, the bacteria disrupt the normal functioning of the cells, causing widespread damage through instigation of the host's inflammatory response, the release of bacterial toxins and host cell invasion (Ketley, 1997). The process of pathogenesis will be outlined below and will include a description of the associated virulence factors where appropriate.

1.8 Colonisation

In order to be able to successfully colonise its host *Campylobacter* has to be capable of sensing and reacting to its environment. Intestinal epithelial cells are covered by a mucus layer which consists largely of mucin glycoproteins produced by the goblet cells (Deplancke & Gaskins, 2001). The bacteria move along chemical concentration gradients by using coupled chemotactic and motility systems. A number of chemicals, including mucin and L-serine have been shown to have a chemo-attractant effect. In contrast various bile acids have been shown to act as chemo-repellants (Hugdahl *et al.*, 1988). Many bacteria have evolved complex systems for movement inside the host and there are several distinctive methods of bacterial motion. For the majority of bacteria, movement is achieved through use of whip-like flagella (Rajagopala *et al.*, 2007). Movements include swimming, tumbling, twitching and gliding (Wall & Kaiser, 1999).

Much of what we know about chemotaxis and signal transduction originates from studies in *E. coli.* In *E. coli* the chemotaxis system has methyl-accepting chemotaxis proteins (MCPs) which are large trans-membrane proteins. The protein complex consists of the linker protein (CheW) and histidine kinase (HK) (CheA). Large numbers of these complexes in one area facilitate the amplification of the signal from the environment (Ames *et al.*, 2002). Ligand-binding is specific for a range of chemoattractants and in their presence the binding of CheY to flagellar proteins influences the direction of rotation of the flagella and the subsequent movement of bacterial cell (Bren & Eisenbach, 2001). This mode of action means that the cell is able to switch between swimming and a tumbling-like motion, thereby responding to the presence of chemo-attractants or –repellents. This response requires the phosphorylation

of the Che- proteins, CheY and CheA and the dephosphorylation by the phosphatase CheZ.

Campylobacter motility is achieved by movement of a single or two polar flagella and would appear to be necessary for colonisation (Black *et al.,* 1988). These structures coupled with the bacteria's spiral morphology allow easy passage though the mucus covering the gut epithelia (Newell *et al.,* 1985). The flagellum structure itself is encoded by the *flaA* and *B* genes both of which are phase-variable (Caldwell *et al.,* 1985). The *Campylobacter* flagellum is made up mainly (80%) of FlaA protein although FlaB is not redundant as has been shown by mutation studies where *flaB* mutants exhibited reduced motility (Guerry *et al.,* 1991; Wassenaar *et al.,* 1994). FlaC was originally thought to be part of the flagellar structure but this was later shown to be secreted and is currently thought to be involved in host cell interaction (Parkhill *et al.,* 2000; Song *et al.,* 2004). Flagellar assembly and glycosylation are complex processes with the involvement of approximately 50 gene products (Parkhill *et al.,* 2000; Wosten *et al.,* 2004). Surprisingly the sequencing of the *Campylobacter* genome revealed that few flagellar genes were arranged into distinct operons (Parkhill *et al.,* 2000), though this does not appear to cause problems with respect to expressing flagellar proteins. Flagellar biosynthesis is closely regulated with timed protein production and gene expression controlled by the two-component FlgS/FlgR signal transduction system (Wosten *et al.,* 2004). The assembly process is coupled with a post-translational modification, *O*-linked glycosylation (Goon *et al.,* 2003a; Goon *et al.,* 2003b). Indications that *Campylobacter* had a system for glycosylating flagella came from studies of *C. coli* flagellin (Logan *et al.,* 1989). Evidence for the process in *C. jejuni* followed when Doig *et al.* (1996) utilised the affinity of flagellin for lectin as a means of identifying glycoproteins. *O*-

linked glycosylation is specific to the flagella and is an intrinsic part of flagellar assembly. Mutational studies where flagellar glycosylation is eliminated shows that flagella formation is prevented and this results in a non-motile phenotype (Szymanski & Wren, 2005). The modification of flagella in this way is likely to be of significant importance to the bacteria. Once the flagellar proteins are secreted and assembled, the glycan is exposed and in a position to interact with other cells. Gene expression is regulated such that the correct flagellar protein is produced when required by the assembly process (Wosten *et al.*, 2004). The flagellar basal body, hook and filament are also involved in the export of FlaC, the flagellar secreted protein (FspA) and a *Campylobacter* invasion antigen (Cia) (Konkel *et al.*, 2004; Poly *et al.*, 2007; Song *et al.*, 2004).

1.9 Iron acquisition and uptake

1.9.1 Iron chemistry

Iron is essential for most organisms as it is necessary for, amongst other processes, DNA synthesis, the TCA cycle, respiration and electron transfer. It is the fourth most abundant element within the Earth's crust. The increase in oxygen levels in the atmosphere about 2.5 billion years ago, heralded a considerable shift in iron chemistry (Andrews *et al.*, 2003). One of the group of transition metals, iron exists in two states, ferrous (Fe^{2+}) and the insoluble ferric (Fe^{3+}). Interactions involving iron and oxygen can lead to the accumulation of potentially mutagenic oxygen species. Highly reactive hydroxyl groups can proceed to other reactions, causing widespread damage throughout the cell, in particular to DNA, lipid membranes and proteins (Andrews *et al.*, 2003).

Some bacteria have been shown not to require iron; the Lactobacilli were the first of the iron-independent organisms to be described with only one or two iron atoms in each cell, compared to around 10^5 atoms per cell in *E. coli* (Andrews *et al.*, 2003). In addition to the Lactobacilli there have since been other organisms shown not to require iron directly, for example *Borrelia burgdorferi* and *Treponema palladium*, the causative agents of Lyme disease and syphilis respectively (Posey & Gherardini, 2000). Conversely, a number of infections are associated with iron overload, for example individuals suffering from hemochromatosis are vulnerable to infection by *Vibrio vulnificus* (Bullen *et al.*, 1991).

1.9.2 Overview of bacterial iron acquisition

In common with many other bacteria, successful colonisation and infection by *Campylobacter* depends upon the bacterium sequestering iron from its host. Acquiring iron poses a problem for bacteria as it is tightly bound by the host iron-binding proteins such as lactoferrin (Lf) and transferrin (Tf) (in mammals) and ovotransferrin in the avian host, or within complexes such as haem and ferritin. Lactoferrin is a multi-functional protein found in tears, milk and saliva. It is also found within the mucosal layer of the small intestine, where it binds free ferric iron, thus rendering it generally unavailable (Ward & Conneely, 2004). It has been shown that the concentration of Lf can increase where there is infection by enteric bacteria (Qadri *et al.*, 2002).

1.9.3 Uptake of ferrous iron

Ferrous iron is soluble at neutral pH and can diffuse through the outer membrane and into the periplasm before any active transport is required (Figure 1.7). The low pH and reduced oxygen levels within the gut are favourable for the persistence of iron in its

ferrous state. This would imply that it is essential for enteric bacteria to have a ferrous iron uptake system if they are to successfully colonise the gut. Ferrous iron uptake was initially described in *E. coli* with the Feo system, which in *E. coli* includes *feoA, B* and *C* (Hantke, 1987; Hantke, 2004). *E. coli* FeoA and C are small, hydrophilic proteins of 75 and 78 residues respectively. The precise function of both FeoA and C have yet to be determined (Cartron *et al.*, 2006). It has been suggested that FeoA may interact with the

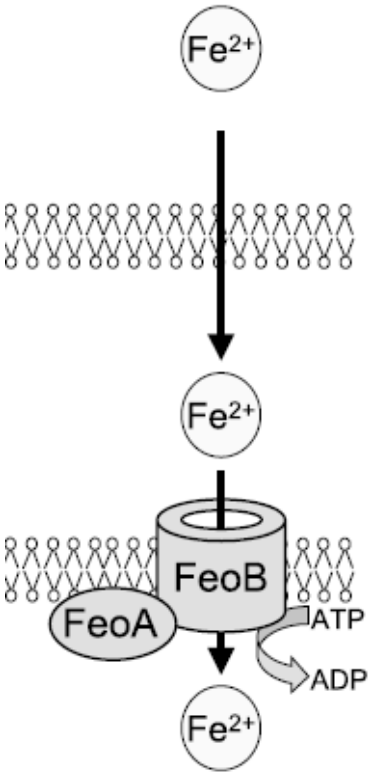


Figure 1.7. Schematic representation of ferrous iron uptake. Ferrous iron is able to diffuse through porins in the outer membrane and into the periplasm. Active transport is required to move the iron into the cytoplasm. This is achieved by the FeoB transporter in an energy-dependent manner. (van Vliet *et al.*, 2002)

negatively charged cytoplasmic membrane as it lacks any recognisable signal sequence (Cartron *et al.*, 2006). It has been shown that *feoA*, *B* and *C* are Fur-repressed with FeoC having a putative role as a transcriptional regulator (Cartron *et al.*, 2006). The key component in the Feo system is FeoB, which is the permease located within the cytoplasmic membrane (Kammler *et al.*, 1993). In *E. coli* the protein has a G-protein region, which has GTPase activity (Marlovitis *et al.*, 2002). Residues 1 to 270 comprise an integral membrane region which contains eight transmembrane domains (Cartron *et al.*, 2006). These membrane-spanning domains are highly conserved between species (Marlovitis *et al.*, 2002).

1.9.4 The Feo system in C. jejuni

C. jeuni NCTC 11168 possesses a *feoAB*-type operon (Parkhill *et al.*, 2000). Initially it was suggested that FeoB was not required for ferrous iron uptake (Raphael & Joens, 2003). However, recently, FeoB has been shown to be necessary for the uptake of ferrous iron in *C. jejuni* strains NCTC 11168, 81-176 and ATCC 43431 (Naikare *et al.*, 2006). Naikare and co-workers also suggested that *feoB* is likely to be PerR-regulated. Previously it had been shown that *feoB* is not Fur-dependent (Holmes *et al.*, 2005). (Fur and PerR are discussed in more detail in section 1.9.20). *Campylobacter* will take up ferrous iron in preference to ferric iron presumably because it is soluble and because of the relative ease with which it is transported; it is not yet known if *C. jejuni* FeoB actually binds ferrous iron, but it is known that FeoB is a cytoplasmic membrane protein of 773 residues (Naikare *et al.*, 2006). Naikare and co-workers showed *feoB* mutants to be restricted in their ability to take up ferrous iron (Naikare *et al.*, 2006). They also made the observation that in the *feoB* mutants the ferrous iron was localised to the

periplasm, suggesting both a role and probable location for the protein (Naikare *et al.*, 2006).

1.9.5 Overview of ferric iron uptake

The transport of iron and its subsequent metabolism were first described in *E. coli* and it is these findings that have provided the basis for the modelling of other bacterial iron uptake systems (Figure 1.8) (Braun, 1985). The known iron uptake systems in *C. jejuni* are shown in Figure 1.9 (Miller, *et al*., 2009). Bacteria express iron uptake proteins depending on the levels of available iron (Andrews *et al.*, 2003). *E. coli* K-12 is known to have the capacity to synthesise no less than six outer membrane receptors. The uptake of ferric iron is dependent upon iron carriers known as siderophores. These compounds have a high affinity for iron in its ferric form (Koster, 2001). Ferri-siderophore complexes then bind to specific outer membrane receptors (Miethke & Marahiel, 2007), where they are transported using energy derived from the cytoplasmic membrane via the TonB-ExbB-ExbD transduction system (Chakraborty *et al.*, 2007; Koster, 2001). The iron is then transported to the cytoplasmic membrane as either free iron or still complexed with the siderophore, by a periplasmic binding protein. Transport across the inner membrane is completed by an ABC transporter system and is ATP-dependent (Moeck & Coulton, 1998).

1.9.6 Siderophore-mediated iron uptake

Under normal physiological conditions there is very little free ferrous iron in the host (Andrews *et al.*, 2003). Having taken up any available ferrous iron and in order to maintain sustained growth, *Campylobacter* must be able to acquire and utilise ferric iron. Many other microbes, when in an iron restricted environment, will synthesise

siderophores. These are low molecular weight iron chelators which sequester bound iron. Analysis of genome sequences has revealed that *Campylobacter* does not possess any genes for the biosynthesis of siderophores (Palyada *et al.*, 2004). A study by Field *et al.* (1986) had suggested that many *Campylobacter* species produced a variety of siderophores though their results have not been confirmed. It has since been shown however that *C. jejuni* is able to exploit a variety of siderophores produced by other organisms (Baig *et al.*, 1986; Palyada *et al.*, 2004; Stintzi *et al.*, 2008).

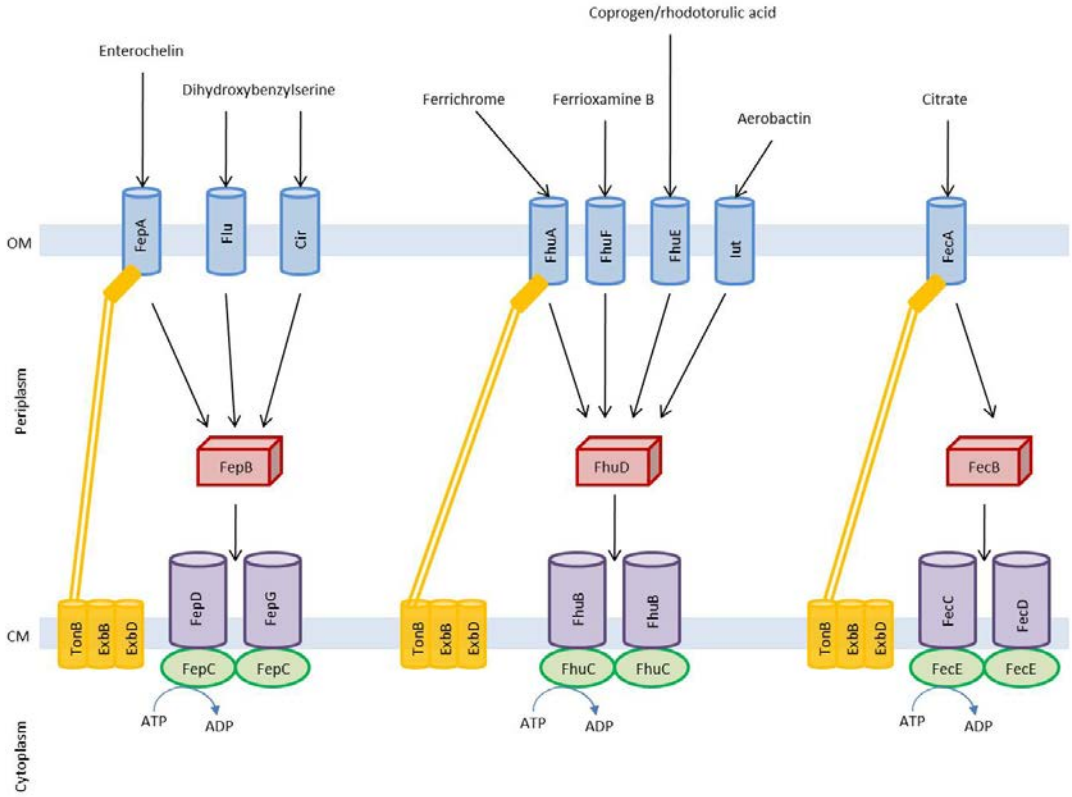


Figure 1.8. Ferric iron transport systems in *E. coli*. Figure adapted from Liang *et al.* (2007). Outer membrane porins (blue shapes), with specificities indicated, transport chelated iron across the outer membrane (OM) using energy transduced via TonB (yellow, illustrated for one member of each group). The chelated iron binds to a periplasmic protein (red) and then is transported across the cell membrane (CM) by ABC transport systems (purple and green).

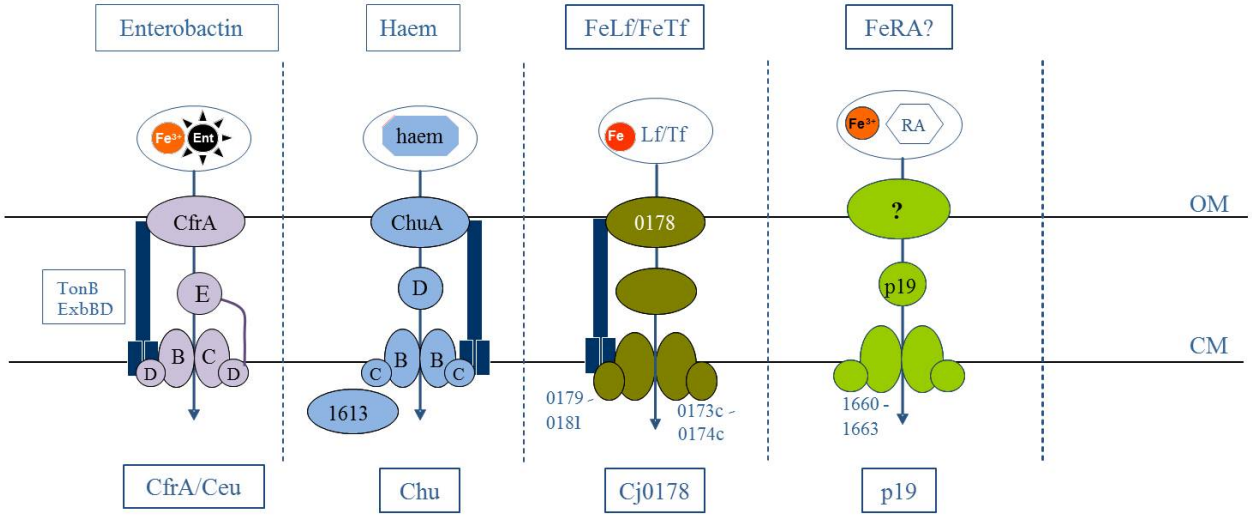


Figure 1.9. Ferric iron uptake systems in *C. jejuni*. Ferric iron is bound by siderophores and enters the periplasm via a ligand-gated porin, using energy derived from a proton motive force (TonB, ExbBD). Once in the periplasm the iron is bound by a periplasmic binding protein and delivered to the cytoplasmic membrane. (Figure adapted from Miller et al., 2009).

The acquisition of iron bound to siderophores means that bacteria need to possess a system for either removing the iron from the chelator or to actively transport the whole complex across the outer membrane prior to removal of the iron intracellularly (figure 1.10). Historically it appears that the earliest direct reference to iron chelators was that of the hydroxamate, aspergillic acid (Dutcher, 1947). Since then approximately 500 siderophores have been described (Andrews *et al.*, 2003). The use of the term 'siderophore' for the whole class of microbial iron chelators was suggested by Lankford (Lankford & Byers, 1973). Siderophores are divided into distinct categories depending on their functional group and/or backbone (catecholate, phenolate or hydroxamate) and within these groups are further sub-groups (Matzanke, 1991). The catecholates include enterobactin and mycobactin (Figure 1.11). The greatest structural variation is observed within the hydroxamate class of siderophores, as they can be either cyclic or linear. Examples within this class are the ferrioxamines (eg. Desferrioxamine B, Desferral™), rhodotorulic acid and the ferrichrome (Figure 1.12).

1.9.7 Enterobactin and the uptake of ferri-enterobactin

Enterobactin is a catecholate siderophore with three functional groups (Figure 1.11). Initially isolated in 1970 from *E. coli* growth medium, it was the first tricatecholate to be described (O'Brien & Gibson, 1970). Biosynthesis of enterobactin has been outlined in several bacterial species including *Salmonella* Typhimurium (Pollack & Neilands, 1970) and *E. coli* (O'Brien & Gibson, 1970). In the latter, chorismic acid is converted to

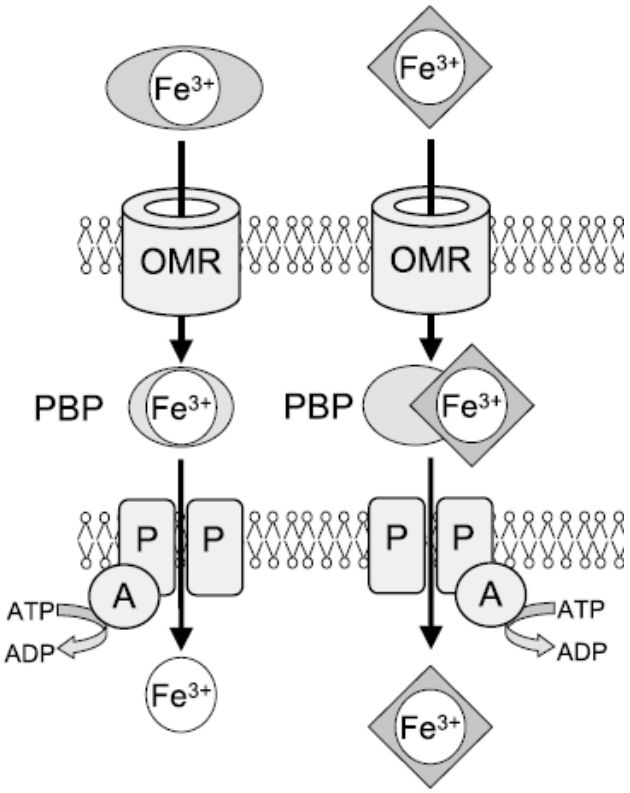


Figure 1.10. General ferric iron uptake. Illustrated (left) is the iron being removed from the chelator at the outer membrane and (right) the ferri-siderophore complex being transported across the membrane and into the periplasm, where it is bound by a periplasmic binding protein (van Vliet *et al.*, 2002).

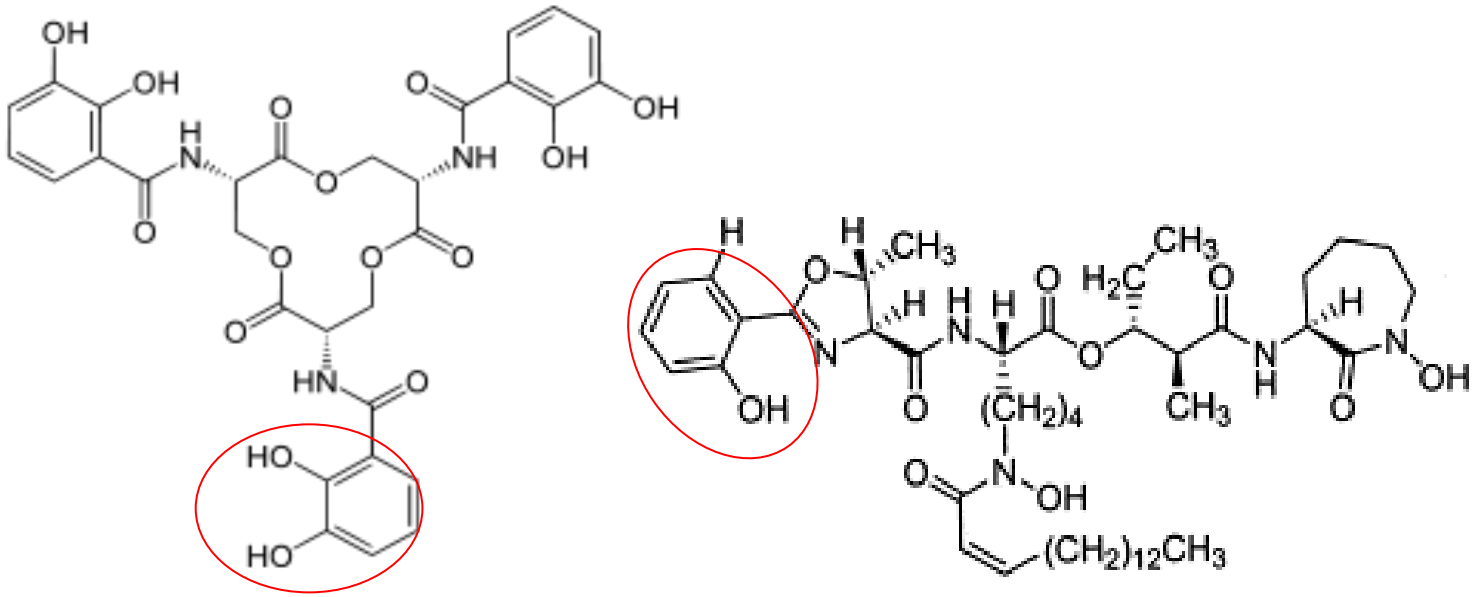
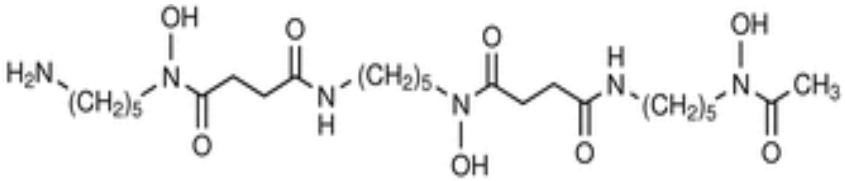
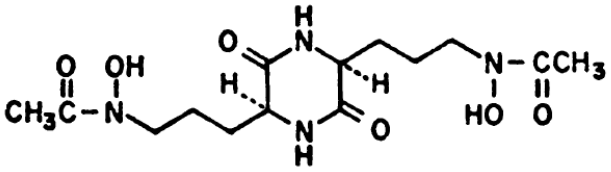


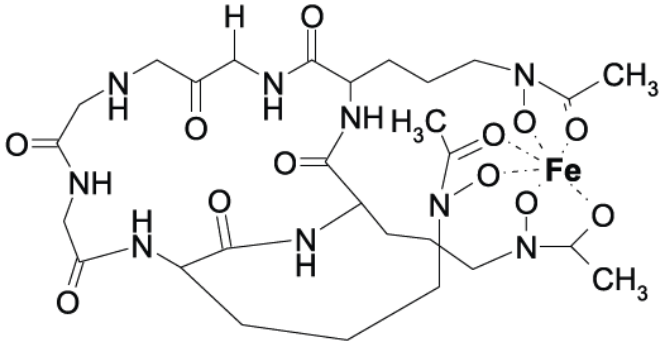
Figure 1.11. Examples of catecholate siderophores, showing enterobactin (left) and mycobactin (right). A single functional group from each siderophore is circled in red



Desferrioxamine



Rhodotorulic acid



Ferrichrome

Figure 1.12. Examples of hydroxamate siderophores. Many hydroxamate siderophores are synthesised by fungal species.

2,3-dihydroxybenzoic acid (DHB) in a pathway involving EntA, B and C (Gehring *et al.*, 1997). In the second part of the pathway, EntE, F and D add L-serine to the DHB to form enterobactin (Shaw-Reid *et al.*, 1999). Enterobactin is the only siderophore to be synthesised endogenously by *E. coli* (Andrews *et al.*, 2003) and not surprisingly the organism has a system to enable the uptake of ferri-enterobactin (Blattner *et al.*, 1997; Cox *et al.*, 1970; Hollifield & Neilands, 1978). The structure of the ferri-enterobactin outer membrane receptor, FepA was reported some time ago (Smith *et al.*, 1998), with the detailed organisation being solved more recently (Buchanan *et al.*, 1999). The residues are arranged into a 22-strand β-barrel with a plug domain in the N-terminus. Ferri-siderophore binding is achieved by the extracellular loops (Raymond *et al.*, 2003) (Figure 1.13). Upon binding, the plug domain undergoes a conformational change which allows the passage of the ferri-siderophore through the channel of the receptor (Usher *et al.*, 2001). Once in the periplasm, the ferri-siderophore complex is bound by the periplasmic binding protein FepB (Sprencel *et al.*, 2000) (Figure 1.8), which delivers the complex to FepD and FepG, the two cytoplasmic membrane transporters (Greenwood & Luke, 1978; Raymond *et al.*, 2003) (Figure 1.8). In common with other periplasmic binding proteins, FepB is able to bind a single ferri-siderophore at any one time, which it shuttles across the periplasm (Koster, 2001).

