

Iron Acquisition from Transferrins by
Campylobacter jejuni

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Abstract

Iron acquisition is vital for intestinal colonisation by *Campylobacter jejuni*. Characterisation of a number of iron uptake systems has occurred recently, allowing advancement in the understanding of the iron sources that *C. jejuni* utilises and how this occurs; however, the molecular basis of iron uptake from host iron-binding glycoproteins, the transferrins, is not known. The research presented here confirms that *C. jejuni* can use iron from the transferrins for growth and further characterises this process and the factors involved.

Iron uptake from the transferrins requires proximity and appears to be receptor specific. Binding of lactoferrin to the cell surface is iron-responsive. Cj0178, a protein similar to TonB-dependent receptors, is required and the involvement of the enterochelin outer membrane receptor protein CfrA, FeoB, the ferrous iron inner membrane transporter, and the ABC transporter system Cj0175c-Cj0173c was also indicated. Less lactoferrin bound to cells without Cj0178 and complementation of the *cj0178* mutation was successful. A role for Cj0178 in the uptake of haem was not demonstrated. Regulation of the genes *cj0176c-cj0173c* and *cj0177-tonB1* was shown to require Fur and promoter activity levels increased under iron-restriction. The presence of two Fur-boxes indicated separate regulation of the operons. The catecholamine stress hormone noradrenaline augmented the growth of *C. jejuni* in the absence and presence of iron and in the presence of the transferrins, but was non-essential.

A model is proposed of how transferrin-bound iron is used by *C. jejuni*. The process appears to be novel, involving a number of systems, but further work is required to confirm how they interact. The system through which noradrenaline may supply iron was investigated; however the mechanisms involved require further characterisation. The involvement of Cj0178 implies that the uptake of transferrin-derived iron is important for successful colonisation, which is vital for establishing an infection.

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Contents

Addenda consisting of one data CD

Format: PC. Software required: Microsoft Word (2003 onwards) and Adobe Acrobat Reader (version 6).

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 3. Raw Growth Curve Data and Statistical Testing of Differences
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List of Abbreviations

16S rRNA	16S ribosomal RNA
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AHT	Anhydrotetracycline
AIDS	Acquired immune deficiency syndrome
Apo-Lf	Apo-lactoferrin
Apo-ovo-Tf	Apo-ovotransferrin
Apo-Tf	Apo-transferrin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
B	Brucella medium
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDT	Cytolethal distending toxin
CEB	<i>Campylobacter</i> electroporation buffer
cfu	Colony forming units
CMH	Chelex-treated Mueller-Hinton medium
CPS	Capsular polysaccharide
Crp	Cyclic AMP receptor protein
CT	Cholera toxin
CTAB	Hexadecyltrimethyl ammonium bromide
DHBA	Dihydroxybenzoic acid
DBS or DHBS	Dihydroxybenzoylserine
DIG	Digoxigenin
DMEM	Dulbecco's Modified Eagles Medium
DMF	N,N'-dimethylformamide
DMT1	Divalent-metal transporter-1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EB	Elution buffer

Abbreviations

ECF	Extracytoplasmic function
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetate
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EMSA	Electrophoretic mobility shift assay
ENS	Enteric nervous system
EP	Extracellular polysaccharide
EPEC	Enteropathogenic <i>Escherichia coli</i>
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FBS	Foetal bovine serum
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
⁵⁵ Fe	Iron-55 radioisotope
⁵⁵ Fe-Lf	Iron-55-loaded lactoferrin
⁵⁵ Fe-ovo-Tf	Iron-55-loaded ovotransferrin
⁵⁵ Fe-Tf	Iron-55-loaded transferrin
Ferri-Lf	Ferri-lactoferrin
Ferri-ovo-Tf	Ferri-ovotransferrin
Ferri-Tf	Ferri-transferrin
FeSO ₄	Ferrous sulphate
Fur	Ferric uptake repressor protein
Fur ^{Cj}	Recombinant <i>C. jejuni</i> Fur protein
× g	Centrifugal force (gravity)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBS	Guillain-Barré syndrome
GC content	Guanine/cytosine content
GTP	Guanosine triphosphate
HABA	2-(4'-hydroxyazobenzene) benzoic acid
HP	Hydroperoxidase
HRP	Horseradish peroxidase
IL-8	Interleukin-8
IPCRM	Inverse PCR mutagenesis
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion sequence

Abbreviations

K _d	Dissociation constant
LB	Luria-Bertani medium
LbpA	Lactoferrin binding protein A
LbpB	Lactoferrin binding protein B
Lf	Lactoferrin
LGP	Ligand-gated porin
LOS	Lipooligosaccharide
M	Molar
MALDI-ToF	Matrix assisted laser desorption/ionisation-time of flight mass spectrometry
mcs	Multiple cloning site
MEM α	Minimal Essential Medium Alpha
MFS	Miller-Fisher syndrome
MH	Mueller-Hinton medium
MHPG	4-hydroxy-3-methoxyphenylglycol-piperazine salt
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	Messenger RNA
NA	Noradrenaline
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate with hydrogen
NCTC	National Collection of Type Cultures
NE	Norepinephrine
NGAL	Neutrophil-gelatinase-associated lipocalin
Nramp	Natural resistance-associated macrophage proteins
O ₂ ⁻	Superoxide
OD ₄₂₀	Optical density at 420 nm
OD ₅₉₅	Optical density at 595 nm
OD ₆₀₀	Optical density at 600 nm
OMRP	Outer membrane receptor protein
ONPG	<i>Ortho</i> -nitrophenol- β -D-galactoside
ORF	Open reading frame
Ovo-Tf	Ovotransferrin
PAGE	Polyacrylamide gel electrophoresis
PBP	Periplasmic binding protein

Abbreviations

PBS	Phosphate-buffered saline
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PK	Proteinase K
PKA	Protein kinase A
PMNL	Polymorphonuclear leucocytes
P-pant	4'-phosphopantetheine
PVDF	Polyvinylidene fluoride
Q-TRAP	Hybrid linear ion trap mass spectrometry
5'-RACE	5' rapid-amplification of cDNA ends
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
spp.	Species
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TbpA	Transferrin binding protein A
TbpB	Transferrin binding protein B
TBS-T	Tris-buffered saline with tween
TCA	Tricarboxylic acid
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	N-[tris(hydroxymethyl)methyl]-2-aminoethanesulphonic acid
Tf	Transferrin
Tfs	Transferrins
UV	Ultraviolet
VNC	Viable-non-culturable
v/v	Volume/volume percent

Abbreviations

w/v	Weight/volume percent
w/w	Weight/weight percent
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

Chapter 1. Introduction

1.1. Introduction

Campylobacter spp. are the most frequent cause of human bacterial enteritis in the developed world. Contracted mainly from contaminated food sources, the ensuing diarrhoea, other clinical symptoms and further complications lead to many lost working days and hours of hospital treatment resulting in a significant economic burden to society. Key to successful colonisation and disease progression, iron acquisition is crucial for bacterial growth and metabolism, with many iron sources available to the colonising micro-organism. *C. jejuni* was previously considered incapable of acquiring iron from the human iron-binding and transport glycoproteins lactoferrin (Lf) and transferrin (Tf) (Pickett *et al.*, 1992). The present study has demonstrated that *C. jejuni* can use Lf- or Tf-bound iron and allowed further in depth characterisation of the process.

1.2. *Campylobacter*

1.2.1. History

In 1886 Theodor Escherich identified curved-spiral rod-shaped bacteria that could not be cultured (Escherich, 1886; Vandamme, 2000). These organisms were subsequently proposed to be campylobacters, and the evidence supporting this hypothesis was outlined in 1986 (Kist, 1986). Identified as members of the *Vibrio* genus in 1913 (McFadyean and Stockman, 1913; Skirrow, 2006), campylobacters were originally associated with abortion in sheep (McFadyean and Stockman, 1913; Skirrow, 2006), cattle (Smith, 1918) and humans (Vinzent *et al.*, 1947; Vinzent, 1949; Vandamme, 2000). Abortion was proposed to have been caused by members of the *C. fetus* species (originally named *Vibrio fetus*; Smith and Taylor, 1919). Members of the *Campylobacter* genus were named as such in 1963 (Sébald and Véron, 1963; Vandamme, 2000). Differences in fermentative metabolism, microaerophilic growth requirements and the GC content of certain ‘vibrio’ species were identified, allowing separation of the newly named campylobacters from other more typical vibrios (Sébald and Véron, 1963; Véron and Chatelain, 1973; Vandamme, 2000). *Campylobacter* spp. remained primarily associated with reproductive problems and

abortion in farm animals until the 1970s (Smith and Taylor, 1919; Florent, 1953). There were earlier reports of campylobacters associated with human diarrhoeal disease (King, 1957), but successful isolation from patients with bacterial enteritis occurred only in the 1970s, prior attempts having used inappropriate culture conditions (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973; Skirrow, 1977; Ketley, 1997). The introduction of a selective medium containing vancomycin, polymyxin B and trimethoprim allowed routine isolation of campylobacters in many microbiology laboratories (Skirrow, 1977; Ketley, 1997). Since the 1980s *Campylobacter* spp. have emerged as the most prevalent and economically damaging food-borne enteric bacterial pathogens in the Western world.

1.2.2. *Campylobacter* Species

There are 16 species within the *Campylobacter* genus that have been organised into groups based on their 16S ribosomal (r) RNA gene sequences. Three significant phylogenetic clades can be formed, each including species sharing common traits. The first clade includes *C. hyointestinalis*, *C. lanienae*, *C. mucosalis* and *C. fetus*, which are generally found in larger animals, cannot hydrolyse indoxyl acetate and are often resistant to nalidixic acid. The second includes *C. concisus*, *C. hominis*, *C. gracilis*, *C. sputorum*, *C. curvus*, *C. showae* and *C. rectus*, which typically require hydrogen and anaerobic conditions for successful culture and are found in the human oral cavity. Some of these species have been shown to be sporadic pathogens of humans (On, 2005). The third clade, described as the thermophilic campylobacters, are discussed below.

1.2.2.1. The Thermophilic campylobacters

C. upsaliensis, *C. helveticus*, *C. lari*, *C. coli* and *C. jejuni* are potential or established enteropathogens typically possessing resistance to 5-fluorouracil and growing optimally at 42°C (On, 2005). This adaptation is proposed to aid successful commensal colonisation of their natural environment, the avian gut.

C. upsaliensis was first isolated from canine stools and lacks the ability to produce catalase (Sandstedt *et al.*, 1983; Moss *et al.*, 1990; Sandstedt and Ursing, 1991). Although relatively uncommon, *C. upsaliensis* has been isolated from human patients with gastroenteritis and septicæmia (Bourke *et al.*, 1998). Mostly commensal in domestic canines and felines, *C. upsaliensis* can sporadically cause disease in these hosts. Most

closely related to *C. upsaliensis* is *C. helveticus*, another *Campylobacter* species that was first identified from cats and dogs, with and without clinical symptoms (Burnens and Nicolet, 1992; Stanley *et al.*, 1992). More common in cats, *C. helveticus* is not yet implicated as a causative agent of human enteritis (Stanley *et al.*, 1992; Bourke *et al.*, 1998). Traits differentiating *C. helveticus* from *C. upsaliensis* are an incapability to survive on potato starch agar or to reduce selenite (Stanley *et al.*, 1992). Because only 80% of *C. upsaliensis* strains achieve successful growth at 42°C when tested (Goossens *et al.*, 1990), ‘thermotolerant’ (as opposed to ‘thermophilic’) may be a more suitable definition of *C. upsaliensis* and *C. helveticus*.

C. lari is typically resistant to nalidixic acid and was therefore separated from the related species *C. coli* and *C. jejuni* by this trait (On, 2005). However, nalidixic acid sensitive *C. lari* strains have been subsequently identified (Endtz *et al.*, 1997). Sources of this species are diverse, including wild birds (predominantly gulls), poultry, and cattle, as well as shellfish and untreated water. *C. lari* is capable of causing bacterial enteritis in humans and infection can prove fatal in immuno-suppressed individuals (Lastovica and Skirrow, 2000).

C. coli and *C. jejuni* are the major pathogens, with *C. jejuni* accounting for 80-95% of all *Campylobacter* infections in humans. *C. coli* is the second commonest, causing up to 50% of reported human cases of infection in certain areas (Skirrow, 1994). These strains were, however, first isolated from animals with diarrhoeal disease (Jones *et al.*, 1931; Doyle, 1948; Ketley, 1997). *C. coli* is most commonly recovered from pigs, but is also found in poultry, cattle and dogs, with most animals not presenting symptoms of enteric disease. Although *C. coli* and *C. jejuni* are very closely related, one differentiating feature is the ability to hydrolyse hippurate, for which *C. coli* is negative (On, 2005). Further identification problems have arisen due to the isolation of hippurate-negative *C. jejuni* strains (Morris *et al.*, 1985), which are impossible to differentiate from *C. coli* using routine testing. The use of minimal medium for growth or recognition of alpha-haemolytic activity may assist with the identification of these two key species, but will probably not solve the problem of providing indisputable differentiation (On, 1996).

1.3. *Campylobacter jejuni*

C. jejuni was originally described in 1931 as ‘*Vibrio jejuni*’ following culture from the intestinal contents of cattle (Jones *et al.*, 1931; On, 2005). Isolation from the blood of

humans suffering gastroenteritis followed (King, 1957; King, 1962; Vandamme, 2000), as well as an association with abortion in sheep (Bryans *et al.*, 1960; Vandamme, 2000). The *C. jejuni* species contains two subspecies known as *jejuni* and *doylei*. *C. jejuni* subsp. *doylei* is very different from the *jejuni* subspecies, as it lacks an animal host, the ability to reduce nitrate, and the ability to survive at 42°C (Steele and Owen, 1988). It has, however, been isolated from patients with enteritis and septicaemia (Lastovica and Skirrow, 2000).

C. jejuni subsp. *jejuni* (which will subsequently be referred to as *C. jejuni* in this study) is recognised as the most common causative agent of bacterial gastroenteritis worldwide (Skirrow, 1994).

1.3.1. Transmission and Epidemiology

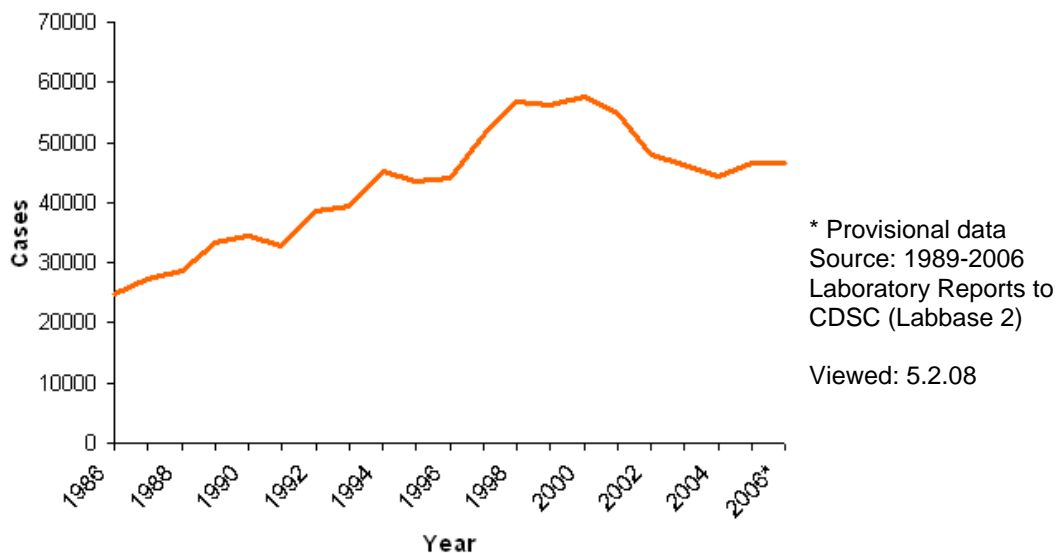
1.3.1.1. Sources of Infection

C. jejuni is a commensal organism in a variety of animals including birds (mainly poultry), cattle, sheep, pigs and dogs (Skirrow, 1994). In poultry, *C. jejuni* (and *C. coli*) carriage is elevated in comparison to other species of bird, with commensal colonisation of the caecum at a minimum of 10^{10} cfu/g of caecal contents (Shreeve *et al.*, 2000). *C. jejuni* colonisation of poultry is common because of the close proximity of birds during rearing and the ingestion of faeces (coprophagy). Subsequent contamination of meat by the transfer of faecal matter onto the surface during processing is frequent. *Campylobacter* numbers present on a fresh chicken carcass have been proposed to be as many as 10^3 - 10^6 cfu (Hood *et al.*, 1988). Freezing of poultry meat and offal has been shown to reduce the presence of *Campylobacter* compared to non-frozen meat (Lee *et al.*, 1998). Poorly handled or improperly cooked poultry is by far the most frequent source of human infection, but bacterial enteritis caused by *C. jejuni* can be acquired from a range of other sources including cattle. *Campylobacter* presence in cattle varies throughout the gut (rumen <100 cells/g, stool 10^4 - 10^6 cells/g; Grau, 1988); it is found in the small intestine, large intestine and caecum, but not the true stomach. *C. jejuni* has also been found in the small intestine of lambs (Stanley *et al.*, 1998a; Stanley *et al.*, 1998b). The low numbers of *Campylobacter* found on beef, pork and lamb are likely to be due to chilling and dehydration of the meat during processing (Grau, 1988), as the occurrence in livestock can be up to 100%. Offal products often contain higher levels of *Campylobacter* due to contamination during the slaughtering process (Bolton *et al.*, 1985; Kramer *et al.*, 2000).

Other sources of *Campylobacter* include raw milk, household pets, and environmental sources such as standing water. Water contamination can be caused by faecal matter from wild birds, farmland run-off, waste from food processing plants and contamination by urban sewage (Miller and Mandrell, 2005).

1.3.1.2. Incidence in Industrialised Countries

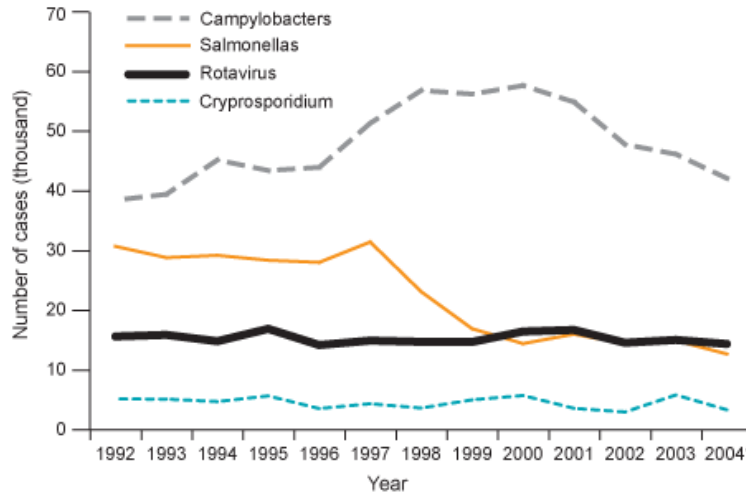
Campylobacter infection is a major public health burden in the developed world. Health protection agency figures show the general increase of reported cases of *Campylobacter* infections in England and Wales from 1986 (Fig. 1.1). It is estimated that actual numbers of cases may be up to 10 times higher than those reported (Adak *et al.*, 2002). Studies in the U. K. and the Netherlands have estimated that the number of actual *Campylobacter* cases may even be as much as 100 times higher than those reported (Friedman *et al.*, 2000). *Campylobacter* is the most common cause of food-borne bacterial enteritis in the United States, with an estimated 2.4 million cases of infection per year (Friedman *et al.*, 2000).



http://www.hpa.org.uk/infections/topics_az/campy/data_ew.htm

Figure 1.1. Number of laboratory reports of faecal isolates of *Campylobacter* spp. reported to the Health Protection Agency Centre for Infections from England and Wales from 1986-2006.

Recorded cases of *Campylobacter* infection are also significantly higher than those reported for other important gastrointestinal pathogens such as *Salmonella* spp., rotavirus and *Cryptosporidium* spp. in England and Wales (Fig. 1.2).



<http://www.hpa.org.uk/cdr/archives/archive05/enteric05.htm>. Viewed: 6.3.08.

Figure 1.2. Annual incidence of selected gastrointestinal pathogens in England and Wales from 1992-2004 reported to the Health Protection Agency.

The general increase in *Campylobacter* infections seen (Fig. 1.1) since 1986 is likely to be due to increased recovery by microbiological laboratories using improved detection and isolation methods and better reporting throughout the 1980s and into the early 1990s (Skirrow, 1991; Friedman *et al.*, 2000). However, over the last 15 years since testing for *Campylobacter* has become routine, the upward trend in cases of infection may be due to a genuine increase.

Campylobacter infections arise in individuals of all ages with peaks noted in the very young (less than 4 years) and young adults. Incidence of infections is 1.2 to 1.5 times higher in males than females, with an exaggeration of this discrepancy in younger adults (below 30 years of age). *Campylobacter* outbreaks are rare, with the majority of all infections sporadic, and associated with an individual. Some developed countries in Europe experience seasonal fluxes, with a clear increase in cases during the summer months (Friedman *et al.*, 2000).

1.3.1.3. *Campylobacter* Incidence in Developing Countries

The epidemiology of *Campylobacter* infections in the developing world, compared to industrialised nations, is quite different. Increased exposure and elevated levels of infection at a very young age lead to notably altered patterns of disease. Severe diarrhoeal disease, mild diarrhoea or asymptomatic carriage can all occur following *C. jejuni* infection. Incidence of *Campylobacter* infection in children is much higher in the developing world (0.4 cases per child each year or *Campylobacter* incidence of 40,000/100,000 population per year for children below the age of 5; Calva *et al.*, 1988; Taylor *et al.*, 1988), compared to developed countries (incidence of 50/100,000 population per year for adults and around 300/100,000 for children between the ages of 1 and 4 years; Skirrow, 1987). Infection rates in young children in developing countries are over 100 times higher than in England, but illness does not often manifest itself (Oberhelman and Taylor, 2000). Infection can lead to severe gastroenteritis in younger children; older children and adults are rarely ill, but shed bacteria frequently. *Campylobacter* is often recovered from infected individuals along with other pathogenic organisms (Taylor *et al.*, 1988); in industrialised nations multiple infections are rare. Infections can be contracted from contaminated food early in life or from contaminated environmental sources. Humans may also contract the disease from animals living in their houses, such as chickens. Cases of disease demonstrate little or no seasonality. High rates of asymptomatic carriage occur, which is not seen in the Western world. Although adaptation and strain variation may partially explain the differences in clinical presentations, a major factor in the variation between industrialised and poorer countries is the immune status of the individual (Oberhelman and Taylor, 2000). Less pronounced clinical outcomes occur after childhood due to developed protective immunity (Oberhelman and Taylor, 2000).

1.3.2. Disease Progression

1.3.2.1. Clinical Features

Bacterial enteritis is the most frequent outcome of human infection by *C. jejuni*. *C. jejuni* has a low infective dose; consumption of as few as 500-800 organisms can cause disease (Robinson, 1981; Black *et al.*, 1988). Cases of disease increase proportionally to increases in dose. Upon consumption, the average incubation time for *C. jejuni* is 3 days;

however the incubation period can range from 18 hours to 8 days (Skirrow and Blaser, 2000). The illness onset and progression depends upon the strain virulence, infective dose and the immune status of the individual. Initially patients suffer severe abdominal cramping, followed by the start of acute diarrhoeal disease. A number of individuals (around 30%) experience non-specific flu-like symptoms including fever (40°C), chills, dizziness, headache, malaise and myalgia prior to diarrhoeal onset. Diarrhoea is copious (around 8-10 events per day) and can be watery, bile stained and bloody. Larger amounts of blood appear for around 15% of individuals following 1 to 2 days of diarrhoeal disease; however faecal leucocytes and erythrocytes occur in almost all episodes. Patients often suffer nausea; however vomiting is rare (15% of all cases). Symptoms usually decline following 3 to 5 days of illness. Bacterial shedding persists for a number of weeks following recovery, but persistent carriage of bacteria and extended symptomatic illness only occur in immuno-suppressed individuals, such as AIDS patients (Black *et al.*, 1988; Skirrow and Blaser, 2000; van Vliet and Ketley, 2001). Most cases are self-limiting with treatment rarely needed. Patients showing little sign of improvement of severe symptoms or who are immuno-compromised can be treated in hospital with antibiotics such as erythromycin or fluoroquinolones such as ciprofloxacin. When erythromycin cannot be used, tetracyclines or chloramphenicol can be (clinical aspects of infection have been reviewed in Skirrow and Blaser, 2000).