1.9.8 Ferri-enterobactin uptake in Campylobacter

C. jejuni possesses a ferri-enterobactin uptake system, the outer membrane receptor being CfrA (Carswell *et al.*, 2008; Palyada *et al.*, 2004; Parkhill *et al.*, 2000). The ferri-enterobactin uptake system was first described in *C. coli* (Richardson & Park, 1995).

Homology has been demonstrated (98.7% identity across 696 residues) between the *C. jejuni* CfrA and the ferri-enterobactin receptor in *C. coli* (Palyada *et al.*, 2004).

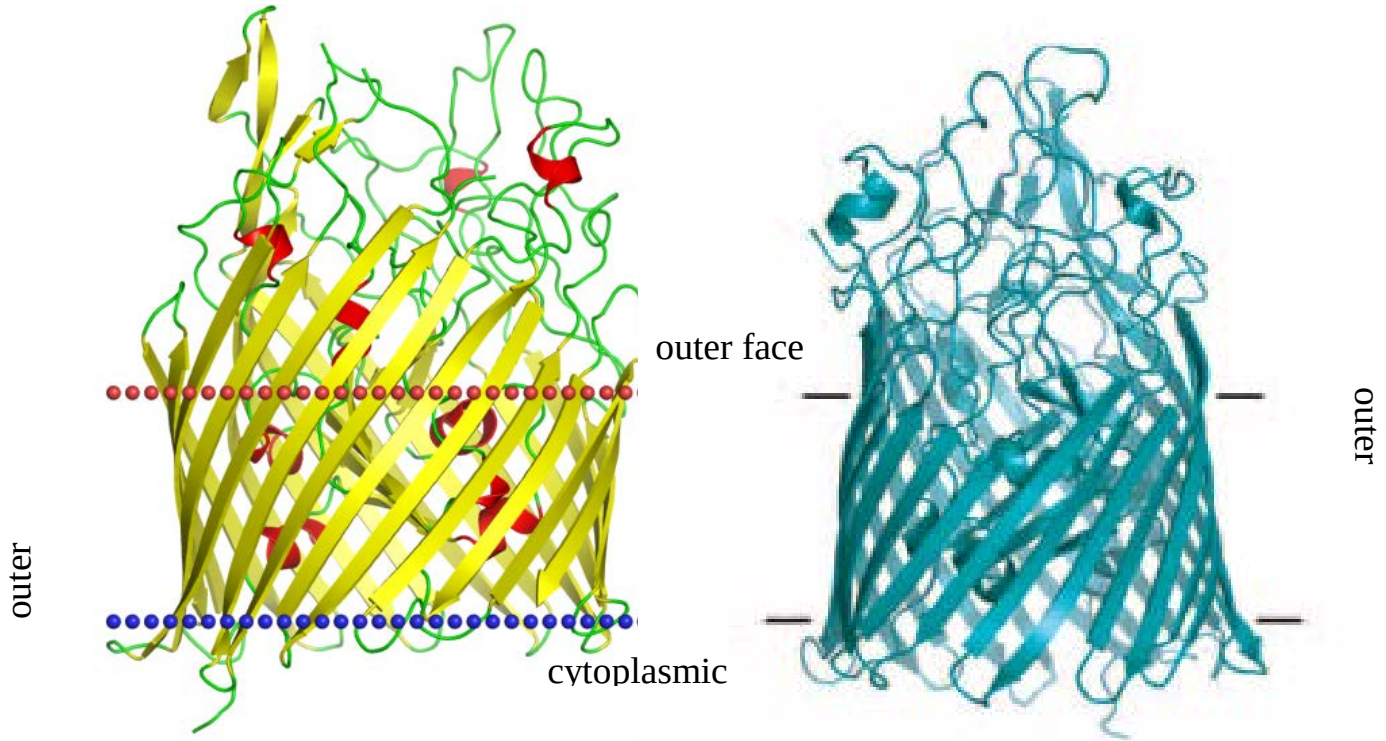


Figure 1.13 Structure of *E. coli* FepA (left) and *C. jejuni* CfrA (right). Both proteins have a similar structure involving a β-barrel with N-terminal plug domain and extracellular loops. FepA and CfrA are involved in the uptake of iron from the siderophore enterobactin (Carswell *et al.*, 2008; Klug *et al.*, 1997).

CfrA has a 24% residue homology to FepA, however they possess the same characteristic long, extracellular loops with shorter periplasmic domains (Carswell *et al.*, 2008) (Figure 1.13). It has also been shown that a *C. jejuni cfrA* mutant is unable to grow where enterobactin was the sole iron source (Palyada *et al.*, 2004). CfrA is typical of many ferri-siderophore transporters in that it has a 22-strand β-barrel structure (Carswell *et al.*, 2008) (Figure 1.13). In addition to the β-barrel, the protein has a plug domain which is thought to be associated with siderophore binding. As its name suggests, the plug is likely to be the part of the receptor that stops the ferri-siderophore from entering the periplasm directly from the environment surrounding the cell. It has also been reported that CfrA may be implicated in the uptake of iron from the transferrins as it is likely that Cj0178 is not exclusively responsible for that role (Miller *et al.*, 2008). One of the three *tonB* genes in NCTC 11168 (*tonB3*) is adjacent to *cfrA* and mutation of *tonB3* removes the strain's ability to utilise enterochelin (A Stintzi, unpublished). CfrA also has an accompanying ABC transport system, CeuB,C and D (Richardson & Park, 1995)(Figure 1.9). Mutations in any of the genes encoding the members of the transport system results in the loss of enterochelin uptake (Richardson & Park, 1995). It is probable therefore that all components of the system are necessary for uptake of iron from enterochelin. CfrA has also been shown to be implicated in growth in *C. jejuni* when grown in an iron-restricted medium supplemented with serum and noradrenaline (Haigh *et al.*, 2008).

1.9.9 Uptake of iron from exogenous siderophores

The uptake of iron from ferrichrome in *E. coli* was described as long ago as 1982 (Neilands, 1982). Ferrichrome (Figure 1.12) is a hydroxamate siderophore synthesised

by the smut fungus *Ustilago sphaerogena* (Emery & Neilands, 1961). In *E. coli*, the uptake of ferrichrome and other ferri-hydroxamates is performed by FhuA, E and F with the periplasmic protein FhuD binding a range of ferri-siderophores and transporting them to FhuB in the cytoplasmic membrane (Figure 1.8) (Ferguson *et al.*, 1998; Koster, 1991). Using these systems, *E. coli* is able to utilise the iron supplied by ferrichrome (FhuA), ferrioxamine B (FhuF), rhodotorulic acid (FhuE) and aerobactin (Iut) (Koster, 1991; Liang *et al.*, 2007). The ABC transporter components FhuB and FhuC each encompass a pair of identical subunits which are located around the cytoplasmic membrane (Andrews *et al.*, 2003). The FhuB modules are integral to the membrane and are protected from proteolysis by the periplasmic FhuD (Mademidis *et al.*, 1997). *E. coli* is also able to utilise citrate, as ferric dicitrate, through FecA, although the organism is known not to produce dicitrate at a high enough concentration to utilise free iron (Braun *et al.*, 1998; Silver, 1996). Having established that *E. coli* can utilise a wide range of ferri-siderophores it is noteworthy that the rate of uptake differs between the hydroxamates, with ferrioxamine B and rhodotorulic acid being transported much slower than ferrichrome and aerobactin (Braun & Hantke, 1991; Winkelmann, 1986). Many bacterial species, including *Campylobacter* engage in so-called siderophore piracy, that is the utilisation of iron through exogenous siderophores, which also increases the competition for resources within the host (Andrews *et al.*, 2003).

1.9.10 Utilisation of hydroxamate siderophores by Campylobacter

Campylobacter lacks any obvious genes for siderophore biosynthesis (Parkhill *et al.*, 2000), although, as stated previously, the organism possesses iron uptake systems which allow it to utilise at least one exogenous siderophore (Baig *et al.*, 1986; Carswell *et al.*,

2008; Palyada *et al.*, 2004). A system which may enable *Campylobacter* to use ferrichrome was described in the strain M129 (Galindo *et al.*, 2001). That study identified a possible *fluA* homologue which was 65% G-C rich (compared to the remainder of the *C. jejuni* genome which is 35% GC). The *C. jejuni* M129 FhuA has 33% homology to the *E. coli* FhuA, but there is no homologue in any of the *Campylobacter* strains sequenced to date (Hofreuter *et al.*, 2006; Parkhill *et al.*, 2000; Pearson *et al.*, 2007).

Recent reports have highlighted a further iron-regulated protein thought to be involved in iron uptake from the hydroxamate siderophore rhodotorulic acid (Stintzi *et al.*, 2008; van Vliet *et al.*, 1998)(Figure 1.9). The p19 system has a 19 kDa periplasmic binding protein, p19, an associated cytoplasmic membrane protein, Cj1658 and a probable ABC transporter system encoded by the genes *cj1661-cj1663* (Parkhill *et al.*, 2000; Stintzi *et al.*, 2008). A corresponding outer membrane receptor has yet to be identified. The p19 system appears to be conserved between sequenced species of *Campylobacter* (Chaudhuri & Pallen, 2006).

1.9.11 Haem utilisation

The majority of iron within the host can be found complexed to haem, which consists of a porphyrin ring with an iron atom in the centre (Andrews *et al.*, 2003; Ridley *et al.*, 2006). Several species of bacteria are able to use haem-based groups as an iron source, indeed in species without the necessary biosynthetic pathways (eg *Bacteroides fragilis*), the requirement for haem becomes absolute (Otto *et al.*, 1990). It is necessary for the haem to be removed from the associated protein before it can be transported. This process has been shown to be siderophore-independent, instead iron uptake relies upon

the action of a number of enzymes, for example haemolysins and proteases which remove the haem and deliver it to the receptor (Andrews *et al.*, 2003; Ghigo *et al.*, 1997). Receptors for haem or haemoproteins have been identified in several bacterial species including HpuAB in *Neisseria meningitidis* (Lewis *et al.*, 1998) and HxuA in *Haemophilus influenzae* (Cope *et al.*, 1998). In Gram-negative bacteria, an ABC transporter system is necessary for the movement of haem across the membrane (Genco & Dixon, 2001). Internalised haem is freed by haem oxygenase (HO) and a number of bacterial HOs have been described including HutZ from *Vibrio cholerae* (Wyckoff *et al.*, 2004) and ChuS from *E. coli* O157:H7 (Suits *et al.*, 2005).

1.9.12 Haem uptake in C. jejuni

C. jejuni is able to use haem, haem complexed with hemopexin, haemoglobin and haemoglobin with haptoglobin (Pickett *et al.*, 1992). An outer membrane receptor of 70 kDa, ChuA, has been identified as being responsible for haem uptake and mutation of *chuA* results in the organism's inability to grow when supplemented with haem alone (Pickett *et al.*, 1992). ChuA has an associated ABC transporter group, ChuB, C and D, although mutation of the genes encoding these proteins does not completely prevent growth where haem is the sole iron source (Ridley *et al.*, 2006). An additional component of the Chu system is ChuZ, a haem oxygenase and an essential enzyme for the removal of iron from haem. *C. jejuni* ChuZ is a homologue of the *Plesiomonas shigelloides* HugZ (Henderson *et al.*, 2001). Mutation of *chuZ* prevents growth where haem is the only source of iron (Ridley *et al.*, 2006). ChuZ has also been shown to break down haem in the presence of ascorbic acid, where the latter is used as an electron donor (Ridley *et al.*, 2006).

1.9.13 The role of the haem oxygenase ChuZ

Haem oxygenase (HO) was first described in 1968, when Schmid and co-workers observed the degradation of haem in the microsomes of rat spleen and liver (Tenhunen *et al.*, 1968). HO binds haem in a ratio of 1:1 and catalyses a reaction which liberates iron from the haem (Yoshida & Kikuchi, 1978; Zhu *et al.*, 2000). From human studies, it is known that the first step in the degradation involves a reductase that reduces ferric iron to its ferrous state (Yoshida *et al.*, 1980). More recently the process has been described in *N. meningitidis* and *N. gonorrhoeae*, where HemO is implicated in protection against iron toxicity (Zhu *et al.*, 2000). In *Neisseria* spp. the end products of the degradation are ferric biliverdin and carbon monoxide (CO) (Zhu *et al.*, 2000). In contrast to *Neisseria*, the breakdown process in *E.coli* O157:H7 produces CO and a ferric-free biliverdin (Suits *et al.*, 2005). In *C. jejuni*, *chuZ* is Fur-regulated, with the region between *chuA* and the divergently arranged *chuZ* containing two possible Fur boxes (van Vliet *et al.*, 2002). This regulation may serve in reducing the risk of iron toxicity through haem build up (Ridley *et al.*, 2006).

1.9.14 Haem biosynthesis

Given the importance of haem in cellular regulatory responses to oxygen and iron it is not surprising that the process of haem biosynthesis is so widespread throughout many forms of life (Woodard & Dailey, 1995). The requirement for haem is generally satisfied by biosynthesis as opposed to acquisition from the extra-cellular environment, although the majority of bacteria possess systems for both uptake and synthesis (Panek & O'Brian, 2002). The precursor to the pathway, δ-aminolevulinic acid or ALA, is synthesised from either glutamate (in the C_5 pathway) or from glycine and succinyl co-

enzyme A (Panek & O'Brian, 2002). As *C. jejuni* possesses *hemA* (*cj0542*), a glutamyl-tRNA reductase that reduces glutamyl-tRNA to glutamate 1-semialdehyde and *hemL* (*cj0853c*), a GSA amino-transferase that converts glutamate 1-semialdehyde to ALA, it is likely that the ALA comes via the C_5 pathway (Panek & O'Brian, 2002). To date no prokaryotic system is known to have both the C_5 and glycine/succinyl CoA pathways (Panek & O'Brian, 2002). The pathway continues in a step-wise manner until it ends with the incorporation of an iron atom into the protoporphyrin ring, the addition performed by ferrochelatase, encoded by *hemH* (Panek & O'Brian, 2002).

1.9.15 Iron uptake from transferrins

Transferrin is found in serum, with lactoferrin being present in saliva, milk and tears (Gray-Owen & Schryvers, 1996; Singh *et al.*, 2002). As stated previously, each protein has the capacity to bind two iron atoms in a reversible reaction (Abdallah & Chahine, 2000; Anderson *et al.*, 1987). Uptake systems for transferrin and lactoferrin have been identified in a number of pathogenic bacteria including *N. gonorrhoeae* and *H.influenzae* (Bruns *et al.*, 1997; Cornelissen & Sparling, 1994). Transferrin/lactoferrin receptors are expressed in low iron environments and have been shown to differentiate between the apo and holo forms of the proteins (Lee & Schryvers, 1988). In *Neisseria* species the receptors are comprised of two subunits that strip iron from the proteins at the surface of the outer membrane (Schryvers & Morris, 1988). The uptake of iron from transferrin and lactoferrin is dependent upon the energy provided by a functional TonB-ExbB-ExbD system with the subunits translocating the iron removed from either lactoferrin or transferrin across the outer membrane (Anderson *et al.*, 1994). Further

transport of the iron is undertaken by a periplasmic binding protein and an associated ABC permease (Andrews *et al.*, 2003).

1.9.16 Utilisation of ferri-lactoferrin and –transferrin by C. jejuni

Initially it was reported that *C. jejuni* was unable to utilise iron from the transferrins (Pickett *et al.*, 1992). Recently however, Cj0178 was identified as a possible outer membrane receptor for lactoferrin- and transferrin-bound iron. Research using liquid growth assays has indicated that the relationship between Cj0178 and transferrin/lactoferrin is proximity-dependent (Miller *et al.*, 2008). This system also has an associated ABC transporter, namely Cj0173c – Cj0175c though mutations in the transporter system genes do not result in the complete abolition of growth where lactoferrin is the sole iron source, suggesting that there may be alternative ABC transport systems available (Miller *et al.*, 2008). The structure of the periplasmic iron-binding protein Cj0175c has recently been solved. Also termed CfbpA, for *Campylobacter* ferric-binding protein, it consists of two domains which are linked by two β-strands (Tom-Yew *et al.*, 2005). It has also been proposed that Cj0178 is implicated in haem uptake as well as the Chu system (Chan *et al.*, 2006), but in contrast to mutations in *chuA*, a *cj0178* mutant is completely unaffected in haem uptake (Miller, personal communication; Miller *et al.*, 2008).

1.9.17 Energy transduction through the TonB-ExbB-ExbD system

The TonB system has been described in numerous Gram-negative bacteria, with the complex being identified as the energy transducer for outer membrane receptors (Larsen *et al.*, 1996). TonB itself cycles between different states which provides the necessary energy required by the outer membrane receptor (Postle, 1993). TonB is connected to

the cytoplasmic membrane by way of an uncleaved signal sequence and interaction between ExbB and TonB, in addition to ExbB and ExbD, influence the conformation of TonB (Letain & Postle, 1997). In *C. jejuni* NCTC11168, a strain that has three *tonB* homologues, the *tonB* gene *cj0753c* is adjacent to *cfrA*, the gene encoding the enterobactin outer membrane receptor (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). Likewise, the gene encoding the lactoferrin/transferrin receptor Cj0178 is adjacent to *cj0179-cj0181*, which encode homologues of TonB, ExbB and ExbD (Parkhill *et al.*, 2000).

1.9.18 The fate of ferri-siderophore complexes

Since the aim of transporting the ferri-siderophore across the outer membrane, periplasm and cytoplasmic membrane is the acquisition of iron, the iron must be liberated from the siderophore once the complex is inside the cell. It is thought that the iron is reduced thereby exploiting the siderophore's lower affinity for ferrous iron (Andrews *et al.*, 2003). This process has been studied extensively in *E.coli*, where the protein product of the *fes* gene, enterobactin esterase, catalyses the hydrolysis of the ferri-siderophore complex before the reductase YgjH, assists in the dissociation of the iron from the siderophore (Zheng *et al.*, 2012). The synthesis and uptake of an endogenous siderophore has resulted in a protein with a number of different functions. However, the processes surrounding the liberation of iron from exogenous siderophores is not so well documented, although it would seem that a reductase is often involved (Hantke, 2001).

1.9.19 Iron storage

With several mechanisms in place to prevent uncontrolled iron accumulation it is useful for bacteria to have the capacity to store iron and thus to avoid the generation of damaging reactive species. Storage also maintains a ready source of iron for times when there is little available. There are three proteins which are known to store iron in bacteria; ferritin, bacterioferritin and a smaller Dps protein. *C. jejuni* has genes which encode ferritin, the Cft protein Cj0612c, and a putative bacterioferritin, Cj1534c (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). Both ferritin and bacterioferritin consist of 24 subunits which have the capacity to bind up to 3000 iron atoms. The proteins take up ferrous iron which is then internally ferroxidised into ferric iron which is then stored (Andrews *et al.*, 2003). Bacterioferritin is exclusive to prokaryotes and contains haem in the form of protoporphyrin IX. It has been reported that in *C. jejuni cj0612c* mutants are more susceptible to oxidative stress indicating a probable role in iron detoxification in addition to iron storage (Wai *et al.*, 1996). It has been shown that the iron stored by ferritin is inactivated preventing the generation of reactive oxygen species (Wai *et al.*, 1995).

1.9.20 The ferric uptake and peroxide stress regulators, Fur and PerR

It is clear that bacteria require systems which are capable of both sensing *and* regulating iron levels in order to grow successfully within the host environment. This regulation is achieved by way of the ferric uptake regulator, Fur. The 'global iron-dependent regulator' has been studied extensively in *E. coli*, where it has been shown to control the expression of in excess of 90 genes (Andrews *et al.*, 2003). Transcription of iron-regulated promoters is inhibited through the binding of Fur to a Fur box which is a

control sequence within iron-regulated genes (van Vliet *et al.*, 2002). *C. jejuni* Fur has been shown to recognise the *E. coli* Fur box, which indicates the extent to which the sequence of the Fur box is conserved (Wooldridge *et al.*, 1994). In addition to transcription from its own promoter *E. coli fur* is transcribed from the promoter of *fldA* (the flaxodoxin gene) at times when the organism is under superoxide stress. When under peroxide stress, transcription from the *E. coli fur* promoter is influenced by OxyR, the peroxide stress regulator (Zheng *et al.*, 1999).

Fur has been shown to regulate the transcription of a total of 53 genes in *Campylobacter* (Holmes *et al.*, 2005; Palyada *et al.*, 2004). Amongst them are the genes which encode the iron transport proteins CeuE, ChuD, p19, ChuA and CfrA (van Vliet *et al.*, 1998), the genes for the TonB-ExbB-ExbD transduction complexes and those for several oxidative stress response proteins (Palyada *et al.*, 2004). The *C. jejuni* response to peroxide stress is mediated by a Fur homologue, PerR and this protein along with Fur may be responsible for the co-regulation of a number of iron-regulated genes, namely *katA* and *ahpC* (Holmes *et al.*, 2005; van Vliet *et al.*, 1999) . As mentioned previously, bacterial cells are likely to come into contact with a number of environmental stressors. These can sometimes be due to a build-up of iron within or around the cells. Reactive oxygen species arise as a result of normal cellular metabolism and are inactivated by superoxide dismutases (SODs). All *Campylobacter* species express one SOD (SodB) which has iron as a co-factor (Pesci *et al.*, 1994). Whilst *sodB* mutants showed no growth defect *in vitro*, they did exhibit reduced cellular invasion and colonisation in a chick model indicating that a functional protein is necessary for successful pathogenesis (Pesci *et al.*, 1994; Purdy *et al.*, 1999). Superoxide dismutation forms peroxides through the activity of SodB, which will produce hydroxyl radicals unless they are effectively

inactivated. *C. jejuni* expresses an enzyme, KatA, which acts upon the peroxide, converting it into water and oxygen. KatA expression is under the control of PerR and is therefore ultimately regulated by iron (van Vliet *et al.*, 1999).

1.10 Stress and the role of noradrenaline

1.10.1 Human gut physiology

In humans the inner lining of the gastro-intestinal (GI) tract is comprised of three layers, namely epithelium, lamina propria (connective tissue) and muscularis mucosae (a thin muscular layer). The epithelium is in direct contact with the contents of the gut and consists largely of columnar, stratified squamous epithelium. Epithelial cells can be considered to be part of the immune response in humans as they possess toll-like receptors (TLRs) which have the ability to recognise LPS and flagellin on the cell surfaces of pathogens (Green & Brown, 2010). Tight junctions form links between the epithelial cells and in doing so prevent leakage from the gut lumen. The lumen surface is an area of high cell turnover, with the sloughing and complete replacement of epithelial cells occurring every five to seven days. The gut is also highly innervated with fibres of the enteric nervous system (ENS) controlling motility and secretions into the lumen, all of which are involuntary (Costa *et al.*, 2000). Sympathetic nerve fibres within the GI tract cause a decrease in gut secretion and motility, thereby slowing the digestive process. These responses are initiated by the neurotransmitter noradrenaline (NA) which binds to α- and β-receptors located throughout the abdomen. NA is synthesised from tyrosine in nerve terminals throughout the body and stored in synaptic vesicles until released through the process of exocytosis. Approximately 50% of the

body's total NA synthesis and use occurs within the ENS (Freestone & Sandrini, 2010). Tyrosine is largely derived from food and consists of a benzene ring with a hydroxyl group and an amine side chain (Freestone *et al.*, 2008). Tyrosine is converted to DOPA through the addition of a hydroxyl group by the enzyme tyrosine hydroxylase, following which DOPA is converted to dopamine by the removal of a carboxyl group by aromatic L-amino acid decarboxylase (Freestone *et al.*, 2008). In the final step of the process, noradrenaline is formed by the addition of a hydroxyl group onto the amine side chain, in a reaction catalysed by dopamine β-hydroxylase (Freestone *et al.*, 2008). Unbound NA overspills and acts upon receptors located throughout the gut. This action inhibits the firing of action potentials and decreases tone and spontaneous nervous activity as well as inhibiting the release of acetyl-choline and other neurotransmitters (Brookes & Costa, 2002). This may account for the higher concentration of NA found within the gut lumen.

1.10.2 The effects of stress

Stress can be physical or psychological, and in some cases both, and can arise through a number of factors including physical illness or injury and fear or other strong emotional reactions. The stress response is controlled by the hypothalamus, which has a direct effect upon the ANS and ENS. This reaction has evolved to initiate a rapid response and is intended to conserve the body's resources in order to aid escape from the stressor. Exposure to prolonged stress will illicit one or more of three distinct stages of behaviour; 'fight-or-flight', resistance reaction and exhaustion. The fight-or-flight is perhaps the most familiar of the responses during which there is dilation of the pupils, increase in heart rate and decrease in blood supply to the GI tract (Reiche, 2004). All

non-essential bodily processes are slowed down in an effort to concentrate the blood supply to vital organs and muscles. If the stressor is not removed the body will progress onto the resistance reaction, during which the body prepares itself for a more prolonged response. This involves the release of cortisol from the adrenal glands which reduces inflammation, triggers the production of glucose for energy and increases gastric acid secretion. If the source of the stress remains, the body will become exhausted. Over-exposure to cortisol quickly results in muscle wastage and ultimately the immune system will become suppressed (Tortora & Derrickson, 2006). At this stage, even if the stressor is removed, the body will not return to normal, rather it stays in the resistance reaction phase for some time, and in doing so remains vulnerable to infection and pathological changes.

1.10.3 Gut physiology and stress in chickens

As the accepted source of many cases of *Campylobacter* infections in developed countries is undercooked or contaminated poultry meat, it is pertinent to discuss how the effects of stress on broiler chickens can manifest in the food chain. As with humans, the avian gut is particularly well innervated and the mechanisms for NA secretion are similar to those outlined above. Previous research has shown that although *Campylobacter* species are a commensal in the avian gut, the organism has the ability to move through the epithelium of the caeca, cloaca or colon to organs such as the liver and spleen (Smith *et al.*, 2008; Young *et al.*, 1999). Translocation has been shown to occur with an inflammatory response in the avian host similar to that seen in human infection (Smith *et al.*, 2008).

As already mentioned, the causes of stress can be physical or psychological. In intensive poultry production, physical causes of stress can arise from over-crowding, physical injury, excessive temperatures, poor hygiene regimes and insufficient or inaccessible food and water. Chickens have a basic socio-psychological need to form distinct social groups and preventing them from doing so can be a cause of acute psychological stress. Other major psychological stressors are the practise of flock-thinning, changes in handlers, the prevention of normal roosting activities and exposure to the 24-hour lighting used in intensive farming facilities. There is now evidence that increases in NA as a result of stress in the host can cause changes in bacteria/host interactions and a resultant increase in bacterial growth. It has been suggested that this is because host stress causes an up-regulation in the expression of tyrosine hydroxylase, normally the point of control in the synthesis of NA (Zhou *et al.*, 2004). Extensive research has been carried out on a number of different bacterial species including *E. coli*, *Pseudomonas*, *Yersinia*, *Salmonella*, and *Listeria* spp, all of which exhibited an increase in growth in the presence of catecholamines (Bailey *et al.*, 1999; Coulanges *et al.*, 1998; Lyte & Ernst, 1992; Sperandio *et al.*, 2003). Much of this early research involved *E. coli* with Lyte and co-workers highlighting the important link between traumatic injury and trauma-induced sepsis (Lyte & Ernst, 1992).

The key to the success of *Campylobacter* infection lies in the organism's ability to invade host cells. The cells of the gut epithelium represent the gateway to the host's tissues and uptake of the bacteria is dependent upon transduction within the host cell itself. The internalisation of bacteria involves the structural re-arrangement of the cell-supporting actin and tubulin. These structural changes are initiated by the bacteria and as a result the bacteria are internalised by the process of endocytosis (Konkel *et al.*,

1992a). Research carried out by Oelschlaeger *et al*. (1993) in which the actin cytoskeleton was disrupted, showed that actin was not necessary for *Campylobacter* invasion. Furthermore, as part of the same study, the authors revealed that internalisation by *Campylobacter* was dependent on microtubules (Oelschlaeger *et al.*, 1993) Extensive studies have suggested that *Campylobacter* invasion is largely dependent upon its ability to bind fibronectin and subsequently utilise microfilaments in order to gain entry directly into the host cell (Konkel & Joens, 1989).

1.10.4 Bacterial growth and catecholamine hormones

As already mentioned, bacterial growth is enhanced by the presence of a number of catechols, including NA. This hormone has also been observed to initiate expression of a number of bacterial virulence genes. Much of the early work in this field involved *E. coli*. Lyte and co-workers first made the observation that trauma patients were at risk of sepsis caused by increases in growth and virulence of indigenous bacteria (Lyte & Ernst, 1992; Lyte & Bailey, 1997). In trauma patients, the release of NA is likely to be as a result of direct neuronal damage in those nerves in and proximal to the site of injury, in addition to the increased release of the hormone from the nerves in the gut as a result of psychological stress caused by pain and fear (Eisenhofer *et al.*, 1996). The widespread therapeutic use of catecholamines in critically ill patients provides an additional supply of the hormones which are likely in turn, to further enhance bacterial growth (Freestone *et al.*, 2002). It was also noted that food withdrawal and surgery increased expression of type I fimbriae in commensal *E. coli*, which presumably led to the observed increase in bacterial attachment to the wall of the gut (Alverdy *et al.*, 1988). The key factor in the promotion of enhanced bacterial growth has been shown to

be the catechol moiety present on the catecholamine hormones NA, adrenaline and dopamine (Freestone *et al.*, 2002). Such is the importance of this structure that modification of the moiety negates the growth enhancing capability of the catecholamine (Freestone *et al.*, 2002). Observed increases in bacterial growth have been remarkable, with one group reporting a 100,000-fold increase in *E. coli* O157:H7 in the presence of NA (Lyte *et al.*, 1996). As detailed in Chapter 3, bacteria have an absolute requirement for iron in order to establish infection and proliferate. It therefore becomes necessary for them to acquire iron from the host. In order to achieve this effectively they must have the ability to interact with host iron-binding proteins. Ordinarily, bacteria would exhibit poor growth in serum-supplemented media due to the presence of transferrin (Tf) which limits the amount of available iron (Freestone & Sandrini, 2010). However, Freestone and co-workers showed that when NA is added to the media, the catecholamine has the ability to complex with the Tf and to provide iron for the growth of *E. coli* (Freestone *et al.*, 2000). The NA-mediated supply of iron to bacteria was also shown to be dependent upon the concentration of iron within the medium and it was reported that there is a decrease in NA-mediated iron provision to *E. coli* with an increase in the concentration of free iron (Freestone *et al.*, 2000). Having established that supplementing media with Tf and physiological levels of NA enhances bacterial growth, the question remained as to whether a similar effect would be observed in the presence of lactoferrin (Lf). Under normal circumstances, the exposure of enteric bacteria to Tf is minimal, therefore it was necessary to investigate the effect of supplementing the growth medium with Lf and NA (Freestone *et al.*, 2000). It was found that *E. coli* also had the ability to utilise iron supplied by a NA-Lf complex (Freestone *et al.*, 2000).

1.10.5 The mechanism of iron provision by NA/Lf and NA/Tf complexes

The first suggestion that NA-enhanced bacterial growth may be connected with enterobactin synthesis or uptake was made by Freestone *et al.* (2000), when it was reported that *E. coli entA* and *entF* mutants failed to grow in a serum-based medium that was supplemented with NA. This observation was supported by Burton *et al*. (2002) when they showed that *E. coli* mutants in both enterobactin synthesis and uptake, *entA* and *fepA* respectively, were unable to respond to NA. The authors also revealed that a *tonB* mutant was similarly affected (Burton *et al.*, 2002). It had previously been shown that radio-labelled hydrogen [^{3}H] complexed with NA was transported into the bacterial cell which led to the proposal of the involvement of a bacterial cell surface receptor (Burton *et al.*, 2002; Freestone *et al.*, 2000). The lack of any obvious specific NA receptors in bacterial genomes (Freestone *et al.*, 2000) had confirmed the likelihood that an iron-uptake system is responsible for the transport of catecholamine-complexes (Burton *et al.*, 2002).

1.10.6 NA-enhanced growth in C. jejuni

The role of NA in enhancing growth in *Campylobacter* has been reported by Cogan *et al* (2007). The increases in growth of *Campylobacter* were comparative to those observed in other bacterial species such as *Pseudomonas aeruginosa*, *Salmonella enterica* and *Yersinia enterolitica* (Bailey *et al.*, 1999; Cogan *et al.*, 2007; Lyte & Ernst, 1992). Cogan and co-workers observed a 50-fold increase in the growth of *C. jejuni* in medium where NA had been added (Cogan *et al.*, 2007). However, although *C. jejuni* lacks any obvious siderophore synthesis mechanisms, the bacterium does have an enterobactin receptor, CfrA (Carswell *et al.*, 2008; Palyada *et al.*, 2004; Parkhill *et al.*,

2000)(Chapter 3). Recent research carried out within the group has shown that the NCTC11168 outer membrane receptor CfrA is responsible for NA-mediated iron uptake (Haigh *et al.*, 2008). The work has also revealed that two other catecholamines, adrenaline and dopamine, can enhance growth of *C. jejuni* in serum-supplemented medium (Haigh *et al.*, 2008).

1.11 Adhesion and invasion

It has been shown that *C. jejuni* and *C. coli* are able to adhere to cells without invading them (Everest *et al.*, 1992), however the opposite may not be true (Ketley, 1997). A number of different surface structures have been implicated in binding to gut epithelial cells. Many other bacterial species rely on fimbrial structures for adhesion but despite early reports of *Campylobacter* fimbrae (Doig *et al.*, 1996b), the lack of any obvious fimbriae-related genes revealed these to be probable artefacts (Gaynor *et al.*, 2001). Two *Campylobacter* surface proteins, PEB1 and PEB3 have been reported as being antigenic (Pei & Blaser, 1993). PEB1 is also able to adhere to HeLa cells and may therefore have a function in colonisation (Pei & Blaser, 1993). Investigation of *Campylobacter* host cell invasion frequently utilises intestinal cell lines such as Caco-2, HeLa and INT407 (Everest *et al.*, 1992; Wassenaar *et al.*, 1991). Internalisation may require the Cia proteins. CiaB was the first to be described, with *ciaB* mutants exhibiting reduced cell invasion (Konkel *et al.*, 1999). Significantly the gene is present in 98% of patients with *Campylobacter* infection (Datta *et al.*, 2003). Host cell processes such as endocytosis may also aid in *Campylobacter* internalisation

(Wooldridge *et al.*, 1996). *Campylobacter* translocation into the epithelial cell has been reported by several groups (Everest *et al.*, 1992; Grant *et al.*, 1993; Konkel *et al.*, 1992b). Some bacteria pass between the epithelial cells using the tight junctions whilst others penetrate the cell layer (Everest *et al.*, 1992; Konkel *et al.*, 1992a). Once inside the cell the bacteria may be met with a lysosomal response, although this can take 3 hours (Demelo *et al.*, 1989). There is generally a decline in the number of bacteria after 6 hours with a significant reduction in the number of bacteria after 36 hours (Demelo *et al.*, 1989).

1.12 Toxins

A number of toxins have been described in *C. jejuni* (Wassenaar, 1997) with cytolethal distending toxin (CDT) being the most widely documented (Pickett *et al.*, 1994; Pickett *et al.*, 1996). CDT in *C. jejuni* was first detailed in 1988 when Johnson and Lior tested cultured cell lines for distension in the presence of the toxin (Johnson & Lior, 1988). CDT interferes with the cell cycle of host cells by blocking the G_2/M stage of the cycle thereby preventing the cell entering mitosis. This is achieved by the toxin sub-unit CdtB eliciting DNA damage and subsequently causing cell death (Bang & Madsen, 2003). This action results in the epithelial damage, inflammation and fluid leakage which characterises a *Campylobacter* infection. CDT consists of three subunits, CdtA, B and C, with CdtB being the active component. The levels of CDT produced appears to vary between *Campylobacter* species and between hosts (Asakura *et al.*, 2007). The toxin causes distension in HeLa and Caco-2 cells which in turn causes the death of the cell (Johnson & Lior, 1988; Whitehouse *et al.*, 1998).

1.13 Hypothesis, aims and objectives

The primary purpose of this project was to investigate the hypothesis that the glycosylation of proteins involved in iron uptake in *Campylobacter* was not necessary for their function in that process. The aims were to investigate the relationship between glycosylation, iron-uptake and stress. Although the topics have been studied singly in the past, there have been no documented reports of the study of their inter-relatedness, in particular the question of whether *C. jejuni* proteins need to be glycosylated in order to function. The aims of the work can be summarised as follows:

1. Using previously well-documented ferric iron uptake systems to ascertain whether glycosylation is a functional necessity in such systems by:
   (a) Constructing relevant glycosylation mutants
   (b) Investigating similarities and differences between wild-type *C. jejuni* and the mutants obtained from 1(a) above.
   (c) Comparing the growth patterns of iron and glycosylation mutants alongside wild-type *C. jejuni*.
2. Investigate the role of the host stress hormone noradrenaline (NA) by further studying the finding that NA-mediated growth response involves iron transport system(s).
3. To establish whether iron-mediated growth involves other known proteins

In consideration of the hypothesis and aims, the objectives were to construct *pgl* mutants and investigate their ability to grow throughout a range of conditions, using available iron-uptake mutants for comparison. In addition, the role of NA was studied and the role of GAPDH in iron acquisition investigated.

Chapter 2: Materials and methods

2.1 Bacterial strains, their growth conditions, media recipes and additives

All media were supplied by Oxoid (Basingstoke, UK) and chemicals by Sigma-Aldrich, UK, unless otherwise stated.

2.1.1 Escherichia coli

The *E. coli* strains used throughout this study were grown routinely aerobically at 37 ºC on Lysogeny Broth (LB or LB agar) (Roth, 1970)(Bertani, 2004) and supplemented, where necessary, with appropriate antibiotics to select those cells harbouring the desired recombinant plasmid. The strains are detailed in Table 2.1. Blue/white colony screening was achieved by the addition of X-gal (5' bromo-4' chloro-3' indolyl β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) to the LB agar. Liquid cultures were generally performed overnight (12-16 hours) on a large flat-bed shaker at 200 rpm (New Brunswick G10 Gyrotory® shaker).

2.1.2 Campylobacter jejuni

The *C. jejuni* strains used in this study were routinely cultured on Mueller-Hinton agar (MHA). Liquid cultures were grown in MH broth or Minimal Essential Medium (MEMα, GIBCO®, Invitrogen, UK) with shaking at 500 rpm (Vibrax VXR, IKA, Germany). All media were supplemented with vancomycin and trimethoprim and the presence of a resistance gene cassette was selected for by the addition of chloramphenicol or kanamycin; the concentrations of the respective antibiotics are shown in Table 2.2. All *C. jejuni* strains were grown under microaerobic conditions

(85% N_2, 10% CO_2 and 5% O_2) at 42 °C in a variable atmosphere incubator (VAIN and VA1000; Don Whitley Scientific, Shipley, UK).

Table 2.1. Bacterial strains and plasmids used throughout this study

Strain	**Description**	**Source**
E.coli		
DH5α	F$^-$Φ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17, (r_K^- m_K^+), *gal*$^-$, *pho*A, *sup*E44, λ$^-$, *thi*$^-$1, *gyr*A96, *rel*A1	Invitrogen
DS941	AB11157 *recF argR ::*5Tn	Summers & Withers, 1990
BL21 (DE3)	F$^-$ *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5])	Derived from B834 (Wood, 1966)
Rosetta (DE3) pLysS	F$^-$ *ompT hsd*S_B(R_B^- m_B^-) *gal dcm* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind*1 *sam*7 *nin*5]) pLysSRARE (Cam^R)	Lab stock strain
BL21 (DE3) CodonPlus-RIL	deficient in *lon* and *ompT* proteases; contains plasmid encoding *argU*, *ileY*, and *leuW*	Stratagene
Rosetta	lactose permease (*lacY*) mutant, deficient in *lon* and *ompT* proteases; contains plasmid encoding *argU*,	Novagen

	argW, glyT, IleX, leuW, metT, proL, thrT, thrU, and *tyrU*	
C. jejuni		
NCTC11168	Genome strain	(Parkhill *et al.*, 2000) National Collection of Type Cultures, Colindale, London, UK
81-176	Clinical isolate	(Black *et al.*, 1988; Korlath *et al.*, 1985)
SLH1	NCTC11168 Δ*pglI*::*cat* (cassette derived from pcatwlaJf (G. Marsden)	This study
SLH2	NCTC11168 Δ*pglB*::*kan* (transformed using pLM35a	This study and LA Millar (Millar, 2003)
SLH3	NCTC11168 Δ*gapA*::*cat* (cassette derived from pAV35)	This study and A van Vliet (van Vliet *et al.*, 1998)
KAR2	NCTC11168 *Cj0178*::*aphA-3* (Km^R)	Miller *et al.* 2008
KAR3	NCTC11168 *p19*::*aphA-3* (Km^R)	Miller *et al.* 2008
JDR5	NCTC11168 Δ*chuA*::*cat* (Cm^R)	Ridley *et al.* 2006

Δ*pglK*	NCTC11168 *pglK*::*kan* (Km^R)	G Thacker, Dept of Infectious and Tropical Diseases, LSHTM.
Δ*Cj1658*	NCTC11168 *Cj1658*::*cat* (Cm^R)	Dr R D Haigh (unpublished)

Plasmids

pUC18	Cloning/suicide vector (Amp^R)	New England Biolabs
pUC19	Cloning/suicide vector (Amp^R)	New England Biolabs
pcatwlaJf	Derived from pUC19. Contains Cm^R cassette with SmaI ends.	G Marsden
pAV35	pBluescript vector containing Cm^R cassette and promoter.	(van Vliet *et al.*, 1998)
pJMK30	pUC19 containing the *C. coli* kan^R cassette flanked by multiple cloning sites	(van Vliet *et al.*, 1998)
pRDH255		R. Haigh
pSH1	Derived from pUC19. Contains NCTC11168 *pglI* region	This study
pSH2	Derived from pUC19. Contains NCTC11168 *pglB* region	This study
pSH3	Derived from pSH1. *pglI* disrupted by Cm^R cassette from pcatwlaJf	This study
pSH4	Derived from pUC19. Contains NCTC11168 *gapA* region	This study
pSH5	Derived from pSH4. *gapA* disrupted by Cm^R cassette from pAV35.	This study and (van Vliet *et al.*, 1998)

pSH7	Derived from pUC19. Contains NCTC11168 *hemE* region	This study
pSH10	Derived from pSH7. Contains *hemE* disrupted by kan^R cassette from pRDH255	This study and R Haigh
pTrcHISB	Expression vector for N-terminal His-tag protein expression (Amp^R)	Invitrogen
pTrc1613c	*Cj1613c*-His_6 expression vector	(Ridley *et al.*, 2006)
pET151D/gapA	pET151D expression vector with NCTC11168 *gapA* gene	P. Moody
pWKS30		Lab stock plasmid

2.1.3 Growth media additives

Table 2.2. Additives to growth media and their concentration

Additive	**Stock concentration**	**Final concentration**
Trimethoprim	5 mg/ml in 50:50 ethanol:dH_2O	5 µg/ml
Vancomycin	10 mg/ml in dH_2O	10 µg/ml
Ampicillin	100 mg/ml in dH_2O	100 µg/ml
Kanamycin	50 mg/ml in dH_2O	50 µg/ml
Chloramphenicol	20 mg/ml in ethanol	20 µg/ml
X-gal	20 mg/ml in DMF	40 µg/ml
IPTG	0.1 M in dH_2O	0.2 mM

2.1.4 Media and buffer recipes

Agarose: To make 400 ml of 0.8% agarose for gel electrophoresis, 3.2 g of LE agarose (Lonza, UK) were added to 400 ml 1 × TAE. Once melted and cooled, ethidium bromide (EtBr; Fisher Scientific, UK) solution (10 mg/ml) was added to a final concentration of 5% (v/v).

Binding buffer for protein purification: 20 mM sodium phosphate buffer, 20 mM imidazole and 500 mM NaCl.

Campylobacter Electroporation Buffer (CEB): 0.272 M sucrose (Fisher Scientific) and sterile glycerol 15% (v/v) added to dH_2O; this was autoclaved before use.

Cell lysis buffer: 50 mM TRIS (pH 8), 1 mM EDTA (pH 8) and 100 mM NaCl.

Electrophoresis loading buffer: 50× TAE 10% (v/v), 70% (v/v) glycerol 18% (Fisher Scientific), Orange G 0.3% (w/v) in dH_2O.

Elution buffer for protein purification: 20 mM sodium phosphate buffer, 500 mM NaCl and 500 mM imidazole.

Induction medium (Sambrook, 2001): 10× MOPS salts 10% (w/w), glucose 0.2% (w/v), casamino acids 0.2% (w/v)(Becton Dickinson (BD)France), 20 µg/ml adenine, 0.5 µg/ml thiamine, 0.1 M neutral phosphate buffer.

Lysogeny broth medium: Contains tryptone 1 % (w/v), yeast extract 0.5% (w/v) and NaCl 1% (w/v) in dH_2O. The medium was adjusted to a pH of 7.5 with 5 M sodium hydroxide (NaOH) and autoclaved before use. Solid medium was achieved by the addition of 1.5% (w/v) Bioagar (Biogene Ltd) before autoclaving.

Mueller-Hinton agar: Contains beef infusion (dehydrate) 30% (w/v), casein hydrolysate 1.75% (w/v), starch 0.15% (w/v) and 1.7% (w/v) agar. This was made up by adding 15.2 g of the prepared powder (Oxoid, Basingstoke, UK) to 400 ml dH_2O, which was then autoclaved and allowed to cool below 55°C before the addition of selective antibiotics.

Mueller-Hinton broth: Contains beef infusion (dehydrate) 30% (w/v), casein hydrolysate 1.75% (w/v) and starch 0.15% (w/v). This was made by adding 8.4 g of prepared powder (Oxoid) to 400 ml dH_2O and autoclaving before use. The broth was subsequently cooled below 55°C before adding antibiotics.

M9 minimal salts medium: 1 × M9 Salts, 40% (v/v) sterile glucose solution, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 4% (v/v) sterile yeast extract solution, thiamine/proline supplement (total 10.16 ml in 400 ml sterile H_2O). The supplement was required where the host strain is Δ*lac_proAB*. These ingredients were added to tap water in order to provide essential trace elements before the medium was autoclaved.

M9 agar: As above for M9 minimal salts medium with the addition of 1.5% (w/v) Bioagar (Biogene Ltd).

SOC medium for recovery of cells following transformation: Bacto-tryptone 2% (w/v), bacto-yeast 0.5% (w/v), NaCl 0.05% (w/v) and 2.5 mM KCl. This was autoclaved before adding 0.36% (w/w) sterile glucose solution and 2 M MgCl.

TES Buffer: 10 mM EDTA in 10 mM TRIS and sucrose 25% (w/v)

T4 DNA Ligase buffer (×10): This buffer was added to the ligation reaction to a final concentration of ×1 and consisted of 660 mM Tris, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP. The pH was adjusted to 7.5 and the buffer stored in 20 µl aliquots at -20 °C.

2 × Rapid ligation buffer: The following were added together: 60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% PEG 8000.

10× MOPS salts (Sambrook, 2001)**:** 400 mM MOPS (pH 7.4), 40 mM tricine (pH 7.4), 0.1 mM $FeSO_4.7H_2O$, 95 mM NH_4Cl, 2.8 mM K_2SO_4, 5 µM $CaCl_2.2H_2O$, 5.3 mM $MgCl_26H_2O$ and 0.5 M NaCl. This was made up in dH_2O then filter-sterilised.

RNase A: Product as supplied by Qiagen was dissolved at 10 mg/ml dH_2O and then boiled for 15 minutes to inactivate the DNases. The solution was stored in 20 µl aliquots at -20 °C.

2.2 Genetic manipulation

2.2.1 Plasmids

The cloning throughout this study was routinely carried out using the plasmids pUC19 and pUC18 (amp^R; New England Biolabs - NEB). The plasmids were maintained in *E. coli* DS941or DH5α and isolated after overnight incubation in 5 ml LB by using a Qiagen Mini-Prep kit (QIAgen) according to the manufacturer's instructions (with minor modifications). A number of plasmids were used in order to obtain their antibiotic resistance cassettes. Protein expression was carried out using the pET and pTrc expression vectors and the *E. coli* BL21, BL21(DE3) and BL21 Rosetta expression strains. All plasmids are listed in Table 2.1 and plasmid maps are shown in the Appendix.

2.2.2 Chloroform extraction of DNA

Extraction of *C. jejuni* genomic DNA was achieved using the following method; *C. jejuni* cells were cultured and passaged once onto three selective MHA plates before

being harvested by adding 3 ml phosphate buffered saline (PBS) and centrifuged for 20 minutes at 3202 *g* at room temperature. The cells were then re-suspended in 9.5 ml PBS, 2 ml 10% SDS, 100 µl 20 mg/ml Proteinase K (NEB) and 20 µl 10 mg/ml RNase A (QIAgen). These were then incubated at 37 ºC for 1 hour after which were added 1.8 ml 5M NaCl (pre-heated to 37 ºC) and 1.5 ml CTAB/NaCl (10% Cetyl Trimethyl Ammonium Bromide) with 0.7 M NaCl). The solution was then incubated for 20 minutes at 65 ºC. To this solution was added an equal volume of 24:1 chloroform:isoamylalcohol; the solutions were then mixed before being centrifuged at 4 ºC for 20 minutes at 3202 *g*. The latter step results in 3 distinct layers, the top layer (clear phase) was removed and the DNA precipitated with 70% isopropanol. The DNA was carefully 'spooled' to a sealed 1000 µl pipette tip before being washed in 70% ethanol and re-suspended in 1 ml elution buffer (EB) (QIAgen) at 37 ºC overnight. The DNA/EB mix was divided into 50 µl aliquots before the concentration was ascertained using a spectrophotometer (Jenway Genova Mk3 Life Science Analyser - Geneflow, UK or Nanodrop – Thermo Scientific). Alternatively, when a quicker protocol was required, the PUREGENE® DNA Purification Kit (Gentra Systems Inc. – QIAgen, UK) was used according to the manufacturer's instructions with the following exceptions; one plate of overnight culture was harvested with 500 µl MHB and centrifuged at 14,000 *g* for 5 seconds to pellet the cells. During the RNase treatment stage, the tube was inverted 50 times and the sample incubated for 30 minutes. During protein precipitation the sample was vortexed for 40 seconds (Vortex – Gilson GV Lab®). This was followed by centrifugation for 5 minutes and incubation on ice for 10 minutes. For

DNA precipitation only fresh 100% Isopropanol was used. DNA yield was measured as stated above.

2.2.3 Ethanol precipitation

To each 20 µl ligation reaction the following were added, 2 µl 3 M sodium acetate (at pH 5.2), 1 µl glycogen (Roche Diagnostics GmbH, Germany) and 50 µl 100% ethanol. These were left at room temperature for 45 minutes before being spun at 14,000 *g* for 15 minutes. The supernatant was discarded and the smeared pellet washed carefully with 1 ml cold 70% ethanol. The pellet was then centrifuged for 1 minute at 14,000 *g*. After the ethanol was discarded, the open tube was placed in the fume hood to allow complete evaporation of any remaining ethanol. The pellet was then re-suspended in 10 µl dH_2O.

2.2.4 DNA ligation

A ligation reaction typically utilised a ratio of 3:1 insert to vector molecules (a 5:1 ratio being used for 'blunt-ended' reactions) and used 0.4 units of T4 DNA ligase (at a concentration of 400 U/ml) per 20 µl reaction. T4 acts by promoting the formation of phosphodiester bonds between the 5' phosphate and 3' hydroxyl groups in DNA (Sambrook, 2001). Ligations with cohesive-ended digest products were left overnight (or longer) at 16 °C. Blunt-ended ligations were carried out overnight at room temperature. The T4 DNA ligase was inactivated by heating to 65 °C for 10 minutes.

2.2.5 Restriction enzyme digests

Digests were typically performed using ~400 ng of DNA. All restriction enzymes and buffers were obtained from New England Biolabs (NEB, UK) the volume used being

appropriate to their unit concentration and reported efficiency. Bovine serum albumin (BSA; NEB) was used at a final concentration of 100 µg/ml. The cutting enzyme was inactivated in accordance with the specific instructions for that enzyme. Where required vectors were dephosphorylated using Shrimp Alkaline Phosphatase (SAP; Roche) to prevent re-ligation. This was achieved by adding 1 µl SAP to the sample following digest and incubating at 37 °C for 30 minutes. The SAP was inactivated by incubation at 65 °C for 15 minutes. Following digest and dephosphorylation (where performed) the reaction was purified using a Minelute column (QIAgen) according to the manufacturer's instructions.

2.2.6 Polymerase chain reaction (PCR)

PCR was performed using an Eppendorf Mastercycler® (Eppendorf, UK) or G-Storm GS1 thermocycler (GRI, UK) with annealing time and temperatures appropriate to the primers used. Reaction volumes were normally 20 µl and the reaction parameters are shown in Table 2.4.

Table 2.3 Polymerase chain reaction volumes

Component	**Volume**
Template DNA	1 µl Concentrations **For genomic DNA:** 10-50 ng for 0.1-3kb targets 50-100 ng for 3-10kb targets **For plasmid DNA:** 0.1-1 ng for 0.1-3kb targets
Forward primer (2 pmol. μl^{-1})	2 µl
Reverse Primer (2 pmol. μl^{-1})	2 µl
10× PCR buffer (contains 25 mM Mg^{2+})	2 µl
dNTPs (contains 10 mM each of dATP, dTTP, dCTP and dGTP)	0.4 µl
Taq polymerase (2.5 u. μl^{-1})	0.2 µl
ddH_2O (molecular biology grade water)	12.4 µl

Table 2.4 Standard PCR reaction

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	94 ºC	2 min	1
Denaturation	94 ºC	15 sec	
Annealing	(variable)	30 sec	29
Extension	72 ºC	(variable)	
Final extension	72 ºC	5 min	1

2.2.7 Colony PCR

Colony PCR was employed as an in initial method of screening for transformant colonies. Transformants were sub-cultured onto 'patch plates' and grown overnight before being selected for screening. It was normal practice to have already applied antibiotic selection to these colonies. A small amount of culture from each colony to be screened was removed with a sterile wooden toothpick (typically an amount ~1 mm across). This was added to 20 µl dH_2O and mixed thoroughly. The cell solution was then heated in a thermocycler for 5 minutes at 96 °C before being centrifuged at 14,000 *g* for 2 minutes. A 2 µl aliquot of the resulting supernatant was used as the template in a PCR reaction (components listed in Table 2.5).