A less frequent outcome of *C. jejuni* infection is bacteraemia which is characterised by fever and rigors. Often the very young, very old or immuno-suppressed individuals succumb to this; however it may temporarily occur for any individual at the start of an infection. *C. jejuni* can also cause a wide range of other disorders, for example, septicaemia, meningitis, abortion, mild hepatitis and various urinary tract and renal diseases (Tee and Mitch, 1998; Skirrow and Blaser, 2000).

1.3.2.2. Influence of Immune Responses on the Progression of Disease

Following 5 days of disease, antibodies specific to *Campylobacter* antigens can be found in the serum of patients (Blaser and Duncan, 1984; Black *et al.*, 1988; Skirrow and Blaser, 2000). Volunteers repeatedly exposed to the same strain showed inflammatory diarrhoea and antibody production upon the first exposure, but only asymptomatic colonisation upon the second exposure (Black *et al.*, 1988; Skirrow and Blaser, 2000). However, sequential infections (within 2 years) have occurred which may be due to an

individual being challenged with a different strain. In developing countries fully protective immunity can be achieved from a small number of infections due to cross-reaction (Skirrow and Blaser, 2000). Protective immunity has also been observed in long-term poultry workers; new workers often demonstrate disease aspects of bacterial enteritis upon commencing work (Cawthraw *et al.*, 2000). Immune responses are important in the progression of infection as demonstrated by the prolonged or invasive nature of disease in immuno-compromised patients (Tee and Mitch, 1998). In addition to a protective role, the host immune system is also involved in the development of major autoimmune complications following *C. jejuni* infection.

1.3.2.3. Guillain-Barré Syndrome and Other Complications

Guillain-Barré syndrome (GBS) is an autoimmune disease that results in symmetrical flaccid paralysis, with individuals losing control of their muscles, reflexes and often respiratory control (Nachamkin *et al.*, 1998). Worsening of symptoms occurs over 2 to 3 weeks, with the disorder usually self-limiting and most patients recovering within several weeks or months (Nachamkin *et al.*, 1998). Miller-Fisher syndrome (MFS) is a similar disorder to GBS, but a loss of reflexes, unsteadiness, and a loss of eye movement are not accompanied by weakness (Fisher, 1956; Nachamkin *et al.*, 1998). Around 30-40% of GBS episodes are proposed to follow on from infection with *C. jejuni*. Symptoms are worse in *C. jejuni*-related GBS, demonstrated by an increase in deaths, the necessity for ventilatory support and more severe neurological damage (*C. jejuni* infection as an antecedent to GBS is reviewed in Nachamkin *et al.*, 1998). *C. jejuni* infection is also a common antecedent to the autoimmune complication Reiter's Syndrome. The onset of pain and inflammation of the joints occurs 3 days to 6 weeks after the start of gastric symptoms. The disorder lasts up to a year; prognosis is full recovery (Skirrow and Blaser, 2000).

1.3.3. Biology

C. jejuni is a member of the bacterial 16S rRNA super family VI (Vandamme 1991). Curved-spiral and rod-shaped (1.5-6.0 µm long and 0.2-0.5 µm wide), this Gram-negative bacterium is microaerophilic (3-15% O₂, 3-10% CO₂ and ~85% N₂) and thermophilic. Growth is optimal at 42°C, but *C. jejuni* is capable of growth between 34 and 44°C (Ketley, 1997). The organism is non-spore-forming, catalase and oxidase positive and

urease negative. Fastidious organisms, *C. jejuni* require complex growth media and cannot ferment or oxidise carbohydrates for use as an energy source, instead deriving their carbon from amino acids such as serine or tricarboxylic acid cycle intermediates. *C. jejuni* are highly motile due to single unsheathed flagella located at one or both cell poles (Vandamme, 2000; van Vliet and Ketley, 2001). The organisms are capable of surviving in a viable-but-non-culturable (VNC) state following exposure to unfavourable conditions such as a lack of nutrients or atmospheric oxygen concentrations. The bacteria become coccoid in shape and often remain undetected by routine culturing methods; disease may nevertheless be caused if the appropriate conditions are encountered (Rollins and Colwell, 1986; Tholozan *et al.*, 1999).

1.3.4. Genomics

The genome of *Campylobacter* spp. is small (1.6-1.7 mega-bases in size) with a relatively low GC content of approximately 30% (Parkhill *et al.*, 2000; Fouts *et al.*, 2005). The extensively studied human intestinal pathogen *Escherichia coli* O157:H7 has a genome size of approximately 5.4 mega-bases and a GC content of around 50% (Perna *et al.*, 2001). The genome sequence of *C. jejuni* strain NCTC 11168 was published in 2000 (Parkhill *et al.*, 2000). The genome included 1,654 predicted ORFs with an average length of 948 bp, resulting in 94.3% of the genome coding for proteins, the densest genome sequenced at the time of publishing (Parkhill *et al.*, 2000). A number of stable RNA species (54) were also predicted (Parkhill *et al.*, 2000). The only repeat sequences present in the genome appear to be the rRNA operon and three other ORFs (Parkhill *et al.*, 2000). Other than *cj0752*, a pseudogene bearing resemblance to an insertion sequence element transposase, there are no functional IS elements, transposons, retrons or prophages encoded (Parkhill *et al.*, 2000). In addition to the ribosomal protein clusters, there are only three other obvious gene clusters present, encoding lipooligosaccharide (LOS) biosynthesis, extracellular polysaccharide (EP) biosynthesis and biosynthesis of the flagellar posttranslational modification proteins. There is also a protein glycosylation locus (Szymanski *et al.*, 1999). Other genes are organised into groups which often lack similar functions. The genome contains very few genes for the breakdown of amino acids, an incomplete glycolytic pathway, but a complete gluconeogenesis pathway and a full tricarboxylic acid cycle (Parkhill *et al.*, 2000). The NCTC 11168 genome is also rich in hypervariable sequences, which are short homopolymeric tracts of bases found within genes. Produced by slipped-

strand mispairing, altered lengths of tracts cause changes in transcription or translation and phase variation of bacterial surface structures (Linton *et al.*, 2000). *C. jejuni* lacks many of the DNA repair systems present in *E. coli*, leading to the noted sequence variation. Most of the homopolymeric tracts are located within the LOS biosynthesis, EP biosynthesis and flagellar modification clusters, allowing rapid adaptation to survival in the fluctuating host environment and defence against host immune responses (Parkhill *et al.*, 2000). The *C. jejuni* genome contains only 3 sigma factors, similar to the related organism and gastric pathogen *Helicobacter pylori* (Tomb *et al.*, 1997; Parkhill *et al.*, 2000). The genome contains a number of two-component regulatory systems and other global regulator proteins. *C. jejuni* appears to have more regulation capability than *H. pylori*, reflecting the wider variety of environments that *C. jejuni* survives in (Parkhill *et al.*, 2000).

Since the original genome sequence publication, several other *Campylobacter* genomes have been completed including *C. lari* strain RM2100, *C. upsaliensis* strain RM3195 and *C. coli* strain RM2228 (Fouts *et al.*, 2005). *C. jejuni* strain RM1221 (Fouts *et al.*, 2005), *C. jejuni* strain 81-176 (Hofreuter *et al.*, 2006) and *C. jejuni* strain 81116 (Pearson *et al.*, 2007) have also been sequenced, allowing comparisons between multiple *C. jejuni* genomes. *C. jejuni* strain RM1221 is highly similar to NCTC 11168; key differences include the presence of four large integrated elements in RM1221 and variation in ORFs found within the EP biosynthesis cluster (Fouts *et al.*, 2005). *C. jejuni* strain 81-176 is a highly pathogenic and invasive strain with a marginally smaller genome than NCTC 11168 due to fewer genes involved in capsular polysaccharide production and flagellar modification. A small number of duplications, deletions or insertions differ between 81-176 and NCTC 11168. 81-176 also differs from NCTC 11168 by the presence of two plasmids, pVir and pTet (Korlath *et al.*, 1985; Bacon, 2000; Bacon *et al.*, 2002; Batchelor *et al.*, 2004). In contrast to NCTC 11168, strain 81116 contains fewer homopolymeric tracts, a novel duplication, and functional ORFs that are pseudogenes in the other strains (Pearson *et al.*, 2007). Strain 81116 is also proposed to be more genetically stable (Manning *et al.*, 2001). As with strains NCTC 11168 and RM1221, strain 81116 does not contain plasmids (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Pearson *et al.*, 2007).

1.3.5. Pathogenesis of Infection

The ability of a micro-organism to cause disease in a host characterises it as a pathogen. The presence of this organism in all cases of disease, but absence from healthy

hosts, the ability to culture the organisms from diseased individuals and the subsequent re-infection of a healthy host with the cultured cells associates an organism with a specific disease. Successful re-isolation from the diseased host and identification of the causative agent as the original organism conclusively proves the association (Koch's Postulates). Both the development of a filtration technique to isolate campylobacters from stool samples of diseased individuals (Butzler *et al.*, 1973; Dekeyser *et al.*, 1972), and the development of a selective supplement for medium (Skirrow, 1977) overcame the isolation problems encountered by diagnostic microbiology laboratories in cases of *Campylobacter* infection (Vandamme, 2000). Subsequent studies with human volunteers demonstrated a link between infection by *C. jejuni* and clinical symptoms (Black *et al.*, 1988).

1.3.5.1. Progression of *C. jejuni* Infection

Upon consumption of contaminated food or water, colonisation of the host intestinal environment follows and is essential for establishing infection. Colonisation results in clinical symptoms ranging from abdominal discomfort to diarrhoea due to damage to the gut epithelium (Konkel *et al.*, 2000). Bacteria that avoid removal by peristalsis (Konkel *et al.*, 2000) colonise the mucosal surface of the lower gastrointestinal tract by initially penetrating the mucosal secretions to reach the epithelial cells below (Szymanski *et al.*, 1995). The shape of the cell and the polar flagellum at one or both ends allow the bacterium to successfully enter the mucus (van Vliet and Ketley, 2001). The flagellum itself is composed of a number of flagellin monomers, encoded by either *flaA* or *flaB*, which are major antigens and together form the filament part of the flagella (Harris *et al.*, 1987; Guerry *et al.*, 1990; Nuijten *et al.*, 1990). Mutations resulting in the loss of flagella cause impaired colonisation and invasion (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993b; Yanagawa *et al.*, 1994; Yao *et al.*, 1994). Chemotactic motility towards or away from a sensed concentration gradient is also vital for colonisation, demonstrated by the inability of organisms with a mutated chemotaxis system to colonise the intestinal cell surface of animal models (Takata *et al.*, 1992). Following penetration of the mucus layer, adherence of *C. jejuni* to host cells is needed for colonisation and disease progression; adherence is proposed to be mediated by the association of *C. jejuni* cell surface proteins with host cell surface receptors (Konkel *et al.*, 1997; Pei *et al.*, 1998; Konkel *et al.*, 1999a; Ziprin *et al.*, 1999; Jin *et al.*, 2001). The flagellum itself may act as a secondary adhesin required for colonisation of the intestine in

the mouse animal model (Grant *et al.*, 1993; Yanagawa *et al.*, 1994; Yao *et al.*, 1994). Other adhesins are also involved and potential candidates include capsular polysaccharide (CPS) and LOS (McSweeney and Walker, 1986; Fauchère *et al.*, 1989; Doig *et al.*, 1996; Bacon *et al.*, 2001). *C. jejuni* is also capable of producing the exotoxin, cytolethal distending toxin (CDT), which is known to cause cell-cycle arrest at G2 phase, damage host DNA and distend HeLa and Caco-2 cells (Johnson and Lior, 1988; Whitehouse *et al.*, 1998; Pickett, 2000). In addition, the acquisition of iron is essential for survival, successful colonisation and infection of the human host (which will be reviewed in detail in Sections 1.4 and 1.5).

Following colonisation and adherence, a subset of *C. jejuni* cells can invade host epithelial cells, leading to further damage and allowing avoidance of host immune responses (Everest *et al.*, 1992; Konkel *et al.*, 2000). The damage caused increases the severity of diarrhoea which may include blood and pus; the gut lining may become ulcerated (Konkel *et al.*, 2000). *C. jejuni* have been shown to invade cell lines of human origin, for example HeLa, HEp-2 or intestinally derived INT407, Caco-2 or HT29 cells (Konkel *et al.*, 1992a; Oelschlaeger *et al.*, 1993). Upon contact with host cells, successful invasion requires the expression of new bacterial proteins and is also influenced by host cell secreted factors (Konkel *et al.*, 1992a; Konkel *et al.*, 1993; Konkel *et al.*, 1999b). *C. jejuni* has also been documented to affect the host cell structure to bring about invasion by influencing the microfilaments (Konkel *et al.*, 1992a), microtubules (Oelschlaeger *et al.*, 1993), or both (Oelschlaeger *et al.*, 1993; Biswas *et al.*, 2003). Once within the cells, *C. jejuni* is mainly found within vacuoles but can reside free in the cytoplasm (Konkel *et al.*, 1992b); it does not appear to replicate freely in either.

Invasion and toxin production trigger a strong inflammatory response during infection which may be crucial to the development of gut ulceration (Ketley, 1997; Konkel *et al.*, 2000). Following inflammatory damage, polymorphonuclear leucocytes (PMNLs) and monocytes can be found in the intestinal epithelium (Everest *et al.*, 1992; Autenreith *et al.*, 1995). PMNLs phagocytose *C. jejuni* resulting in killing; phagocytosis is enhanced by opsonisation. Ingestion of *C. jejuni* can also occur without opsonisation; *C. jejuni* can survive for up to 7 days following this internalisation (Wooldridge *et al.*, 1996). CDT production induces secretion of IL-8, a proinflammatory cytokine, from the basolateral surface of the epithelium (Pickett, 2000). IL-8 release also induces influx of PMNLs from the lamina propria, further aiding removal of *C. jejuni* and control of bacterial spread (Autenreith *et al.*, 1995). The strong immune response may also damage the epithelial

organisation further and cause inflammatory lesions, aiding infection by allowing bacteria access to the lamina propria resulting in more severe symptoms (Konkel *et al.*, 2000).

Bacteria are often found at the intercellular junctions of host cells and can translocate across epithelial cell barriers. *C. jejuni* travels from the apical to basolateral surfaces of polarised Caco-2 cells. Bacteria are found within vacuoles in the cells and later transcytosing between cells, sometimes without invasion, via tight junctions. Transepithelial electrical resistance is not lost for the first 6 hours of translocation, demonstrating that tight junctions are in fact only transiently open during penetration by *C. jejuni* unlike during *Salmonella* infection (Finlay and Falkow, 1990; Everest *et al.*, 1992; Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993; Brás and Ketley, 1999; MacCallum *et al.*, 2005).

C. jejuni is a pathogen of humans as colonisation of the human gastrointestinal tract epithelium results in disease progression as described above and the presentation of clinical symptoms. Colonisation of chickens by *C. jejuni* is not associated with pathological damage and is therefore considered to be commensal, even though antibody responses are triggered (Humphrey, 2006). *C. jejuni* is found in the caeca in chickens, in the mucosal secretions in the crypts of the epithelium, but direct association with the cell surface does not appear to occur (Beery *et al.*, 1988; Newell and Wagenaar, 2000). Therefore, *C. jejuni* colonisation results in a different presentation of symptoms in different hosts.

1.4. Iron

1.4.1. The Role of Iron

Iron is an essential micronutrient for life, necessary for almost all organisms' survival; it plays a central role in the host-pathogen relationship. As the fourth most abundant element present in the crust of the Earth, it has been readily available throughout evolution, leading to the wide range of processes that are dependent upon it today (Beinert *et al.*, 1997; Andrews *et al.*, 2003). Iron is important because of its role as a cofactor or prosthetic group in many proteins involved in metabolism and basic cellular pathways in both pathogens and the host (Schaible and Kaufmann, 2004). Iron is important for photosynthesis and respiration, nitrogen fixation, methanogenesis, hydrogen generation and consumption, the TCA cycle, oxygen transport, the regulation of gene expression and RNA and DNA synthesis. Iron also participates in electron-transfer chains, is found within the

redox centre of iron-sulphur proteins and acts to stabilise protein structures (Andrews *et al.*, 2003; Faraldo-Gómez and Sansom, 2003). Iron can exist in two redox states as either reduced ferrous (Fe^{2+}) or oxidised ferric (Fe^{3+}) forms, with the $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox potential spanning from -300 mV to +700 mV. By incorporation into proteins, iron allows the necessary biological roles of the proteins to be fulfilled by taking on the required redox potential, geometry or spin state (Andrews *et al.*, 2003). The limited number of organisms thought not to depend upon iron, include lactobacilli (Archibald, 1983; Weinberg, 1997), *Borrelia burgdorferi*, the causative agent of Lyme disease (Posey and Gherardini, 2000), and *Treponema pallidum* which causes syphilis. However, the latter two exploit the host metabolism, which uses iron, because of their obligate intracellular lifestyle (Andrews *et al.*, 2003).

1.4.2. Iron Availability in the Human Host

Iron is not freely available in the host environment, unlike nutrients such as carbon, nitrogen, phosphate, potassium and magnesium (Ratledge and Dover, 2000). Originally, in the anoxic environments of the past, iron usage would have been free from problems. Upon the photosynthetic production of oxygen, soluble Fe^{2+} (0.1 M at pH 7) was readily oxidised to Fe^{3+} (Andrews *et al.*, 2003). Under aqueous and aerobic conditions at physiological pH (~7), iron is predominantly present as Fe^{3+} , which forms ferric oxide hydrate complexes that are largely insoluble and therefore poorly available for use by cells (solubility = 1.4×10^{-9} M; Ratledge and Dover, 2000; Miethke and Marahiel, 2007). To combat this insolubility and availability problem, the host has evolved a number of iron binding and transport processes to allow successful delivery of the necessary Fe^{3+} to cells. Therefore, the amount of free iron available in the host is extremely low and often stated as 10^{-18} M at pH 7 (Andrews *et al.*, 2003).

Following dietary intake of iron, the nutrient travels into the blood and becomes attached to the host iron-binding glycoprotein Tf, which transports the iron around the body (Ratledge and Dover, 2000). The key properties of Tf and the related glycoproteins Lf and ovotransferrin (ovo-Tf) are reviewed in Section 1.4.5. Tf and Lf are usually only partially (25-35% and 5-9% respectively) iron-saturated (Weinberg, 1978) in the host allowing emergency binding of excess and unexpected iron in the tissue fluids and blood when needed, removing free iron from the body. Iron is also found bound to ferritin, an intracellular iron storage compound (Ratledge and Dover, 2000; Schaible and Kaufmann,

2004), or complexed to haem and haemophores and other iron proteins (Wandersman and Stojiljkovic, 2000), all of which limit iron availability.

The reduction of iron by gastric secretions has been proposed (Bezwoda *et al.*, 1978) implying that Fe^{2+} is the more abundant form in the stomach (Wooldridge and van Vliet, 2005). In the intestinal environment, iron can exist as either Fe^{2+} or Fe^{3+} depending on the pH and oxygen tension. Within the gut lumen the environment is anaerobic, resulting in a larger amount of Fe^{2+} than Fe^{3+} . Closer to the gut mucosa, the environment becomes more oxidising, resulting in increased Fe^{3+} (Wooldridge and van Vliet, 2005). At the gut surface, iron can be reduced from Fe^{3+} to Fe^{2+} by an intestinal ferric reductase (for example, duodenal cytochrome *b*) and be acquired by intestinal cells via the permease, divalent-metal transporter-1 (DMT1), which allows the passage of Fe^{2+} from the lumen into the cytoplasm. Alternatively, iron can be bound by iron chelators such as haemophores or Lf (Hentze *et al.*, 2004; Schaible and Kaufmann, 2004). If necessary, Fe^{2+} can be oxidised to Fe^{3+} by an intestinal membrane-bound oxidase, hephaestin, or by an oxidase found in the plasma, caeruloplasmin, allowing binding to Tf, which then transports the nutrient around the body.

The uptake of iron from Tf by host cells occurs by receptor-mediated endocytosis. Ferri-Tf binds to the Tf receptor at the cell surface. Once internalised, iron is removed inside an acidified endocytic vesicle and released into the cytoplasm by DMT1, where excess iron is stored by ferritin. Apo-Tf is released from the cell surface via endocytic vesicle/cell membrane fusion. The Tf receptor becomes available for re-use after recycling to the cell surface (Hentze *et al.*, 2004). In addition, a neutrophil-gelatinase-associated lipocalin (NGAL/24p3) has been identified as an iron uptake pathway that is independent of Tf (Kaplan, 2002). NGAL is secreted by epithelial cells and neutrophils, complexes iron and is then moved to late endosomes (Kaplan, 2002).

Iron is recycled in the host mainly by reticuloendothelial macrophage phagocytosis and lysosomal degradation of erythrocytes. It can also be recycled by the uptake of free haem-complexed iron using the macrophage haemoglobin-scavenger receptor CD163 (Hentze *et al.*, 2004). Loss of the peptide hepcidin-1 leads to iron overload in mice, while over-expression causes anaemia (Ganz, 2003), implying a key role for this peptide in the regulation of iron absorption and release in the host (Schaible and Kaufmann, 2004).

1.4.3. Iron and Reactive Oxygen Species

The combination of an excess of iron with oxygen can be toxic. The mixture of iron and oxygen leads to the production of toxic reactive oxygen species (ROS) from either the Fenton reaction of Fe^{2+} with H_2O_2 , or the Haber-Weiss reaction of oxygen radicals with H_2O_2 . The reactive hydroxyl and superoxide radicals are known to damage cellular lipids, proteins and DNA by reacting within the cell, which can cause prokaryotic and eukaryotic cell death. The generation of ROS within phagocytes is successfully used as a mechanism for killing ingested bacteria (Pierre and Fontecave, 1999; Braun and Braun, 2002). This process is presented in Figure 1.3. To prevent damage, all cells tightly regulate intracellular iron concentration.

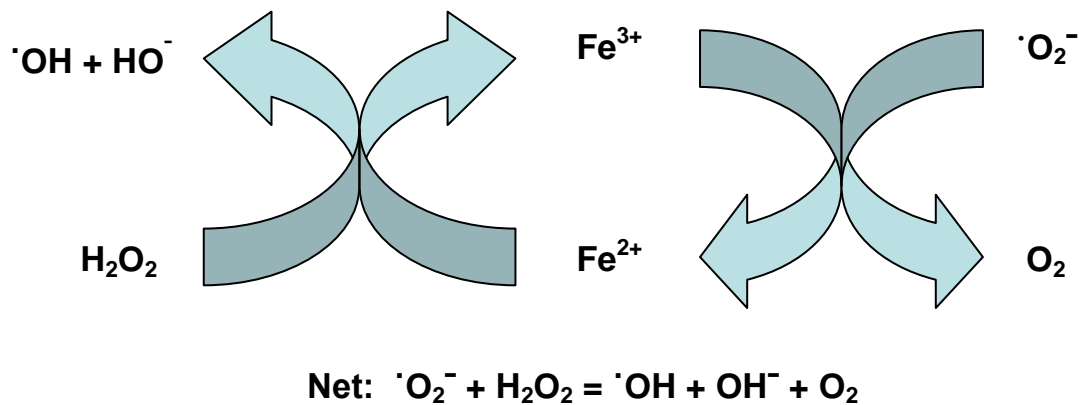


Figure 1.3. Haber-Weiss chemistry involved in the production of reactive oxygen species from oxygen and an excess of iron. In total, the reactions shown are the Haber-Weiss cycle; the left side of the schematic is the Fenton reaction. (Diagram adapted from Ratledge and Dover, 2000).

1.4.4. Iron Sources for the Invading Pathogen

The low availability of iron in the human host acts as a non-specific defence mechanism against invading micro-organisms. Micro-organisms generally require 10^{-6} to 10^{-7} M iron for survival and replication (Weinberg, 1978). Therefore, most have evolved specialised and highly adapted mechanisms to scavenge iron from the iron-limited host environment, principally involving chelation or reduction. These include Fe^{3+} ion chelators known as siderophores and their associated specific ferri-siderophore uptake systems (Miethke and Marahiel, 2007). Alternatively, ferric reduction of Fe^{3+} to the more soluble Fe^{2+} by adjusting the pH of the surroundings (Andrews *et al.*, 2003) or by specific reduction

using ferric reductases, coupled with Fe^{2+} transport systems (Kammler *et al.*, 1993), allows uptake. Haem acquisition systems (Bullen, 2006) and receptor-based uptake systems for the removal and internalisation of iron from host proteins such as Tf and Lf also exist (Ekins *et al.*, 2004).