Table 2.5 Components of colony PCR reaction

Component	**Volume**
Template: supernatant from colony 'boilates'	2 µl
Forward primer (2 pmol. μl^{-1})	4 µl
Reverse Primer (2 pmol. μl^{-1})	4 µl
10× PCR buffer (contains 25 mM Mg^{2+})	2 µl
dNTPs (contains 10 mM each of dATP, dTTP, dCTP and dGTP)	0.4 µl
Taq polymerase (2.5 u. μl^{-1})	0.2 µl
ddH_2O (molecular biology grade water)	7.4 µl

2.2.8 PCR product purification

PCR clean-up was carried out using Qiagen QIAquick, MinElute, or DNEasy columns according to the manufacturer's instructions.

2.2.9 Visualising DNA by gel electrophoresis

All PCR products, plasmid preparations, restriction enzyme digests, DNA ligations and chloroform extraction of DNA was visualised by gel electrophoresis using the recipe as described previously. LE agarose was added to 1 × TAE before being melted in a standard microwave oven. This was then allowed to cool before adding ethidium bromide as per recipe. The molten agarose was stored medium-term in a 55 °C oven. In order to visualise the DNA a sample was loaded into wells along with 2 µl gel electrophoresis loading buffer per 10 µl sample.

2.2.10 Primers

Primers were used at a concentration of 2 pmol/µl. The primers used during this study are detailed in Table 2.6 below.

Table 2.6 Primers used throughout this study

Primer name	**5' – 3' sequence**	**Comments**
wlaDf_PstI	AACTGCAGGCAGGAGATGGACAGTTAAG	Amplification of *pglI*
wlaDr_EcoRI	GGAATTCCTTAGAATTAGGCAAGATTAAATCAG	Amplification of *pglI*
pglI_test_fwd	AGTGATAAGGTGTATTATGAAAG	Verification
wlaDinv1_SmaI	TCCCCCGGGCATGAGCATAACCCTCATTTG	Inverse PCR - *pglI*

wlaDinv2_SmaI	TCCCCCGGGGGCAAGATAGCGTGACTTTAG	Inverse PCR - *pglI*
wlaFfwd_BamHI	CGGGATCCGCCCTTGAAGACATAGC	Amplification *pglB*
wlaFrev_EcoRI	GGAATTCGTCCTAAATTCCGCATAAAC	Amplification *pglB*
pglBtest	TGTATTCATCTTGCGGCACTATAAC	Verification
wlaFinv1_SmaI	TCCCCCGGGAAGGATACCACCAAAGATAAATTC	Inverse PCR – *pglB*
wlaFinv2_SmaI	TCCCCCGGGGCAAGACTTAGTGTAGAATATACAG	Inverse PCR – *pglB*
wlaFinva	ATTCGCACTTTCATCGCTTC	Inverse PCR – *pglB*
pglK_fwd	GTGCACATTTAGCGGCTTTGG	Mutant verification
pglK_rev	CGTGTCAAATCTAAATTGTTC	Mutant verification
M13_F	GTTGTAAAACGACGGCCAGTG	Insert verification
M13_R	GGAAACAGCTATGACCATGATTAC	Insert verification
CatR_Kpn	GGGGTACCGAATTCAGCTGCGCCCTTTAG	Cat-specific primer (C E Miller)
cminvF_SmaI	TCCCCCGGGCTAATACAATCATCGAAACAAAAC	Inverse PCR - *pglB*

cminvR_SmaI	TCCCCCGGGGGTGATTAATTCTAGAGATGCTAAAG	Inverse PCR - *pglB*
pglB_ver1	GGCGCTTTATAGTTGTAAATATGG	Mutant verification
kan	CAAGTGGTATGACATTGCCTTCTG	Kan-specific primer
SkanR	GGTTATTGTCCTGGGTTTCAAGCATTAG	Kan-specific primer
STM invkan-F	CTGGGGATCAAGCCTGATTG	Kan-specific primer
invkanF	GATGGCAAAGTAAGTGGC	Inverse primer for Kan – L. Millar
invkanR	CCTCAAATGGTTCGCTG	Inverse primer for Kan – L. Millar
Pav35F_NotI	ATAAGAATGCGGCCGCCGGCGGTGTTCCTTTCC	pAV35 primer with *Not*I site
PAV 35_NOT I	ATAAGAATGCGGCCGCAGCTGCGCCCTTTAGTTCC	pAV35 primer with *Not*I site
gapAfwd	CTTGCTCGCTCATGTCAATGG	Amplifying *gapA*
gapArev	GAATAGTTTGTAGGTCGTGCAGGCCTAGGGC	Amplifying *gapA*
gapAinv1	GCCGTAAAGTTAGCAAAGATG	Inverse PCR *gapA*
gapAinv2	CTGCCTTTAAATTCACCATGTACTG	Inverse PCR *gapA*
hemEfwd	GGGGTACCGTTTTAGCAAGATTGGGTATAG	Amplifying *hemE*

hemErev	CTTCTTAAACTTCGTGAATTAGCTGCAGCCAATGCATTGGAA	Amplifying *hemE*
hemEinv1NotI	ATAAGAATGCGGCCGCCGTAAATTCATACCCATTTCAAGAG	Inverse PCR *hemE*
hemEinv2NotI	ATAAGAATGCGGCCGCAATTTTATTCCCTAAAGGCATTAGC	Inverse PCR *hemE*
pTrcHISBf	GTATCGATTAAATAAGGAGG	Verifying pTrcHis vector
pTrcHISBr	CTACTCTCTCGCATGGGGAGAC	Verifying pTrcHis vector
TetF	GATCCCGGGATGCGGTAGTTTATCACAG	Verifying pET151 vector (R Haigh)
pACYCR	CGATGGATCCGGCGTAGAGG	Verifying pET151 vector (R Haigh)

All primers were custom made by Invitrogen or Sigma-Aldrich. Restriction enzyme sites and 5' clamps are underlined.

2.3 Transformation and related procedures

2.3.1 Preparation of competent cells

Two ml of an overnight culture were grown to an OD_{600} of 0.4 – 0.6 and then centrifuged for 15 minutes at 3202 *g* at 4 ºC. Cells were washed in a total of 100 ml of (ice-cold) 50 mM $CaCl_2$. These were centrifuged once more at 3202 *g* at 4 °C, before

being re-suspended in a total of 10 ml 50 mM $CaCl_2$ and 20% (v/v) glycerol. A similar method was employed to prepare competent *E. coli* cells for electroporation.

2.3.2 Chemical transformation

Chemical transformation of *E. coli* relies on heat shocking the cells in order to facilitate the uptake of the plasmid. Transformation was achieved by mixing ~1 ng of plasmid and 50 µl chemically-competent *E. coli*, leaving on ice for 20 minutes and heat-shocking by placing in a 42 ºC water bath for 50 seconds. The samples were cooled on ice for 2 minutes. The cells were incubated at 37 ºC for 90 minutes in 450 µl SOC medium. The cells were spun for 90 seconds at 14,000 *g* and re-suspended in 100 µl SOC.

2.3.3 Electroporation

Electroporation of *E. coli* was performed using electrocuvettes with a 2 mm gap (Cell Projects Ltd, Geneflow, UK) and a BioRad GenePulser (BioRad, UK) set at 2.5 kV, 200 Ω and 25 µF. Electrocuvettes were pre-chilled in a -20 ºC freezer for at least 30 minutes before use. Cells were recovered by immediately flushing out the cuvette with 1 ml SOC (pre-warmed to ~37 °C) and incubating with shaking at 37 ºC for 1 hour. Electroporation of *C. jejuni* is performed in a similar manner and recovery was carried out by adding 0.1 ml SOC and plating the cells onto MHA (with trimethoprim and vancomycin and 5% de-fibrinated horse blood (Oxoid, Basingstoke, UK) and incubating overnight at 37 ºC under microaerobic conditions.

2.3.4 Natural transformation

Natural transformation was achieved by passaging 4 MHA plates with the strain requiring transformation and incubating overnight at 37 ºC under microaerobic conditions. The cells were then harvested using 2 ml MHB and an initial OD_{600} reading was taken. This reading was then used to calculate the 'starting suspension' to add to MHB to make a final volume of 5 ml. Subsequently 0.5 ml of this 'final suspension' was added to 1 ml MHA (plus antibiotics where appropriate) in a 15 ml tube. This was then incubated for 3 hours under microaerobic condtions. Following the incubation, 5 µg 'donor' DNA was added, the tubes agitated gently and placed in the VAIN for a further 4 hours. After the 4 hour incubation, the cells were carefully removed from the surface of the MHA and spread onto MHA plates with the appropriate selection.

2.4 Sequencing

DNA sequencing is performed 'in house' by PNACL (Protein and Nucleic Acid Chemistry Laboratory) using an ABI 3730 DNA Analyser (ABI Applied Biosystems®). The sequencing reaction is carried out using a BigDye®Terminator v1.1 Cycle Sequencing Kit (ABI Applied Biosystems®) on a standard Eppendorf Mastercycler®. The standard settings for sequencing reactions are shown in Table 2.7.

Table 2.7 Sequencing reaction

Step	Temperature	Duration
1	96 ºC	10 seconds
2	96 ºC	10 seconds
3	(5-10 ºC below T_m of primer)	10 seconds
4	60 ºC	4 minutes
5	(Steps 2 → 4 29 times)	
6	60 ºC	5 minutes
7	End	End

Following the reaction it is necessary to perform an SDS clean-up by adding 2.2% SDS (2 µl to a 20 µl reaction) and placing in a thermal cycler at 98 ºC for 5 minutes then 25 ºC for 10 minutes. Any unincorporated dye from the sequencing reaction was removed by using a Qiagen Dye-Ex kit in accordance with the manufacturer's instructions with the exception of drying the sample.

2.5 Growth assays

2.5.1 Liquid growth assays

Liquid growth assays were carried out using either MEMα (GIBCO®, Invitrogen, UK) or chelated (20 μM Deferoxamine mesylate (Sigma)) MHB (Oxoid, UK) (cMHB). Initial overnight cultures were prepared by harvesting cells from 4 confluent swabbed plates with 2 ml PBS and centrifuging at 14,000 *g* for 2 minutes. Pellets were re-suspended in 500 μl of selective medium before samples of the same strain were pooled and their collective OD_{600} readings taken. Overnight cultures were started at an OD_{600} of 0.1 (5 ml cultures, selective MHB, with shaking at 500 rpm (Vibrax VXR IKA, Germany)).

Following overnight incubation, the cells were harvested once more by centrifugation at 3202 *g* for 20 minutes. Pellets were re-suspended in 1 ml selective medium and their OD_{600} readings taken once more. Growth assays were started at an OD_{600} of 0.025 (in 10 ml selective medium. The OD_{600} readings were recorded at 0, 4, 8, 12 and 24 hour time points.

Table 2.16. Additives to growth assay media

Additive*	Strain/s	Concentration†	Comments
Ferri-rhodotorulic acid (FeRA)	*Δp19, ΔpglB, ΔCj1658,* NCTC11168	1 μM	used to compare *ΔpglB* with controls
$FeCl_3$	all	1 μM	positive growth control
RA	all	1 μM	negative control
Haem	*ΔchuA, ΔpglB,* NCTC11168	25 μM	iron source
$FeSO_4$	all	10 μM	iron source/positive control

†Concentration was calculated to provide strains with an adequate iron source to grow successfully. The 1 μM concentrations were calculated as being equivalent to an iron concentration of 10^{-6} M (between 10^{-7} and 10^{-5} M having been established as being necessary for bacterial growth (Andrews *et al.*, 2003)).

*Suppliers: FeRA and RA, Biophore Research Products, EMC microcollections GmbH, Tübingen, Germany. $FeCl_3$, Sigma-Aldrich, UK. Haem and $FeSO_4$ Fluka (Sigma-Adrich, UK). Malate and pyruvate, (Sigma-Adrich)

2.5.2 Plate assays

Plate assays were carried out using chelated (50 μM Deferoxamine mesylate (Sigma)) Brucella agar (BA; BD, France). Cells were harvested from each of 2 confluent plates with 2 ml chelated Mueller-Hinton broth (cMHB). The cells were then centrifuged for 2 minutes at 14,000 *g* and re-suspended in 500 μl cMHB. Cells were added to cooled, chelated BA at 1×10^7 colony-forming units (cfu)/ml (approximate OD_{600} of 0.005). The plates were then incubated under microaerobic conditions for 2 hours. Following the incubation, the surface of the agar was spotted with 5 μl of the additives being tested and the halos measured once the cells had grown (overnight – 24 hours). The method used is similar to that of Pickett *et al.* (1992).

2.5.4 Noradrenaline-utilisation assays

The noradrenaline assays were carried out in MHB, chelated with 5% (w/v) Chelex (Sigma) (cMHB) and supplemented with 10% (v/v) bovine serum (BS). *C. jejuni* cells from 2 confluent plates were harvested by adding 2 ml PBS and centrifuging for 2 minutes at 14,000 *g*. These were re-suspended in 500 μl MHB, Following which samples of the same strain were pooled and their OD_{600} values taken. Initial overnight cultures were set up by using a starting OD_{600} of 0.1. Cells from these initial cultures were added at a cfu of 10^4/ml of medium. The assays were carried out in 24-well plates (one for each time point) and continued over 6, 24 and 48 hours. For each sample reading a plate was removed from the VAIN, the OD_{600} readings were taken and a 200 μl aliquot serially diluted (to 10^{-8}) in a 96-well plate. Following this 100 μl of each dilution were spotted onto plates containing selective MHA. The plates were then incubated in the VAIN overnight. It should be noted that as each additive varied in

colour over time, it was necessary to include 'blanks' in each of the 24-well plates. It also became apparent that there was slight evaporation from the samples from approximately 6 hours onwards. In order to reduce the chances of the evaporation confounding the results the plates were covered with Nescofilm® (Bando Chemical Industry Ltd, Kobe, Japan) at 3 hours.

2.6 Computational methods and software

Initial gene information was obtained from the on-line *Campylobacter* database CampyDB (http://www.xbase.ac.uk/campydb/). Further analysis, primer design, plasmid visualisation and construct design were performed with the aid of Clone Manager Professional Suite v.8 and v.9 (Scientific and Educational Software, 2005). Returned sequence data were analysed using the ABI Sequence Scanner v1.0 (Applied Biosystems® - available at www.appliedbiosystems.com). Additional information about *E. coli* was obtained from ColiBase (http://www.xbase.ac.uk/colibase/). Growth assay data tabulation, graph plotting and statistical analyses were performed using GraphPad Prism®Version 5 (GraphPad Software Inc, San Diego, California, USA). Protein analysis and prediction were performed with the aid of PSORT (http://psort.ims.u-tokyo.ac.jp/form.html), the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do) and the ExPASy Proteomics Server (http://www.expasy.ch/).

Chapter 3: Glycosylation of *C. jejuni* iron uptake systems and the role of noradrenaline

As stated in Chapter 1, the aims of this project were to investigate any association between glycosylation, iron-uptake and stress. There has been no previous study documented as to whether glycosylation is in fact a functional necessity. To re-state the aims from the Introduction:

1. Using previously well-documented ferric iron uptake systems to ascertain whether glycosylation is a functional necessity in such systems by:
   (a) Constructing relevant glycosylation mutants
   (b) Investigating similarities and differences between wild-type *C. jejuni* and the mutants obtained from 1(a) above.
   (c) Comparing the growth patterns of iron and glycosylation mutants alongside wild-type *C. jejuni*.
2. Investigate the role of the host stress hormone noradrenaline on previously investigated growth patterns by further studying the finding that NA-mediated growth response involves iron transport system(s).
3. To establish whether iron-mediated growth involves other known proteins or systems.

The construction of the *pgl* mutants was performed using standard mutagenesis techniques and is outlined in section 3.1. Comparisons in growth between the mutants and wt NCTC11168 were achieved largely through the use of growth assays; these are shown in sections 3.2 and 3.3. The involvement of the host stress hormone NA is

presented in section 3.4 and an outline of the involvement of another protein is detailed in section 3.5.

3.1 The construction of glycosylation mutants in C. jejuni - aim 1(a)

3.1.1 *Mutagenesis of pglI*

Having identified the *pglI* gene using the online database CampyDB, cloning primers were designed with the aid of the Clone Manager program. The primers wlaDf_PstI and wlaDr_EcoRI shown in Table 2.6 were used to amplify the *pglI* gene from chromosomal DNA of *C. jejuni* NCTC11168 in addition to flanking regions of approximately 500 base pairs on either side of the gene. The incorporation of these flanks is important for the successful recombination of the mutated gene in the final stage of the mutagenesis procedure. A different restriction site was added to the 5' end of each of the primers to allow directional cloning of the *pglI* amplicon into the cloning plasmid pUC19. Following purification, separation by gel electrophoresis showed a band of the desired product size of 1.8 kb. Both pUC19 and the resulting PCR product were subjected to restriction enzyme digest using *Pst*I and *Eco*RI. Each stage of the cloning process was checked by agarose gel electrophoresis (Figure 3.1). During planning of the cloning process, thorough checks were made to establish that the chosen restriction sites were not duplicated anywhere, either within the *pglI* amplicon or within other regions of the cloning vector.

The *pglI* amplicon (insert) was ligated with the digested pUC19 (vector) and afterwards the reactions were purified using the ethanol precipitation. Controls were used throughout the cloning process, the ligation control being pUC18 singly digested with

*Bam*HI. This control was subjected to an identical ligation reaction and afterwards was used as a positive transformation control. Distilled water was used as a negative control for the transformations. The purified ligation products were used to transform *E. coli* DH5α cells as per the method outlined in Chapter 2. The transformed cells were grown overnight on LB agar supplemented with ampicillin, X-gal and IPTG to allow blue/white screening. White colonies were picked for patching onto a plate containing selective medium (LB agar plus ampicillin) and grown overnight. These patches were then screened using colony PCR with M13 primers, before recombinants were purified using the method outlined in Chapter 2. Plasmids from the purification were further screened using M13 primers in a PCR reaction in combination with the original cloning primers. The M13 primers were then used in a PCR reaction to sequence any potential recombinants . The sequencing of the initial construct verified it as pSH1 (shown in Figure 3.2). Following the construction of pSH1 containing the *pglI* gene and flanks a portion of the open reading frame (ORF) was deleted and a selective marker inserted. For *pglI* and indeed for most of the mutants made as part of this study, an established inverse PCR mutagenesis strategy was adopted (Dorrell *et al.*, 1996; Wren *et al.*, 1994).

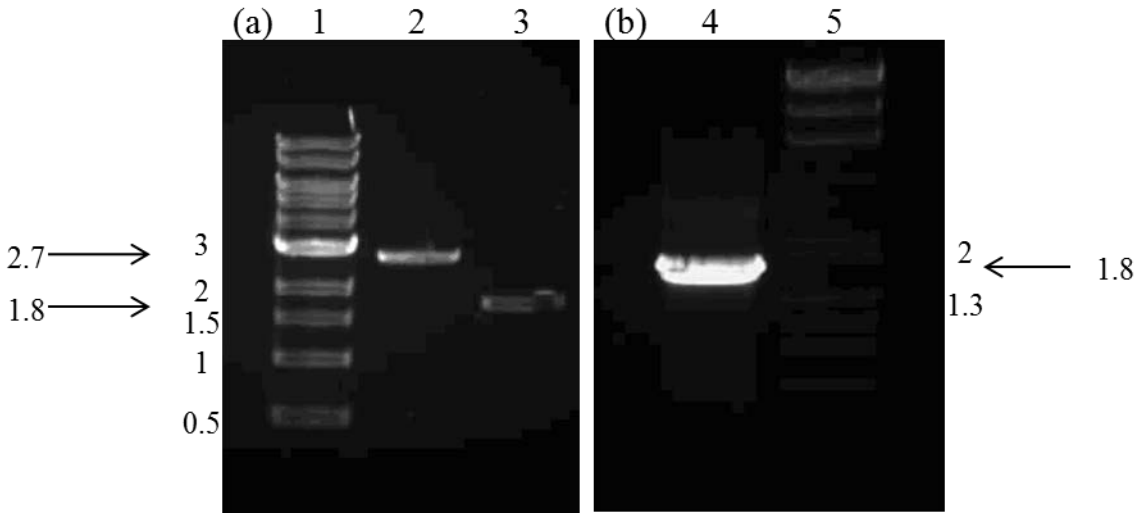


Figure 3.1. Gel electrophoresis of the stages of construction of the mutant strain NCTC11168 Δ*pglI::cat*. Markers are in lanes 1 and 5 and marker sizes are shown in kb. Bands are indicated by the arrows with sizes shown in kb. (a) The result of the digest of pUC19 is shown in lane 2 and the restriction enzyme digest of the *pglI* amplicon (using primers wlaDf_PstI and wlaDr_EcoRI) in lane 3. Both fragments were digested with *Pst*I and *Eco*RI in a double digest reaction. Lane 1 contains 0.5 µg of 1 kb ladder. Fragment sizes are as predicted with pUC19 being 2.7 kb and the *pglI* amplicon 1.8 kb. (b) Lane 4 shows the result of a PCR of the ligation of the digested *pglI* amplicon and digested pUC19 (initial construct). The PCR reaction was conducted using M13 forward and reverse primers which gave the product size of 1.8 kb. Lane 5 contains 1 µg of λφ molecular weight marker for reference.

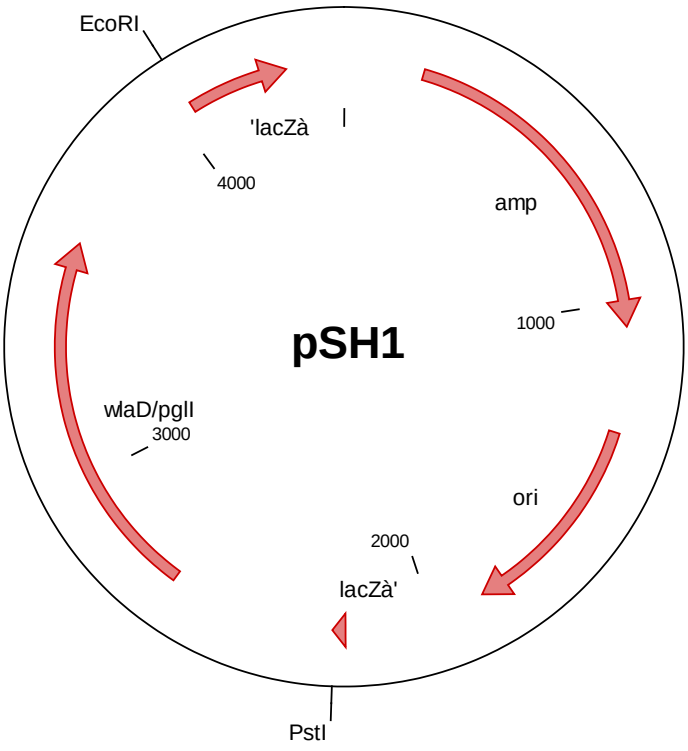


Figure 3.2. Map of the initial construct pSH1. The construct is the result of ligating the *pglI* amplicon and pUC19, both previously restriction enzyme digested with *Pst*I and *Eco*RI. The position of the two restriction sites is indicated on the map. The red arrows represent open reading frames.

A pair of inverse primers, wlaDinv1_SmaI and wlaDinv_SmaI, were designed with the aid of the Clone Manager program. The primers had *Sma*I restriction sites added to the 5' ends and were designed to anneal to regions within *pglI* and facing outwards, and in doing so, delete a total of 309 bp of the gene. The resulting linear product was 4.2 kb and had a *Sma*I restriction site at each end. The amplicon also contained the flanking regions incorporated by the initial PCR reaction. The product of the inverse PCR reaction was visualised using gel electrophoresis and is shown in Figure 3.3(a). The addition of the *Sma*I sites enabled the insertion of a previously chosen selectable marker in the form of a chloramphenicol resistance cassette. The cassette was derived from another construct, pcatwlaJf (G. Marsden) and consisted of the resistance cassette without a promoter. This promoterless cassette was chosen in order to minimise the likelihood of polar effects within the *pgl* locus. The amplified and *Sma*I-restricted chloramphenicol resistance cassette and the *Sma*I-digested inverse PCR product of pSH1 were ligated together. The ligation reactions were purified using the ethanol precipitation method as before. These purified constructs were transformed by electroporation into *E. coli* DH5αe as outlined in Chapter 2. The transformed cells were grown overnight on selective medium (LB agar with ampicillin and chloramphenicol) after which twenty colonies were selected to patch onto a further plate for screening by colony PCR. M13, gene-specific and cat^R cassette primers were used for the screening and the colonies that produced PCR products of the correct size were chosen for overnight culture and purification to obtain the transformant plasmids. The transformants were then verified by sequencing and the result was the final construct pSH3, shown in Figure 3.4.

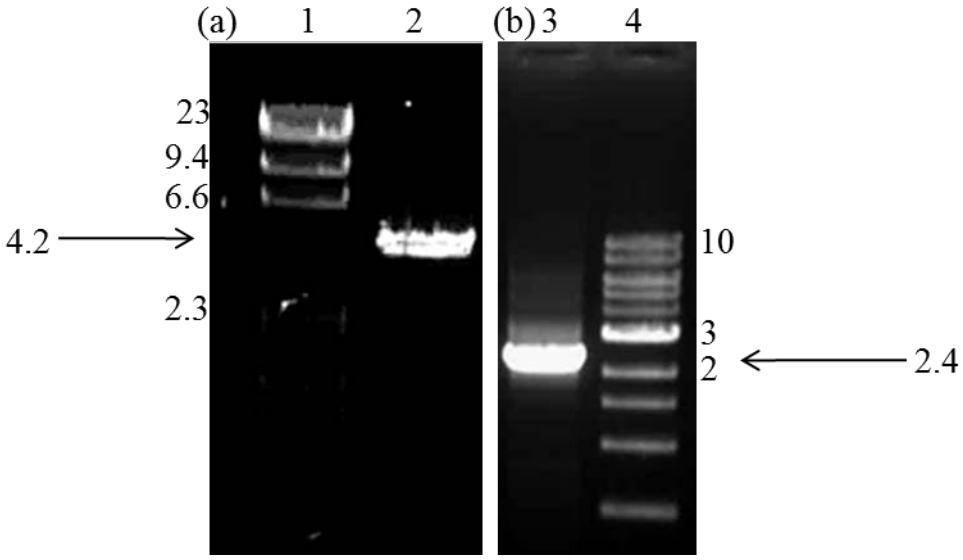


Figure 3.3. Gel electrophoresis of the construction of the mutant strain NCTC11168 Δ*pglI::cat*. All band sizes are given in kb and arrows indicate the position and size of the sample band. (a) Lane 1 contains λφ marker and lane 2 contains the linear product of the inverse PCR of pSH1 with the predicted size being as shown. (b) Lane 4 contains 1 kb marker and lane 3 the band from the colony PCR of *C. jejuni* NCTC11168 *pglI* mutants, with the band being the predicted size of 2.4 kb.