1.4.4.1. Ferri-Siderophores

Bacterial siderophores are low molecular weight (<1000 Da), high affinity (for example the $K_d = 10^{52} \text{ M}^{-1}$ for ferri-enterochelin) Fe^{3+} binding compounds (Andrews *et al.*, 2003). Uptake pathways for these small iron binding compounds can be found in bacteria, fungi and even monocotyledonous plants (Andrews *et al.*, 2003; Miethke and Marahiel, 2007). Siderophores are typically generated and secreted by iron-starved bacteria (intracellular iron concentration of $<10^{-6} \text{ M}$ which prevents proliferation), they bind extracellular Fe^{3+} , for example from host protein Tf, and are then taken back into the cell as a siderophore-iron complex. Alternatively, the iron is reduced by free extracellular or membrane-bound ferric reductases and taken up as Fe^{2+} (Andrews *et al.*, 2003; Miethke and Marahiel, 2007). The dissociation constant of siderophores ranges from 10^{22} to 10^{50} , resulting in successful removal of iron from Tf, Lf or ferritin, but not usually from haemophores (Ratledge and Dover, 2000). Atypically, siderophores of mycobacteria are found on the bacterial cell envelope attached by aliphatic chains (Ratledge and Dover, 2000; Faraldo-Gómez and Sansom, 2003). Some organisms can also perform siderophore piracy, using specific acquisition systems to gain siderophore-associated iron from those produced by other organisms without the need for synthesising the siderophore itself. Baker's yeast can use several siderophores as sources of iron but does not produce any (Miethke and Marahiel, 2007). If the iron has not been separated from the siderophore at the cell surface, it can be removed from the ferri-siderophore complex within the cell by intracellular ferric reductases or by hydrolysis of the siderophore using ferri-siderophore hydrolases (Faraldo-Gómez and Sansom, 2003; Miethke and Marahiel, 2007).

There are more than 500 known siderophores (Ratledge and Dover, 2000; Andrews *et al.*, 2003) which typically possess three oxygenated chelating groups that form hexadentate octahedral complexes with Fe^{3+} (Andrews *et al.*, 2003; Faraldo-Gómez and Sansom, 2003). They are grouped according to the chemical nature of the chelators as catecholates, hydroxamates or α -hydroxycarboxylates (Andrews *et al.*, 2003; Miethke and Marahiel, 2007). Combinations of chelators or other chelating groups also arise in a

smaller subset of siderophores (Faraldo-Gómez and Sansom, 2003). Siderophores can be produced from a range of precursors including ornithine (catecholates), lysine (hydroxamates), salicylic acid (mycobactin), citrate and amino acids (Brown and Holden, 2002; Winkelmann, 2002).

Iron uptake is best characterised in enteric bacteria such as *E. coli*, *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp (Ratledge and Dover, 2000). The catecholate siderophore enterochelin is known to be a key iron source for all enterics (Ratledge and Dover, 2000; Faraldo-Gómez and Sansom, 2003). Enterochelin (Fig. 1.4a) is a 669 Da catecholate, with the highest dissociation constant of any natural compound of 10^{52} (Ratledge and Dover, 2000).

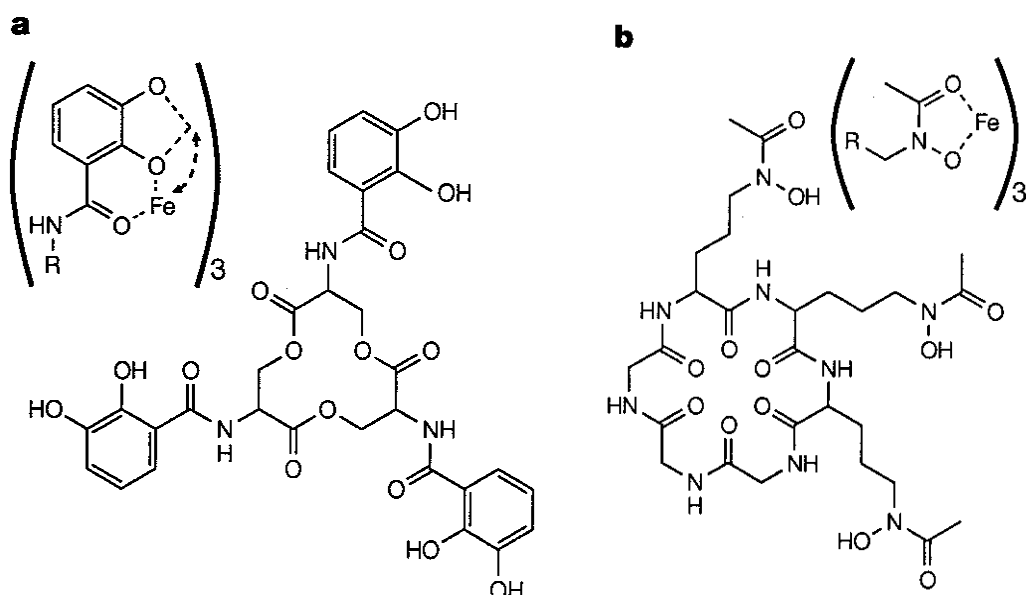


Figure 1.4. The structures of the siderophores **a**) enterochelin and **b**) ferrichrome (Faraldo-Gómez and Sansom, 2003).

In *E. coli*, enterochelin synthesis is achieved by the expression of *entABCDEF* (Fig. 1.5; Ratledge and Dover, 2000). EntC, EntB and EntA convert chorismate to dihydroxybenzoic acid (DHBA). EntD, a 4'-phosphopantetheine (P-pant) transferase post-translationally modifies EntB and EntF. The DHB-AMP ligase EntE activates DHBA transferring it to the P-pant of EntB. EntF activates L-serine transferring it to the P-pant of its own PCP domain. EntF then accepts DHBA from EntB and catalyses the formation of an amide bond with serine producing a DHBA-ser-S-EntF thioester. The DHB-ser (DBS) is then transferred to another domain of EntF. Further rounds of DBS synthesis result in a

DBS trimer joined by ester linkages, which is then cyclised into enterochelin and released from EntF (Fig. 1.5; Earhart, 2004).

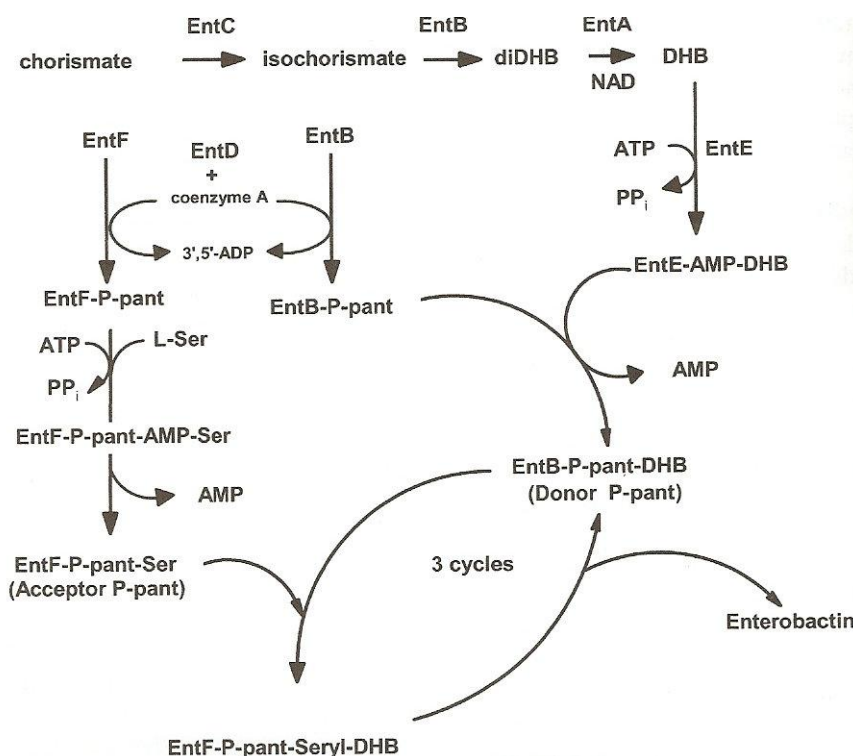


Figure 1.5. Biosynthesis of enterochelin in *E. coli* from dihydroxybenzoic acid and serine catalysed by EntA-F (taken from Earhart, 2004).

Once synthesised, enterochelin is secreted across the inner membrane, a process mediated by EntS, a major facilitator super-family protein with 12 α -helical transmembrane segments (Furrer *et al.*, 2002). Release across the outer membrane proceeds via the channel-forming protein TolC (Bleuel *et al.*, 2005).

In addition to enterochelin, enteric bacteria such as *E. coli*, *S. flexneri* and *K. pneumoniae* can also produce the hydroxamate aerobactin (Wooldridge and Williams, 1993; Ratledge and Dover, 2000). Aerobactin is proposed to be produced as well as enterochelin in order to obtain iron from sources other than Tf, such as ferritin (Ratledge and Dover, 2000). However, some *Salmonella* spp. do not produce aerobactin at all (Ratledge and Dover, 2000). The 740 Da hydroxamate siderophore ferrichrome (Fig 1.4b) is produced by fungi and can be used by *E. coli* (Faraldo-Gómez and Sansom, 2003). Additional fungal siderophores hijacked by *E. coli* spp. include coprogen and rhodotorulic acid (Ratledge and Dover, 2000; Faraldo-Gómez and Sansom, 2003). *E. coli* are also

capable of acquiring iron from ferri-citrate (Hussein *et al.*, 1981). *Salmonella* spp. are capable of using the carbon from citrate but they cannot derive iron from ferri-citrate (Ratledge and Dover, 2000). One further hydroxamate siderophore group, ferrioxamines B, E and G are used by *Salmonella* spp. (Luckey *et al.*, 1972; Kingsley *et al.*, 1999) but not synthesised by them. *E. coli* are not thought to be capable of using ferrioxamines (Ratledge and Dover, 2000).

Originally described as supplying iron to bacteria of the genera *Proteus*, *Providencia* and *Morganella* (Drechel *et al.*, 1993), α -ketoacids and α -hydroxyacids can provide iron for *Pasteurella haemolytica*, *Staphylococci*, and *E. coli* (Reissbrodt *et al.*, 1994; Heuck *et al.*, 1995; Kingsley *et al.*, 1996). *Salmonella* spp. are also capable of using these primary metabolites as an iron source for growth under iron-limited conditions when there are no siderophores present (Kingsley *et al.*, 1996).

The uptake of ferri-siderophores in Gram-negative bacteria involves the use of highly specific outer membrane receptor proteins (OMRP). Energy is provided by proton-motive force transduced from the inner cytoplasmic membrane by the ExbBD-TonB complex, which spans the periplasm. Within the periplasm, periplasmic binding proteins bind and concentrate the substrate delivering it to ATP-binding cassette (ABC) transporters located within the inner membrane. The complex is transported across the inner membrane and into the cell cytoplasm where the iron is removed and used (Fig. 1.6, using the ferri-enterochelin uptake system as an example).

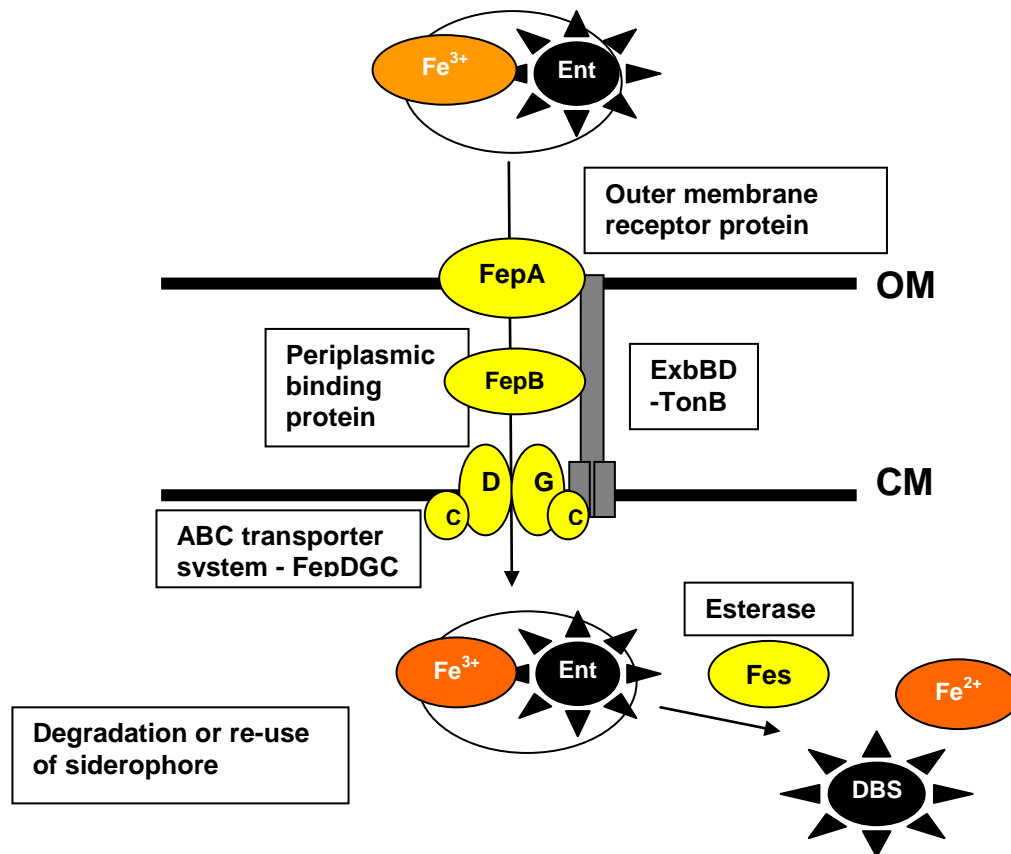


Figure 1.6. Process by which iron is taken up from ferri-siderophores in Gram-negative bacteria using ferri-enterochelin as an example. The ferri-enterochelin complex is taken up via a specific, high-affinity energised outer membrane receptor protein, FepA. Concentration in the periplasm occurs by FepB binding of ferri-enterochelin. FepB delivers the ligand to the ABC transporter system FepDGC. Located within the cytoplasmic membrane, FepC subunits bind and hydrolyse ATP and FepD and G form the channel spanning the membrane. Ferri-enterochelin passes through the hydrophobic channel into the cytoplasm. Within the cytoplasm hydrolysis of the ester bonds (producing dihydroxybenzoyl serine) and reduction of the associated iron liberates the iron for use in the cell.

Unlike Fe²⁺, ferri-siderophores cannot travel through the outer membrane porins (limit = ~600 Da) by passive diffusion, instead requiring active transport across the outer membrane (Andrews *et al.*, 2003). Because of this, bacteria have developed a number of high affinity OMRPs for siderophores that are typically expressed under iron-limitation. Extensively studied, the OMRPs of *E. coli* include FepA for ferri-enterochelin (Fig. 1.6), FhuA for ferrichrome and FecA for ferri-citrate (Ferguson *et al.*, 1998; Locher *et al.*, 1998; Buchanan *et al.*, 1999; Ferguson *et al.*, 2002). The structures consist of two domains. The first domain is a β -barrel structure formed by 22 antiparallel strands with short periplasmic loops and much longer extracellular loops that can vary in size. The second domain is an

N-terminal globular domain ‘plugging’ the barrel at the periplasmic end, covalently linked to the first β -strand providing part of the substrate binding site that regulates movement through the channel (Ratledge and Dover, 2000; Braun and Braun, 2002). The structure was demonstrated by X-ray crystallography of the ligand gated porins FhuA and FecA with and without bound substrate. The binding of substrate caused major conformational changes (Ferguson *et al.*, 1998; Locher *et al.*, 1998; Ferguson *et al.*, 2002; Braun and Endriß, 2007).

Changes occur in the external loops of FecA upon association of ferri-citrate resulting in exposure of the binding site and closing of the newly formed cavity around ferri-citrate allowing the next stage of transport to occur (Ferguson *et al.*, 2002). FhuA does not experience the external loop movement seen with FecA, but this may still occur *in vivo* (Ferguson *et al.*, 1998). In addition, FecA and FhuA experience unwinding of a short periplasm exposed α -helix, resulting in a new flexible extended structure (Ferguson *et al.*, 1998; Ferguson *et al.*, 2002). The N-terminal region of FecA, FhuA and FepA is not fixed, so binding of the substrate results in the mobilisation of this region which is proposed to be necessary for interaction with TonB. The crystal structure of FepA bound to ferri-enterochelin has not been determined (Ferguson *et al.*, 1998; Buchanan *et al.*, 1999; Ferguson *et al.*, 2002; Braun and Endriß, 2007).

The amount of TonB protein present in the cell is limited, and in *E. coli* TonB is thought to directly interact with all TonB-dependent receptor proteins through their TonB boxes (Postle, 1993; Cadieux and Kadner, 1999). TonB boxes are found at the N-terminal end of receptor proteins and associate with the C-terminal domain of TonB allowing TonB-mediated ferri-siderophore uptake. TonB is attached to the inner membrane by its N-terminal domain and spans the periplasmic space (Braun and Braun, 2002; Andrews *et al.*, 2003).

ExbB and ExbD proteins are located in the inner membrane and complex with TonB in a 1:7:2 ratio of Tonb:ExbB:ExbD (Higgs *et al.*, 2002). The chemiosmotic gradient of the inner membrane is used by ExbBD to energise and conformationally change TonB, thus transducing energy to the OMRP. The energy transduction changes the binding site affinity and causes structural changes that allow a channel to open within the OMRP; the plug domain may be expelled into the periplasm with bound ferri-siderophore or the siderophore may travel into the periplasm by surface-diffusion through the channel (Ferguson and Deisenhofer, 2002). Although *E. coli* possesses one ExbBD-TonB system,

other bacteria can have more than one; for example *Vibrio cholerae* has two TonB proteins required for energy transduction to different OMRPs (Meyer and Payne, 2001).

Once in the periplasm, ferri-siderophores are delivered to inner membrane ABC-transporter systems by periplasmic binding proteins (PBP, for example FepB, Fig. 1.6). Ferri-siderophores may be bound by PBPs adjacent to the OMRP or collected within the periplasm (Köster, 2001). The PBP FhuD of *E. coli* has been extensively characterised and shown to be bilobal from its crystal structure (Clarke *et al.*, 2000; Clarke, 2002). FhuD binds a range of ferri-hydroxamates in the periplasm, including ferrichrome, at a binding site between the two lobes. Specificity is achieved by the iron-hydroxamate centre interacting with residues in the binding site, although the siderophore backbone itself does not interact (Köster, 2001; Ferguson and Deisenhofer, 2002). Other PBPs include FepB, involved in the transport of ferri-enterochelin (Fig. 1.6), and FecB, which transports ferri-citrate (Ferguson and Deisenhofer, 2002).

ABC transporters (for example FepDGC, Fig. 1.6) hydrolyse ATP to ADP to provide the energy needed for active transport of many compounds including ferri-siderophores across membranes (Davidson and Maloney, 2007). Typically the hydrophobic pore is produced by two permease proteins and these can be two different monomers (for example, in *E. coli* FepD and FepG for ferri-enterochelin transport, Fig. 1.6), or one large two-domain polypeptide (for example in *E. coli* FhuB for ferrichrome transport). The channel can also be formed by two copies of the same permease subunit (Ferguson and Deisenhofer, 2002; Andrews *et al.*, 2003). On the inner surface of the inner membrane, two ATPases bind and hydrolyse ATP (Davidson and Maloney, 2007). Examples in *E. coli* include FhuC (ferrichrome), FepC (ferri-enterobactin, Fig. 1.6) and FecE (ferri-citrate; Ferguson and Deisenhofer, 2002). Degeneracy has been noted in the ABC systems of bacteria with highly specific OMRPs for a single iron source, but ABC systems that can complement each other. *E. coli* K-12 has six OMRPs, but three ABC transporter systems. *Pseudomonas aeruginosa* possesses 35 OMRPs and four ABC transporter systems (Köster, 2001). In Gram-positive organisms that do not possess an outer membrane, ferri-siderophores cross the inner membrane using binding-protein-dependent ABC permeases, which are anchored to the outside surface of the inner membrane by a binding protein (Köster, 2001).

Once in the cytosol, Fe^{3+} is believed to be liberated from the siderophores by intracellular ferric reductases or cleavage of the siderophore. Reduction of Fe^{3+} to Fe^{2+} leads to dissociation from the complex. In *E. coli*, the esterase encoded by *fes* hydrolyses

the ester bonds of ferri-enterochelin yielding dihydroxybenzoyl serine (DBS) and can reduce the Fe^{3+} bound by enterochelin allowing its dissociation from the complex as Fe^{2+} (Andrews *et al.*, 2003). As siderophore production is induced by a lack of iron, upon sufficient iron acquisition, production will cease to avoid the chance of toxic ROS (iron and oxidative stress defence and the regulation of this are discussed in Sections 1.4.7 and 1.4.8).

Finally, the binding of a ferri-siderophore to an OMRP may induce transcription (Schalk *et al.*, 2004). TonB-dependent OMRP expression is regulated by the ferric uptake repressor protein (Fur, discussed in detail in Section 1.4.8), but further regulation of iron uptake genes is achieved through siderophores binding to an OMRP. Binding can send a signal to the cytoplasm via an inner membrane regulatory protein (anti-sigma factor) and a sigma factor in the cytoplasm from the extracytoplasmic function (ECF) family, inducing transcription (Braun, 1997). The process requires energy transduced by the ExbBD-TonB complex (Kim *et al.*, 1997).

1.4.4.2. Ferrous Iron

The uptake of Fe^{2+} is an important pathway used by many bacteria to acquire the iron they need for cellular processes and growth. Only an inner membrane transporter protein is necessary for the uptake of Fe^{2+} by Gram-negative bacteria, as the Fe^{2+} is small enough to diffuse through outer membrane porins into the periplasm. The Fe^{2+} -specific transport system is designated Feo and was first identified and characterised in *E. coli* (Hantke, 1987; Kammler *et al.*, 1993). Part of a small anaerobically induced and iron repressed operon *feoABC*, *feoA* encodes a protein proposed to be similar to the C-terminal region of DtxR, an iron repressor of *C. diptheriae* (Hantke, 1987; Kammler *et al.*, 1993). The *feoB* gene encodes an inner membrane protein most likely acting as a Fe^{2+} permease, originally thought to be an ATPase capable of providing the energy for Fe^{2+} transport (Kammler *et al.*, 1993). The N-terminal region of FeoB has been more recently demonstrated to have GTPase ability (Marlovits *et al.*, 2002). This domain also has a guanine nucleotide binding site which is necessary for successful Fe^{2+} uptake (Marlovits *et al.*, 2002). The system is important under anaerobic conditions due to the higher levels of Fe^{2+} compared to Fe^{3+} present in the gut environment. Strains of both *E. coli* and *Salmonella* spp. carrying mutated copies of *feoB* have reduced colonisation capability in the mouse intestine (Stojiljkovic *et al.*, 1993; Tsolis *et al.*, 1996). Active transport of Fe^{2+} across the inner membrane is therefore likely to depend upon energy derived from both

ATP and/or GTP hydrolysis. FeoC (YhgG) is a small protein found in *E. coli* and other γ -proteobacterial systems. The start codon of *feoC* overlaps the stop codon of *feoB* which may indicate that they are translationally coupled. FeoC is proposed to act as a metal/redox-dependent transcriptional regulator of *feoABC* (Cartron *et al.*, 2006).

There are *feoB* homologues in several bacteria, including *H. pylori* (Velayudhan *et al.*, 2000), and *feoB* is frequently present without *feoA*. In *H. pylori* a *feoB* mutant was growth deficient under iron-limitation, was deficient in mouse intestine colonisation, and had lower cellular iron levels than wild-type cells (Velayudhan *et al.*, 2000). Extracellular ferric reductase activity has been discovered in bacteria such as *E. coli* and *H. pylori*, but the reductases themselves have not been identified (Worst *et al.*, 1988; Cowart, 2002). Anaerobic organisms such as *Clostridium perfringens* are capable of producing a microanaerobic environment in the host, allowing the reduction of chelated Fe^{3+} to Fe^{2+} and its subsequent uptake, which they also enhance by the creation of an acidic environment (Ratledge and Dover, 2000).

1.4.4.3. Haem

Haem-containing proteins are the most abundant source of iron in the host environment. Haem (Fig. 1.7) is the reduced Fe^{2+} protoporphyrin IX form and haemin refers to the oxidised or Fe^{3+} form of the molecule (Attardo-Genco and White-Dixon, 2001). Haem is bound in the host serum to proteins such as haemoglobin, haemopexin, albumin and lipoproteins, with haemoglobin present circulating in the blood within erythrocytes (Attardo-Genco and White-Dixon, 2001).

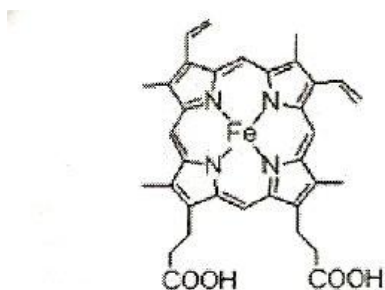


Figure 1.7. The structure of haem (Attardo-Genco and White-Dixon, 2001).

Gram-negative bacteria have evolved specific haemolysins and proteases to release haem from erythrocytes. Iron may then be bound by host proteins or by bacterial proteins such as haemophores. Haemophores or haem-binding proteins are produced, secreted, bind haem and return it to the bacterial cell surface where binding by an OMRP precedes transport into the cell (Attardo-Genco and White-Dixon, 2001; Andrews *et al.*, 2003). Free haemin, haemoglobin or haemopexin-haem complexes also serve as sources of haem for bacteria. Following binding to an OMRP, transport across the outer membrane occurs in an energy dependent manner as previously described for ferri-siderophore transport. Transport across the inner membrane follows, with iron released from haem in the cytoplasm by haem oxygenase-dependent degradation, yielding biliverdin, carbon monoxide and iron as the end products (Attardo-Genco and White-Dixon, 2001; Kikuchi *et al.*, 2005).

Haem is an important source of iron for many bacteria including *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *S. flexneri*, *Yersinia pestis*, enterohaemorrhagic *E. coli*, *P. aeruginosa* and *V. cholerae* (Ratledge and Dover, 2000; Wandersman and Stojiljkovic, 2000). *V. cholerae*, *Y. pestis*, *H. influenzae* and isolates of *Neisseria* can use haemoglobin as an iron source (Wandersman and Stojiljkovic, 2000). *N. meningitidis* possesses a haemoglobin and haemoglobin-haptoglobin receptor, HpuAB. *H. influenzae* has a haemopexin receptor, HxuA. HasR from *S. marcescens* functions as a haem receptor that may or may not be coupled with HasA, a haemophore (Cope *et al.*, 1998; Lewis *et al.*, 1998b; Letoffe *et al.*, 2004). The crystal structure of the haem oxygenase ChuS of *E. coli* 0157:H7 has recently been determined and, based on sequence comparison, orthologues are likely to be present in many other bacteria including *Shigella* spp., *Yersinia* spp., *Enterobacter* spp. and *Bordetella* spp. (Suits *et al.*, 2005).

1.4.5. Transferrin and Lactoferrin

Members of the Tf iron-binding protein family are glycosylated, bilobed, monomeric proteins of around 80 kDa. Similar N-terminal and C-terminal regions each consist of two domains that are separated by an iron binding cleft. Each protein reversibly binds two atoms of Fe^{3+} in conjunction with two bicarbonate anions, which allow coordination of the Fe^{3+} in the binding cleft (Weinberg, 1978; Anderson *et al.*, 1987; Day *et al.*, 1992; Abdallah and El Hage Chahine, 2000). The two domains adopt a closed conformation when the protein is iron-loaded; however the domains show some separation when the protein is in various iron-free states (Baker *et al.*, 2002).

1.4.5.1. Roles in the Host

Tf is synthesised by the liver and found primarily in serum (at 20 to 30 μM), transporting iron in blood-plasma to and between erythropoietic bone marrow cells, the spleen, the small intestine and muscles (Weinberg, 1978). Synthesised by polymorphonuclear leukocytes and glandular epithelial cells, Lf is present in exocrine secretions, principally milk, and found at infection sites, playing a vital role in non-specific host defence mechanisms, and demonstrating bacteriostatic and bactericidal properties (Masson and Heremans, 1966; Masson *et al.*, 1966; Van Snick *et al.*, 1974; Anderson *et al.*, 1987; Ellison III *et al.*, 1988; Singh *et al.*, 2002). Lf secretion has been shown to rise in response to enteric pathogen infection (Qadri *et al.*, 2002). Present in blood serum, the avian iron transport and delivery protein ovo-Tf demonstrates 51% homology with human Tf and 49% with human Lf (Giansanti *et al.*, 2002). Ovo-Tf was originally described in 1889; however its capability to inhibit microbial growth was not identified until much later (Schade and Caroline, 1944; Weinberg, 1978)

Lf binds iron with greater affinity (at pH 6.4 to 6.7) than Tf (Aisen and Leibman, 1972) and can bind iron down to at least pH 4, acidic conditions that result in the loss of iron from Tf (dissociation at pH 4-6; Van Snick *et al.*, 1974; Ward *et al.*, 1996). Lf and Tf demonstrate high amino acid identity (60%), with almost matching structures (Querijnje *et al.*, 1971; Baker *et al.*, 1998; Baker *et al.*, 2002); however no immunological cross-reaction occurs unless the proteins are denatured (Van Snick *et al.*, 1974).

1.4.5.2. Transferrin and Lactoferrin as Bacterial Iron Sources

A conformational change is believed to mediate iron release from Tf caused by domain separation. Bacteria can induce this by proteolytic cleavage by surface associated proteases (Okujo *et al.*, 1998). Siderophore-mediated iron acquisition from the protein may also occur (Ratledge and Dover, 2000). Ferric reduction by specific ferric reductases or alteration of the pH of the environment results in Fe^{3+} becoming Fe^{2+} leading to dissociation of the iron from the protein due to lower affinity (Vartivarian and Cowart, 1999). Bacterial receptor proteins coupled to a specific uptake system have also evolved to exploit this iron source.

P. aeruginosa employs a siderophore-mediated mechanism of iron acquisition from Tf (Sriyosachati and Cox, 1986). *Bordetella pertussis* and *Bordetella bronchiseptica* are

proposed to be capable of both a siderophore-mediated and a direct contact method of iron uptake resulting in internalisation of the acquired iron (Redhead, 1987; Gorringer *et al.*, 1990; Menozzi *et al.*, 1991). The gastric pathogen *H. pylori* (Husson *et al.*, 1993; Dhaenens *et al.*, 1997), the nosocomial pathogen *S. aureus* (Park *et al.*, 2005), the human fungal pathogen *Candida albicans* (Knight *et al.*, 2005), *H. influenzae* (Schryvers, 1989) and *Pasteurella haemolytica* (Ogunnariwo and Schryvers, 1990) can also acquire iron from this source. The most extensive characterisation of the uptake of iron from Tf or Lf by binding to a bacterial receptor has occurred in *Neisseria* spp. (Perkins-Balding *et al.*, 2004).

Specific cell surface receptors that bind Tf in *Neisseria* spp. consist of two proteins, Tf binding protein A (TbpA, 98 kDa) and Tf binding protein B (TbpB, 68 kDa). Originally identified in *N. meningitidis* as Tbp1 and Tbp2 (Schryvers and Morris, 1988b), they were renamed later as TbpAB in all *Neisseria* spp. after the genes that encode them, *tbpA* and *tbpB*, which are found in a bicistronic operon (Legrain *et al.*, 1993). TbpA demonstrates homology to TonB-dependent siderophore-receptor outer membrane proteins, suggesting similar structure and function, and is present at the cell surface as a homo-dimer (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993). TbpB is tethered to the outer membrane by N-terminal cysteine-associated fatty acyl groups, and is a bilobed lipoprotein (Legrain *et al.*, 1993; Anderson *et al.*, 1994). There appears to be no phase variation of the receptor, which requires only iron-restriction for expression, possibly resulting in constitutive expression *in vivo* (Schryvers and Morris, 1988b; Legrain *et al.*, 1993).

Isogenic neisserial mutants in *tbpA* and *tbpB* showed *tbpA* to be essential in the process of iron uptake from Tf for growth (Cornelissen *et al.*, 1992; Irwin *et al.*, 1993). The *tbpB* mutants were capable of only limited iron acquisition and growth (Irwin *et al.*, 1993; Anderson *et al.*, 1994). Although *Neisseria* spp. are expected to encounter mainly Lf upon infection, a *tbp* mutant gonococcal strain was avirulent in the human infection model indicating the importance of Tf *in vivo* (Cornelissen *et al.*, 1998). TbpA binds all iron-states of Tf, but TbpB can recognise and preferentially bind ferri-Tf. TbpA and TbpB are proposed to function together as a Tf receptor *in vivo* (Cornelissen and Sparling, 1996). TbpA is proposed to form the pore allowing iron transport into the periplasm; TbpB augments the process, increasing receptor affinity and specificity, but is non-essential (Cornelissen and Sparling, 1996). The receptor genes are transcriptionally regulated by Fur. The receptors require energy transduced by the ExbBD-TonB complex to transport Tf-derived iron across the otherwise unenergised bacterial outer membrane (Biswas *et al.*, 1997). Binding of ferri-Tf induces conformational change. The receptor obtains energy

from TonB, inducing iron release from Tf and opening of the gated pore; iron transport and apo-Tf release follow. Once in the periplasmic space, iron is bound by the periplasmic binding protein FbpA (Chen *et al.*, 1993) and delivered to the inner membrane transport system FbpBC, which facilitates iron transport into the cytoplasmic compartment (Adhikari *et al.*, 1996). The loss of TbpB or both Tbps in the human pathogen *H. influenzae*, which is capable of using ferri-Tf as a sole source of iron (Herrington and Sparling, 1985; Pidcock *et al.*, 1988), resulted in an inability to grow when iron was supplied in this form. However, at high Tf concentrations, limited growth was observed with a *tbpA* mutant (Gray-Owen *et al.*, 1995).

The Lf receptor is not as well characterized as the Tf receptor, but is also composed of two proteins, LbpA and LbpB (Lf binding protein A and B). LbpA was originally identified in *N. meningitidis* as a 98 kDa Lf-binding protein (Schryvers and Morris, 1988a; Pettersson *et al.*, 1993; Pettersson *et al.*, 1994). LbpB was identified as an 84 kDa protein that could also bind Lf (Pettersson *et al.*, 1998; Schryvers *et al.*, 1998). The genes *lbpA* and *lbpB* are organised as an operon and induced by iron-restriction as seen with *tbpAB* (Lewis *et al.*, 1998a; Pettersson *et al.*, 1998). A *N. meningitidis lbpB* mutant was shown to bind slightly less Lf than wild-type cells; an *lbpA* mutant was less capable of binding Lf than the *lbpB* mutant. A double mutant bound almost no Lf. Plate feeding assays showed that the *lbpA* mutant was incapable of exploiting Lf as a sole source of iron. In contrast, an *lbpB* mutant was unaffected (Pettersson *et al.*, 1998). Interestingly, Lf is predicted to be the more abundant of the two glycoproteins in the neisserial niche, but a gonococcal strain lacking Lbp is still virulent in the human infection model (Cornelissen *et al.*, 1998).

1.4.6. Iron Storage

Bacteria possess iron storage proteins that they can use as an iron source for growth and cellular processes when iron is limited in the extracellular environment (Andrews, 1998). They consist of ferritins which are also found in eukaryotes, bacterioferritins which contain haem, and Dps proteins (Andrews, 1998). The ferritins, bacterioferritins and Dps proteins are separate families but share structural similarities, a major trait being the ability to store iron. The central iron storage hollow is created by 12 (Dps proteins) or 24 (ferritins and bacterioferritins) subunits aggregating to produce a spherical casing. Up to 4500 atoms of iron can be stored by the 500 kDa ferritins and bacterioferritins, with around 500 atoms stored by the 250 kDa Dps proteins. Fe²⁺ iron is taken up by bacterial storage proteins and

oxidised by ferrooxidation inside the conserved ferroxidase active site of the proteins. The Fe^{3+} is transferred to the central hollow where it is stored until required (Andrews, 1998; Andrews *et al.*, 2003).

The loss of the *E. coli* ferritin A (FtnA) leads to less intracellular iron at stationary phase after iron-replete growth and less growth when iron-restricted after iron-replete growth (Abdul-Tehrani *et al.*, 1999). As this only occurred following growth in the presence of enough iron, FtnA is proposed to store iron following exponential growth when iron is in excess, to be used if iron-restriction is encountered. Storage also avoids the toxicity associated with accumulating large amounts of free intracellular iron (Abdul-Tehrani *et al.*, 1999).

The function of bacterioferritins is less well characterised than that of ferritins and the function of the associated haem is unclear. In *E. coli*, loss of the haem caused no change in iron uptake, but storage of more iron (4-fold) than wild-type cells, implying a regulatory role for the haem in reduction of the stored iron (Andrews *et al.*, 1995).

Dps proteins are not only iron storage proteins, they have also been shown to play a role in protection of DNA from ROS. Originally isolated from *E. coli* (Almiron *et al.*, 1992), the Dps protein primarily prevents oxidative damage of DNA, but is still capable of iron storage (Zhao *et al.*, 2002). A similar protein capable of storing iron has also been found in *Listeria* spp. (Bozzi *et al.*, 1997).

1.4.7. Iron and Oxidative Stress Defence in Bacteria

Host proteins Nramp1 and Nramp2 (natural resistance-associated macrophage proteins) are produced by macrophages allowing modification of cellular iron levels (Wardrop and Richardson, 1999). Nramp1 is proposed to remove and acquire iron from Tf, enhancing intracellular iron concentrations within the macrophage in response to infection by phagosomal pathogens (Cellier *et al.*, 1996; Skamene *et al.*, 1998; Canonne-Hergaux *et al.*, 1999; Kuhn *et al.*, 1999; Zwilling *et al.*, 1999). The build up of iron within the macrophage results in the production of toxic ROS (Section 1.4.3) (Zwilling *et al.*, 1999). *Mycobacterium avium* can cause increased infections in mice with mutated Nramp1 (Hackan *et al.*, 1998; Zwilling *et al.*, 1999).

Micro-organisms can avoid phagocytosis to evade killing by phagocyte oxidants. The production of toxins to destroy phagocytes is documented for Gram-positive organisms *Streptococcus* spp. (streptolysin; Bernheimer and Schwartz, 1960) and *Staphylococcus*

aureus (leukocidin; Rogolsky, 1979). Phagocytosis can also be avoided by the production of a hydrophobic capsule as seen in *N. meningitidis* (Hendley *et al.*, 1975; Richardson and Sadoff, 1977). *S. flexneri* produces cytolysins which cause phagosome membrane damage resulting in bacterial escape (Sansonetti *et al.*, 1986). Examples of specific defences against ROS include inhibition of respiratory burst within macrophages by *Leishmania* spp. (Frankenburg *et al.*, 1990; Brandonisio *et al.*, 1994). *Legionella pneumophila* can also inhibit respiratory burst in neutrophils (Sahney *et al.*, 1990). In addition, microbes can produce antioxidant scavenging substances, such as the exopolysaccharide alginate produced by *P. aeruginosa* (Learn *et al.*, 1987; Simpson *et al.*, 1989).

Specific resistance to oxidative stress is catalysed by a number of enzymes including superoxide dismutase (SOD), catalases and peroxidases. SOD is found in many bacteria and decreases intracellular superoxide (O_2^-) to an acceptable level (Imlay and Fridovich, 1991). SOD catalyses the breakdown of superoxides to H_2O_2 and O_2 , and is named according to the metal cofactor used. There are a number of SODs which can use copper-zinc, nickel, manganese or iron (van Vliet *et al.*, 2002). Superoxide is proposed to reduce iron producing a hydroxyl radical by the transfer of an electron to H_2O_2 ; this radical then causes DNA damage (McCord and Day, 1978). Mutation of the genes encoding SOD in a number of organisms, for example *E. coli* and *S. mutans*, results in increased DNA damage and subsequent cell death (Carlioz and Touati, 1986; Farr *et al.*, 1986; Nakayama, 1992). This was confirmed to be dependent upon iron, as cell-permeable iron chelators prevented cell death (Imlay and Linn, 1988). In *E. coli*, regulation of superoxide oxidative damage defence is achieved by SoxR/SoxS. The genes *soxS* and *soxR* are divergently transcribed and induce expression of oxidative stress defence genes (Chan and Weiss, 1987; Tsaneva and Weiss, 1990; Bauer *et al.*, 1999) by a two stage transcriptional activation. SoxR, a redox sensing protein, induces *soxS* transcription in the presence of superoxide (Tsaneva and Weiss, 1990; Amabile-Cuevas and Demple, 1991; Wu and Weiss, 1991; Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). SoxS binds to particular promoter regions inducing the expression of target genes (Amabile-Cuevas and Demple, 1991; Fawcett and Wolf, 1994; Li and Demple, 1994; Li and Demple, 1996). SoxR achieves sensing of superoxide due to an iron sulphur centre which is normally reduced. Upon encountering superoxide the iron sulphur centre oxidises; only the oxidised SoxR is capable of activating *soxS* transcription (Ding *et al.*, 1996; Gaudu and Weiss, 1996; Bradley *et al.*, 1997; Ding and Demple, 1997).

Catalases and peroxidases are produced by many bacteria and they function by converting H_2O_2 (produced by aerobic growth or superoxide dismutation) to H_2O and O_2 . Inactivation of peroxides is important as they play a key role in the formation of hydroxyl radicals. Most obligate anaerobes do not synthesise these enzymes, but almost all aerobes and facultative anaerobes do (McCord *et al.*, 1971). *E. coli* possesses two catalases, encoded by *katG* and *katE*. Hydroperoxidase I (HPI) is encoded by *katG* and is a bifunctional catalase-peroxidase found in the periplasm. Encoded by *katE*, hydroperoxidase II (HPH) is a monofunctional catalase and is located only in the cytoplasm (Clairborne and Fricovich, 1979; Clairborne *et al.*, 1979; Loewen *et al.*, 1985).

Regulation of hydrogen peroxide induced oxidative stress responses is achieved in *E. coli* and *Salmonella* spp. by the transcription factor OxyR (Christman *et al.*, 1985; Morgan *et al.*, 1986; Storz *et al.*, 1987). OxyR regulates a number of genes including *katG*, *ahpC* and *ahpF*. The genes *ahpC* and *ahpF* encode alkyl hydroperoxide reductase that reduces hydroperoxides to alcohols using NADP/NADPH as an electron donor (Storz *et al.*, 1989; Altuvia *et al.*, 1994; Zheng *et al.*, 2001a; Zheng *et al.*, 2001b). OxyR can be reduced or oxidised and senses oxidants directly. Upon exposure to H_2O_2 , oxidised OxyR will induce transcription by interacting directly with RNA polymerase (Zheng *et al.*, 1998; Choi *et al.*, 2001). As well as activating the expression of a number of genes, OxyR also negatively autoregulates (Christman *et al.*, 1989; Tartaglia *et al.*, 1989) and is reduced and therefore inactivated by GrxA (glutaredoxin 1), a member of the regulon, allowing negative feedback (Zheng *et al.*, 1998; Åslund *et al.*, 1999). As well as OxyR, *E. coli* also produces a small RNA, OxyS, upon exposure to H_2O_2 . Abundant and stable, OxyS acts as a regulator, activating and repressing gene expression and reducing mutation rates (Altuvia *et al.*, 1997).

Regulation of peroxide-induced stress responses in the Gram-positive organism *Bacillus subtilis* is achieved by the regulator protein PerR (Bsat *et al.*, 1998). PerR is a homologue of the Fur protein, which is discussed in detail in Section 1.4.8. PerR and related peroxide sensors have since been found in a number of Gram-negative and Gram-positive organisms (Mongkolsuk and Helmann, 2002). PerR binds two metal ions per monomer (Herbig and Helmann, 2001), one zinc ion, which is proposed to function mainly for maintenance of protein structure, and either a manganese (Mn^{2+}) ion or Fe^{2+} ion as a co-repressor (Chen *et al.*, 1995; Herbig and Helmann, 2001). In *B. subtilis*, PerR represses genes involved in oxidative stress defence such as *katA* (the major catalase), *ahpCF* (alkyl hydroperoxide reductase), *fur*, *perR* itself, *zosA* (zinc uptake) and a haem biosynthesis

operon (Fuangthong *et al.*, 2002; Mongkolsuk and Helmann, 2002) by binding to operator sequences known as Per boxes in the promoter regions. H_2O_2 causes PerR oxidation and, in turn, dissociation from the DNA and derepression of the oxidative stress defence genes; however only PerR with Fe^{2+} associated reacts with H_2O_2 (Bsat *et al.*, 1998; Herbig and Helmann, 2001; Fuangthong *et al.*, 2002). *B. subtilis* possesses two catalases that have been shown to be similar to HPI and HPII from *E. coli* (Loewen and Switala, 1987; Loewen, 1989). As iron and the production of ROS are so closely connected, OxyR, SoxRS and PerR also interact with other iron-dependent regulators such as Fur, interlinking iron acquisition, storage and metabolism with oxidative stress defence.

1.4.8. Fur Regulation

1.4.8.1. Fur

Fur is a global bacterial regulatory protein and primary member of the Fur super-family of metalloregulatory proteins (Mongkolsuk and Helmann, 2002). In *E. coli*, Fur is a 17 kDa protein and classically operates by repressing transcription of genes under the control of iron-responsive promoter regions using Fe^{2+} as a corepressor (Escolar *et al.*, 1999). Fur was originally discovered in *E. coli* and *S. enterica* serovar Typhimurium by the identification of mutant cells in which iron-dependent repression was lost (Ernst *et al.*, 1978; Hantke, 1981). Fur is a homodimer under aqueous conditions (Coy and Neilands, 1991) and when iron is in excess a Fe^{2+} ion binds to each subunit. Each subunit is proposed to have two domains (Coy and Neilands, 1991; Stojiljkovic and Hantke, 1995), with the C-terminal domain involved in aiding dimerisation and binding Fe^{2+} and the N-terminal domain responsible for DNA binding (Coy and Neilands, 1991; Stojiljkovic and Hantke, 1995). In addition, the *E. coli* Fur also binds zinc at the C-terminal domain which is important for function; not all Fur proteins are capable of doing this (Jacquamet *et al.*, 1998; Althaus *et al.*, 1999; Lewin *et al.*, 2002). Binding of Fe^{2+} alters the conformation of Fur, resulting in an increase in affinity for the Fur binding site (Fur box) within the promoter regions of Fur-regulated genes (Fig. 1.8). This blocks transcription by RNA polymerase, so inhibiting expression of those genes regulated by iron. Upon iron restriction, Fe^{2+} is lost from the Fur dimer resulting in the dissociation of Fur from promoter regions, allowing RNA polymerase to contact the promoter (Fig. 1.8). RNA

polymerase then transcribes the iron-repressed genes allowing the cell to acquire the essential nutrient when needed (Escobar *et al.*, 1999).

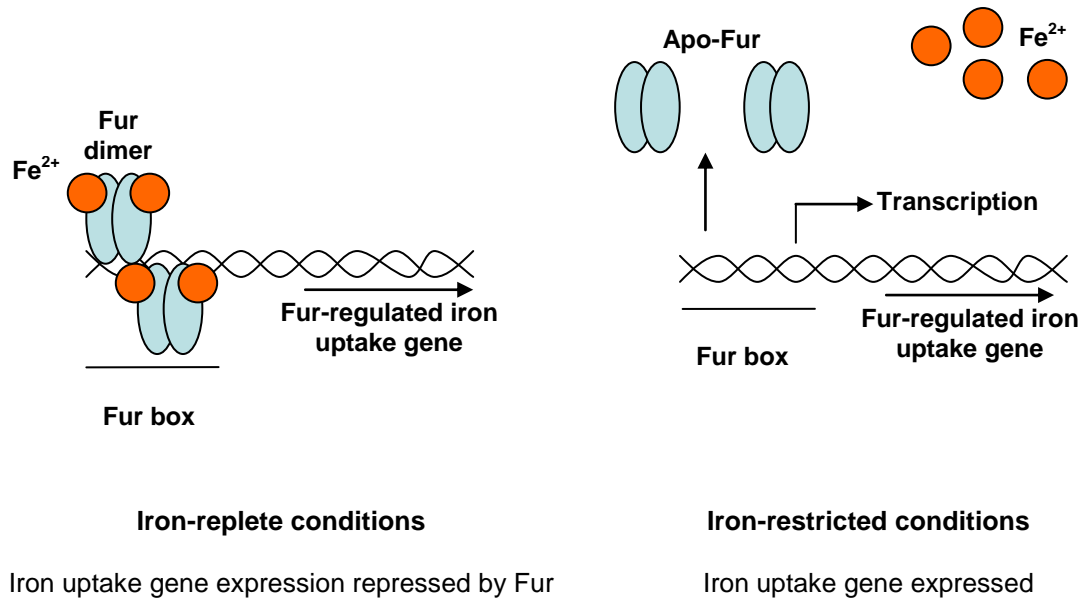


Figure 1.8. Classical regulation by Fur. Under iron-replete conditions, Fe^{2+} acting as a corepressor associates with the Fur dimer which can then bind to iron-regulated promoter regions due to a conformational change. Binding of the complex to the Fur-box blocks transcription resulting in repression of gene expression. Under iron restriction, Fe^{2+} dissociates from Fur which can then no longer bind the Fur box. The loss of Fur allows RNA polymerase access to transcribe the previously repressed gene. Fur regulation is common for genes involved in iron uptake and iron dependent processes.