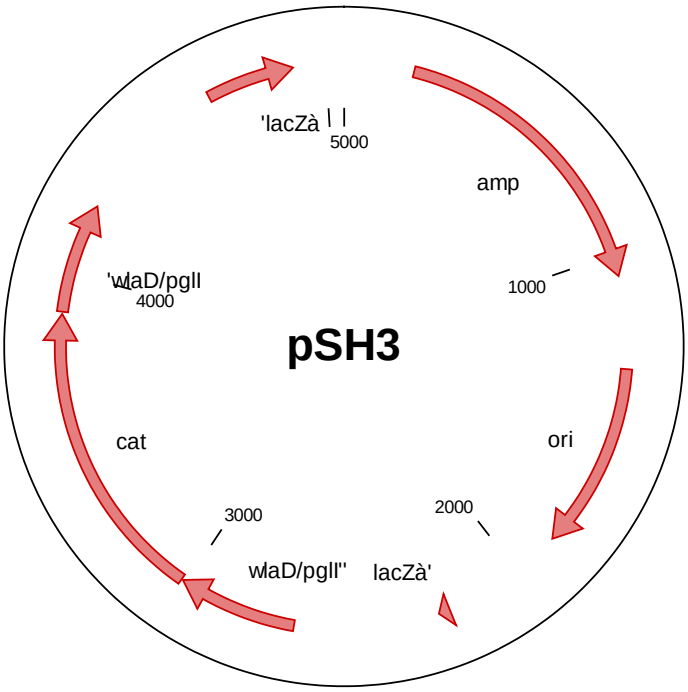


Figure 3.4. Map of the *pglI* final construct pSH3. Construct was made by ligating the enzyme-restricted inverse PCR amplicon of pSH1 and chloramphenicol-resistance cassette derived from pcatwlaJf. Red arrows represent open reading frames.

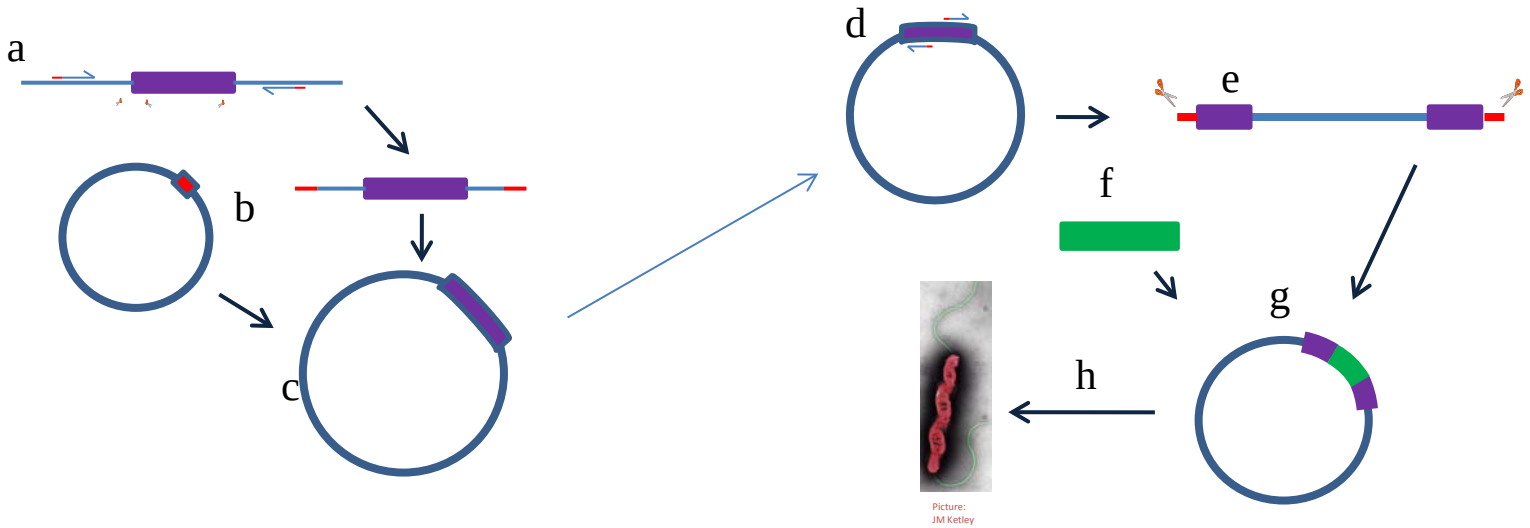


Figure 3.5. Overview of the mutagenesis strategy employed throughout this study. (a) PCR amplification of the gene of interest (shown in purple) plus ~500 bp of flanking region on either side of the gene. (b) Amplicon is digested at the PCR-incorporated restriction sites. The chosen cloning vector (eg. pUC19) was also digested with the same enzyme pair. (c) The amplicon and vector were ligated to form the initial construct. (d) Primers annealed within the gene and amplified outwards to give a linear product with added restriction sites and in so doing, deleted a portion of the gene. (e) Inverse PCR amplicon was digested (indicated by scissors) to match ends of desired antibiotic resistance cassette. (f,g) Inverse PCR amplicon and resistance cassette (indicated in green) were ligated together. (h) Final construct was used to transform *C. jejuni*.

Following the successful sequencing of pSH3, the construct was used to transform wild-type NCTC11168 using electroporation. The cells were recovered overnight on MHA plates containing just vancomycin and trimethoprim. Following this initial overnight recovery, the cells were plated onto medium containing vancomycin, trimethoprim and chloramphenicol. The pUC19-based final construct acts as a suicide vector in NCTC11168 as the strain will not allow stable plasmid maintenance. The previously incorporated flanking regions of *pglI* allow for the replacement of the wild-type gene by double homologous recombination. Successful transformants grew on the selective medium and these colonies were patched onto new plates and screened by colony PCR. Probable transformants were cultured and chromosomal DNA prepared using the technique outlined in Chapter 2. This DNA was then PCR amplified and sequenced to verify that the Δ*pglI::cat* mutant strain had been obtained.

This mutagenesis strategy was adopted throughout the course of this study and for reference is summarised in Figure 3.5. Where mutant strains or constructs were obtained from other members of the group or from other labs, these were verified by screening and sequencing in a similar way to those mutants made specifically for this study.

3.1.2 Mutagenesis of pglB

The identification and amplification of *pglB* was performed in a similar manner to the strategy employed for *pglI*. Cloning primers *wlaF*fwd_*Bam*HI and *wlaF*rev_*Eco*RI shown in Table 2.6 were used to amplify the *pglB* region and flanks from a *C. jejuni* NCTC11168 chromosomal preparation. These primers were also used to introduce terminal *Bam*HI and *Eco*RI restriction sites. Restriction enzyme digestion was carried

out with *Bam*HI and *Eco*RI on pUC19 and the *pglB* amplicon and following purification, the fragments were ligated and the ligation product used to transform *E. coli* DH5α. The initial construct was verified by colony PCR and sequencing and the resulting initial construct was named pSH2. A schematic diagram of pSH2 is shown in Figure 3.6.

Numerous attempts were made to perform inverse PCR on the initial construct pSH2.as this was to form an integral part of the mutagenesis strategy to obtain the mutant *pglB*. A product was obtained however only with a template concentration in excess of 150 ng, which is between 150 and 300 × greater than is routinely used. Excessive template concentrations resulted in multiple bands on the agarose gel and meant that follow-up procedures would be more difficult to perform. The original intention had been that a defined *pglB* deletion mutant would be constructed specifically for this study. However due to problems, it was decided to use a previously-made final construct (pLM35 - (Millar, 2003); see Appendix B for construct). During the verification of pLM35, it transpired that the available construct was not the deletion mutation previously described and further, more detailed verification was required. The construct was expected to have a 1.4 kb deletion, however this was found not to be the case and instead a 294 bp deletion with an inserted kanamycin cassette was in fact determined. The result of this verification was a re-mapped plasmid, pLM35a which can be seen in Appendix B. As the construct contained the desired mutated *pglB* it was transformed by electroporation into *C. jejuni* NCTC11168 and successful transformation was confirmed by colony PCR. The observed band size of 4.3 kb (data not shown) was consistent with an insertion mutation, not the deletion mutation as had been originally described (Millar, 2003).

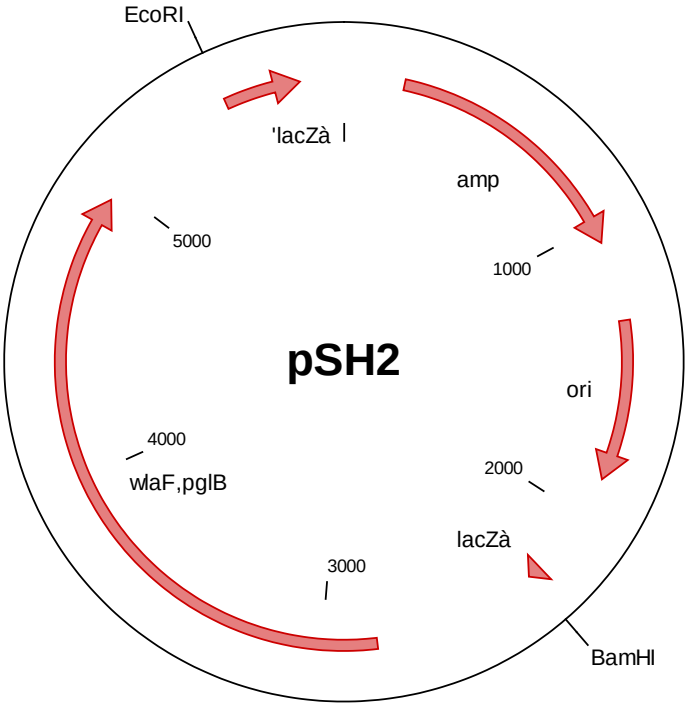


Figure 3.6. Map of the pglB initial construct pSH2 made from the ligation of the *pglB* region amplicon with pUC19, both previously restriction enzyme digested with *Bam*HI and *Eco*RI. The position of the two restriction sites is indicated on the map. The red arrows represent open reading frames.

3.2 Verifying glycosylation mutants

3.2.1 Investigating the phenotypic effects of mutating pglK

As mentioned previously it was decided to include the 'flippase' PglK in this research following the publication by Nothaft *et al*. (2009). In order that the study remained on schedule it was decided to obtain the Δ*pglK* strain from another group (G. Thacker, Dept of Infectious and Tropical Diseases, LSHTM). To verify the mutant strain, two primers were designed; pglK_fwd and pglK_ rev (Table 2.6), which anneal to the flanks surrounding the disrupted gene. These were predicted to give a product of 2151 bp in the wild-type NCTC11168 and 3.1 bp in the *pglK* mutant. The *pglK* locus in the mutant strain was sequenced to complete the verification process. Chromosomal DNA was extracted from the cultured Δ*pglK* strain using the procedure outlined in Chapter 2. Verification revealed that the mutant was constructed by the insertion of the kanamycin cassette into the *Swa*I site 656 bp into the *pglK* gene.

3.2.2 Investigating pgl mutant growth in liquid culture (1b)

The *pglB* and *pglI* mutants were tested in MHB using the liquid growth method as outlined in Chapter 2. Growth was compared with that of the wild-type NCTC11168. Growth was tested over a period of 24 hours with samples taken at 4, 8, 12 and 24 hours. No supplements were added to the MHB. The results of the growth assay are shown in Figure 3.7. The *pgl* mutant strains grew well in MHB with final OD_{600} readings reaching between 0.7 and 1.0. Growth of Δ*pglB*::*kan* was particularly good, exceeding that of the wild-type throughout the course of the assay. These results indicated that there were no adverse effects caused by the mutations under normal growth conditions.

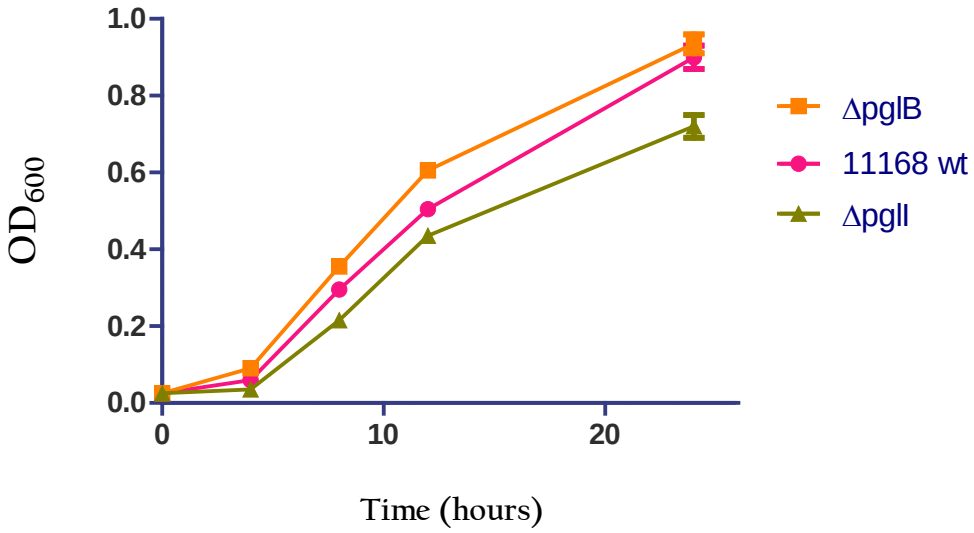


Figure 3.7. Comparison of growth of *pglB* and *pglI* mutants with wild type. Liquid growth of *pglB* and *pglI* mutants with a wild-type NCTC11168 control in iron-replete MHB, under microaerobic conditions with shaking at 500 rpm. All samples were grown in volumes of 15 ml MHB with 1 ml samples taken at 0, 4, 8, 12 and 24 hours. Optical density (at 600 nm) readings were taken and recorded with the starting OD_{600} being 0.025 for each sample. In this instance strains were tested in duplicate with SEM shown. The growth of each mutant compared to that of the wild-type was not significantly different; wt vs Δ*pglI*, $P = 0.742$ and wt vs Δ*pglB* $P = 0.852$, two-tailed T test used.

3.2.3 Comparing levels of natural competence

In the absence of a growth phenotype in the NCTC11168 glycosylation mutants, the research carried out by Larsen *et al.* (2004) with *C. jejuni* 81-176 raised the question that a significant decrease in natural competence might also be observed in NCTC11168 Δ*pglB* and Δ*pglK*. The bi-phasic natural transformation protocol employed for this investigation into the natural competence is outlined in Chapter 2. Duplicate samples of NCTC11168 wild-type, Δ*pglB* and Δ*pglK* were prepared by harvesting 4 passaged MHA plates for each strain with 2 ml MHB and centrifuging at 14,000 × *g* for 3 minutes. It was decided to omit the *pglI* mutant due to the absence of any clear differences observed between the mutant and the wild-type. Cells were re-suspended and added to an OD_{600} of 0.5 to 1 ml MHA in a 15 ml tube. Following incubation under microaerobic conditions for 3 hours, the samples were each inoculated with ~5 µg of Δ*cj0444*::*ermC* chromosomal DNA (R. Haigh) and incubated for a further 3 hours. *Cj0444* is a pseudogene in NCTC11168 and uptake of the Δ*cj0444*::*ermC* DNA would therefore confer erythromycin resistance without affecting a functional gene. After the 3 hour incubation time the samples were removed from the VAIN and spread onto selective MHA before being returned to the VAIN for a total of 7 days, after which the total number of colonies were counted for each sample. A graphical representation of the results is shown in Figure 3.8. The *pglB* mutant exhibited the most marked deterioration in the level of natural competence (2.4% compared to 11168 wt), with the *pglK* mutant having 11.7% of the level of natural competence of wild-type NCTC11168.

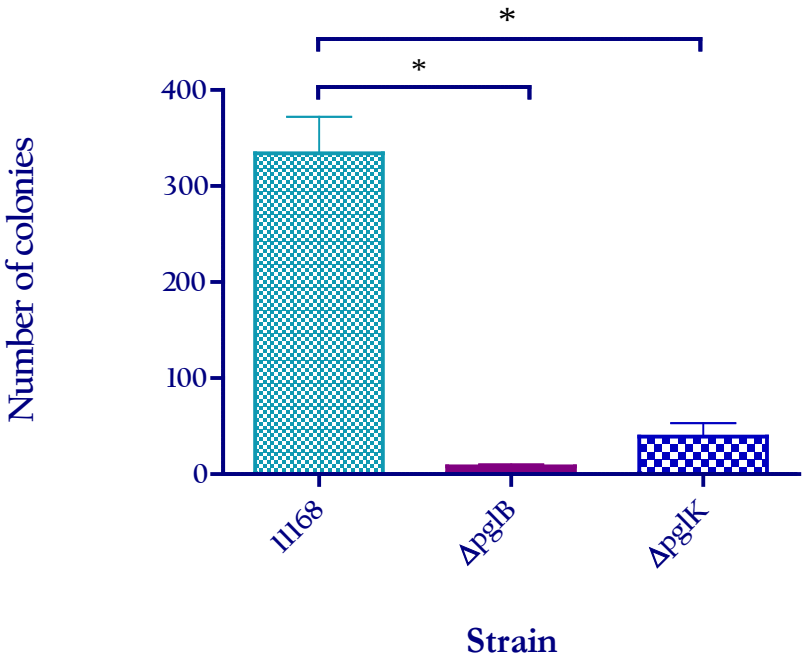


Figure 3.8. The natural competence of *pgl* mutants. NCTC11168 wild-type and two *pgl* mutants (*pglB* and *pglK*) were compared with respect to their level of natural competence. The investigation was carried out by harvesting 4 MHA plates for each strain, with 2 ml MHB and centrifuging at 14,000 × *g* for 3 minutes. Following incubation in the VAIN for 3 hours, the samples were each inoculated with ~5 µg of Δ*cj0444*::*ermC* chromosomal DNA and incubated for a further 3 hours. After the incubation, samples were removed from the VAIN and spread onto selective MHA before being returned to the VAIN for a total of 7 days, after which the total number of colonies were counted for each sample. The graph shows the total number of colonies for two MHA plates obtained for the three strains. Each condition was tested in duplicate with SEM shown. Wt vs ΔpglB $P = 0.0134$, wt vs ΔpglK $P = 0.0183$, two-tailed T test used.

3.3 Comparing the growth of glycosylation and iron-uptake mutants 1(c)

3.3.1 Establishing the normal growth patterns for the pgl mutants by comparison with iron-uptake mutants

A variety of growth and nutrient utilisation assays were employed in order to test for any effects of the *pgl* mutations. Initially liquid growth assays were performed using MEMα (GIBCO®, Invitrogen, UK), though these were often found to yield inconsistent results with the different supplements being used. For example when FeRA was added to MEMα, the *p19* mutant would grow in a way comparative to wild-type in one growth assay and then show impaired growth in the following assay under exactly the same conditions. Therefore, in an attempt to obtain consistent results, Mueller-Hinton broth was used (MHB, Oxoid, UK) which was chelated with either 20 μM Deferoxamine mesylate (Sigma) or 5% (w/v) Chelex (Sigma) to reduce the iron levels. Figure 3.9 shows the strains growing in iron replete MHB. This assay was carried out in order to establish normal growth patterns for each of the strains and to ensure that any variation was therefore due to the conditions being tested. The *pgl* mutants were compared to mutants in the known ferric-uptake systems, namely Δ*chuA*, Δ*p19*, Δ*0178* and Δ*cfrA*, as predictable growth patterns for these mutants had previously been established.

3.3.2 Investigating specific iron-uptake systems

3.3.2.1 Utilisation of ferric-rhodotorulic acid

Recent reports indicate a possible role for p19 in iron uptake from the fungal siderophore ferric-rhodotorulic acid (FeRA) (Stintzi *et al.*, 2008), with the periplasmic protein p19 being implicated in that role. In view of this, several growth assays were conducted to compare the growth of the *pgl* mutants with a *p19* mutant when given

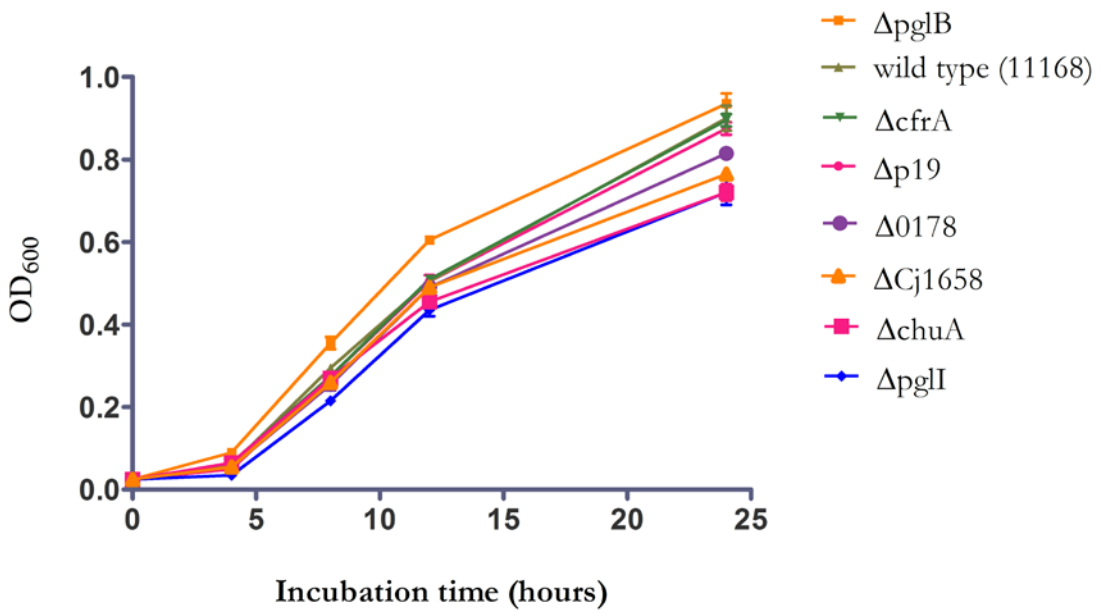


Figure 3.9. Comparing growth of the *pgl* mutants and mutants of the four known ferric uptake systems. The investigation was conducted using iron-replete MHB and was used as a 'standard' for subsequent growth assays. The investigation was carried out using 15 ml sample sizes with 1 ml taken for optical density readings at 0, 4, 8, 12 and 24-hour time points. Samples were cultured in the VAIN with shaking at 500 rpm. The graph shown is the result of two replicates with SEM shown. In all instances any differences in growth between Δ*pglB* and the iron mutants was not significant (Δ*pglB* vs Δ*p19* (P = 0.815), Δ*cfrA* (P = 0.842), Δ*0178* (P = 0.749), Δ*chuA* (P = 0.666) and Δ*Cj1658* (P = 0.714), two-tailed T test used.

FeRA as the sole iron source (figure 3.10). The inner membrane protein Cj1658 has also been reported to be involved in the uptake of iron from FeRA (Stinzi et al., 2008) and thus a further growth assay was performed in order to investigate this finding (figure 3.11). From the results shown in the two figures, it would suggest that neither p19 nor Cj1658 are solely responsible for the uptake of iron from FeRA. Considering that the siderophore is synthesised and released by strains of the smut fungus *Rhodotorula* it seems unlikely that *Campylobacter* would come into contact with rhodotorulic acid within its usual niche environment.

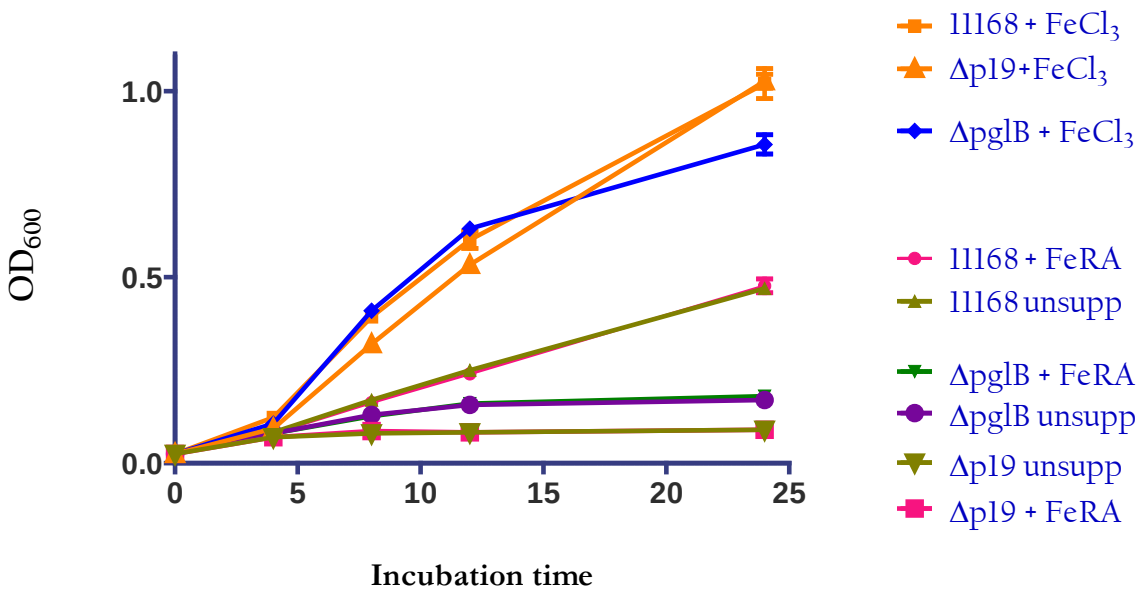


Figure 3.10. Liquid growth assay investigating growth in iron-restricted MHB supplemented with $FeCl_3$ as a positive control and FeRA as the test additive. Strains were grown in iron-restricted MHB supplemented with either 1 µM FeRA or 1 µM $FeCl_3$.All conditions were tested in triplicate with SEM shown. Differences in growth between the positive control ($FeCl_3$) and the test additive (FeRA) were not significant. *P* values were: 11168 (0.262), Δ*pglB* (0.104) and Δ*p19* (0.107), two-tailed T test used.

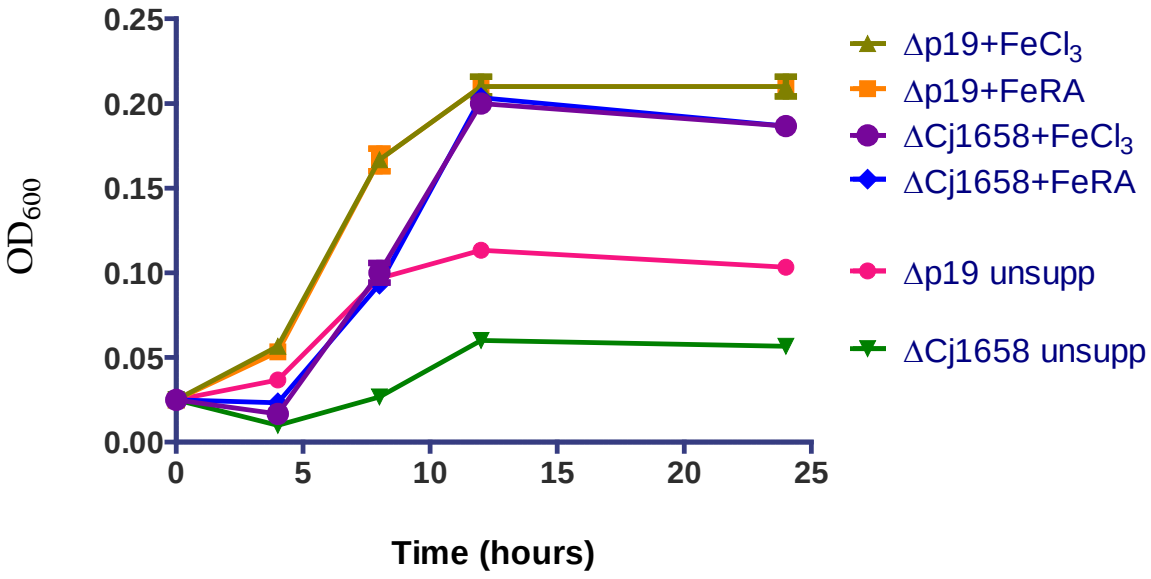


Figure 3.11. Liquid growth assay testing growth in MEMα. Strains were grown in MEMα supplemented where shown with either 1 µM FeRA or 1 µM $FeCl_3$. All conditions were tested in triplicate with SEM shown. Differences in growth between the positive control ($FeCl_3$) and the test additive (FeRA) were shown to be non-significant, with *P* values being Δ*p19* (0.991) and Δ*Cj1658* (0.991), two-tailed T test used.