Since the discovery of *fur* in *E. coli*, *fur* homologues have been identified in many other micro-organisms such as *Neisseria* spp., *Yersinia* spp., *Salmonella* spp., *H. pylori*, *P. aeruginosa*, *V. cholerae*, as well as the Gram-positive organisms *B. subtilis* and *Staphylococcus* spp. (Ernst *et al.*, 1978; Staggs and Perry, 1991; Litwin *et al.*, 1992; Berish *et al.*, 1993; Prince *et al.*, 1993; Thomas and Sparling, 1994; Heidrich *et al.*, 1996; Thomas and Sparling, 1996; Bereswill *et al.*, 1998; Bsat *et al.*, 1998).

1.4.8.2. Fur Boxes and the Interaction of Fur with DNA

The *E. coli* Fur box was originally identified by investigating where Fur associated within iron-regulated promoters, and is normally found between the -10 and -35 regions of Fur-regulated promoters. The operator site was first identified by DNase I footprinting of a number of Fur binding regions in *E. coli* as a 19 bp inverted repeat consensus sequence

(Fig. 1.9a; De Lorenzo *et al.*, 1987). In addition, this consensus sequence allowed iron regulation when cloned downstream of a different promoter region (Calderwood and Mekalanos, 1988). The homodimeric structure of the Fur protein and the inverted repeat operator sequence indicated that Fur was likely to interact with DNA and repress transcription in a classical manner. Following this, Fur binding to the aerobactin operon promoter region was shown to occur in three repeats of two protected and four unprotected bases within the main binding region (Bindereif and Neilands, 1985; De Lorenzo *et al.*, 1986; De Lorenzo *et al.*, 1988a; Escolar *et al.*, 1999). Other iron responsive promoter regions contain more than one Fur box which may overlap (Griggs and Konisky, 1989; Tardat and Touati, 1993; Hunt *et al.*, 1994). Electron microscopy and hydroxyl radical footprinting demonstrated that polymerised Fur can helically wrap around the binding region of the DNA (De Lorenzo *et al.*, 1988b; Fréchon and Le Cam, 1994; Le Cam *et al.*, 1994) and across regions outside the specific Fur box covering much larger regions of DNA (De Lorenzo *et al.*, 1988b; Le Cam *et al.*, 1994). Because of this, the consensus sequence was subsequently proposed to be made up of a series of 6 bp (5'-NAT (A/T) AT-3') repeats, resulting in the 19 bp palindrome becoming three repeats of 6 bp (Fig. 1.9b; Escolar *et al.*, 1998; Escolar *et al.*, 1999). This allowed an increase in the number of 6 bp repeats in a Fur operator sequence, such as in the long operator region in the aerobactin promoter region of *E. coli*, which is composed of a string of up to eighteen tandem hexamers (Escolar *et al.*, 2000). Three repeats is the minimum required for a functional operator sequence with the specific hexamer sequence essential for Fur interaction (Escolar *et al.*, 1998). In addition to sequence recognition and binding of Fur to each hexamer, protein-protein interaction is also necessary across at least three of the hexamers before the complex can function successfully (Escolar *et al.*, 1998). Fur dimers first bind to a higher affinity site facilitating Fur binding to other sites allowing polymerisation of the protein along the DNA (De Lorenzo *et al.*, 1988b; Escolar *et al.*, 2000). If the Fur protein binds to DNA in this way it demonstrates similarity to eukaryotic factors and nucleoid-associated proteins rather than classical prokaryotic repressor proteins (Escolar *et al.*, 1999; Escolar *et al.*, 2000). More recently, the Fur box has once again been reinterpreted, with the 19 bp consensus sequence proposed to contain two 15 bp inverted repeats that overlap and are both capable of binding a Fur dimer (Fig. 1.9c; Baichoo and Helmann, 2002). Fur is predicted to bind sequences offset by 6 bp in adjacent major grooves, therefore binding the DNA on opposite sides demonstrating similarity to *B. subtilis* Fur binding sites (Baichoo and Helmann, 2002). In addition, a similar model was proposed by another group that involves the 19 bp consensus

as two overlapping Fur binding regions of 13 bp each (Fig. 1.9d). Again, two Fur dimers could bind on opposite sides of the DNA, shifted by around half a turn of the helix (Lavrrar *et al.*, 2002).

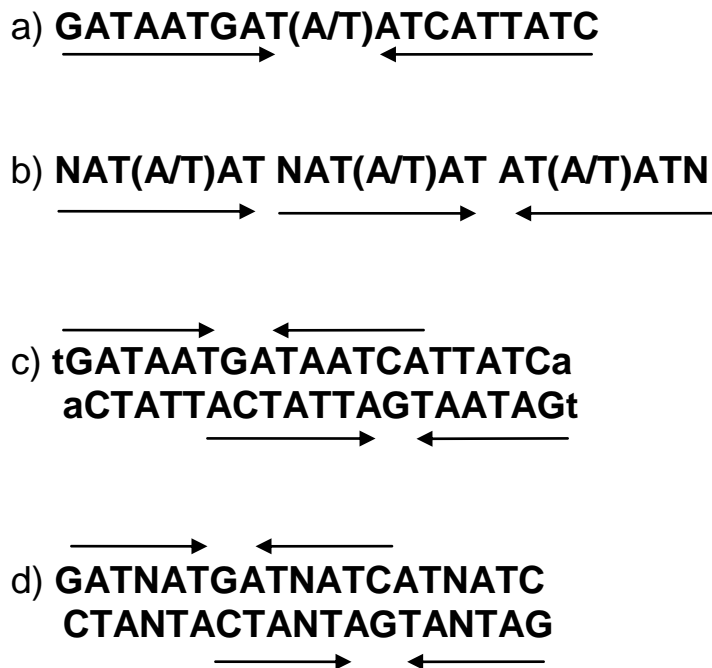


Figure 1.9. The Fur box consensus sequence as proposed by **a)** de Lorenzo *et al.*, 1987; **b)** Escolar *et al.*, 1998; **c)** Baichoo and Helmann, 2002; **d)** Lavrrar *et al.*, 2002.

1.4.8.3. Related Metalloregulators

In addition to Fur, there are also other members of the Fur super-family that bind metal ions and regulate gene expression. The Fur homologue PerR has been discussed as part of the oxidative stress section (Section 1.4.7) and is best characterised in *B. subtilis* as the regulator of peroxide induced stress responses (Bsat *et al.*, 1998). Zur is a Fur homologue and zinc binding regulatory protein that has been described in *E. coli* and the Gram-positive organism *B. subtilis* (Gaballa and Helmann, 1998; Patzer and Hantke, 1998). Zur binds zinc in a similar concentration-dependent manner as seen with Fur and Fe^{2+} . Zinc is required by the cell, but becomes toxic at too high a concentration and so uptake and usage must be regulated. The regulatory mechanisms used by Zur are, however, less well characterised than Fur (Gaballa and Helmann, 1998; Patzer and Hantke, 1998). In total, *B. subtilis* possesses three Fur homologues; Fur itself, PerR and Zur (Bsat *et al.*, 1998; Gaballa and Helmann, 1998; Escolar *et al.*, 1999).

1.4.8.4. The Fur Regulon

The major role played by Fur is repression. However, the Fur protein is responsible for the regulation of many genes in both a negative and positive manner, including genes other than those involved directly in iron uptake and metabolism. Fur has been shown to be involved in acid shock response in *S. Typhimurium* (Hall and Foster, 1996). In *E. coli* Fur has been associated with oxidative stress defence (Niederhoffer *et al.*, 1990; Tardat and Touati, 1993), chemotaxis (Karjalainen *et al.*, 1991), and virulence factor expression (Litwin and Calderwood, 1993). Fur represses a number of genes in *E. coli* including *cyoA*, *flbB*, *fumC*, *gpmA*, *metH*, *nohB*, *purR* and *sodA* which are involved in respiration, motility, the TCA cycle, glycolysis, biosynthesis of methionine, phage-DNA packaging, purine metabolism and oxidative stress defence, respectively (Touati, 1988; Stojiljkovic *et al.*, 1994; Park and Gunsalus, 1995; Vassinova and Kozyruv, 2000; McHugh *et al.*, 2003). This demonstrates the global regulatory role that Fur plays in the cell.

In *E. coli* there are also several genes that are activated by Fur, such as *acnA* (aconitase A), *bfr* (bacterioferritin for iron storage), *finA* (ferritin for iron storage), *fumA* and *fumB* (fumerases), *sdhCDAB* (TCA cycle) and *sodB* (oxidative stress defence; Park and Gunsalus, 1995; Tseng, 1997; Massé and Gottesman, 2002; McHugh *et al.*, 2003). The promoter regions of these genes do not appear to contain a Fur box. Activation of *sodB* appears to be iron dependent and requires a palindromic sequence centred on the +1 position and a large downstream sequence that is AT-rich (Dubrac and Touati, 2000). Fur was found to increase the stability of the *sodB* transcript. A histone-like protein H-NS was shown to interact with the same sequence as Fur within the *sodB* promoter causing repression of *sodB* expression (Dubrac and Touati, 2000). Fur and H-NS did not, however, seem to directly influence *sodB* transcription or the resulting transcript. The mechanism of Fur induction of the *sodB* transcript and the Fur-dependent post-transcriptional increase in stability was not explained (Dubrac and Touati, 2002).

Additional regulation was demonstrated by the discovery of *ryhB*, a classically Fur repressed gene responsible for the production of a small non-coding RNA (Massé and Gottesman, 2002). The *ryhB* sRNA (RyhB) was shown to directly repress at least 18 other transcripts which all encode proteins involved in iron usage (Massé *et al.*, 2005). This was proposed to aid the cell by decreasing iron requirement when this essential nutrient was in short supply (Massé and Gottesman, 2002; Massé and Arguin, 2005). RyhB is proposed to act as an antisense RNA to the ribosome binding sites of the mRNAs that it regulates, such

as those of *sodB*, *fumA*, *acnA*, *bfr* and *ftnA*. This induces degradation of both the mRNA target and RyhB itself caused by recruitment of the RNA degradosome (Massé and Gottesman, 2002; Massé *et al.*, 2003). However, without degradation, RyhB can also block translation of the mRNA target (Morita *et al.*, 2006). RyhB represses a number of transcripts, but also represses a set of genes that are regulated by Fur as well (Massé *et al.*, 2005). RyhB overproduction causes an increase in the amount of free iron in the cell, shown to be acquired from the external environment. The acquired iron binds to and activates the regulatory protein Fur resulting in classical Fur repression of many genes. A *ryhB* mutant was also shown to be impaired in cellular growth and viability under iron restriction, implying a role for RyhB in managing recently acquired iron in times of iron depletion, allowing only the most essential proteins access to this nutrient (Jacques *et al.*, 2006). RyhB homologues have been found in other bacteria, such as *Salmonella* spp., *Klebsiella* spp. and *Shigella* spp. (Massé and Gottesman, 2002; Wilderman *et al.*, 2004). An example of a gene known to be directly activated by Fur is the *pfr* (ferritin) gene of *H. pylori* (Delany *et al.*, 2001). Apo-Fur represses *pfr* transcription by binding to the promoter region at a unique site. Derepression occurs upon association of Fe²⁺ with Fur, resulting in expression of *pfr* (Delany *et al.*, 2001).

In *E. coli*, *fur* is induced 10-fold under oxidative stress by the SoxRS regulatory system and OxyR, resulting in intracellular Fur levels 2-fold higher than normal (Zheng *et al.*, 1999). Fur is also autoregulated in *E. coli* (De Lorenzo *et al.*, 1988b). The expression of *fur* has also been shown to be regulated by the cAMP receptor protein (Crp) which is the main transcriptional regulator of carbon metabolism (De Lorenzo *et al.*, 1988b; Zhang *et al.*, 2005).

1.5. *C. jejuni* and Iron

The iron uptake systems and iron homeostasis mechanisms characterised in *C. jejuni* so far will now be reviewed.

1.5.1. Iron Sources, Uptake Systems and the Genes Involved

C. jejuni has been demonstrated to be capable of acquiring iron from a number of external sources including siderophores, haemin and Fe²⁺.

1.5.1.1. Siderophore Uptake Systems

C. jejuni has long been thought to be incapable of synthesising siderophores (Field *et al.*, 1986; Pickett *et al.*, 1992), and the inability to locate any siderophore biosynthesis genes within the genomes of *C. jejuni* NCTC 11168, RM1221 and 81-176 supports this (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006). A previous study indicated that the organism is capable of utilising exogenous siderophores as sources of iron, including enterochelin and ferrichrome. Aerobactin, rhodotorulic acid and desferrioxamine B were not shown to promote growth (Field *et al.*, 1986).

1.5.1.1.1. Ferri-Enterochelin

C. jejuni is capable of acquiring iron from ferri-enterochelin using the TonB-dependent OMRP CfrA (Cj0755) and the binding-protein dependent inner membrane ABC-transporter system encoded by *ceuBCDE* (*cj1352-cj1355*; Fig. 1.10; Parkhill *et al.*, 2000). CeuE is a lipoprotein with homology to periplasmic binding proteins of ferri-siderophore transport systems, and was shown to have a signature sequence for siderophore-binding proteins and to confer haemolytic activity upon *E. coli* (Park and Richardson, 1995). Following additional investigation, genes encoding parts of the enterochelin transport system were identified in *C. coli* (Richardson and Park, 1995). The *C. jejuni* operon *ceuBCDE* demonstrates a high level of similarity to the *C. coli* ferri-enterochelin inner membrane transport system, with *ceuBC* proposed to encode the inner membrane permease proteins, *ceuD* proposed to encode an ATPase and *ceuE* encoding the periplasmic binding protein (Parkhill *et al.*, 2000; Palyada *et al.*, 2004).

The OMRP was not immediately obvious as it is located elsewhere on the *C. jejuni* chromosome. The gene *cfrA* was originally identified in *C. coli* where, interestingly, a *cfrA* mutant strain could utilise ferri-enterochelin as a sole iron source (Guerry *et al.*, 1997). However more recently, a *cfrA* mutant *C. jejuni* NCTC 11168 strain was shown to be unable to grow when supplied only with ferri-enterochelin as an iron source, identifying CfrA as the OMRP for this system (Palyada *et al.*, 2004). A *ceuE* mutant was slightly impaired in its ability to grow when provided with ferri-enterochelin as a sole iron source, indicating that CeuE is involved in, but not essential for, the use of iron from ferri-enterochelin for growth (Palyada *et al.*, 2004). Mutants in *cfrA* and *ceuE* were also significantly impaired in avian gastrointestinal tract colonisation (Palyada *et al.*, 2004).

1.5.1.1.2. Ferrichrome

C. jejuni strain M129 was shown to have an 80 kDa OMP expressed under low iron conditions; however only six out of eleven other *C. jejuni* strains studied had a homologue (Galindo *et al.*, 2001). The protein was found to be encoded within an operon of three ORFs demonstrating homology to the ferrichrome uptake system of *E. coli* and *P. aeruginosa* (Galindo *et al.*, 2001). The *cfhuABD* operon encodes CFhuA, homologous to FhuA, the *E. coli* ferrichrome receptor, CFhuB, which demonstrates homology to the permease FhuB, and CFhuD, which shows homology to FhuD, the periplasmic binding protein (Galindo *et al.*, 2001). The region is astonishingly GC rich (65%) considering that the genome of *C. jejuni* has an average GC content of 30-35% (Parkhill *et al.*, 2000; Galindo *et al.*, 2001; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006). The sequenced *C. jejuni* genomes lack a *cfhuABD* operon (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007) and experimental confirmation of the role of these proteins in the strains that do possess them is still required (Galindo *et al.*, 2001).

1.5.1.1.3. Rhodotorulic Acid

Although previously thought to be incapable of utilising the fungal hydroxamate siderophore ferri-rhodotorulic acid as an iron source (Field *et al.*, 1986), recent unpublished data has indicated a role for the as yet uncharacterised Cj1658-Cj1663 iron uptake system of *C. jejuni* NCTC 11168 in the uptake of iron from this source (Fig. 1.10; Ketley, J. M., unpublished data). A 19 kDa periplasmic protein was first identified by purification from

C. jejuni 81-176 (Janvier *et al.*, 1998); this protein was designated P19 (Cj1659; Janvier *et al.*, 1998). Found within an operon in NCTC 11168, *cj1658* encodes a membrane associated protein that does not resemble a classical TonB-dependent OMRP. P19 (Cj1659) is the putative periplasmic siderophore-binding protein, and Cj1661-Cj1663 demonstrate homology to the components of an inner membrane ABC transporter system (Parkhill *et al.*, 2000). Cj1658-Cj1663 was suggested to play a role in iron uptake, as Cj1658 and P19 homologues are found in *Y. pestis* on an iron-uptake pathogenicity island (Carniel, 2001). Mutants in either *cj1658* or *p19* were unable to utilise iron supplied as ferri-rhodotorulic acid for growth (Ketley, J. M., unpublished data), indicating a role for this system in the uptake and use of rhodotorulic acid-derived iron, contradicting earlier reports that *C. jejuni* cannot use this siderophore (Field *et al.*, 1986).

1.5.1.2. Haem Utilisation

C. jejuni was shown to be capable of using iron derived from haem-containing compounds for growth, including haemin, haemoglobin, haemin-haemopexin and haemoglobin-haptoglobin (Pickett *et al.*, 1992). Mutants were isolated that were consistently unable to grow when supplied only with haemin, haem-containing substances and also high concentrations of haemin and haemoglobin (Pickett *et al.*, 1992). The loss of a ~71 kDa OMRP had occurred in these mutant strains (Pickett *et al.*, 1992). The gene, named *chuA*, which encodes a 70 kDa OMRP showing similarity to other bacterial OMRPs, was shown to be iron regulated (Fig. 1.10; van Vliet *et al.*, 1998).

In *C. jejuni* NCTC 11168, *chuA* (*cj1614*) is encoded as part of an operon, *chuABCD* (*cj1614-cj1617*; Parkhill *et al.*, 2000). ChuB is predicted to be the permease protein, ChuC the ATPase and ChuD the periplasmic binding protein of the inner membrane ABC transporter system (Fig. 1.10; Parkhill *et al.*, 2000; Ridley *et al.*, 2006). Mutation of each of the genes *chuABCD* and subsequent growth of the mutant strains supplied only with haemin or haemoglobin as an iron source showed that the *chuA* mutant strain grew significantly less than wild-type. This implies that ChuA is essential for growth on haemin/haemoglobin. Although there was only a slight decrease in the growth of the *chuB*, *chuC* or *chuD* mutant strains, this was still statistically significant (Ridley *et al.*, 2006), implying that the ABC transport system is involved in, but not essential for growth, implying redundancy (Ridley *et al.*, 2006). Upstream of *chuA* and separated by a divergently transcribed promoter region, *chuZ* (*cj1613c*) encodes an iron responsive haem

oxygenase (Ridley *et al.*, 2006). ChuZ is an orthologue of a haem uptake system protein, HugZ, from *Plesiomonas shigelloides* (Henderson *et al.*, 2001) and has been shown to function as a haem oxygenase within the cell (Ridley *et al.*, 2006). Expression of the Chu system is not essential for colonisation of the avian gut, but is upregulated in both the rabbit ileal loop model and the chick intestine (Palyada *et al.*, 2004; Stintzi *et al.*, 2005).

1.5.1.3. Ferrous Iron Uptake

The *C. jejuni* NCTC 11168 genome contains a homologue of the *E. coli* *feoB* gene (*cj1398*, 29% identity) adjacent to a smaller gene (*cj1397*) which demonstrates limited identity (16%) to the *feoA* gene of *E. coli* (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). An initial study of a *C. jejuni* strain carrying a mutation in *feoB* showed that the mutant strain did not differ from the wild-type strain in the amount of Fe^{2+} taken up by the cells. The authors concluded that FeoB is therefore not required for Fe^{2+} uptake in *C. jejuni* (Raphael and Joens, 2003). As *C. jejuni* strains M129 and F38011 were used to draw this conclusion, a second study further investigated the role of FeoB in *C. jejuni* strains NCTC 11168, 81-176 and ATCC 43431 (Naikare *et al.*, 2006). It was proposed that as the genome sequence has been published for NCTC 11168 and not for strains M129 and F38011, it could be confirmed that NCTC 11168 does not contain another unidentified *feoB* homologue or similar Fe^{2+} uptake system that could complement *feoB* (Naikare *et al.*, 2006). It was shown that by knocking out *feoB* in NCTC 11168, Fe^{2+} transport was significantly disrupted. The mutant strain acquired about half the Fe^{2+} that was acquired by wild-type cells during growth. Fe^{2+} remained in the periplasm in the mutant cells, and the mutant could not compete with wild-type cells to colonise or survive in the rabbit ileal loop model. Colonisation of the chick caecum was also compromised, and wild-type cells outcompeted the mutant strains in infection models using colostrum-deprived piglet intestine. Co-transcription of the *feoB* and *feoA* homologues was also shown in strain NCTC 11168, confirming *feoAB* to be an operon (Naikare *et al.*, 2006). FeoB was therefore proposed to transport Fe^{2+} in *C. jejuni* NCTC 11168 (Fig. 1.10). The *feoB* gene is non-functional in some strains (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007).

1.5.1.4. Other Iron Uptake Systems and Additional Genes

1.5.1.4.1. The Cj0173c-Cj0178 System

The *C. jejuni* NCTC 11168 genome also contains one more Fe³⁺ uptake system encoded by *cj0173c-cj0178*. Putatively organised into two operons separated by a promoter region, *cj0176c-cj0173c* and *cj0177-cj0178* demonstrate homology to a siderophore-dependent iron uptake system (Fig. 1.10; Parkhill *et al.*, 2000). The genes *cj0175c-cj0173c* were originally identified as an Fe³⁺ transporting inner membrane associated periplasmic binding protein-dependent transport system, similar to those described in *Serratia marcescens* that do not appear to depend upon an OMRP (Zimmermann *et al.*, 1989). The operon was named *cfbpABC* and demonstrates homology to the *hitABC* operon found in *H. influenzae* and *Y. pestis* (van Vliet *et al.*, 2002). In *H. influenzae*, the loss of *hitC* results in an inability to utilise protoporphyrin IX or iron; the authors concluded that the system was therefore likely to be involved in Fe²⁺ uptake (Sanders *et al.*, 1994). CfbpC (Cj0173c) shows similarity to an ATPase, with CfbpB (Cj0174c) labelled as the permease; the periplasmic binding protein is CfbpA (Cj0175c; Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). Upstream of *cj0175c* and encoded in the same orientation, *cj0176c* starts the operon and appears to encode a small lipoprotein of unknown function (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). More recently, the crystal structure of CfbpA has been solved showing an unusual preference for binding free Fe²⁺ over free Fe³⁺ (Tom-Yew *et al.*, 2005). There is no requirement for a synergistic anion as seen with other ferric transport proteins, indicating that this is a new type of binding protein, suggesting a novel role in iron uptake (Tom-Yew *et al.*, 2005). CfbpA is proposed to be similar to neisserial FbpA. Neisserial FbpABC transport systems function to transport Tf or Lf derived iron from the periplasm into the cytoplasm. Since *C. jejuni* is proposed to be incapable of utilising iron derived from Tf and Lf (Pickett *et al.*, 1992), the authors suggest that CfbpABC are not likely to play this role (Tom-Yew *et al.*, 2005).

The genes *cj0177* and *cj0178* are adjacent to *cj0176c-cj0173c* on the *C. jejuni* NCTC 11168 chromosome, encoded divergently as an operon (Parkhill *et al.*, 2000). The first gene *cj0177* encodes a lipoprotein that demonstrates homology to *phuW* from *P. aeruginosa*. PhuW is expressed along with a haem uptake ABC transporter system; it is located to the inner membrane and is necessary, but not essential, for fully efficient haem uptake (Ochsner *et al.*, 2000). There is also similarity to *cjrA* associated with colicin Js

uptake in *E. coli* and *Shigella* spp. (Šmajš and Weinstock, 2001), although the CjrA protein is not essential for colicin Js uptake (Šmajš and Weinstock, 2001). Cj0178 shows homology to the haemin receptor, PhuR (Ochsner *et al.*, 2000) and the colicin Js receptor, CjrB (Šmajš and Weinstock, 2001), but identification of an iron source that Cj0178 can bind or take up is still lacking.

Because of the similarity of *cj0177* and *cj0178* to genes involved in haem uptake and usage, the Cj0177 crystal structure was solved and shown to bind two cofacial haem groups in a pocket formed by the Cj0177 dimer (Chan *et al.*, 2006). The authors suggested that Cj0177 and Cj0178 may function as a haem uptake system as well as ChuABCD, multiple haem uptake systems being required due to the diversity of haem sources available. They proposed that Cj0178 could be the haemopexin-haem receptor protein, but no experimental haem uptake or growth data were presented to confirm this role; only the binding of haem to Cj0177 was demonstrated (Chan *et al.*, 2006). Interestingly, Cj0178 was also demonstrated to be required for both chick colonisation and colonisation of the rabbit ileal loop model, revealing the importance of this OMRP *in vivo* (Palyada *et al.*, 2004; Stintzi *et al.*, 2005). This is in contrast to ChuA and so redundancy in haem uptake is unlikely.

The *C. jejuni* NCTC 11168 genome also contains *cj0444*, which is a pseudogene that demonstrates homology to the genes encoding the major iron OMRPs. Interestingly, in strains where *cj0444* is a pseudogene, the OMRP encoding genes *cfrA* and *cj0178* are functional (NCTC 11168, RM1221), while in *C. jejuni* strains 81-176 and 81116, which contain a functional copy of *cj0444*, the OMRPs CfrA and Cj0178 are lacking (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007). There seems to be no such inconsistency with the *chu* or *p19* systems, which appear to be conserved across strains (Ridley *et al.*, 2006).

1.5.1.4.2. ExbBD-TonB Energy Transduction Systems

Located downstream of *cj0178* is *cj0179-cj0181* which encode TonB1, ExbB1 and ExbD1 homologues (Parkhill *et al.*, 2000). There are three *tonB* (*cj0753c*, *cj1630* and *cj0181*), *exbB* and *exbD* (*cj0179-80*, *cj1628-29*, *cj0109-10*) homologues present in the *C. jejuni* NCTC 11168 genome (Parkhill *et al.*, 2000). The TonB proteins may function with specific uptake systems; for example *cj0753c* is found divergent to *cfrA* and *cj0181* is found immediately downstream of the *cj0173c-cj0178* operons. Alternatively, the different

TonB proteins may function with any uptake system (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). Two sets of *exbBD* are found transcriptionally coupled to a *tonB*; the other (*cj0109-10*) to the ATP synthase operon (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002).

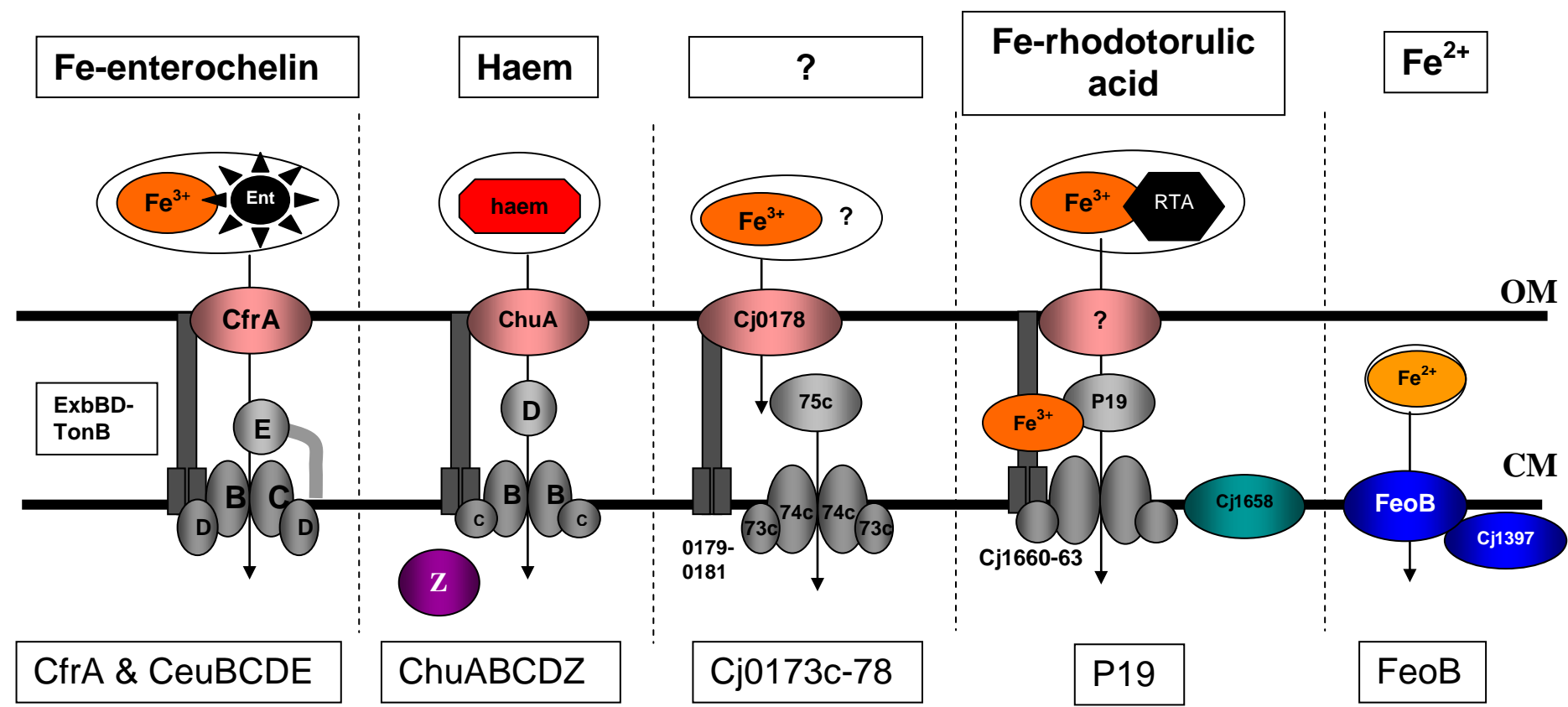


Figure 1.10. Functional iron uptake systems of *C. jejuni* NCTC 11168.

1.5.2. Iron Homeostasis in *C. jejuni*

C. jejuni is exposed to oxygen during transmission between hosts, and to ROS when surviving oxidative bursts within cells that it has invaded in the human host. Two separate but possibly overlapping iron-responsive regulatory systems are encoded within the *C. jejuni* genome regulating iron acquisition and oxidative stress defence. The two regulatory proteins involved in this process are the Fur protein, responsible for regulation of iron uptake genes and the peroxide stress regulator protein PerR.

1.5.2.1. Regulation of Iron Uptake

In *C. jejuni*, the Fur protein is encoded by a gene (*cj0400*) homologous to *E. coli fur* (Parkhill *et al.*, 2000). The protein demonstrates 40% identity to *E. coli* Fur (Schaffer *et al.*, 1985) and is 18 kDa in size. Originally identified by two groups (Wooldridge *et al.*, 1994; Chan *et al.*, 1995), *C. jejuni fur* was shown to complement a *fur* mutant in *E. coli* strain H1780 (Wooldridge *et al.*, 1994). The *fur* gene is located upstream of *lysS* and *glyA* in *C. jejuni*, an organisation not usually seen in Gram-negative organisms (van Vliet *et al.*, 2000). The *lysS* and *glyA* genes encode the housekeeping proteins lysyl-tRNA synthetase and serine hydroxymethyltransferase, respectively (Chan and Bingham, 1991; Chan and Bingham, 1992). The three genes do, however, seem to be transcribed together and therefore comprise an operon (Chan *et al.*, 1995). This operon is also found in *C. upsaliensis* (Bourke *et al.*, 1996). Fur and Crp boxes were found within the region upstream of *fur* in *C. jejuni*, which is interesting as *E. coli* Fur autoregulates, and is also regulated by Crp (De Lorenzo *et al.*, 1988b; Wooldridge *et al.*, 1994; Chan *et al.*, 1995). *C. jejuni* Fur has also been shown to bind to synthetic DNA carrying potential Fur boxes (Chan *et al.*, 1995) and to putative Fur boxes within the Cj1613c-*chuA* intergenic spacer region (Ridley *et al.*, 2006).

A later study showed that *fur* is expressed from two separate promoter regions, neither of which is iron-regulated, located upstream of the first and second ORFs before *fur* (van Vliet *et al.*, 2000). Using a *lacZ* reporter system in *C. jejuni* strain 480, the region immediately upstream of *fur* in strain 81116 (this region is identical in NCTC 11168) was shown to have no promoter activity. When a transcriptional fusion of this region to *lacZ* was made, no expression of *lacZ* was detected (van Vliet *et al.*, 2000). β -galactosidase activity was detected from transcriptional fusions of fragments containing either or both the

gatC (*cj0398*) and the *cj0399* promoter regions from the upstream genes (van Vliet *et al.*, 2000). Expression was higher when both promoters were present, indicating a potential role for both promoters in the expression of *fur* (van Vliet *et al.*, 2000). *C. jejuni fur* has interesting novel characteristics as, without its own promoter region, *fur* is not autoregulated (van Vliet *et al.*, 2000).

Mutation of *C. jejuni fur* was achieved by the insertion of a selectable marker towards either the 3' end or the 5' end of *fur*, resulting in truncated Fur proteins in the different mutants (van Vliet *et al.*, 1998). The mutated *fur* was shown to no longer complement the *E. coli* H1780 *fur* mutation (van Vliet *et al.*, 1998). Only *C. jejuni* mutant strains carrying the selectable markers in the forward orientation with respect to the *fur* gene could be maintained, indicating a polar mutational effect (van Vliet *et al.*, 1998). The *C. jejuni fur* mutant strain exhibited poorer growth rates than wild-type in both iron-replete and iron-restricted conditions, but showed similar trends (van Vliet *et al.*, 1998). Iron-replete growth did not seem harmful to the *C. jejuni fur* mutant strain, which was interesting as the mutation of *fur* in other bacteria such as *E. coli* and *Y. pestis* is detrimental to cells grown under iron-replete conditions due to iron overload (Staggs *et al.*, 1994; Touati *et al.*, 1995; van Vliet *et al.*, 1998). The poorer growth rates seen may also be partly due to interruption of expression of the rest of the operon and not wholly due to Fur loss (van Vliet *et al.*, 1998). Following growth of *C. jejuni* NCTC 11168 and the *fur* mutant strain under iron-replete and iron-restricted conditions, the protein profiles were found to differ (van Vliet *et al.*, 1998). Several key proteins were found to be derepressed in the *C. jejuni fur* mutant under high-iron conditions. Within the cytoplasmic fraction of wild-type cells, two iron-regulated proteins were found which were 26 and 55 kDa in size and proposed to be AhpC and KatA based on earlier identification (Grant and Park, 1995; Baillon *et al.*, 1999). Their expression was higher, but not derepressed in the Δfur background (van Vliet *et al.*, 1998). Both genes have a potential Fur box operator sequence in their promoters (Grant and Park, 1995; Baillon *et al.*, 1999). It was proposed that an additional oxidative stress defence regulatory system may be acting upon these genes that had not yet been identified (van Vliet *et al.*, 1998). Isolated from the periplasmic portion, CeuE, ChuD and P19 were shown to be iron-regulated in the wild-type cells and their expression was derepressed in the *fur* mutant strain (van Vliet *et al.*, 1998). Within the outer membrane fraction of wild-type cells, iron regulated OMRPs were identified as ChuA, CfrA and Cj0178 (Iro80; van Vliet *et al.*, 1998). The iron regulation of OMRPs has been shown in earlier studies for both *C. jejuni* and *C. coli* strains (Field *et al.*, 1986;

Pickett *et al.*, 1992; Guerry *et al.*, 1997). Derepression of ChuA, CfrA and Cj0178 expression occurred in the *fur* mutant strain (van Vliet *et al.*, 1998).

Two studies have demonstrated differences in the transcriptome and proteome of *C. jejuni* NCTC 11168 in response to iron-restriction or high-iron conditions. The expression of many iron transport and storage and oxidative stress defence genes was shown to be higher under iron-limitation (Palyada *et al.*, 2004; Holmes *et al.*, 2005). Microarray studies of a *fur* mutant strain have shown that 53 genes are Fur regulated in *C. jejuni*, including 29 genes of interest that are classically repressed by iron and Fur (Palyada *et al.*, 2004). These include genes involved in haem uptake, enterochelin uptake, the P19 system, *cj0173c-cj0178* and ExbBD-TonB components, but not *fur* itself (Palyada *et al.*, 2004). Upon addition of iron to iron-restricted cells, iron acquisition genes were down-regulated, but metabolic proteins were expressed to a higher level (Palyada *et al.*, 2004; Holmes *et al.*, 2005). Protein glycosylation was boosted when iron was restricted, indicating the importance of iron in *C. jejuni* virulence (Palyada *et al.*, 2004). A *C. jejuni* NCTC 11168 *fur* mutant strain was also shown to be significantly reduced in chick cecum colonisation when compared to wild-type cells, indicating the importance of Fur regulation *in vivo*, not only due to the loss of control of iron homeostasis but also to the loss of expression of important Fur-activated genes (Palyada *et al.*, 2004).

A second study demonstrated an increase in transcript levels for the iron uptake systems highlighted above under high-iron conditions in a *fur* mutant strain, indicating derepression of these genes (Holmes *et al.*, 2005). The genes were shown to be expressed to low levels under high-iron conditions and higher levels under iron-restriction in wild-type cells (Holmes *et al.*, 2005). Other genes shown to be derepressed in the *fur* mutant strain again included components of the multiple ExbBD-TonB systems and the pseudogene *cj0444* (Holmes *et al.*, 2005). Expression of the succinate dehydrogenase operon in *E. coli* was lower under iron-restriction due to RyhB repression (Massé and Gottesman, 2002). In *C. jejuni* succinate dehydrogenase expression was also lower under iron-restricted conditions (Holmes *et al.*, 2005). The genes were not repressed in the *fur* mutant strain when iron-replete leading the authors to conclude that a RyhB regulatory system is not likely to be involved in the overall regulation in *C. jejuni* (Holmes *et al.*, 2005).

The use of the *E. coli* Fur box consensus sequence as a basis for locating *C. jejuni* Fur boxes was not fully successful and did not correlate with promoters that were found to be Fur regulated based on experimental data (van Vliet *et al.*, 2002). This was unexpected

as *C. jejuni* Fur had been previously demonstrated to bind to the *E. coli* Fur box sequence (Wooldridge *et al.*, 1994). The *C. jejuni* Fur box was therefore redefined based on work with *H. pylori* to six 5'-NAT-3' trimers, requiring a match to a minimum of four (van Vliet *et al.*, 2002). Fur box sequences were located in the promoter regions of a number of genes including *chuA*, *cfrA*, *p19*, *feoB*, *ceuB*, *cj0177*, *cfhuA* and *exbB*, all involved in iron transport. Genes for iron storage and binding were also identified including *cft* and *cj1534c*, encoding a bacterioferritin/Dps homologue. Fur boxes were also found within the promoters of a number of genes possibly involved in electron transport or oxidative stress defence (van Vliet *et al.*, 2002). A later study redefined the Fur box consensus sequence in *C. jejuni* using computational analysis of the upstream regions of a number of genes shown to be upregulated when iron was limited (Palyada *et al.*, 2004). A 19 bp consensus sequence was proposed based on the relative frequencies of bases at certain positions (5'-ATTTTGATAATTAATATTA-3'); however other bases were present at many of the positions in much lower frequencies. This consensus does not match the *E. coli* consensus sequence well (Palyada *et al.*, 2004). The authors propose further experimental investigation to confirm or refute the sequence (Palyada *et al.*, 2004).

1.5.2.2. Oxidative Stress Defence and Iron Storage

C. jejuni can respond to oxidative stress caused by superoxide using a single SOD requiring iron as a cofactor (Pesci *et al.*, 1994; Purdy and Park, 1994). SodB (Cj0169) is found in the cytoplasm and a *sodB* mutant strain could not survive intracellularly within epithelial cells or colonise chicks as well as wild-type (Pesci *et al.*, 1994; Purdy *et al.*, 1999). Interestingly SOD activity is induced by iron, but was not affected in *perR* or *fur* mutant strains, and is therefore possibly not regulated by either (van Vliet *et al.*, 1999).

Peroxides are inactivated in *C. jejuni* cells by peroxidases such as catalase (KatA) and alkyl hydroperoxide reductase (AhpC; Grant and Park, 1995; Baillon *et al.*, 1999). AhpC breaks down alkyl hydroperoxide intermediates and the loss of *ahpC* causes susceptibility to atmospheric oxygen and cumene hydroperoxide (Baillon *et al.*, 1999). KatA protects cells from H₂O₂ and can contribute to *C. jejuni* survival within macrophages, but not epithelial cells (Day *et al.*, 2000). The expression of *katA* and *ahpC* was reduced under low-iron conditions in the *fur* mutant, but not derepressed in the mutant strain under high-iron conditions in comparison to wild-type cells (van Vliet *et al.*, 1998). The genes are therefore expressed under iron-restriction, but do not appear to be regulated by Fur. A

Fur homologue was subsequently discovered in *C. jejuni* (PerR) demonstrating 32% identity to *B. subtilis* PerR (van Vliet *et al.*, 1999). The mutation of *perR* in both the wild-type and a *fur* mutant strain allowed investigation of the relationship between the regulators (van Vliet *et al.*, 1999). The *perR* mutant strain grew comparably to wild-type, whereas the double *perR fur* mutant grew similarly to the single *fur* mutant strain (van Vliet *et al.*, 1998; van Vliet *et al.*, 1999). Proteins previously shown to be Fur repressed remained iron-regulated when *perR* was mutated, but were not in either the single *fur* mutant strain or the *perR fur* mutant strain. Therefore, the loss of PerR did not seem to dramatically interfere with Fur regulation (van Vliet *et al.*, 1999). The *kata* and *ahpC* genes were shown to be derepressed in the *perR* or *perR fur* mutant strains, but remained iron repressed in either the single *fur* mutant strain or wild-type cells (van Vliet *et al.*, 1999). This demonstrated PerR regulation of *ahpC* and *kata* expression in *C. jejuni*; however, expression levels were higher in the *perR* mutant strains than under normal iron-limitation (van Vliet *et al.*, 1999). Mutation of *perR* was also shown to cause hyper-resistance to H₂O₂ and cumene hydroperoxide, demonstrating that the over-expression of KatA and AhpC resulted in functional enzymes and conferred a phenotype (van Vliet *et al.*, 1999). KatA activity was found to be near zero in wild-type cells when iron-replete; activity was found to be increased under both high- and low-iron conditions in the *perR* mutant strain. In the *fur* mutant background, activity was lower than wild-type (4-fold) when iron-restricted, but higher when iron-replete. This indicates joint regulation by Fur and PerR of *kata*, verified by increased KatA levels in the *fur perR* mutant strain, but loss of iron-responsive regulation (van Vliet *et al.*, 1999). The authors suggest that this may also be the case for regulation of AhpC. No OxyR homologue was discovered in the *C. jejuni* genome, leading to the proposition that PerR replaces OxyR in *C. jejuni* for the regulation of peroxide stress defence (van Vliet *et al.*, 1999; Parkhill *et al.*, 2000). PerR boxes appear to be a subset of Fur boxes, based on comparisons with proposed PerR boxes in *B. subtilis* and *S. aureus* (Chen *et al.*, 1995; Bsat *et al.*, 1998; Herbig and Helmann, 2001; Horsburgh *et al.*, 2001). The presence of PerR boxes in the promoter regions of genes can be proposed for *C. jejuni*, but may result in confusion due to similarity of predicted Fur and PerR box sequences. A model was proposed in which Fur may recognise PerR boxes as they are smaller, but PerR is unlikely to recognise Fur boxes, allowing cooperation between the regulators (van Vliet *et al.*, 2002). Putative PerR boxes were found in the promoters of *kata*, *ahpC*, *sodB*, *cj1534c* and *perR* itself (van Vliet *et al.*, 2002). The necessity for a metal cofactor for successful DNA binding by Fur or PerR is not known (van Vliet *et al.*, 2002).

C. jejuni also possesses a ferritin (Cft, Cj0612c) that contains iron, and when mutated, causes a growth defect under iron-restriction, indicating a role as a storage protein (Wai *et al.*, 1995; Wai *et al.*, 1996). The mutant strain also demonstrated increased susceptibility to oxidative stress showing the importance of ferritin for detoxification (Wai *et al.*, 1996). A gene (*cj1534c*) encoding a bacterioferritin (Bft) in the *C. jejuni* genome is also possibly Fur/PerR regulated. Bft may function in iron storage and/or oxidative stress defence (Andrews, 1998; van Vliet *et al.*, 2002). A Dps is also encoded in the *C. jejuni* genome. *C. jejuni* Dps was shown to bind up to 40 iron atoms per monomer, and when mutated, the strain lacking Dps was susceptible to H₂O₂-induced stress (Ishikawa *et al.*, 2003); Dps was shown to be constitutively expressed. The Dps protein was therefore proposed to protect cells from H₂O₂ induced stress by binding free iron in the cell (Ishikawa *et al.*, 2003)

C. jejuni possesses a gene (*fdxA*) encoding a ferredoxin located upstream of *ahpC* and divergently transcribed (Baillon *et al.*, 1999; van Vliet *et al.*, 2001). Identified in strain 81116, *fdxA* is identical to *cj0333c* in strain NCTC 11168 (Parkhill *et al.*, 2000; van Vliet *et al.*, 2001). Ferredoxins are key metabolic proteins that act as electron carriers and have iron-sulphur clusters (Bruschi and Guerlesquin, 1988). *C. jejuni* *fdxA* expression was induced in response to iron. The loss of *fdxA* resulted in decreased aerotolerance, but no growth defects and no reduction in peroxide stress defence indicating a potential role for *C. jejuni* FdxA in oxidative stress defence (van Vliet *et al.*, 2001)

1.5.3. The use of Ferri-Lactoferrin or Ferri-Transferrin as an Iron Source by *C. jejuni*

Originally shown to be incapable of growth when supplied solely with ferri-Lf or ferri-Tf as an iron source (Pickett *et al.*, 1992), *C. jejuni* also contains no significant *tbpA/B* or *lbpA/B* homologues (Parkhill *et al.*, 2000). Although Cj0178 and Cj0177 have been suggested to be involved in the use of haem (Chan *et al.*, 2006), Cj0178 has slightly greater homology to the TonB-dependent OMRP components of Tf- and Lf-binding systems (Wooldridge and van Vliet, 2005)

Preliminary work (Rock, 2003) demonstrated that *C. jejuni* cells in contact with ⁵⁵Fe-Tf or ⁵⁵Fe-Lf were able to obtain ⁵⁵Fe from the Tf or Lf. Cells partitioned away from the iron source accumulated far lower amounts of ⁵⁵Fe implying that the process of iron uptake is contact dependent. More iron accumulated in cells supplied with ⁵⁵Fe-Lf than

with ^{55}Fe -Tf. This was suggested to be due to an adaptation by *C. jejuni* to iron acquisition from the most prevalent source encountered in the intestinal niche. Competition assays between ^{55}Fe -Tf and cold Fe-Tf showed that *C. jejuni* cells acquired about half the level of iron accumulated by cells incubated with ^{55}Fe -Tf alone, while cells incubated with ^{55}Fe -Tf and bovine serum albumin (BSA) demonstrated little difference in the level of radioactivity obtained. Therefore, cold Fe-Tf was proposed to compete with ^{55}Fe -Tf for specific binding sites on the *C. jejuni* cell surface. Tf binding was therefore proposed to be a specific process and not due to non-specific adherence of protein to the cell surface. A comparable phenotype was noted with Lf. In addition, 94% of cell-associated radioactivity was found to be in the soluble cell fraction suggesting that the ^{55}Fe had passed through the outer membrane, and probably, the inner membrane, and signalled the existence of a Tf and/or Lf bound iron uptake system (Rock, 2003).

1.6. Aims

Although the characterisation of a number of iron uptake systems has occurred in *C. jejuni* NCTC 11168, an ability to use Lf- or Tf-bound iron had not been shown (Pickett *et al.*, 1992). Preliminary evidence generated by another member of the group indicated that NCTC 11168 may be able to use iron from Lf or Tf, but the mechanism involved was not elucidated (Rock, 2003). NCTC 11168 has a number of outer membrane receptor proteins encoded in the genome, but only Cj0178 has not been associated with a particular iron source. A role for Cj0178 in the uptake of Lf-derived iron was indicated by the previous study (Rock, 2003). A suggestion has also been made that Cj0178 could act as a haem receptor protein (Chan *et al.*, 2006). Cj0178 is proposed to be involved in iron uptake because of similarities to other outer membrane receptors, and is important *in vivo* as loss of *cj0178* resulted in impaired chick and rabbit ileal loop colonisation (Palyada *et al.*, 2004; Stintzi *et al.*, 2005).