3.3.2.2 Uptake of haem

It has been established that the Chu group of proteins are responsible for the utilisation of haem in *C. jejuni* (Parkhill *et al.*, 2000; Ridley *et al.*, 2006), therefore using a *chuA* mutant as a negative control in a growth assay using haem would provide a suitable comparison between that strain and the *pgl* mutants. There is no evidence that ChuA is glycosylated (Nothaft *et al.*, 2008; Young *et al.*, 2002), moreover there seems to be no adverse effect on the transcription of *chuA* in *pglB*, *K* or *I* mutants (Nothaft *et al.*, 2008). The graphical representation of the results of the plate assay conducted is shown in Figure 3.12. The plate assay provided a clear indication as to the phenotype of the mutants. The three *pgl* mutants responded in a similar way to the wild-type with clear halos of growth being observed around the haem- and ferric chloride-supplemented plates (data not shown for $FeCl_3$ control). In contrast there was no growth around the haem on the plate containing the *chuA* mutant. A comparable result was observed during a repeat plate assay (data not shown) with the *chuA* mutant failing to grow with haem as the sole iron source and the *pgl* mutants growing to equivalent levels to that of the wild-type. The observations with respect to the growth of the *chuA* mutant are consistent with those of Ridley *at al*. (2006).

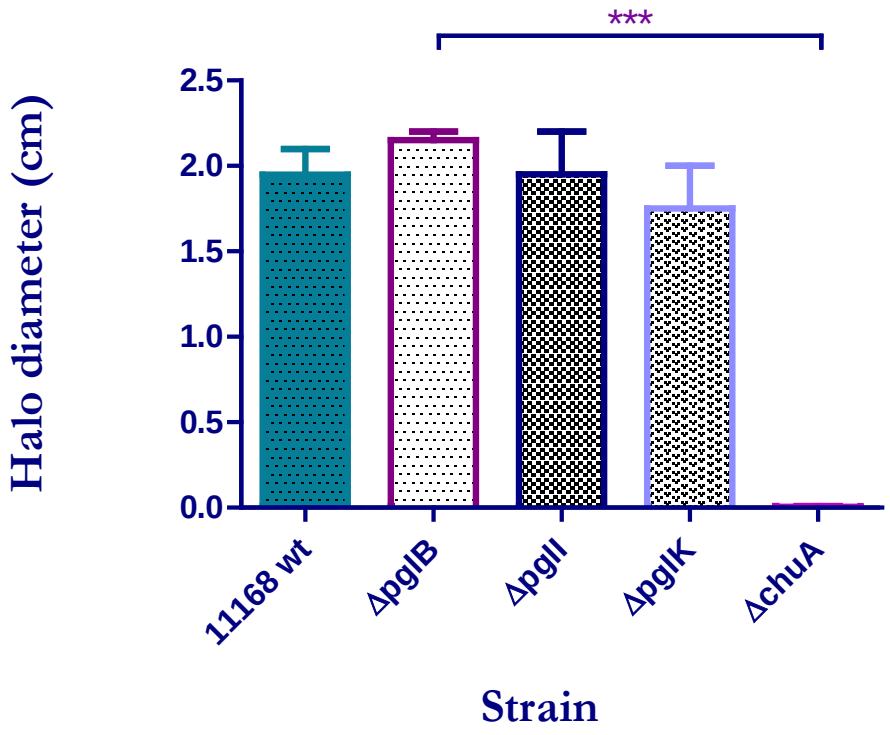


Figure 3.12. Plate assay using iron-restricted Brucella agar with 5 µl aliquots of 100 µM haem dotted in the centre of the medium. The graph shows the combination of two replicates with SEM shown. Differences in halo diameter size between the wild type 11168 and the three *pgl* mutants were tested and shown to be not significant, *P* vales were: wt vs Δ*pglB* = 0.33, wt vs Δ*pglI* = 1.00 and wt vs Δ*pglK* = 0.56. The *P* value for Δ*pglB* vs Δ*chuA* was 0.0005, two-tailed T test used.

3.3.2.3 Uptake of iron from transferrins

The ability of *C. jejuni* to utilise iron from host iron-binding proteins has been widely reported, with the operon *cj0173c – cj0178* having been shown as primarily responsible for the uptake of iron from lactoferrin, transferrin and ovotransferrin (Miller *et al.*, 2008). Of those proteins encoded by the locus, only the periplasmic protein Cj0175c would appear to be glycosylated (Young *et al.*, 2002). Iron uptake from lactoferrin has been shown to be proximity-dependent in liquid growth assays (Miller *et al.*, 2008). The growth assays carried out as part of this study were conducted to compare the growth of the three *pgl* mutants with the wild-type and a *cj0178* mutant using ferri-lactoferrin as the sole iron source. A graphical representation of the results is shown in Figure 3.13. All strains tested responded well to the positive control using $FeCl_{3,}$ producing a growth halo with a diameter of approximately 2 cm (data not shown). All three *pgl* mutants exhibited growth in response to the lactoferrin spot. There was no halo observed with the *cj0178* mutant. As a visual example of this type of assay, Figure 3.14 shows an example of the halo of growth observed around an added iron source, in this instance $FeCl_3$.

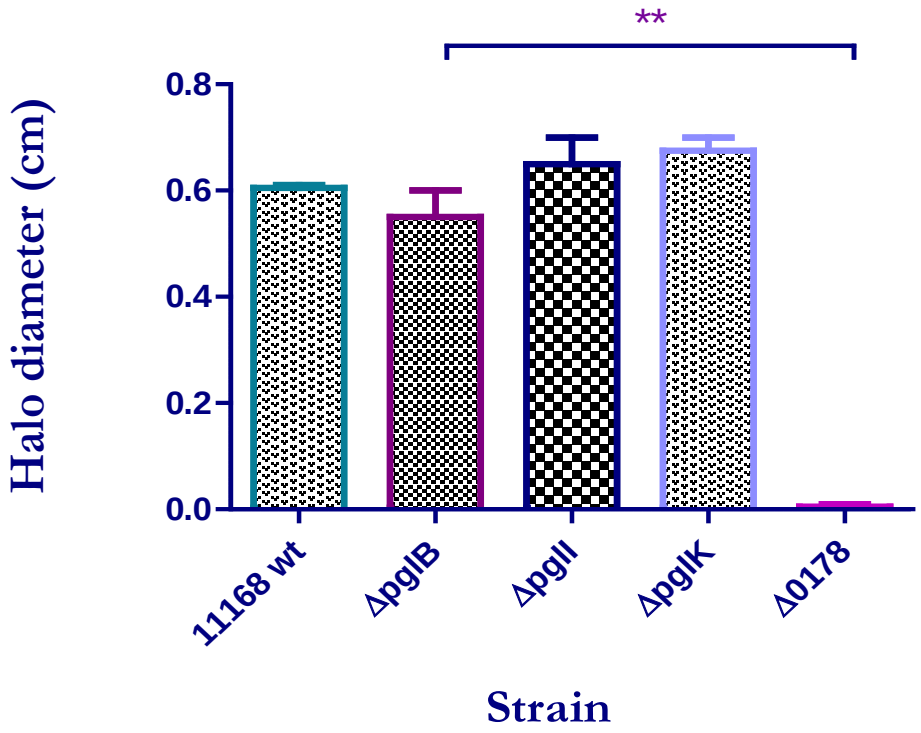


Figure 3.13. Plate assay using iron-restricted Brucella agar with 5 µl aliquots of 25 µM human ferri-lactoferrin dotted in the centre of the medium. The graph shows the combination of two replicates with SEM shown. Differences in halo diameter size between the wild type 11168 and the three *pgl* mutants were tested and shown to be not significant, *P* vales were: wt vs Δ*pglB* = 0.39, wt vs Δ*pglI* = 0.47 and wt vs Δ*pglK* = 0.11. The *P* value for Δ*pglB* vs Δ*0178* was 0.0084, two-tailed T test used.

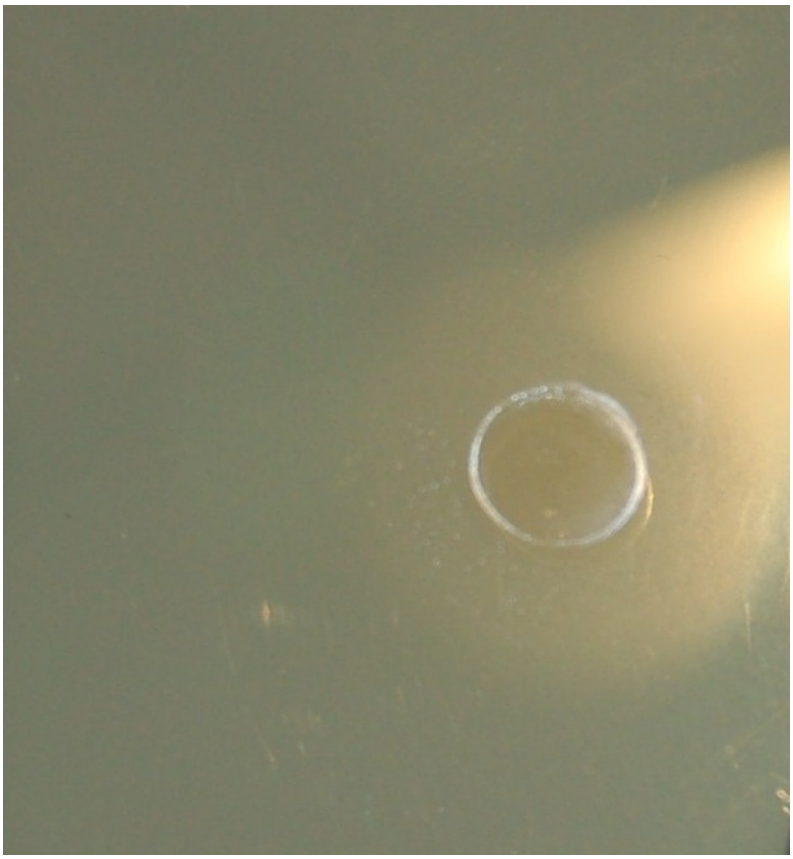


Figure 3.14. Example of a halo of growth around a 5 µl spot containing $FeCl_3$. The circle around the spot shows the area of bacterial growth. The diameter of the halo was measured and recorded in centimetres. The results of such measurements are shown in Figures 3.12 and 3.14.

3.3.2.3 Uptake of iron from enterochelin

The *C. jejuni* outer membrane protein CfrA has been shown to be involved in iron uptake from the bacterial siderophore enterochelin (Palyada *et al.*, 2004). CfrA does not appear to be glycosylated (Young *et al.*, 2002) and there is no apparent change in transcription levels in *pgl* mutants in *C. jejuni* (Nothaft *et al.*, 2008). Enterochelin can exhibit instability during routine use in the laboratory so a catecholate, noradrenaline (NA), that uses the same receptor, was used as an alternative in the growth assays. A *cfrA* mutant has been shown to exhibit restricted growth in a serum-supplemented medium with added NA (RD Haigh, unpublished results). In view of this, growth of the *pgl* mutants was compared to that of a *cfrA* mutant in a range of conditions. Initially, typical growth patterns in both iron replete and iron restricted media were established. The graphical representation of the results of the iron replete assay is shown in figure 3.15. The growth of the *pgl* mutants was then compared to that of Δ*cfrA* in a medium where bovine serum and NA were added to iron-restricted MHA. Under these conditions, the growth of the two *pgl* mutants was comparable to that of the wild-type with both *pgl* mutant strains attaining a final cfu/ml of around 10^9. It is therefore predicted that uptake of iron from enterochelin would not be diminished in a *pgl* mutant

In the light of previous work carried out within the group and given that the structure of enterochelin has some similarity to NA, in that they both possess catechol side chains, it is reasonable to predict that CfrA may also be involved in the utilisation of iron from NA. Initially an attempt was made to investigate enterochelin uptake by using a feeder assay. This was done in order to establish a negative control for use in growth/nutrient utilisation assays for the *pgl* mutants. The assay was to have provided a convenient method of supplying the *Campylobacter* cells with enterochelin. The method exploited the phenotypes of two *E coli*. mutants, Δ*fes* and Δ*entA – fes* mutants do not synthesise

enterochelin esterase and therefore over-produce enterochelin as a result of the inability to breakdown the siderophore, whereas *entA* mutants are unable to synthesise enterochelin. The use of both strains provided positive (Δ*fes*) and negative (Δ*entA*) controls for the assay. Briefly, the *E. coli* strains were added to cooled M9 agar and a 200 µl aliquot placed into a petri dish and allowed to set. The *C. jejuni* strains were harvested from three confluent plates into 2 ml iron-restricted MHB and centrifuged for 3 minutes at 14,000 g. The cell pellet was then re-suspended in 500 µl iron-restricted MHB and the cells added to cooled, iron-restricted Brucella agar at a starting OD_{600} of 0.005. Initially 20 ml of *C. jejuni* in BA were overlaid onto each plate containing the *E. coli* feeder strain but, after an overnight incubation under microaerobic conditions, the *E. coli* had swarmed across the surface of the BA. It was decided to add 30 ml of *C. jejuni* suspension to each plate to ensure that the M9 agar was completely covered. However the *E. coli* repeatedly proliferated across the plate swamping the *Campylobacter*, despite adding vancomycin to the Brucella agar containing the *E. coli* mutant strains, they continued to overgrow the plate (data not shown). Repeated attempts resulted in continued overgrowth of *E. coli* so the feeder assays were abandoned in favour of the noradrenaline utilisation liquid growth assays. The results of those assays are shown in figures 3.15 and 3.16.

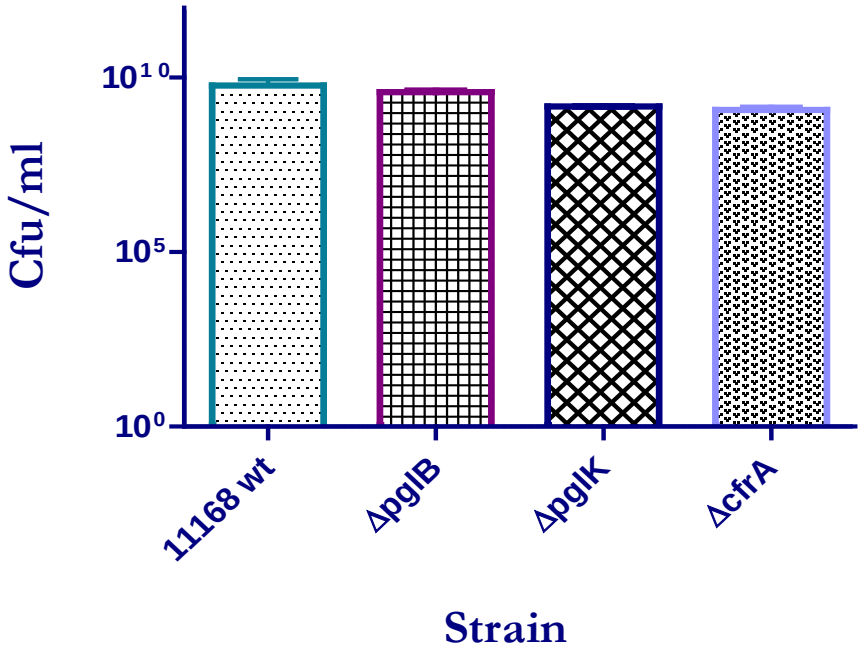


Figure 3.15. Growth assay conducted in iron-restricted MHB (cMHB) supplemented with 1 µM $FeCl_3$. Here samples were taken after a time period of 48 hours and viable counts performed. Results are from duplicate samples with SEM shown. Differences in growth between the 11168 wild type and the other strains are not statistically significant. *P* values were: 11168 wt vs Δ*pglB* = 0.55, Δ*pglK* = 0.27 and Δ*cfrA* = 0.25, two-tailed T test used.

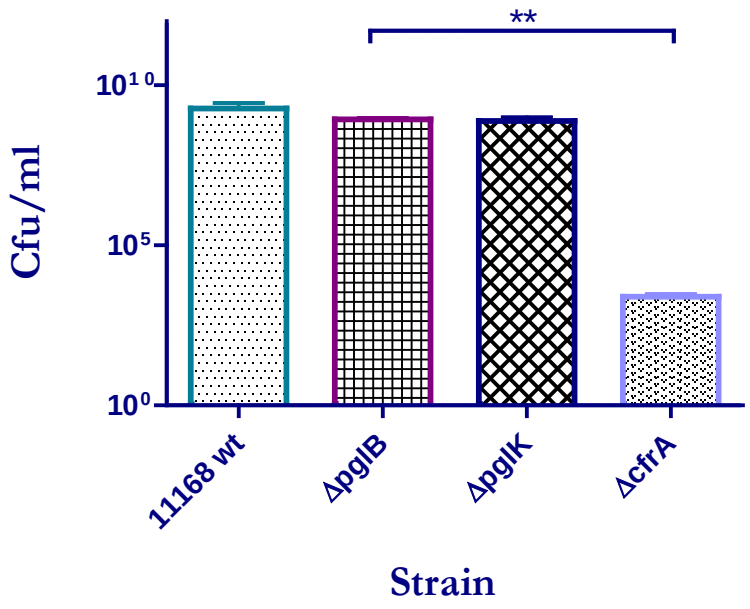


Figure 3.16 Growth assay conducted in cMHB supplemented with 10% (v/v) bovine serum and 100 µM NA. The 48-hour time point is shown here after viable counts were performed. All are from duplicate samples with SEM shown. Differences in growth between Δ*pglB* and Δ*cfrA* are statistically significant. *P* values are: Δ*pglB* vs Δ*cfrA* 0.0095, two-tailed T test used.

3.4 Investigating the uptake of iron from noradrenaline via CfrA (aim 2)

There is no evidence to support the synthesis of siderophores by *C. jejuni*, however it is known that the organism is able to utilise iron provided by the siderophores synthesised by other bacterial species. It was shown as long ago as 1986 that *C. jejuni* is able to utilise iron provided by enterochelin, an *E. coli* siderophore (Field *et al.*, 1986). A number of groups have since confirmed this ability and a standardised growth assay has been developed (Baig *et al.*, 1986). The involvement of *C. jejuni* CfrA in iron uptake from enterochelin was reported in 1986, when sequence similarities were noted between CfrA and BfrA, an iron transport protein in *Bordetella bronchiseptica* (Field *et al.*, 1986). Further confirmation came from studies using a *cfrA* deletion mutant, where it was reported that the mutant was unable to use enterochelin as a sole iron source (Palyada *et al.*, 2004). Moreover, *cfrA* mutants are unable to colonise chickens (Palyada *et al.*, 2004).

3.5 The possible dual role of glyceraldehyde-3-phosphate dehydrogenase in C jejuni (aim 3)

The role of glyceraldehyde-3-phosphate dehydrogenase or GAPDH has been described in many organisms including bacteria and yeast (Gozalbo *et al.*, 1998; Schaumburg *et al.*, 2004). The protein was initially believed to be involved solely in energy metabolism (EM) where it converts glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate in an NAD^+-dependent reversible reaction that is part of the EM pathway (Davidson *et al.*, 1967; Martin & Cerff, 1986). In *E. coli*, GAPDH is encoded by two genes *gapA* and *gapC* but in laboratory strains of *E. coli* K-12, *gapC* has accrued several mutations that produce a truncated protein (Espinosa-Urgel & Kolter, 1998; Hidalgo *et al.*, 1996); however, these mutations are absent in pathogenic strains and natural isolates (Egea *et al.*, 2007). Two *gap* genes have been characterised in *Bacillus subtilis*, with *gapA* being

required for glycolysis and *gapB* being necessary for gluconeogenesis (Fillinger *et al.*, 2000). In *C. jejuni* NCTC11168, GAPDH is encoded by a single gene, *gapA*, which is the first gene in an operon that also contains *pgk*, the gene that encodes phosphoglycerate kinase, the next enzyme in the EM pathway (Parkhill *et al.*, 2000) (Figure 3.17). GAPDH is highly conserved amongst bacteria and is tetrameric in structure, consisting of four identical subunits (Fillinger *et al.*, 2000; Skarzynsji *et al.*, 1987) (Figure 3.18). Recent reports highlight further roles for GAPDH and describe a cell wall location for the protein, despite it lacking any recognisable export signal sequence (Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992; Pancholi & Chhatwal, 2003). Initially the investigations involved only Gram-positive organisms and fungi until a first report was published describing a non-EM role of GAPDH in *E. coli* (Egea *et al.*, 2007). Egea and co-workers outlined the ability of pathogenic *E. coli* to bind human fibrinogen and plasminogen via GapA and showed the organism's capacity to interact with intestinal epithelial cells (Egea *et al.*, 2007). These findings may have implications for the study of infection in other pathogenic bacteria. Egea *et al.* (2007) also noted variations in conformation of GAPDH with all observed forms of the protein being attributable to the *gapA* gene. Although the *gapC* open reading frame is intact in the strains they were studying, the authors could find little evidence of its expression in the extracellular medium (Egea *et al.*, 2007). Alternative forms of GAPDH resulting from post-translational modification have been reported previously in other organisms, including *Streptococcus pneumoniae* (Cash *et al.*, 1999), *Paracoccidioides brasiliensis* (Barbosa *et al.*, 2006) and lactic acid bacteria (Pessione *et al.*, 2005).

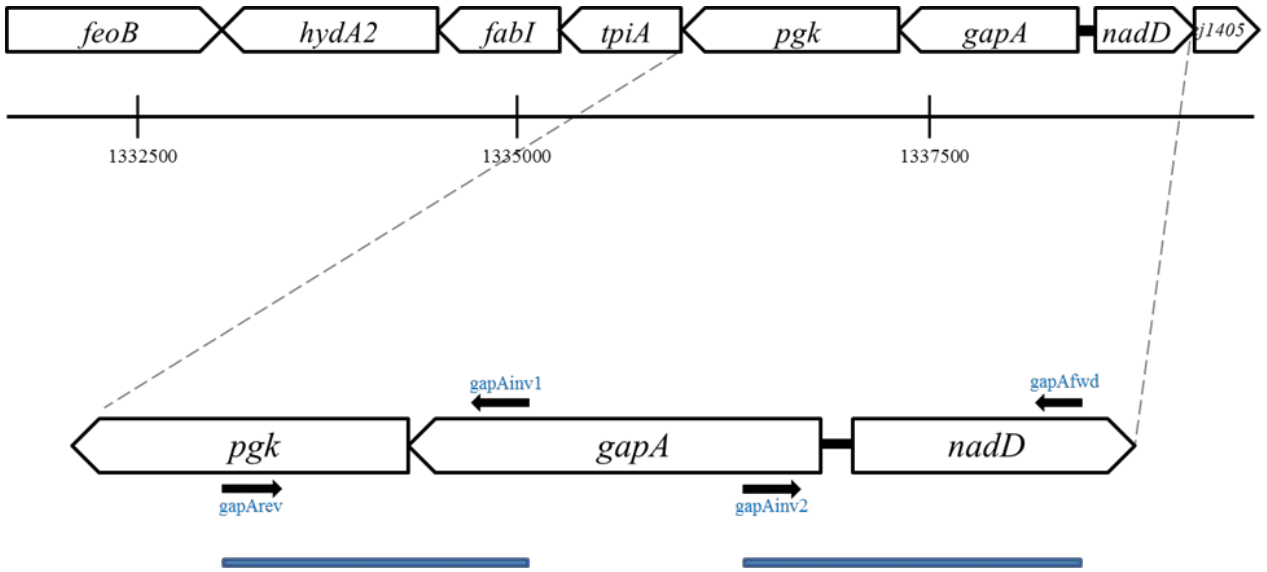


Figure 3.17. Genomic context of *gapA* and indication of primer sites. The directions of the arrows show the direction of transcription. Upper figure shows *gapA* in its genomic context with the genome positions indicated in base pairs. The lower portion of the figure shows *gapA* and the two adjacent genes *nadD* and *pgk* plus the position of the primer pairs used for the *gapA* mutagenesis. The two amplicons produced by the two pairs of primers were utilised in the three-way ligation and are indicated in the figure by the two blue lines. The genes encode the following proteins: *feoB* – ferrous iron transport protein, *hydA2* – putative Ni/Fe hydrogenase, *fabI* – enoyl reductase, *tpiA* – triosephosphate isomerise, *pgk* – phosphoglycerate kinase, *gapA* – glyceradehyde-3-phosphate dehydrogenase, *nadD* – putative nicotinate-nucleotide adenyltransferase, *cj1405* – conserved hypothetical protein.

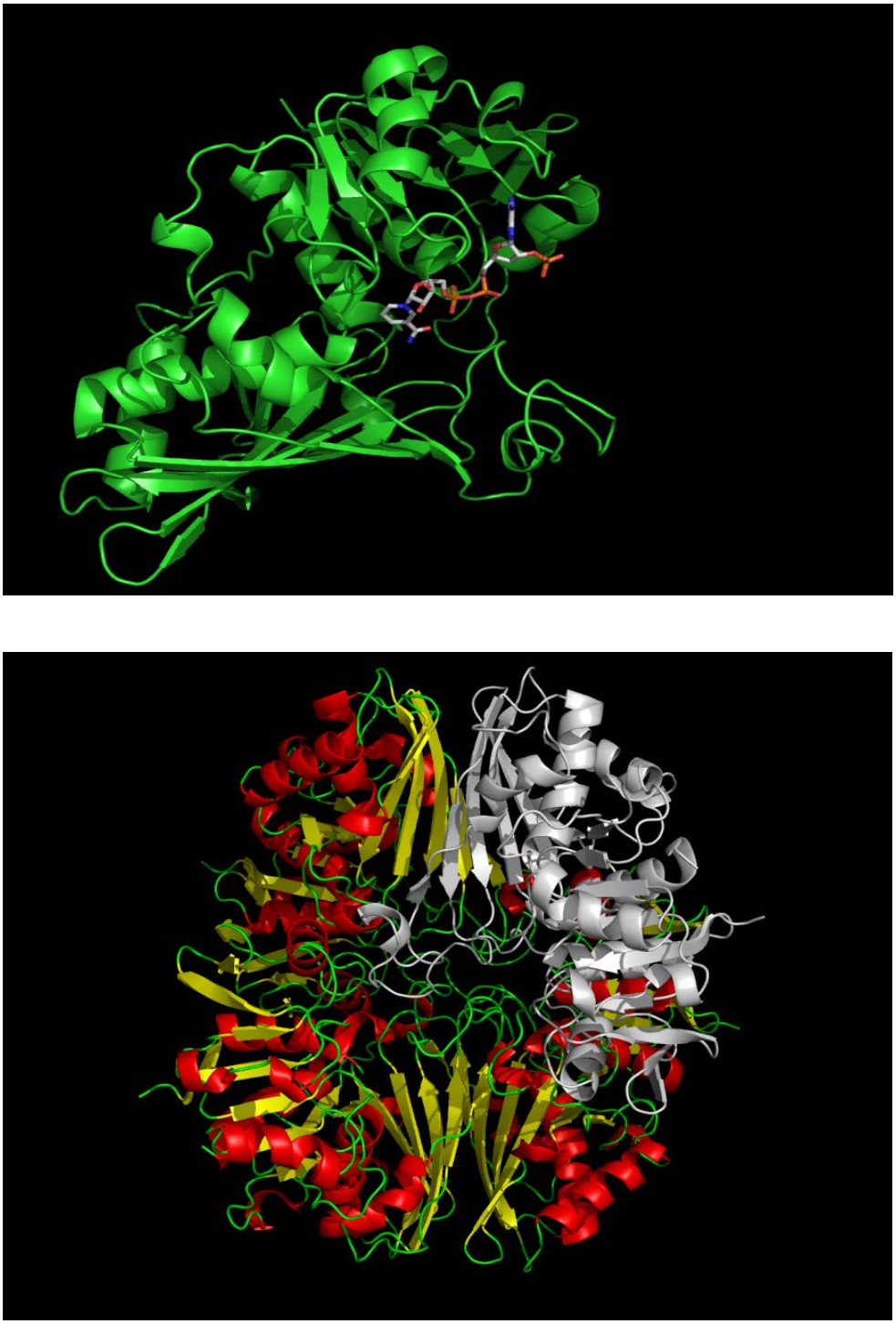


Figure 3.18. *Campylobacter* GAPDH structure. Overviews of the tetramer (below) and monomer showing NADP binding (above). Colours on the tetrameric structure indicate red: alpha helices and yellow: beta-sheets. A single monomer is highlighted in white. (Tourigny *et al*. 2010 and P. R. Elliott, unpublished)

3.5.1 GAPDH and the acquisition of iron

Despite the established role of GAPDH as an anchorless, cytoplasmic enzyme, there are numerous reports describing the alternative roles of this multifunctional protein. Repeatedly investigations reveal the protein's ability to bind transferrin, indeed one report highlights this ability even when GAPDH is not in its native tetrameric conformation (Modun *et al.*, 1994). The capacity of GAPDH to bind host proteins is not confined to pathogenic species; macrophage GAPDH has been reported as binding transferrin in humans and mice (Knutson & Wessling-Resnick, 2003). A transferrin-binding protein (Tpn) has been identified in staphylococci that appears to be host-species specific in that binding is not blocked by non-human transferrin (Modun *et al.*, 1994). This protein was later reported as being a cell wall GAPDH using binding studies where the protein was shown to bind human transferrin (Modun & Williams, 1999). In group A streptococci both forms of the GAPDH protein are encoded by the same gene (Winram & Lottenberg, 1996). Interestingly the organic phosphate, 1,3-diphosphoglycerate, formed by the GAPDH-mediated phosphorylation of glyceraldehyde-3-phosphate, aids in the removal of iron from transferrin (Morgan, 1977). This finding may be central to discovering the mechanism for iron release from Tf and Lf by cell-surface GAPDH and may point to a connection between GAPDH-mediated iron release and glycolysis (Modun *et al.*, 2000).

3.5.1.1 Construction of the gapA mutant strain

As with the previous mutagenesis work, the *gapA* region was identified with the aid of the online database, CampyDB. The gene plus flanking regions of approximately 500 bp were amplified by PCR with primers designed using the Clone Manager program (Figure 3.17). The primers gapAfwdEcoRI and gapArevBamHI are shown in Table 2.6.

The resulting PCR product was purified and with pUC19 was digested using the restriction enzymes *Bam*HI and *Eco*RI. The digested *gapA* amplicon and pUC19 were ligated and the reactions purified using the ethanol precipitation method described in Chapter 2. The purified ligation product was used to chemically transform *E. coli* strain DH5α. The transformed cells were grown overnight on LB agar supplemented with X-gal and IPTG. A number of white colonies were patched onto LB agar supplemented with ampicillin and grown overnight. Selected colonies were then screened by colony PCR using M13 primers as outlined in Chapter 2. Constructs were purified from the colonies and sequenced using M13 primers, however, the initial sequencing revealed four point mutations, two within each flanking gene, *nadD* and *pgk*. The initial cloning step was therefore repeated using a high-fidelity, proof-reading polymerase, Phusion in accordance with the manufacturer's instructions. The purified construct was sequenced once more, but different point mutations were found again within *nadD* and *pgk*. Considering possible toxic effects of *Campylobacter* genes upon *E. coli*, it was decided to use a low copy number plasmid for the initial cloning step. The chosen vector, pWKS30 (Table 2.1) was previously modified by removal of the *Not*I recognition site (R. Haigh, personal communication) and the map of this construct is shown in the Appendix. The removal of the *Not*I site was necessary as initially this was the chosen restriction site added to the inverse primers. The purified *gapA* amplicon was ligated with *Eco*RI- and *Bam*HI-digested pWKS30Δ*Not*I; however, after numerous attempts, the procedure failed to yield the desired clone.

3.5.1.2 Three-way ligation technique

With the failure of previous procedures to yield a *gapA* construct, it was decided to adopt a three-way ligation protocol. This method uses the two separate inserts of the flanking regions as opposed to one whole gene fragment when ligating into the vector, thereby also eliminating the necessity to perform an inverse PCR reaction. The two

inserts are obtained by carrying out two PCR reactions using the gene-specific primers, gapAfwdEcoRI and gapArevBamHI against their corresponding inverse PCR primers, gapAinv1KpnI and gapAinv2KpnI (Figure 3.17). The products were visualised by agarose gel electrophoresis and the resulting amplicons are shown in Figure 3.17. The PCR products were then purified and ligated with the digested vector DNA. Successful ligation would create an internal *Kpn*I restriction site for subsequent insertion of the kanamycin resistance cassette obtained from pJMK30 (Table 2.1). The reactions were then purified using the ethanol precipitation method, before being used to transform into *E. coli* strain DH5αe by electroporation, as outlined in Chapter 2. The transformed cells were grown overnight on selective LB agar and colonies taken and patched for further screening. Colony PCR using both gene-specific and M13 primers again failed to reveal any of the desired clones.

3.6 Discussion

During the course of this study mutant strains in *pglI* and *pglB* have been constructed and verified. A number of growth assays have been performed as part of the investigation and the results of these suggest that glycosylation is not a functional requirement of iron uptake proteins in *C. jejuni*. This study has also shown *pgl* mutants to have a reduced natural competence.

3.6.1 Natural competence

Despite nine years having passed since the sequencing and annotation of the *C. jejuni* NCTC11168 genome, many questions remain as to the organism's ability to colonise its host. It has been shown that protein glycosylation is fundamental to invasion and colonisation (Hendrixson & DiRita, 2004; Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002). Another study showed that glycosylation mutants exhibit a reduced natural competence in 81-176 (Larsen *et al.*, 2004). The results of the investigation into natural competence in NCTC11168 carried out as part of this study are in agreement with the findings of Larsen and co-workers. The results themselves are interesting because unlike 81-176, NCTC11168 does not harbour a plasmid and it was the glycosylation recognition sites on the VirB10 protein in particular that were the subject of Larsen's study. The 81-176 *virB10* has significant homology to *H. pylori comB3*, the products of which need to be glycosylated at a particular recognition site in order to function correctly in natural transformation. A search for possible candidate genes involved in transformation in NCTC11168 reveals several possibilities. Firstly, there is the Campylobacter Transformation System, Cts. The proteins involved in this system are encoded by six genes arranged in a locus which is conserved across a number of *C. jejuni* strains, although *ctsF* (*cj1470c*) is thought to be a pseudogene in NCTC11168 (Parkhill *et al.*, 2000). None of the Cts proteins appear to be glycosylated (Nothaft *et al.*, 2008). Amongst other proteins linked to natural competence are DprA, RecA and

GalE (in 81-176), but again none of which has been shown to be glycosylated (Nothaft *et al.*, 2008). No further information is currently available as to which protein or proteins may be implicated in natural transformation and competence. However, as mutants in the *pgl* system are reported as having variable transcription levels (Nothaft *et al.*, 2008), it is feasible that the difference in competence may be attributable to decreased transcription of a gene not thought to be previously involved.

3.6.2 Growth assays

Although overall the trends were as expected, the liquid growth assays exhibited some inconsistencies (data not shown). These may have been partly due, but not limited to, problems with the different supplements in conjunction with the growth medium. These variations were largely observed when using MEMα with ferri-rhodotorulic acid (FeRA). Initially an existing lab stock of RA was used in the assays – this was in the form of apo-RA to which ferric chloride was added. As the study progressed the decision was made to use ferri-RA (as $Fe_2(RA)_3$ at pH >3), though this too sometimes gave unexpected results, with the strains used as negative controls growing equally as well as the wild-type as had been previously observed. It was the failure to achieve repeated, reliable results that necessitated the use of alternative media such as iron-restricted MHB and different assays such as the plate and 24-well nutrient-utilisation assays. Consistently throughout the assays, the *pgl* mutants grew to a similar final optical density at 600 nm as wild-type NCTC11168. The *pgl* mutants were markedly different to the iron uptake mutants that were used as negative controls. It is known that p19 and Cj0175c are glycosylated (Linton *et al.*, 2005), however no difference was observed between the growth of the wild-type and the *pglB* mutant when investigating the RA and Cj0178 iron-uptake systems.

3.6.3 Protein glycosylation

Initially it was believed that although bacteria produce more glycan chains than was first thought, these chains did not appear to undergo any modification. Subsequent research has shown that not only does *C. jejuni* possess a system for flagellar glycosylation but that the organism also glycosylates other proteins. The exact purpose of glycosylation in prokaryotes generally can only be speculated upon but *C. jejuni* glycosylation mutants exhibit a significant reduction in adhesion and invasion. These effects can be observed in the laboratory using Caco-2 and INT407 cells adhesion and invasion assays (Karlyshev *et al.*, 2004; Szymanski *et al.*, 2002) and *in vitro* effects have been reported whereby there is a significant reduction in the organism's natural competence (Larsen *et al.*, 2004). Glycoprotein function has recently been investigated in more detail by Kakuda and DiRita (Kakuda & DiRita, 2006) in which the authors made a mutation in the gene encoding a putative periplasmic protein (*cj1496c*) which resulted in a reduced ability to invade INT407 cells. The authors concluded that there was no detrimental effect on the protein's stability as a result of the lack of glycosylation. As previously reported by Kelly *et al.* (2006), and as found in this study, there are no observable differences between a *pglI* mutant and wild-type NCTC11168. It is possible that effects due to the loss of protein glycosylation may be as a result of variable transcription. As mentioned earlier, mutations in several *pgl* genes affect the transcription of the *pgl* genes themselves (Nothaft *et al.*, 2008). It is thought that there may be in excess of 250 genes whose regulation is affected by changes in glycosylation although the level of change is often low. There are a number of systems which show variable gene expression in different *pgl* mutants. Of direct relevance to this study is the regulation of those genes which are involved in iron acquisition, namely those from the Cj0178 (lactoferrin), Chu (haem), p19/Cj1658 (rhodotorulic acid) and CfrA (enterochelin) uptake systems.

3.6.4 The importance of protein glycosylation in iron uptake systems

It was the intention of the study to investigate all iron uptake systems in NCTC11168 in particular, as it was this strain that was to be used throughout the study. It has been suggested that p19 and Cj1658 are involved in the uptake of iron from the fungal hydroxamate rhodotorulic acid (Stintzi *et al.*, 2008), and p19 has been shown to be iron-regulated (van Vliet *et al.*, 1998). Good data are available with respect to other iron uptake systems in NCTC11168 namely, Tf/Lf (Miller *et al.*, 2008), haem (Ridley *et al.*, 2006) and catecholamines (this study and Haigh *et al*. 2008).

3.6.5 Uptake of iron from ferrichrome

It has been reported previously that *C. jejuni* was able to utilise iron from ferrichrome (Galindo *et al.*, 2001). Attempts made to repeat the data produced by that group failed to confirm the previous findings (J. McNicholl-Kennedy, personal communication). This needs to be extended to other strains but it is unlikely that any of the sequenced *C. jejuni* strains will be able to utilise ferrichrome. Overall the plate assays gave more consistently repeatable results than had been previously seen in the liquid growth assays. The precise reasons for the difference are unclear; however it has been recently reported that there is a significant up-regulation of genes associated with iron acquisition and uptake in *Campylobacter* grown on agar compared to the growth of bacteria cultured in a liquid growth medium (Sampathkumar *et al.*, 2006). The authors also suggested that culturing *Campylobacter* on agar may be more representative of the organism's growth during the initial phases of infection and that liquid medium did not accurately characterise the normal environment of *Campylobacter*. These observations may indicate that using solid agar medium results in a more consistent, predictable growth pattern and may also explain why the use of a liquid medium, particularly if nutritionally limited, results in a variety of responses.

It is appreciated that plate assays, where the size of a growth halo is measured, can only be semi-quantitative at best, however the aim of the procedure was to examine growth trends in response to the different supplements and the approach taken with the plate assays appears to have largely satisfied that aim. There are several observations that have emerged from the plate assays. Despite the previous report (Galindo *et al.*, 2001), it appears that *C. jejuni* NCTC11168 at least, is not able to utilise ferrichrome in a solid medium (data not shown).

3.6.6 Uptake of iron from rhodotorulic acid

In this study the ability of *C. jejuni* to utilise iron from FeRA appeared to be linked to the growth medium and there is no consistent evidence from this work that either p19 or Cj1658 are essential for uptake of iron from FeRA. The periplasmic protein, p19, is iron-regulated (Holmes *et al.*, 2005; Palyada *et al.*, 2004) and has recently been shown to be capable of binding copper (Chan *et al.*, 2010). Interestingly, Chan *et al*. (2010) solved the structure of p19 and found two distinct metal-binding sites within the protein which they speculate are unlikely to interact with each other (Chan *et al.*, 2010). In the NCTC11168 genome *p19* is adjacent to *cj1658* which encodes a membrane protein (Parkhill *et al.*, 2000). Cj1658 is similar to a permease which would suggest that it has a role as an inner membrane protein rather than being the outer membrane receptor for rhodotorulic acid. The proximity of *p19* and *cj1658* to each other within the genome and their inferred protein products also suggest a possible role in the uptake of ferrous iron. The role of p19 and Cj1658 in the uptake of iron from different sources could be further investigated by using a *C. jejuni* 81-176 background as that strain lacks a functional *feoB* gene (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006).

Ferrichrome and rhodotorulic acid are hydroximate siderophores synthesised by the smut fungi *Ustilago sphaerogena* and *Rhodotorula pilimanae* respectively (Baig *et al.*, 1986) and as such it seems doubtful that *C. jejuni* would encounter them within their usual niche environment. As discussed earlier, it is unlikely that NCTC 11168 can utilise ferrichrome and there is little information available with respect to the exact relationship between RA and the p19 system. As RA is synthesised by a number of different species of smut fungi it is not surprising that the siderophore is found in the soil. In particular, when *Rhodotorula minuta* is in a low iron environment it has been shown to produce 'large' amounts of RA (Atkin *et al.*, 1970). Theoretically this could result in the ferri-siderophore being available indirectly to *Campylobacter* in the environment. The FeRA complex is not transported across the fungal cell membrane. Instead the RA acts as a so-called 'taxi service' to deliver iron to the cell. At pH values of between 4 and 11 the ferri-siderophore is present as Fe_2RA_3 and iron uptake from it is energy-dependent - there appears to be no reduction of the compound (Carrano & Raymond, 1978). *R. minuta* does not utilise ferrichrome but it has been shown that in that organism, citrate is as effective as RA at delivering iron to the cell (Winkelmann, 1991). In structure investigations, it was apparent that the diketopiperazine ring in RA can be replaced with methylene groups without significant loss of iron transporting capability (Muller *et al.*, 1985). This would suggest that uptake in *C. jejuni* may not be RA but a related compound.

The question remains as to the function of p19 and Cj1658. Sequence comparisons with other bacteria reveal similar 19 kDa periplasmic proteins to be linked with the uptake of ferrous iron in those organisms. As p19 has no apparent associated outer membrane receptor it is plausible that p19 and Cj1658 may not be involved in the uptake of ferric

iron from FeRA. The inconsistency of results when investigating FeRA throughout this study would seem to suggest that this is the case.

3.6.7 Bacterial siderophores

Some bacterial siderophores, for example aerobactin, can be present in concentrations in the order of 200 mg/l during infection (Andrews *et al.*, 2003). The ability to utilise exogenous siderophores can potentially create a competitive environment and therefore any organism which is able to use those siderophores produced by others is likely to be at an advantage. Many bacteria have a number of receptors for different ferri-siderophore complexes, in addition to those for which they synthesise and secrete. Siderophore piracy is not confined to *Campylobacter* and the acquisition of iron via siderophore production and/or piracy is only part of an arsenal of virulence factors. So-called ‘stealth’ siderophore production is achieved in such a way as to evade the host immune system, in particular siderocalin, the protein responsible for binding apo- and holo-siderophores in order to prevent iron sequestration (Hoette *et al.*, 2008).

Chapter 4: General discussion

4.1 Overview

The aim of the present study was to address the question of whether *N*-linked glycosylation is necessary for the function of iron uptake proteins and to investigate the involvement of host stress hormones. Whilst addressing these research questions it has also been possible to consider the probable dual role of the enzyme GAPDH.

4.2 Glycosylation is not necessary for the function of iron uptake proteins

Young *et al*. (2002) identified p19 and Cj0175c as binding soy-bean agglutinin and therefore possessing the glycan moiety. Interestingly both p19 and Cj0175c are periplasmic binding proteins, which rules out any possibility that glycosylation is solely a prelude to export of the protein from the cell. The work carried out as part of this study would suggest that the glycosylation of iron uptake proteins is not necessary for their function. This was demonstrated in growth assays involving *pglB* and *pglK* mutants, where the mutant strains grew as well as the wild-type in each assay. Indeed, in a number of assays, the survival time of the *pgl* mutants in unsupplemented medium was better than that of the wild-type. This essentially raises a question; how could a fundamental procedure such as iron uptake be independent of an important biosynthetic process? From eukaryotic biology we know that glycosylation assists with the folding and stability of the newly-formed polypeptide and the folding can to some extent protect the mature protein (Imperiali & O'Connor, 1999). With respect to the iron uptake proteins, both known glycoproteins are small; p19 is 19 kDa and Cj0175c is slightly larger at 37 kDa (Parkhill *et al.*, 2000), the structures of both proteins have recently been solved (Chan *et al.*, 2010; Tom-Yew *et al.*, 2005). Cj0175c has the ‘classical’

periplasmic binding protein structure in that it resembles a Venus Flytrap (Felder *et al.*, 1999), p19 is similar in structure but has a somewhat flatter morphology and is likely to exist as a homo-dimer *in vivo* (Chan *et al.*, 2010). Knowing the structure and the influence exerted by the process of glycosylation, may allow comparison between similar glycoproteins, and in so doing will help determine a role for glycosylation.

4.3 Protein glycosylation is not required for the NA-mediated growth response in C. jejuni

The second aim of this project was to investigate any connection between NA, glycosylation and iron-mediated growth response. It has recently been shown that the enterobactin uptake system in *C. jejuni* is involved in the acquisition of iron from catecholamines (Haigh *et al.*, 2008). The *cfrA* mutant was available as a negative control for the growth assays carried out as part of this study. In all assays performed, the *pgl* mutants grew as well as the wild-type and in some instances had better survival rates over the 48-hour duration of the investigation. These findings suggest that the NA-mediated growth response in *C. jejuni* is not associated with protein glycosylation.

It has been noted in some instances in this study that the *pgl* mutants grow better than the wild-type NCTC11168, which may be due in part to the elimination of various stages of a biosynthetic pathway. A similar observation was reported by Szymanski *et al*., (2002), when they performed growth comparisons between *pglB* and *pglE* mutants and wild-type *C. jejuni* 81-176. The group showed that both mutants had a faster doubling time than the wild-type, with the difference being statistically significant only in the *pglE* mutant, although the reason for the observation was unclear. They went on to report that the wild-type growth was re-established with complementation (Szymanski *et al.*, 2002).

4.4 Why does C. jejuni glycosylate proteins?

The conclusions that iron uptake and NA-enhanced growth are independent of protein glycosylation raised the question as to why the process occurs in *C. jejuni*. Although it has been shown that glycosylation aids protein folding and protects against cleavage and breakdown (Bosques *et al.*, 2004), the situation is more complex. In eukaryotes protein glycosylation is known to affect cell-cell interaction, for example, in the type IIc congenital disorder of glycosylation (CDG-IIc), there is a reduction in leukocyte binding and a resulting increase in the number of infections (Freeze & Aebi, 2005). It has been shown by several investigations that *C. jejuni* glycosylation mutants are impaired with respect to host cell interaction (Szymanski *et al.*, 2002). Szymanski and co-workers considered the effects of mutating *pglB* in *C. jejuni* 81-176 and observed a reduction in adhesion and invasion of INT407 cells (Szymanski *et al.*, 2002). In addition it was found that the *pglB* mutant's ability to colonise the intestines of mice was significantly reduced (Szymanski *et al.*, 2002). Other groups have reported similar findings; Karlyshev *et al*. (2004) studied the importance of protein glycosylation in attachment to human cells and the colonisation of chicks. Using two *C. jejuni* strains, 81116 and the hyper-motile 11168H, the group mutated *pglH* in both strains and observed a reduction in attachment to and invasion of Caco-2 cells, with the reduction being more marked in 81116 than in 11168H; 23-fold to 6-fold with respect to attachment (Karlyshev *et al.*, 2004). From these studies it can be concluded that protein glycosylation is essential for the effective adhesion and invasion of host cells. In order to further verify the *pgl* mutants used in this study, it would be useful to carry out invasion assays, which it is anticipated would demonstrate a non-invasive phenotype for the mutants.

There are other reasons for the glycosylation of proteins by *C. jejuni*. Recently it was reported that protein glycosylation is necessary for competence 81-176 (Larsen *et al.*, 2004). Larsen and co-workers observed a greater decrease in natural competence of 81-176 in *pglB* and *pglE* mutants than had been previously reported by Bacon *et al*. (2000). The investigation into the effects of mutating *pglB* and *pglK* on natural competence that was performed as part of this study saw similar effects. However since NCTC11168 does not have plasmids, the mechanism underlying the process of natural competence must be different in that strain.

Transcriptional profiling revealed altered transcription levels in *pgl* mutants and indicated a close relationship between protein glycosylation and the expression of proteins involved in iron uptake (Nothaft *et al.*, 2008). The changes in transcription levels however are small (average ~2-fold) which would point to a more subtle relationship between the two systems. Variations in transcription level do not seem to be of significance in this study; there is no change in *cfrA*, *chuA* is only down-regulated in a *pglD* mutant, *cj0178* is slightly down-regulated (~ 2-fold) in *pglB* and *pglK* mutants, although no changes were observed in this study and *p19* is down-regulated in only *pglD*, *E* and *F* mutants (Nothaft *et al.*, 2008). Therefore, if these small changes to the level of transcription of iron uptake genes are verified and reproducibly due to changes in glycosylation, the evidence presented in this dissertation suggest that they have no functional consequence in relevant *in vitro* assays.

4.5 Metabolism and the roles of GAPDH

The glycolytic role of GAPDH was described nearly fifty years ago (Davidson *et al.*, 1967), however more recently it has been shown that the enzyme is localised on the cell surface where it is able to interact with host proteins, for example transferrin and

plasminogen (Egea *et al.*, 2007; Raje *et al.*, 2007). It is known that *C. jejuni* is unable to utilise glucose as a carbon source (Smibert, 1984). The fact that *C. jejuni* possesses a pyruvate kinase (PYK) homologue would suggest that the pathway may at some point have functioned in glycolysis, as PYK catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate which is the irreversible, final step in glycolysis (Parkhill *et al.*, 2000; Velayudhan & Kelly, 2002). The translocation of GAPDH to the cell surface was first described in group A streptococci (Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992). The plethora of reports that followed described the binding of GAPDH to actin/myosin, fibronectin and plasminogen (Gozalbo *et al.*, 1998; Pancholi & Fischetti, 1992; Schaumburg *et al.*, 2004; Seifert *et al.*, 2003). More recently a human pharyngeal cell membrane receptor was reported to interact with *S. pyogenes* GAPDH (Jin *et al.*, 2005). Many of these interactions clearly increase the repertoire of pathogens. The proposal that 1,3-diphophoglycerate assists with the removal of iron from transferrin (Morgan, 1977) raises an interesting issue with respect to the multiplicity of roles of GAPDH. Secreted GAPDH has been shown to be enzymatically active irrespective of its location (Egea *et al.*, 2007), therefore the enzyme's location at the cell surface may result in both the binding of host iron transport proteins and the subsequent removal of iron. Further studies could determine the precise location of the iron removal. It is anticipated that if surface-localised GAPDH binds Tf/Lf with the 1,3-diphosphoglycerate removing the iron from the host protein, the latter may deliver the iron to an outer membrane receptor which either disassociates the iron or transports the ferri-complex across the membrane into the periplasm. The mutagenesis of *gapA* that was to be a part of this study has to date proved unsuccessful.

4.6 Campylobacter protein glycosylation and its impact on future disease control

The success of glycoconjugate vaccines has been exploited for some time, with vaccines now being available against *Haemophilus influenzae, Neisseria meningitidis* and *Streptococcus pneumoniae*. Until recently the production of these vaccines relied upon chemical coupling of a pathogen-derived capsular polysaccharide to a suitable protein carrier. A new method, known as protein glycan coupling technology (PGCT), uses an OST, such as PglB to transfer a glycan to a carrier protein. Plasmids carrying the genes for synthesising the glycan, the OST plus the glycosylation sequon are then transformed into *E. coli*, which will then express the recombinant glycoprotein. The resulting protein can then be purified in a single step. The downside of this method is the fact that PglB will only transfer glycans that have a very specific configuration. However recent work carried out on the structure of *C. lari* PglB has shown the potential of engineering structural changes in order to relax these specificities (Terra *at al*, 2012).