The aims of this study were to confirm that NCTC 11168 can take up Lf- or Tf-derived iron and to extend the study to demonstrate that this iron is available to the cells for growth. Investigation of the mechanisms involved was intended to determine whether the process is specific and whether contact between the Lf or Tf and the bacterial cell surface is required for successful acquisition of the iron. The evidence gathered would indicate whether the process is receptor specific, as seen in *Neisseria* spp. (Gray-Owen and Schryvers, 1996; Perkins-Balding *et al.*, 2004), or whether a different mechanism of uptake

is occurring in *Campylobacter*. Mutation of components of the major iron uptake systems followed by growth testing would establish whether any known iron uptake systems were important; the primary candidate appeared to be Cj0178. If a role for Cj0178 was found, further investigation of the region of the genome surrounding *cj0178* would allow better characterisation of this unknown iron uptake system.

Chapter 2. Materials and Methods

2.1. Bacterial Growth Conditions, Antibiotics and Supplements

All media were purchased from Oxoid and all chemicals were purchased from Sigma-Aldrich unless stated otherwise. *Escherichia coli* strains were routinely cultured aerobically at 37°C in Luria-Bertani (LB; Roth, 1970) medium or M9 minimal salts medium (Smith and Bidochka, 1998; Sambrook and Russell, 2001). Liquid cultures were incubated with shaking (200-250 rpm, G10 Gyrotory Shaker, New Brunswick Scientific Co., U.S.A.) for 12-16 hours (overnight). Media were supplemented with antibiotics (routinely stored at 4°C) where necessary to select for strains carrying recombinant plasmids (Table 2.1). To enhance plasmid DNA replication, *E. coli* cultures were incubated for 3 hours, after which chloramphenicol was added to a final concentration of 100 µg/ml and the culture was incubated for a further 12-16 hours (Sambrook and Russell, 2001). *E. coli* strains containing recombinant plasmids were identified by α -complementation on medium containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside), and the lactose analogue and *lac* operon transcriptional inducer IPTG (isopropyl- β -D-thiogalactopyranoside, Table 2.1; Barkley and Bourgeois, 1978; Sambrook and Russell, 2001).

Campylobacter jejuni strains were routinely cultured at 37°C in a variable atmosphere incubator (VAIN, Don Whitley Scientific, Shipley, U.K.) with an atmospheric gas concentration of 85% N₂, 10% CO₂ and 5% O₂ in Mueller-Hinton (MH) medium, Brucella medium (Becton, Dickenson and Company, U.K.) or Minimal Essential Medium alpha (MEM α , Invitrogen) supplemented with vancomycin and trimethoprim (Table 2.1). Blood agar was made by supplementation of MH agar with defibrinated horse blood to a final concentration of 5% (v/v). Liquid cultures were incubated with shaking (500 mot 1/min, Vibrax VXR Basic, IKA) for 12-16 hours (overnight). Where necessary, the appropriate antibiotic was added to the medium to select for strains with mutant alleles (Table 2.1).

Table 2.1. Stock and working concentrations of antibiotics and other additives routinely used for bacterial selection and screening.

Supplements	Stock Concentration	Working Concentration
Vancomycin	10 mg/ml in dH ₂ O	10 µg/ml
Trimethoprim	5 mg/ml in 50% ethanol	5 µg/ml
Kanamycin (Km)	50 mg/ml in dH ₂ O	50 µg/ml
Chloramphenicol (Cm)	20 mg/ml in 100% ethanol	20 µg/ml
Erythromycin (Ery)	5 mg/ml in 50% ethanol	5 µg/ml
Ampicillin (Amp) (Sodium salt)	100 mg/ml in dH ₂ O	100 µg/ml
X-gal (Section 2.2.2.)	20 mg/ml in DMF	40 µg/ml
IPTG (Section 2.2.2.)	0.1 M in dH ₂ O	0.2 mM

The addition of FeSO₄ to cultures to a final concentration of 10 µM ensured iron-replete conditions (van Vliet *et al.*, 1998b). Haem was prepared as described previously (Dyer *et al.*, 1987) and supplemented to a final concentration of 25 µM (Ridley *et al.*, 2006). Human ferri-lactoferrin (Lf), human ferri-transferrin (Tf), ferri-ovotransferrin (ovo-Tf), human apo-Tf and apo-ovo-Tf (90 to >98 % purity) were dissolved in 10 mM Tris-HCl (pH 7.5) to a final concentration of 10 mg/ml and added to cultures to either 0.27 µM or 1.11 µM (Rock, 2003); concentrated NaCl (~5 M) was added to the pure protein solution if there was precipitation to achieve physiological salt concentrations (133-164 mM NaCl, Pellaud *et al.*, 1999; Falt *et al.*, 2001). Apo-Lf was produced from iron-saturated Lf using a method based on those used to deferrate iron-binding glycoproteins prior to radioactive iron-labelling for radiolabeled iron uptake assays (Simonson *et al.*, 1982; Freestone *et al.*, 2000). Iron-saturated human Lf was dissolved to an initial concentration of 10 mg/ml in 10 mM Tris-HCl (pH 7.5) and deferrated by sequential dialysis (in narrow bore dialysis tubing, 6 kDa cut-off, GRI) against two changes of 0.2 M citric acid (pH 2.3), and two changes of distilled water at 4°C. Following this, the protein was dialysed against two changes of 100 mM Tris-HCl (pH 7.5). The protein was concentrated and desalted using Microcon YM-50 (*Microcon*[®] Centrifugal Filter Devices, 50 kDa molecular weight cut-off, Millipore, as per the manufacturer's instructions) and the final protein concentration was quantified using the Bradford Assay (Section 2.15.8). Because of the high binding affinity of Lf for iron it is difficult to completely iron-deplete protein preparations (Freestone *et al.*, 2000). To induce iron-restricted conditions in MH or Brucella medium, deferoxamine

mesylate salt (DesferalTM) was added to a final concentration of 20 μ M (van Vliet *et al.*, 1998b). Growth in unsupplemented MEM α , a defined iron-limited growth medium, induced iron-limitation as described previously (van Vliet *et al.*, 1998b).

2.1.1. Storage of Bacterial Strains

E. coli and *C. jejuni* strains were routinely stored long-term at -80°C in cryo-tubes as glycerol stocks (Sambrook and Russell, 2001). Bacteria were recovered from frozen stocks maintained on dry ice by scraping cells from the surface of the frozen culture followed by incubation. *E. coli* strains were grown overnight in LB broth liquid cultures and *C. jejuni* strains were grown overnight on confluent spread MH agar plates (supplemented with appropriate antibiotics where necessary, Table 2.1). *E. coli* cells were harvested by centrifugation (at $3202 \times g$ for 5 minutes at room temperature, Eppendorf centrifuge 5810 R). *C. jejuni* cells were washed from each plate with 2 ml of sterile phosphate buffered saline (PBS) and harvested (at $3202 \times g$ for 20 minutes at room temperature), washed again with PBS, and harvested as before. *E. coli* and *C. jejuni* cells were resuspended in 0.5 ml of fresh LB broth or MH broth, respectively, and mixed with an equal volume of sterile 25% (v/v) glycerol in a dry ice/ethanol bath.

2.2. Media Recipes, Buffers and Solutions

Reagents were of analytical grade and sterilisation, where necessary, was achieved by autoclaving at 121°C , 15 lb.inch^{-2} for 15 minutes. All media were stored long-term at 4°C except SOC which was aliquotted and stored at -20°C . Antibiotics were added to media that had been cooled to 55°C or below; Petri dishes were supplied by Sterilin. Antibiotics (Table 2.1) and any other solutions that required sterilisation by filtration were passed through a $0.22 \mu\text{m}$ filter membrane using either Stericups (Millipore) with a vacuum pump (Fisher Scientific) or Pall Life Sciences acrodisc syringe filters with BD Plastipak 5 ml or 10 ml syringes (Becton, Dickenson and Company, U.K.).

2.2.1. Media Recipes

Standard media recipes were prepared as previously described (Sambrook and Russell, 2001).

Brucella (B) Broth: 1% (w/v) pancreatic digest of casein, 1% (w/v) peptic digest of animal tissue, 0.1% (w/v) dextrose, 0.2% (w/v) yeast extract, 0.5% (w/v) sodium chloride (NaCl) and 0.01% (w/v) sodium bisulphite (NaHSO₃) in dH₂O. B broth was prepared by the addition of 28 g of medium (Becton, Dickenson and Company, U.K.) to 1 litre of dH₂O, followed by sterilisation. B agar was made by addition of 1.5% (w/v) *Bioagar* (15 g/l, Biogene Ltd.) prior to sterilisation.

Luria-Bertani (LB) Medium: 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl. LB broth was prepared by the addition of 10 g of bacto-tryptone, 5 g of bacto-yeast extract and 5 g of NaCl to 900 ml of dH₂O. The medium was adjusted to pH 7.2 with 5 M sodium hydroxide (NaOH), the final volume was adjusted to 1 litre with dH₂O and sterilised. Solid medium was made by addition of 1.5% (w/v) *Bioagar* (15 g/l, Biogene Ltd.) prior to sterilisation.

M9 Minimal Salts Medium: M9 minimal salts medium was prepared with 1 × M9 salts, 40% (v/v) sterile glucose, 0.1 mM CaCl₂, 2 mM MgSO₄, 4% (v/v) yeast extract and 10.16 ml thiamine/proline supplement (see below) in sterile tap water. Tap water is essential to provide a source of all necessary trace elements. The MgSO₄ and CaCl₂ solutions were made and sterilised separately and added last to the medium. M9 minimal salts agar was produced by adding 1.5% (w/v) *Bioagar* (15 g/l, Biogene Ltd.) to the tap water prior to sterilisation (Smith and Bidochka, 1998; Sambrook and Russell, 2001).

Thiamine/proline supplement: The addition of thiamine/proline was required to maintain *E. coli* strains carrying a deletion of the proline biosynthetic operon [$\Delta(lac_proAB)$] in the bacterial chromosome and the complementing *proAB* on the F' plasmid. Supplement was prepared with 0.4% (w/v) glucose, 5 mM magnesium sulphate (MgSO₄), 0.01% (w/v) thiamine and 0.01% (w/v) proline in 10.16 ml of dH₂O and sterilised by filtration.

5 × M9 salts: 5 × M9 salts was made by the addition of 56.4 g of pre-prepared commercial powder to 1 litre of dH₂O followed by sterilisation.

Mueller-Hinton (MH) Medium: Beef dehydrated infusion (30%, w/v), 1.75% (w/v) casein hydrolysate, and 0.15% (w/v) starch in dH₂O. MH broth was prepared by the addition of 21 g of medium to 1 litre of dH₂O, followed by sterilisation. MH agar includes the addition of 1.7% (w/v) agar (17 g/l) to the components of MH broth and was prepared by the addition of 38 g of medium to 1 litre of dH₂O and sterilised.

SOC Medium: SOC medium was prepared with 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 0.05% (w/v) NaCl, 2.5 mM potassium chloride (KCl) and 10 mM magnesium chloride (MgCl₂) in 180 ml of dH₂O. The medium was adjusted to pH 7.0 with 5 M NaOH and sterilised. Filter-sterilised glucose was added to 0.36% (v/v) after sterilisation and the final volume was adjusted to 200 ml with sterile dH₂O.

2.2.2. Buffers and Solutions

Calcium chloride (50 mM): CaCl₂ (1.47 g) was dissolved in dH₂O (200 ml) and sterilised.

Calcium chloride (50 mM) with glycerol: CaCl₂ (1.47 g) was dissolved in dH₂O (160 ml) and 20% (v/v) glycerol (40 ml of 100% glycerol) was added. The solution was sterilised.

Campylobacter Electroporation Buffer (CEB): 272 mM sucrose and 15% (v/v) glycerol in dH₂O. The buffer was sterilised.

Chloroform:Iso-amyl alcohol (24:1): Chloroform was overlaid with iso-amyl alcohol in a ratio of 24:1 and mixed prior to use in chromosomal DNA extractions.

Citrate Buffer (0.2 M, pH 2.3): 0.175 M citric acid and 0.025 M trisodium citrate (Na₃C₆H₅O₇) in dH₂O. If the pH was not already at 2.3, small adjustments were made using either citric acid or trisodium citrate.

Citric acid (1 M): Citric acid was dissolved in dH₂O and adjusted to pH 3.2 with Na₃C₆H₅O₇ (1 M). The solution was filter sterilised and stored in aliquots at −20°C.

CTAB/NaCl: 0.7 M NaCl in dH₂O with 10% (w/v) hexadecyltrimethyl ammonium bromide (CTAB). CTAB was dissolved by stirring and heating. The solution was not autoclaved.

EB Buffer: Elution buffer, 50 mM Tris-Cl (pH 8.1-8.2), 1.4 M NaCl and 15% (v/v) ethanol in dH₂O.

EDTA (0.5 M): Disodium ethylene diamine tetra acetate-2H₂O (Na₂EDTA-2H₂O, 74.45 g) was dissolved in dH₂O (320 ml) and adjusted to pH 8.0 with NaOH pellets (approximately 8 g). The disodium salt of EDTA will dissolve only when the pH of the solution is adjusted to 8.0. The final volume was adjusted with dH₂O to 400 ml and the solution was sterilised.

IPTG (0.1 M): 0.119 g of isopropyl-β-D-thiogalactopyranoside was dissolved in 4 ml of dH₂O, the volume was adjusted to 5 ml with dH₂O and sterilised by filtration. Aliquots were stored at -20°C for up to 3 months.

L-lysine (50 mg/ml): 0.5 g of L-lysine was dissolved in 10 ml of dH₂O. The solution was sterilised by filtration and stored at 4°C for up to 3 months.

L-phenylalanine (50 mg/ml): 0.5 g of L-phenylalanine was dissolved in 10 ml of 1 M ammonium hydroxide. The solution was sterilised by filtration and stored at 4°C for up to 3 months.

L-tryptophan (50 mg/ml): 0.5 g of L-tryptophan was dissolved in 10 ml of 0.5 M HCl. The solution was sterilised by filtration and stored at 4°C for up to 3 months.

L-tyrosine (50 mg/ml): 0.5 g of L-tyrosine was dissolved in 10 ml of 1 M HCl. The solution was sterilised by filtration and stored at 4°C for up to 3 months.

Noradrenaline (100 mM): 0.32 g of norepinephrine bitartrate salt was dissolved in 10 ml of dH₂O, the solution was sterilised by filtration. Aliquots were stored at -20°C.

Phosphate Buffered Saline (10 × PBS): 1.37 M NaCl, 0.27 M KCl, 0.015 M potassium phosphate (KH_2PO_4) and 0.08 M sodium phosphate (Na_2HPO_4) in dH_2O . The buffer was adjusted to pH 7.4 with 2 M HCl. The final volume was adjusted with dH_2O and the buffer was sterilised.

Proteinase K (20 mg/ml): Proteinase K was dissolved in dH_2O , aliquotted for single use and stored at -20°C .

RNase A (10 mg/ml): RNase A was dissolved in dH_2O and boiled for 15 minutes to inactivate contaminating DNase activity. Aliquots were stored at -20°C .

SDS (10% or 2.2% w/v): Sodium dodecyl sulphate was dissolved in dH_2O with warming and stirring until all of the powder had dissolved. The solution was not autoclaved.

Sodium acetate (3 M, pH 5.2): Sodium acetate (CH_3COONa ; 40.8 g) was dissolved in dH_2O (60 ml). The solution was adjusted to pH 5.2 with glacial acetic acid and the final volume was adjusted with dH_2O to 100 ml.

Solubilisation Buffer: 10 mM Tris-HCl (pH 7.5), 7 mM EDTA and 0.6% (w/v) N-lauroyl sarcosine sodium salt (sarcosyl) in dH_2O . The final volume was adjusted with dH_2O and the buffer was filter sterilised.

TE Buffer: 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA in dH_2O . The final volume was adjusted with dH_2O and the buffer was sterilised.

TES Buffer: 10 mM Tris-HCl (pH 7.5), 10 mM EDTA and 25% (w/v) sucrose in dH_2O . The final volume of the buffer was adjusted with dH_2O and sterilised by filtration.

Tris-acetate-EDTA (TAE) Electrophoresis Buffer: 0.04 M Tris (hydroxymethyl) aminomethane and 1 mM EDTA in dH_2O . The buffer was adjusted to pH 7.8 with glacial acetic acid and the final volume was adjusted with dH_2O . The buffer was sterilised.

Tris-HCl buffers: Tris Base was dissolved in dH₂O (50% of the required final volume) to achieve a final concentration of 1 M. The buffer was then adjusted to the desired pH values of 6.8 (with approximately 100 ml of concentrated HCl), 7.5 (with approximately 65 ml of concentrated HCl) or 8.8 (with approximately 5 ml of concentrated HCl). Buffers were left to equilibrate with stirring overnight, and then final pH adjustments were made. To achieve the required final volume additional dH₂O was added. Buffers were then sterilised.

Trisodium citrate (1 M): Na₃C₆H₅O₇ was dissolved in dH₂O and the solution was adjusted to pH 5 using citric acid (1 M). The solution was filter sterilised and stored in aliquots at –20°C.

X-gal (20 mg/ml): 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside was dissolved in 5 ml of N,N'-dimethylformamide and stored in aliquots at –20°C protected from light for up to 3 months.

2.3. Bacterial Strains and Plasmids

The bacterial strains and plasmids used and constructed during this study are listed in Table 2.2.

Table 2.2. Bacterial strains and plasmids.

Bacterial strain/plasmid	Characteristics	Reference/source
<i>E. coli</i>		
DH5αe	Cloning host strain: F ⁺ Φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (r _K [–] m _K ⁺) <i>gal[–] phoA supE44 λ[–] thi[–]1 gyrA96 relA1</i> .	(Hanahan, 1983), Invitrogen
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proABlacI^qZ</i> ΔM15 Tn10 (Tet ^R)].	Stratagene, (Holmes <i>et al.</i> , 2005)
<i>C. jejuni</i>		
NCTC 11168 (11168-GS)	Wild-type genome strain.	(Parkhill <i>et al.</i> , 2000), NCTC ¹

480 (NCTC 12744)	Clinical isolate used as host strain for maintaining plasmids/reporter assays.	(King <i>et al.</i> , 1991), NCTC ¹
81-176	Clinical isolate.	(Korlath <i>et al.</i> , 1985; Black <i>et al.</i> , 1988)
KAR2	NCTC 11168 <i>cj0178::aphA-3</i> . (Km ^R).	(Miller <i>et al.</i> , 2008)
KAR3	NCTC 11168 <i>p19::aphA-3</i> . (Km ^R).	(Miller <i>et al.</i> , 2008)
JDR5	NCTC 11168 $\Delta chuA::cat$. (Cm ^R).	(Ridley <i>et al.</i> , 2006)
JDR6	NCTC 11168 $\Delta chuB::cat$. (Cm ^R).	(Ridley <i>et al.</i> , 2006)
JDR20	NCTC 11168 $\Delta tonB1::cat$. (Cm ^R).	(Rock, 2003)
JDR21	NCTC 11168 $\Delta cj0178::cat$. (Cm ^R).	(Rock, 2003; Miller <i>et al.</i> , 2008)
11168 derived from pRDH224	NCTC 11168 $\Delta feoB::ermC'$. (Ery ^R).	(R. D. Haigh)
81-176 derived from pRDH225	81-176 $\Delta cj0444::aphA-3$. (Km ^R).	(R. D. Haigh)
CEM1	NCTC 11168 $\Delta cj0172c::aphA-3$. (Km ^R).	This study
CEM2	NCTC 11168 $\Delta cj0173c::aphA-3$. (Km ^R).	This study
CEM3	NCTC 11168 $\Delta cj0174c::aphA-3$. (Km ^R).	(Miller <i>et al.</i> , 2008)
CEM4	NCTC 11168 $\Delta cj0177::ermC'$. (Ery ^R).	This study
CEM5	NCTC 11168 $\Delta cfrA::aphA-3$. (Km ^R).	(Miller <i>et al.</i> , 2008)
CEM6	NCTC 11168 $\Delta ceuE::aphA-3$. (Km ^R).	This study
CEM7	NCTC 11168 $\Delta ceuE::aphA-3$. (Km ^R , cassette in reverse orientation with respect to deleted gene).	This study

CEM8	KAR2 mutant strain (Km ^R) complemented with a wild-type copy of <i>cj0178</i> , plus <i>cat</i> (Cm ^R), into <i>cj0752</i> .	(Miller <i>et al.</i> , 2008)
CEM9	KAR2 $\Delta cj0177::ermC'$. (Km ^R , Ery ^R).	This study
CEM10	CEM8 $\Delta cj0177::ermC'$. (Km ^R , Cm ^R , Ery ^R).	This study
CEM11	KAR2 $\Delta cfrA::aphA-3$. (Km ^R , Km ^R).	This study
CEM12	KAR2 $\Delta feoB::ermC'$. (Km ^R , Ery ^R).	This study
CEM13	NCTC 11168 <i>cj0178::aphA-3</i> . KAR2 chromosomal DNA naturally transformed into the NCTC 11168 background that all other mutant strains have been constructed in. (Km ^R).	This study
CEM14	NCTC 11168 $\Delta cj0178::cat$. JDR21 chromosomal DNA naturally transformed into the NCTC 11168 background that all other mutant strains have been constructed in. (Cm ^R).	This study
CEM15	JDR6 $\Delta cj0174c::aphA-3$. (Cm ^R , Km ^R).	This study. By R. Patel under the supervision of C. Miller.
CEM16	JDR6 $\Delta ceuE::aphA-3$. (Cm ^R , Km ^R).	This study. By R. Patel under the supervision of C. Miller.
CEM17	JDR6 <i>p19::aphA-3</i> . (Cm ^R , Km ^R).	This study. By R. Patel under the supervision of C. Miller.
Plasmids		
pUC19	Cloning/suicide vector, Amp ^R (<i>amp</i>).	New England Biolabs
pMW10	<i>E. coli/C. jejuni</i> shuttle <i>lacZ</i> reporter vector, Km ^R (<i>aphA-3</i>).	(Wösten <i>et al.</i> , 1998)

p23E5	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>metK</i> promoter region inserted into the <i>Bam</i> HI site of pMW10. (Km ^R).	(van Vliet <i>et al.</i> , 1998b; Wösten <i>et al.</i> , 1998)
pJDR13	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>chuA</i> promoter region inserted into the <i>Bam</i> HI site of pMW10. (Km ^R).	(Ridley <i>et al.</i> , 2006)
pAV35	pBluescript vector containing a Cm ^R (<i>cat</i>) cassette with corresponding promoter.	(van Vliet <i>et al.</i> , 1998b)
pJMK30	pUC19 containing <i>C. coli</i> Km ^R gene (<i>aphA-3</i>) flanked by multiple cloning regions.	(van Vliet <i>et al.</i> , 1998b)
pcam193b8 ²	pUC19 containing a <i>C. jejuni</i> NCTC 11168 genomic fragment (bases 174132-175858) flanked by <i>Sma</i> I sites. (Amp ^R).	(Parkhill <i>et al.</i> , 2000)
pRDH224	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>feoB</i> genomic fragment (bases 1330810-1333652) with a deletion of 1775 bp in the <i>feoB</i> ORF (bases 1331317-1333092) and insertion of an erythromycin resistance cassette (<i>ermC'</i>) into a <i>Bam</i> HI site created at the point of deletion. (Amp ^R , Ery ^R).	(R. D. Haigh)
pKAR2	pcam193b8:: <i>aphA-3</i> . Insertion of a Km ^R cassette (<i>aphA-3</i>) into the distal <i>Psi</i> I site of <i>cj0178</i> (base position 1128).	(Miller <i>et al.</i> , 2008)
pGEMCWH01	<i>cj0752</i> flanks with an internal multiple cloning region in a pGEM-T easy vector backbone. (Amp ^R).	(Elvers <i>et al.</i> , 2005) ³
pASK-IBA7	N-terminal Strep-tag II, factor Xa site, Amp ^R (<i>amp</i>).	IBA (Holmes <i>et al.</i> , 2005)
pJMcK1	<i>C. jejuni fur</i> gene from NCTC 11168 cloned into <i>Bsa</i> I site of pASK-IBA7. (Amp ^R).	(Holmes <i>et al.</i> , 2005)
pRRE	pRR (Karlyshev and Wren, 2005) containing cloned <i>ermC'</i> (Ery ^R).	(O. Bridle)