4.6.1 Dealing with Campylobacter infection – preventing disease in chickens

We now know that there is a direct link between the welfare of food animals and public health. The data produced in this study, along with other work (RD Haigh, personal communication), show that the presence of NA enhances bacterial growth. The cost of the burden of *Campylobacter* infection (in 1995) in the UK alone was £1315 per case and in excess of £113 million in total during 2000 (Roberts *et al*., 2003). There is an obvious need to address the problem and this requires a full understanding of the process of disease progression in poultry. Currently the primary method of infection control is through increased biosecurity but more will need to be done at the stock-keeping level to reduce infection rates. Changes in methods of intensive food production are not possible without some financial cost; with decreased flock size, improved feed and less stressful handling and transport all resulting in a decrease in

infection rates. These stressors have the effect of increasing the synthesis and release of NA and lowering the animals' ability to fight infection. Whilst *Campylobacter* have long thought to be non-pathogenic commensal organisms in chickens, it is now accepted that this is the case only if the birds are kept in 'ideal' conditions (Humphrey, 2011). Routine practices in intensive farming expose the birds to a variety of stressful situations; flock thinning and the associated food withdrawal, changes in handlers and overcrowding are all factors in the proliferation of bacterial infection (Humphrey, 2007). The broiler house is not the end of the story – with levels of *Campylobacter* in chicken faeces being higher following the birds' transport to the slaughterhouse (Humphrey, 2011). It is clear that if human infection by *Campylobacter* is to be controlled, then levels of infection in the avian host have to be effectively reduced. Ultimately the increased financial burden that comes with improvements in animal welfare will be have to be passed onto the consumer.

Appendix A

Gene number	Gene product
Cj0081	Cytochrome oxidase subunit
Cj0114	Periplasmic protein †
Cj0143c	Periplasmic solute-binding protein
Cj0147c	Thioredoxin TrxA
Cj0152c	Membrane protein †
Cj0169	Superoxide dismutase SodB
Cj0175c	Periplasmic iron-binding protein
Cj0178	Membrane protein Lf uptake
Cj0200c	Membrane protein †
Cj0289c	Antigenic peptide PEB3
Cj0313	Membrane protein †
Cj0332c	Nucleoside diphosphate kinase Ndk
Cj0334	Alkyl hydroperoxide kinase AhpC
Cj0367c	AcrA
Cj0376	Periplasmic protein †
Cj0397c	Unknown
Cj0399	Membrane protein †
Cj0414	CmeC
Cj0415	Oxidoreductase sub-unit †
Cj0420	Periplasmic protein †
Cj0493	Trans. elongation factor FusA
Cj0511	Secreted proteinase †

Cj0530	Periplasmic protein †
Cj0599	Periplasmic protein †
Cj0610	Periplasmic protein †
Cj0638c	Inorganic pyrophosphatase Ppa
Cj0648	Unknown
Cj0694	Periplasmic protein †
Cj0715	Periplasmic protein
Cj0734c	HisJ
Cj0779	Thioredoxin peroxidise Tpx
Cj0835c	Aconitate hydratase AcnB
Cj0843c	Transglycosylase †
Cj0906c	Periplasmic protein †
Cj0944c	Periplasmic protein †
Cj0958c	Periplasmic protein †
Cj0982c	Periplasmic protein †
Cj0998c	Periplasmic protein †
Cj1018c	Periplasmic binding protein
Cj1032	Membrane fusion component †
Cj1053c	Membrane protein †
Cj1126c	PglB
Cj1181c	Translation elongation factor Tsf
Cj1214c	Unknown
Cj1221	Chaperonin Cpn60

Cj1345c	Periplasmic protein †
Cj1380	Periplasmic protein †
Cj1444c	Periplasmic protein, caps polysacc export KpsD †
Cj1496c	Periplasmic protein †
Cj1534c	Bacterioferritin
Cj1565c	Paralysed flagellum protein PflA
Cj1621	Periplasmic protein †
Cj1643	Periplasmic protein †
Cj1659	Periplasmic protein p19
Cj1670c	Periplasmic protein CgpA †

† Indicates putative or probable role of the protein.

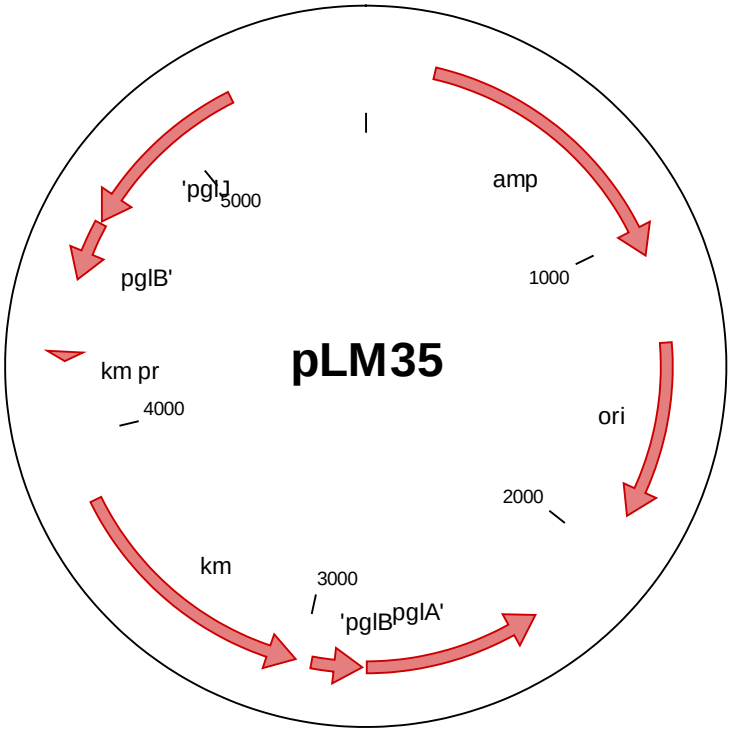


Figure A1. Diagrammatic representation of the *pglB* final construct as described in Millar (2003).

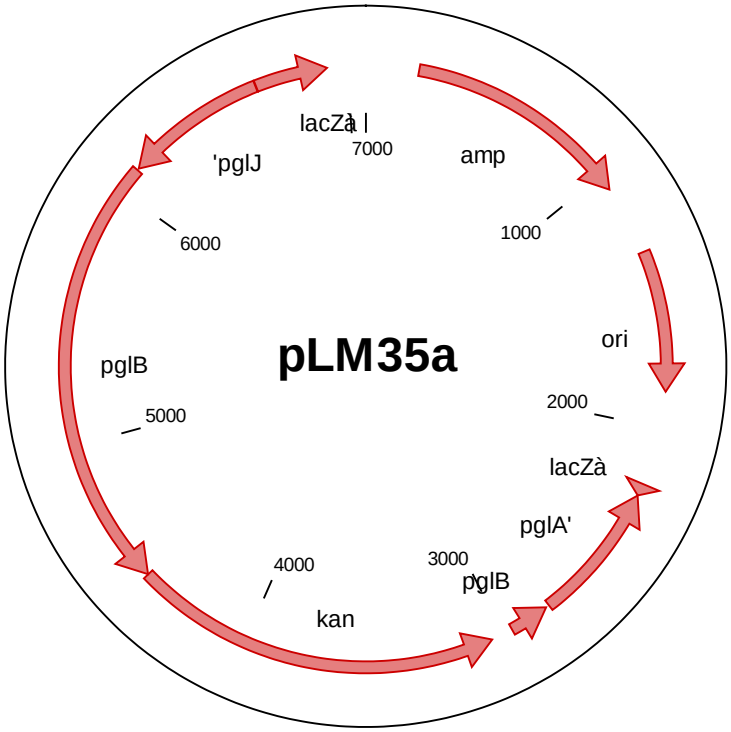


Figure A2. Diagrammatic representation of the *pglB* final construct as mapped for the purposes of this study.

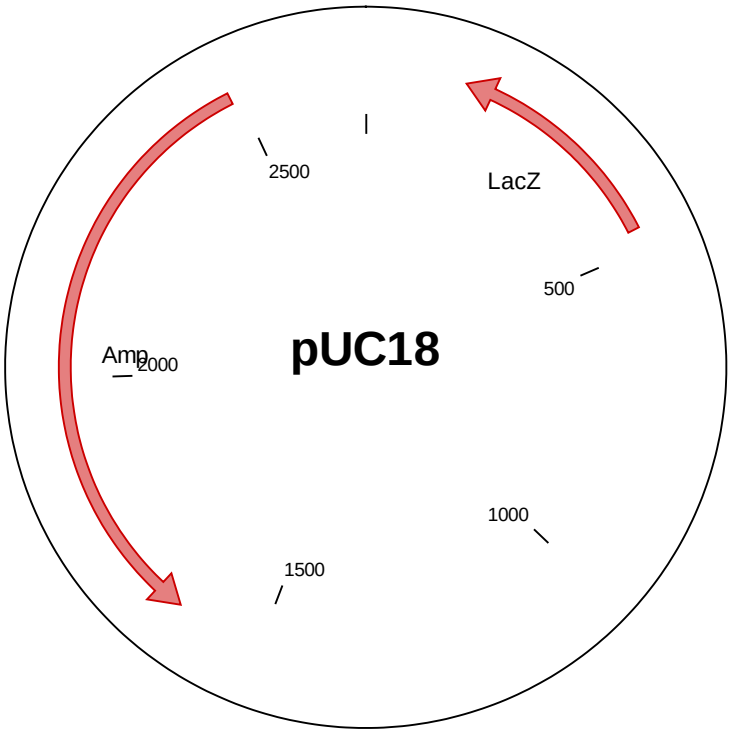


Figure A3. Diagrammatic representation of the pUC18 cloning/suicide vector used routinely throughout this study

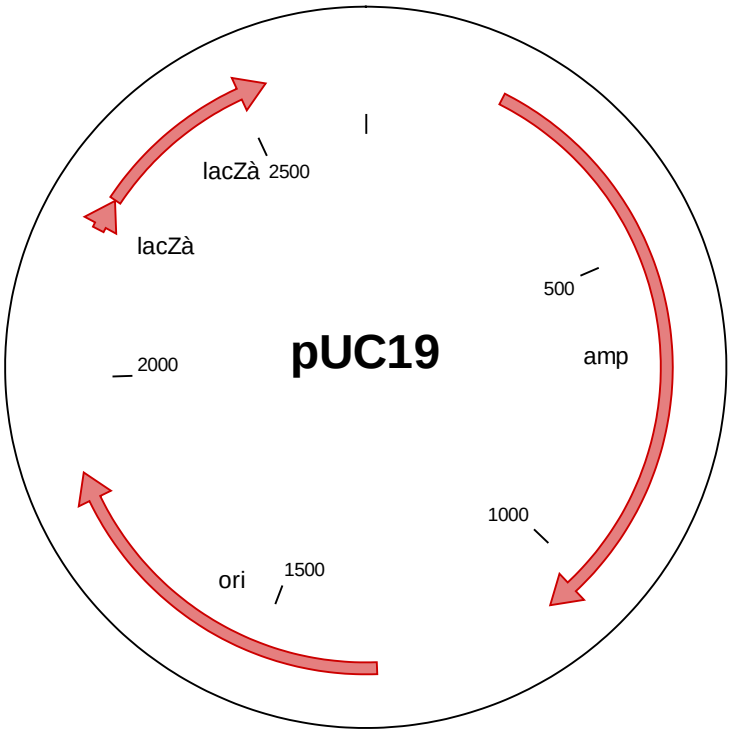


Figure A4. Diagrammatic representation of the pUC19 cloning/suicide vector used routinely throughout this study.

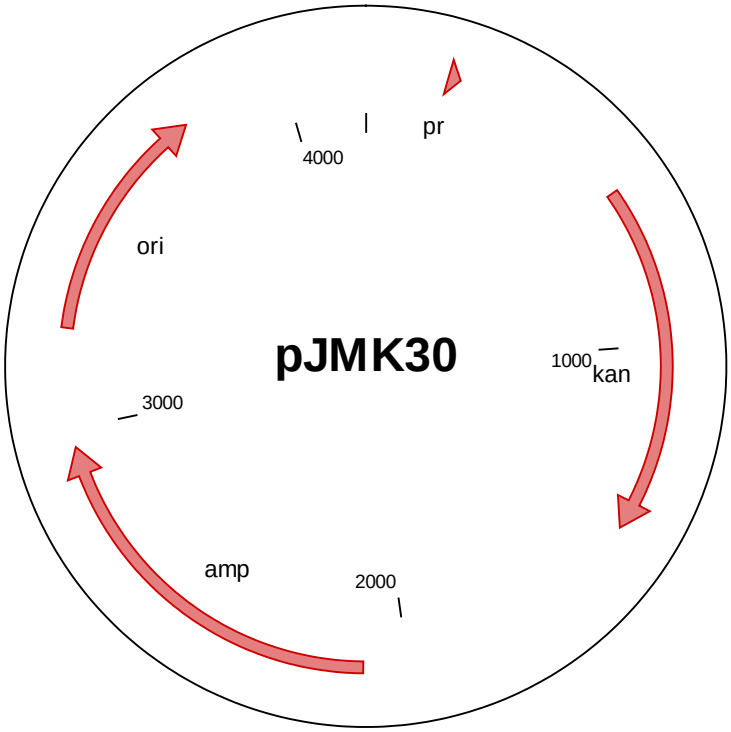


Figure A5 Diagrammatic representation of the pJMK30 plasmid used for its kanamycin cassette – pUC19 with the C. coli kan^{R} cassette flanked by multiple cloning sites (van Vliet *et al.*, 1998)

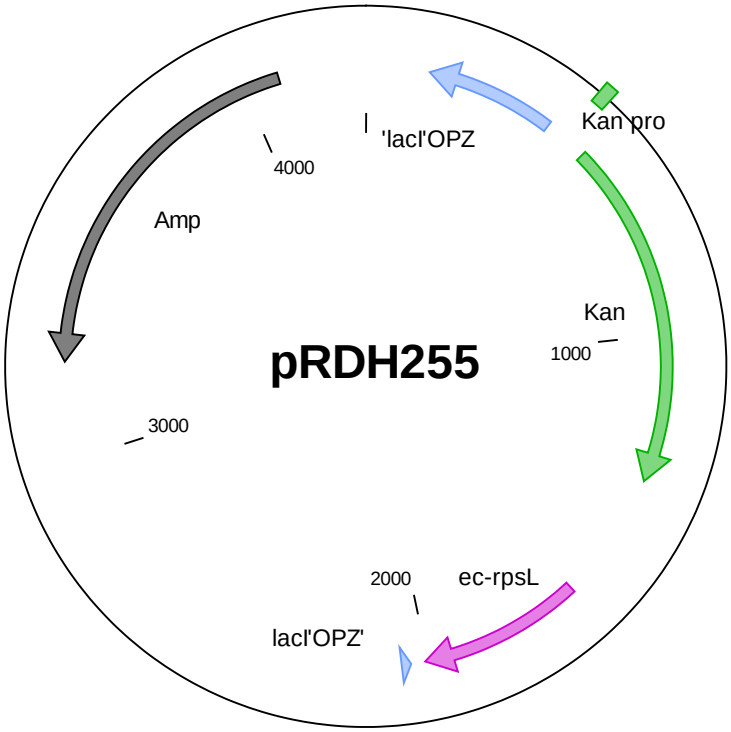


Figure A6 Diagrammatic representation of the pRDH255 plasmid used as a donor for its kan/ec-rpsL cassette (R Haigh)

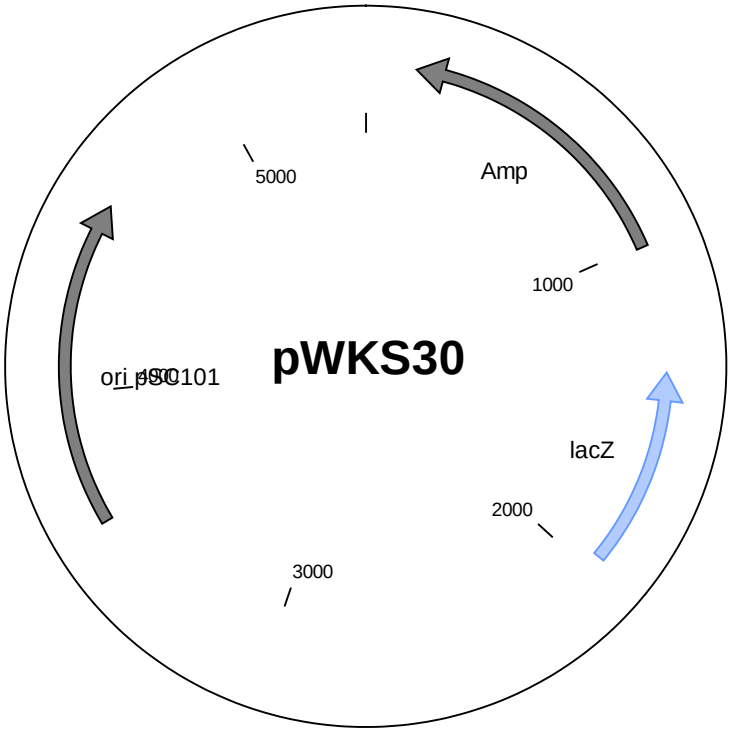


Figure A7 Lab stock plasmid used in cloning

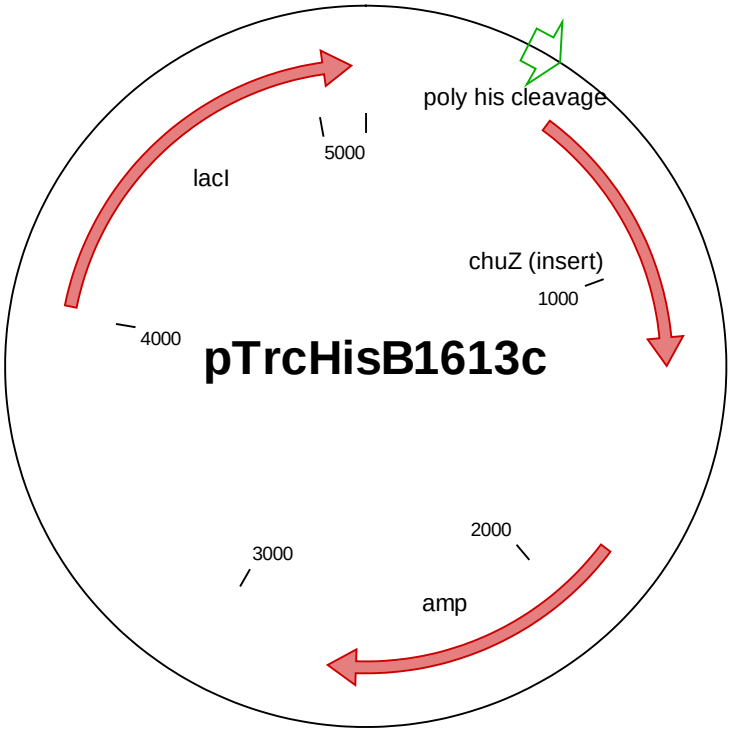


Figure A8 pTrc plasmid used as the Cj1613c-His_6 expression vector for ChuZ structure (Ridley *et al*., 2006)

Work carried out but not included in the results

Investigating haem uptake in Campylobacter – the role of ChuZ

Haem oxygenase (HO) was first described in 1968, when Schmid and co-workers observed the degradation of haem in the microsomes of rat spleen and liver (Tenhunen *et al.*, 1968). HO binds haem in a ratio of 1:1 and catalyses a reaction which liberates iron from the haem (Yoshida & Kikuchi, 1978; Zhu *et al.*, 2000). From human studies, it is known that the first step in the degradation involves a reductase that reduces ferric iron to its ferrous state (Yoshida *et al.*, 1980). More recently the process has been described in *N. meningitidis* and *N. gonorrhoeae*, where HemO is implicated in protection against iron toxicity (Zhu *et al.*, 2000). In *Neisseria* spp. the end products of the degradation are ferric biliverdin and carbon monoxide (CO) (Zhu *et al.*, 2000). In contrast to *Neisseria*, the breakdown process in *E.coli* O157:H7 produces CO and a ferric-free biliverdin (Suits *et al.*, 2005). In *C. jejuni*, *chuZ* is Fur-regulated, with the region between *chuA* and the divergently arranged *chuZ* containing two possible Fur boxes (van Vliet *et al.*, 2002). This regulation may serve in reducing the risk of iron toxicity through haem build up (Ridley *et al.*, 2006). The function of ChuZ in *C. jejuni* has been described by Ridley *et al* (2006); however the structure of the protein has yet to be determined. ChuZ has been shown to be an important protein in the haem uptake (Chu) system (Ridley *et al.*, 2006) and sequence comparison would suggest that ChuZ is not a simple homologue of the *E. coli* haem oxygenase, ChuS (Suits *et al.*, 2005). Competent cells of a number of *E. coli* expression strains were prepared and transformed with a ChuZ expression vector. Potential transformants were screened first by colony PCR and then by sequencing. The ChuZ expression vector pTrcHisB1613c in *E. coli* BL21 was already available for use in the protein structure investigation (Ridley

et al., 2006). Having achieved a sufficiently high concentration and purity it was necessary to test the purified ChuZ in a range of buffers which would not interfere with crystal formation. The elution buffer used in the purification protocol had an excessively high concentration (500 mM) of imidazole. The buffer exchange was accomplished with Amicon Ultra-15 Centrifugal Filter Units (Millipore), used in accordance with the manufacturer's instructions. The filter units allowed both buffer exchange and the concentration of the protein in a simultaneous process. The sample was diluted 1:4 initially with binding buffer without imidazole It was calculated that a total of three washes through the column would reduce the final imidazole concentration to ~20 µM which was considered to be sufficiently low to prevent interference with crystal formation. The purified, buffer-exchanged ChuZ samples were aliquoted into JCSG (Emerald BioSystems) screening plates in preparation for crystallisation trials. The aliquoting was performed by a Cartesian Dispensing System robot (Cartesian Technologies Inc USA, Genomic Solutions Company). Once completed, the plates were placed into crystal-growing rooms at room temperature and 4 °C.

Investigating haem biosynthesis

C. jejuni has recognisable homologues of the genes involved in haem biosynthesis (Panek & O'Brian, 2002; Parkhill *et al.*, 2000), therefore using mutants in the haem biosynthesis pathway would assist in determining the requirement of haem in *C. jejuni*. The aim was also to determine whether haem acquired via ChuA is completely broken down by ChuZ and to address the question of whether iron utilisation from haem can provide haem for metabolism as well as being a source of iron.

Appendix C

Construction of the hemE mutant strain

The *hemE* gene was identified using CampyDB and cloning primers designed with the aid of the Clone Manager program. The two cloning primers namely hemEfwdKpnI and hemErevPstI were used to amplify the gene and flanking regions from chromosomal DNA of *C. jejuni* NCTC11168. Both the PCR product and pUC19 were digested using *Kpn*I and *Pst*I before being ligated. Ligation products were transformed by electroporation into *E. coli* DH5αe, and cells grown overnight. Transformants were screened by PCR using M13 primers and sequenced to verify the construct as pSH7, shown in Figure A1. The construct was then subjected to inverse PCR using the primer pair hemEinv1NotI and hemEinv2NotI. The reaction produced a product of the predicted size of 4.5 kb which was then digested with *Not*I. The digested inverse PCR product was ligated to a similarly digested kanamycin resistance cassette along with the *C. jejuni rpsL* promoter and ribosome binding site plus *E. coli rpsL* gene from pRDH255 (R. Haigh). A method to construct an *rpsL* positive selection system was originally published by Hendrixson *et al.* (2001). The gene itself encodes the L12 protein of the 30s ribosomal subunit (Parkhill *et al.*, 2000) and point mutations confer streptomycin resistance. The resulting construct was screened by colony PCR and sequenced to verify it as the desired product. The map of the construct, pSH10 is shown in Figure A2.

Repeated attempts to obtain a *hemE* mutant have proved unsuccessful. The standard electroporation protocol was employed and following overnight recovery, cells were plated onto selective medium supplemented with vancomycin, trimethoprim and kanamycin plus either 100 µM or 1 mM haem. To date it has not been possible to obtain the *C. jejuni hemE* mutant strain despite having added haem to the medium when recovering the transformed *C. jejuni.* Upon further consideration, it was apparent that if

adding haem it would be necessary to have ChuA expressed which would not be the case if the cells were being grown under iron-replete conditions. It was therefore decided to transform pSH10 into a *fur* mutant background so ChuA would be expressed without having to use an iron-restricted medium. There is an appropriate mutant available within the group and this work is on-going. If the *hemE* mutagenesis is successful, the effects of the mutation will be investigated with growth assays. In addition, other genes within the haem biosynthesis pathway could be targeted to compare effects from the loss of different enzymes involved in the pathway.

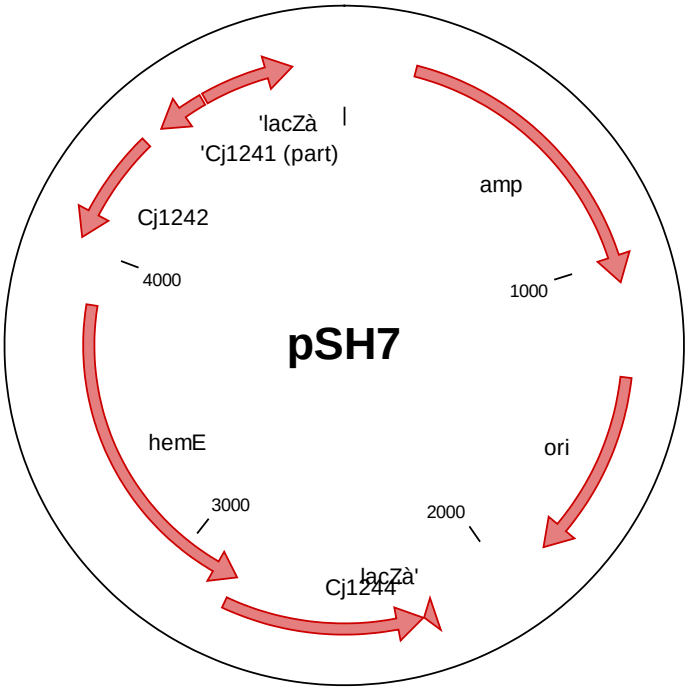


Figure A1. The plasmid pSH7, the result of the ligation of the amplified *hemE* region of NCTC11168 with pUC19.

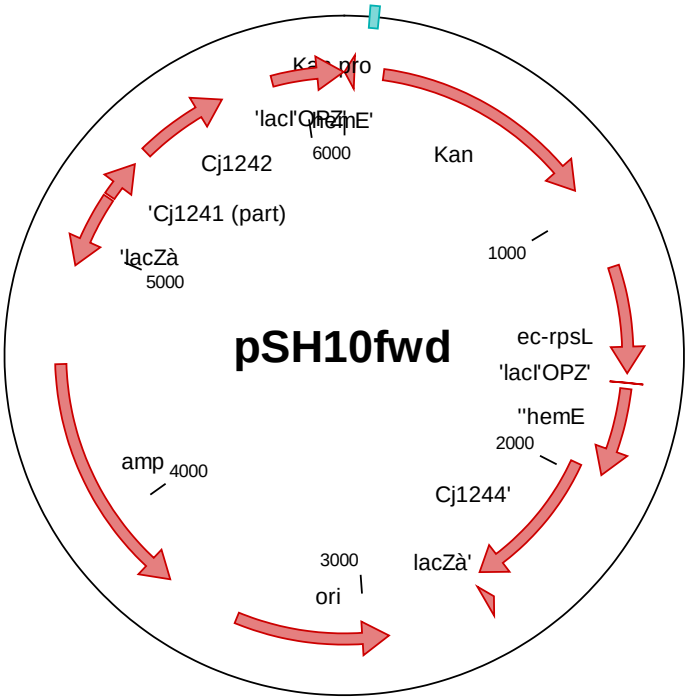


Figure A2. The plasmid pSH10, resulting from the ligation of pSH7 with the kanamycin resistance cassette from pRDH255.

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