pCEM1	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>cj0172c</i> genomic fragment (bases 169571-167306) cloned between <i>KpnI</i> and <i>PstI</i> sites. (Amp ^R).	This study
pCEM2	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>cj0173c</i> genomic fragment (bases 170551-168481) cloned between <i>KpnI</i> and <i>PstI</i> sites. (Amp ^R).	This study
pCEM3	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>cj0174c</i> genomic fragment (bases 171984-169436) cloned between <i>KpnI</i> and <i>PstI</i> sites. (Amp ^R).	(Miller <i>et al.</i> , 2008)
pCEM4	pUC19 containing the <i>C. jejuni</i> NCTC 11168 genomic fragments (bases 172625-173291 and 173690-174389) cloned between <i>KpnI</i> and <i>PstI</i> sites with an internal <i>SmaI</i> site. (Amp ^R).	This study
pCEM5	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>cfrA</i> genomic fragment (bases 704900-708177) cloned between <i>KpnI</i> and <i>PstI</i> sites. (Amp ^R).	(Miller <i>et al.</i> , 2008)
pCEM6	pUC19 containing the <i>C. jejuni</i> NCTC 11168 <i>ceuE</i> genomic fragment (bases 1286177-1288186) cloned between <i>KpnI</i> and <i>PstI</i> sites. (Amp ^R).	This study
pCEM7	pCEM1, $\Delta cj0172c::aphA-3$. Deletion of 1097 bp of the <i>cj0172c</i> ORF (bases 168946-167849) and insertion of a Km ^R cassette (<i>aphA-3</i>) into a <i>BglIII</i> site created at the point of deletion. (Amp ^R , Km ^R).	This study
pCEM8	pCEM2, $\Delta cj0173c::aphA-3$. Deletion of 661 bp of the <i>cj0173c</i> ORF (bases 169877-169215) and insertion of a Km ^R cassette (<i>aphA-3</i>) into a <i>BglIII</i> site created at the point of deletion. (Amp ^R , Km ^R).	This study
pCEM9	pCEM3, $\Delta cj0174c::aphA-3$. Deletion of 1477 bp of the <i>cj0174c</i> ORF (bases 171520-170042) and insertion of a Km ^R cassette (<i>aphA-3</i>) into a <i>BglIII</i> site created at the point of deletion. (Amp ^R , Km ^R).	(Miller <i>et al.</i> , 2008)

pCEM10	pCEM4, $\Delta cj0177::ermC'$. Insertion of an Ery ^R cassette (<i>ermC'</i>) into a <i>Sma</i> I site created between the genomic fragments (bases 172625-173291 and 173690-174389). (Amp ^R , Ery ^R).	This study
pCEM11	pCEM5, $\Delta cfrA::aphA-3$. Deletion of 1966 bp of the <i>cfrA</i> ORF (bases 705484-707451) and insertion of a Km ^R cassette (<i>aphA-3</i>) into a <i>Bgl</i> III site created at the point of deletion. (Amp ^R , Km ^R).	(Miller <i>et al.</i> , 2008)
pCEM12	pCEM6, $\Delta ceuE::aphA-3$. Deletion of 800 bp of the <i>ceuE</i> ORF (bases 1286798-1287599) and insertion of a Km ^R cassette (<i>aphA-3</i>) into a <i>Sma</i> I site created at the point of deletion. (Amp ^R , Km ^R).	This study
pCEM13	pCEM6, $\Delta ceuE::aphA-3$. Deletion of 800 bp of the <i>ceuE</i> ORF (bases 1286798-1287599) and insertion of a Km ^R cassette (in reverse orientation with respect to the deleted gene, <i>aphA-3</i>) into a <i>Sma</i> I site created at the point of deletion. (Amp ^R , Km ^R).	This study
pCEM14	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>cj0176c-cj0177</i> promoter region (bases 172915-172695) inserted into the <i>Bam</i> HI site of pMW10 (<i>cj0176c</i> orientation). (Km ^R).	(Miller <i>et al.</i> , 2008)
pCEM15	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>cj0176c-cj0177</i> promoter region (bases 172695-172915) inserted into the <i>Bam</i> HI site of pMW10 (<i>cj0177</i> orientation). (Km ^R).	(Miller <i>et al.</i> , 2008)
pCEM16	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>cj0178</i> putative promoter region (bases 173477-173763) inserted into the <i>Bam</i> HI site of pMW10. (Km ^R).	(Miller <i>et al.</i> , 2008)
pCEM17	Reporter construct. <i>C. jejuni</i> NCTC 11168 <i>exbB1</i> putative promoter region (bases 175801-176042) inserted into the <i>Bam</i> HI site of pMW10. (Km ^R).	(Miller <i>et al.</i> , 2008)

pCEM18	pUC19 containing the <i>C. jejuni</i> NCTC 11168 genomic fragment (bases 172686-176113; promoter region, <i>cj0177</i> , <i>cj0178</i>) cloned between <i>Kpn</i> I and <i>Pst</i> I sites. (Amp ^R).	(Miller <i>et al.</i> , 2008)
pCEM19	pCEM18, Δ <i>cj0177</i> . Deletion of 764 bp of the <i>cj0177</i> ORF (bases 172943-173706). (Amp ^R).	(Miller <i>et al.</i> , 2008)
pCEM20	pCEM19 plus <i>cat</i> . Insertion of a Cm ^R cassette (<i>cat</i>) at the <i>Pst</i> I site found downstream of <i>cj0178</i> . (Amp ^R , Cm ^R).	(Miller <i>et al.</i> , 2008)
pCEM21	Wild-type copy of <i>cj0178</i> plus promoter region and downstream Cm ^R cassette (<i>cat</i>) from pCEM20, inserted at the <i>Kpn</i> I site within the multiple cloning region of pGEMCWH01 (in forward orientation with respect to <i>cj0752</i> flanks). (Amp ^R , Cm ^R).	(Miller <i>et al.</i> , 2008)

All mutant strains and plasmid constructs carry selectable markers inserted in forward orientation with respect to the gene unless stated otherwise.

¹ National Collection of Type Cultures, Colindale, London, U.K.

² The cam clone was kindly provided by J. Parkhill, PSU, Sanger Centre, Cambridge, U.K.

³ A gift from C. Holmes, University of Birmingham, Edgbaston, Birmingham, U.K.

2.4. Primers

The oligonucleotide primers constructed and used during the course of this study are listed in Table 2.3.

Table 2.3. Oligonucleotide primers.

Primer	Nucleotide Sequence 5'- 3' (Restriction enzyme sites are highlighted in bold)	Product and target gene/construct
Cloning		
<i>cj0172cF</i>	GGGGT ACCGAGCTATGGCAAGAGGATGTAAG	pCEM1 insert <i>cj0172c</i>
<i>cj0172cR</i>	AAA ACTGCAGGAGCCTTATGGCATTGATATAG	
<i>cj0173cF</i>	GGGGT ACCGCTTTAATCACTGCTTTAATTACC	pCEM2 insert <i>cj0173c</i>
<i>cj0173cR</i>	AAA ACTGCAGCATAGCCATGATCACCTGCATTG	
<i>cj0174cF</i>	GGGGT ACCGATAGAGATCAAGCCAGACAAG	pCEM3 insert <i>cj0174c</i>
<i>cj0174cR</i>	AAA ACTGCAGGTACCATAATAGCCGTAATGCC	

<i>cj0177F1</i>	GGGGTACCGAGAATGTTTCAGAGCAAGCAAAAG	pCEM4 insert 1
<i>cj0177R1</i>	TCCCCCGGGCATTAGTAAGTTCATTGGCTTTAATGG	<i>cj0177</i>
<i>cj0177F2</i>	TCCCCCGGGGTTGTGGTGAATTTAAGCTATGG	pCEM4 insert 2
<i>cj0177R2</i>	AAA ACTGCAG CCCACATATCCATTATTTTCAAGTTC	<i>cj0177</i>
<i>cfrAF</i>	GGGGTACCCATTTCGAAGGCTCTTGTATAG	pCEM5 insert
<i>cfrAR</i>	AAA ACTGCAG CTTCTACCTCCATAAACAAGAC	<i>cfrA</i>
<i>ceuEF</i>	GGGGTACCGTTGAAGAATTAGTCGCTTTTG	pCEM6 insert
<i>ceuER</i>	AAA ACTGCAG GGTATAACAATAGGTGGAAGTC	<i>ceuE</i>
Inverse PCR		
<i>cj0172cInvF</i>	GAAGATCTCCTTGGAAGATTATCGAAATG	<i>cj0172c</i> inverse PCR
<i>cj0172cInvR</i>	GAAGATCTCATTGCGCATTTTACAGTAG	product pCEM1
<i>cj0173cInvF</i>	GAAGATCTCTTAAAATAGGAAATCGCTTTGAG	<i>cj0173c</i> inverse PCR
<i>cj0173cInvR</i>	GAAGATCTCTCCTTCTTTGACATGAAGATTG	product pCEM2
<i>cj0174cInvF</i>	GAAGATCTGATGCAGCTTTGCCTTCCTTG	<i>cj0174c</i> inverse PCR
<i>cj0174cInvR</i>	GAAGATCTGTAAAATTGCCCCGAGTTTATAG	product pCEM3
<i>cfrAInvF</i>	GAAGATCTTCGCAAGTGGGAGTGGTAG	<i>cfrA</i> inverse PCR product
<i>cfrAInvR</i>	GAAGATCTTCCCAATGGCGCAAAGTC	pCEM5
<i>ceuEInvF</i>	TCCCCCGGGGGAGCAAGTGGAAATGGACTAGAG	<i>ceuE</i> inverse PCR product
<i>ceuEInvR</i>	TCCCCCGGGGGACTTTCACCGTAGCAGTGTTG	pCEM6
KAR2 complementation		
<i>cj0752upF</i>	GGAATAATCAAGCCTACAAAATC	pGEMCWH01
<i>cj0752upR</i>	GATTTTGTAGGCTTGATTATTCC	
<i>cj0752downF</i>	GTCAATCCACAATATACAAGCAAAAC	
<i>cj0752downR</i>	GTTTTGCTTGATATTGTGGATTGAC	
<i>cj0178compF</i>	GGGGTACCCATAGACATTAATTCCTTTTAGATATTTT G	pCEM18 insert
<i>cj0178compR</i>	A ACTGCAG GTATCTGTTGCATTTGCTTCTG	pCEM18
<i>cj0177compInvF</i>	GAAGATCTGCTATGGAGAAATAGATTTAAAAG	
<i>cj0177compInvR</i>	GAAGATCTGAATGCTTAAAATAGTATAAAATTTC	
CatR_ <i>KpnI</i>	GGGGTACCGAATTCAGCTGCGCCCTTTAG	Chloramphenicol resistance (<i>cat</i>) cassette (pAV35)

Promoter cloning and gel shifts		
<i>cj0176c</i> -77promF	CGGGATCCTAATTCCTTTTAGATATTTTG	pCEM14/15 insert
<i>cj0176c</i> -77promR	CGGGATCCTATTATTGTTACTTTCTTAG	
<i>cj0178</i> promF	CGGGATCCGACATTATATCCTTAAGCCATAAGC	pCEM16 insert
<i>cj0178</i> promR	CGGGATCCTCCTCCCCCTTATAAATCAAAAC	
<i>exbB1</i> promF	CGGGATCCCAAAAATTCCTATTATAGTAGATTTATA TTG	pCEM17 insert
<i>exbB1</i> promR	CGGGATCCTTGTATATTCCTTAATATTTATATTCAA AC	
<i>cj0176c</i> -p-split	TTATTATAATAAAAATGAAAATATTTATC	pCEM14/15 insert
<i>cj0177</i> -p-split	CATTTTATTATAATAAATTTTGATAATA	
Screening		
<i>p19F</i>	GGTGCTATAGATGCTATATCAAAC	<i>C. jejuni</i> genome 1582447-1582470 bp
<i>p19R</i>	CTAAAGAACTTATACCTTCGCTATCG	<i>C. jejuni</i> genome 1583920-1583895 bp
<i>feoB1</i>	CCAGAATTCAAGATGGACGCATAATC	<i>C. jejuni</i> genome 1330824-1330840 bp
<i>feoB4</i>	AAACTGCAGAACAAATAGATTTTGAATTTGAAAGC	<i>C. jejuni</i> genome 1333597-1333572 bp

2.5. Preparation of DNA from Bacterial Cells

2.5.1. Large-Scale Preparation of *C. jejuni* Chromosomal DNA

Preparation of *C. jejuni* chromosomal DNA from both wild-type and mutant strains was completed as described previously (Ausubel *et al.*, 1992). *C. jejuni* cells were grown for 2 days on 4 confluent spread MH agar plates (supplemented with appropriate antibiotics where necessary, Table 2.1). Cells were washed from each plate with 3 ml of sterile PBS, harvested (at $3202 \times g$ for 20 minutes at 4°C) and subsequently resuspended in 9.5 ml of PBS with 2 ml of 10% (w/v) SDS, 100 µl of 20 mg/ml PK and 20 µl of 10 mg/ml RNase A. Preparations were then incubated at 37°C for 1 hour before the addition of 1.8 ml of 5 M NaCl and 1.5 ml of 10% (w/v) CTAB/0.7 M NaCl, which was followed by mixing and incubation of the preparation at 65°C for 20 minutes. The CTAB/protein complexes

were then extracted by the addition of an equal volume of 24:1 chloroform:iso-amyl alcohol and centrifugation (at $3202 \times g$ for 20 minutes at 4°C, Jones and Walker, 1963). The aqueous layer was then harvested and the DNA was precipitated by the addition of 0.7 volumes of isopropanol (Jones and Walker, 1963; Wilson, 1987). Chromosomal DNA was recovered by spooling with a sealed Gilson p1000 tip, washed in 70% (v/v) ethanol and air-dried for around 5 minutes to allow evaporation of the alcohol. The DNA was resuspended in 1-2 ml of EB buffer overnight at 37°C to ensure that it had redissolved completely, aliquotted and stored long-term at -20°C.

2.5.2. Small-Scale Preparation of *C. jejuni* Chromosomal DNA

Small-scale preparation of *C. jejuni* chromosomal DNA was completed using the *Puregene Genomic DNA Purification Kit* (Gentra Systems) according to the manufacturer's instructions. The method was adapted from a previous protocol used by Buffone and Darlington (1985). The Gram-negative bacteria protocol allows the preparation of 10-35 µg of chromosomal DNA. Buffer recipes were not provided with the kit. *C. jejuni* cells were grown overnight on a confluent spread MH agar plate (supplemented with appropriate antibiotics where necessary, Table 2.1). Cells were washed from the plate with 0.5 ml of sterile PBS and harvested (at $15,700 \times g$, Heraeus Sepatech Biofuge 13, for 10 seconds) to form a pellet. Cells were resuspended in 300 µl of *Cell Lysis Solution* and incubated at 80°C for 5 minutes to lyse the cells. Samples were treated with 1.5 µl of *RNase A Solution* (4 mg/ml) and mixed thoroughly by inverting 50 times, followed by incubation at 37°C for 15-30 minutes. Samples were cooled to room temperature by incubation on ice for 1 minute. Proteins were precipitated by the addition of 100 µl of *Protein Precipitation Solution* to the cell lysate and mixed by vortexing at high speed for 40 seconds to uniformly distribute the solution within the cell lysate. Samples were centrifuged (at $15,700 \times g$ for 5 minutes) to pellet the precipitated proteins. If the protein pellet was not tight, samples were vortexed again, incubated on ice for 10 minutes and centrifuged again as before. The supernatant containing chromosomal DNA was decanted into a clean 1.5 ml microfuge tube containing 300 µl of 100% (v/v) isopropanol to precipitate the DNA. Samples were centrifuged (at $15,700 \times g$ for 1 minute) to pellet the DNA. DNA pellets were washed with 300 µl of 70% (v/v) ethanol by inverting several times. Samples were centrifuged for a further 1 minute ($15,700 \times g$) and pellets were allowed to air-dry for 5-10 minutes. *DNA Hydration Solution* (TE Buffer) was added to the

tubes (50 µl to give a final DNA concentration of 500 µg/ml if the yield was 25 µg). Samples were incubated at 65°C for 1 hour, followed by overnight at room temperature to rehydrate the DNA. Periodic tapping of each tube aided dispersion of the DNA which was then stored at 4°C.

2.5.3. Preparation of Plasmid DNA from *E. coli* Strains and *C. jejuni* 480

Small-scale preparation of plasmid DNA was completed using the *QIAprep Miniprep Kit* (QIAGEN) using a microfuge (Eppendorf) according to the manufacturer's instructions. Buffer recipes were provided with the kit are stated. The principle is based on the modified alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979; Birnboim, 1983). *E. coli* strains or *C. jejuni* strain 480 were grown overnight in either LB or MH broth liquid cultures (5 ml) set up from single bacterial colonies (supplemented with appropriate antibiotics where necessary, Table 2.1). Cells were harvested (at $3202 \times g$ for 20 minutes at 4°C). All subsequent centrifugation steps were performed at $15,700 \times g$ and prepared DNA was stored at -20°C. Pelleted bacterial cells were resuspended in 250 µl of *Buffer P1* (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Lysis was achieved by the addition of 250 µl of *Buffer P2* (200 mM NaOH, 1% (w/v) SDS) to induce alkaline conditions, which was followed by neutralisation of the samples and conversion to high-salt binding conditions by the addition of 350 µl of *Buffer N3*. Samples were mixed at each stage by inverting 4-6 times. Centrifugation for 10 minutes pelleted the precipitated chromosomal DNA, denatured proteins, cellular debris and SDS, and the supernatant containing renatured plasmid DNA was then decanted into a *QIAprep Spin Column*. Centrifugation for 1 minute allowed adsorption of the DNA to the *QIAprep silica-gel membrane*. DNA was washed by the addition of 0.75 ml of *Buffer PE* (containing 80% (v/v) ethanol) to the column followed by centrifugation for 1 minute to remove salts. Residual wash buffer was removed by centrifugation for an additional minute. DNA was eluted under low-salt conditions by the addition of 50 µl of *Buffer EB* (10 mM Tris-HCl, pH 8.5) to the centre of the membrane, which was left to stand for 1 minute followed by centrifugation for 1 minute.

Larger-scale preparations from *E. coli* strains were completed using the *HiSpeed Plasmid Midi Purification Kit* (QIAGEN). *E. coli* strains were grown for 8 hours in LB broth starter cultures (5 ml) set up from single bacterial colonies (supplemented with appropriate antibiotics where necessary, Table 2.1). Starter cultures were diluted (1/500-

1/1000) into selective LB broth (50 ml) and grown overnight. Cells were harvested (at $3202 \times g$ for 20 minutes at 4°C) with all subsequent centrifugation steps performed at $15,700 \times g$. Bacterial pellets were resuspended in 6 ml of *Buffer P1*, followed by the addition of 6 ml of *Buffer P2* with mixing as before. Samples were incubated at room temperature for 5 minutes. Cell lysate was neutralised by the addition of 6 ml of *Buffer P3* (3.0 M potassium acetate (CH_3COOK), pH 5.5), mixed, poured into a *QIAfilter Cartridge* and incubated at room temperature for 10 minutes. A *HiSpeed Midi Tip* was equilibrated by the addition of 4 ml of *Buffer QBT* (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100). Cell lysate was filtered into the *HiSpeed Tip* which removes unwanted cellular debris; lysate passed through the column resin by gravity flow. DNA bound to the resin was washed by the addition of 20 ml of *Buffer QC* (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol) to the *HiSpeed Tip* and eluted with 5 ml of *Buffer QF* (1.25 M NaCl, 50 mM Tris-Cl pH 8.5, 15% (v/v) isopropanol). Precipitation of the DNA was achieved by the addition of 3.5 ml (0.7 volumes) of isopropanol, followed by incubation at room temperature for 5 minutes. The eluate/isopropanol mixture was then transferred to a 20 ml syringe and filtered through a *QIAprecipitator Midi Module*. The DNA was washed by the filtration of 2 ml of 70% (v/v) ethanol through the *QIAprecipitator*. Residual alcohol was expelled by forcing air through the *QIAprecipitator* and the DNA was eluted from the membrane with 1 ml of *Buffer TE*.

2.6. Agarose Gel Electrophoresis and Visualisation of DNA

Nucleic acids were routinely analysed using agarose gel electrophoresis (Sambrook and Russell, 2001). Agarose gels were prepared by dissolving *SeaKem LE Agarose* (Cambrex Bio Science Rockland Inc., Rockland, ME, USA) in TAE buffer to 0.8 - 2.0% (w/v) depending on the range of fragment sizes being separated. Ethidium bromide (10 µg/ml, Fisher Scientific) was added to molten agarose (cooled to 55°C) to achieve a final concentration of 0.5 µg/ml. All horizontal gel electrophoresis tanks, gel combs and gel casting trays were produced as standard by the School of Biological Sciences workshop (The University of Leicester). Gels were cast in casting trays with a gel comb or combs depending on gel size inserted to a depth of 5-7.5 mm. After the gel had set it was placed in a gel electrophoresis tank and covered with TAE buffer. DNA samples were mixed with 5 × gel loading buffer (5 × TAE buffer, 15% (v/v) glycerol, 0.3% (w/v) orange G) to a final

concentration of $\times 1$ prior to loading into wells created by the removal of the gel comb. DNA samples were analysed against molecular weight standards consisting of commercially prepared λ DNA (250 ng) restricted with *Hind*III (23130, 9416, 6682, 4361, 2322, 2027 and 564 bp fragments, Gibco-BRL) and Φ X174 DNA (100 ng) restricted with *Hae*III (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 bp fragments, Gibco-BRL). Gels were electrophoresed at a constant voltage between 5-10 V/cm until the orange dye had almost reached the end of the gel (around 80-85% total migration). DNA was visualised using ultraviolet (UV) light at 260 nm and photographed to allow estimation of DNA fragment sizes and concentrations (Syngene Gene Genius Bioimaging System with Genesnap software).

2.7. Purification of DNA

2.7.1. Ethanol Precipitation

Ethanol precipitation (Ausubel *et al.*, 1992; Sambrook and Russell, 2001) was used to concentrate DNA, to remove salt or to purify DNA. DNA was precipitated by the addition of sodium acetate (3 M, pH 5.2) to $1/10^{\text{th}}$ of the total volume, 1 μ l of glycogen (20 mg/ml, Roche Diagnostics) as a carrier for DNA precipitation and 2-3 volumes of room temperature 100% (v/v) ethanol to each sample. Samples were incubated at 4°C for 1 hour or more and centrifuged (at $15,700 \times g$ for 30 minutes at room temperature). The supernatant was aspirated with a pipette to remove as much salt as possible from the DNA. The DNA was washed by the addition of 1 ml of 70% (v/v) ethanol, followed by centrifugation (at $15,700 \times g$ for 1 minute at room temperature). This step was repeated 2-5 times depending on the original sample volume. DNA pellets were allowed to air-dry for 5-10 minutes and redissolved in 10 μ l of *Buffer EB* or dH₂O depending on subsequent applications. Alternatively, when concentration was not required, the salt was removed from the DNA using nitrocellulose filter discs. A nitrocellulose disc was placed (shiny side upwards) on dH₂O and reactions were pipetted onto the surface of the disc. Following 30 minutes incubation at room temperature the purified reaction was aspirated from the disc surface.

2.7.2. Purification of Double-Stranded DNA Fragments

Double-stranded DNA fragments ranging from 100 bp to 10 kb were purified using the *QIAquick PCR Purification Kit* (QIAGEN) and fragments ranging from 70 bp to 4 kb were purified using the *MinElute PCR Purification Kit* (QIAGEN) according to the manufacturer's instructions. Buffer recipes were not provided with the kit. The method is based on the adsorption of nucleic acids to silica-gel surfaces in the presence of high chaotropic salt concentrations (Vogelstein and Gillespie, 1979). All centrifugation steps were performed at $15,700 \times g$ and purified DNA was stored at -20°C . *Buffer PB* (5 volumes) was added to each sample to adjust the solution to $<\text{pH } 7.5$ and induce high salt conditions; samples were mixed. The samples were decanted into either a *QIAquick Spin* or a *MinElute* column and centrifuged for 1 minute to bind the DNA to the membrane. DNA was washed by the addition of 0.75 ml of *Buffer PE* (containing 80% (v/v) ethanol) to the column, followed by centrifugation for 1 minute to remove salts. Residual wash buffer was removed by centrifugation for an additional minute. DNA was eluted under low-salt conditions by the addition of either 30 μl (*QIAquick*) or 10 μl (*MinElute*) of *Buffer EB* or dH_2O to the centre of the membrane, columns were left to stand for 1 minute followed by centrifugation for 1 minute.

2.7.3. Purification of Double-Stranded DNA Fragments from Agarose Gels

Double-stranded DNA fragments ranging from 70 bp to 10 kb were purified from up to 400 mg of low-melt agarose in TAE buffer using the *QIAquick Gel Extraction Kit* (QIAGEN). Fragments ranging from 70 bp to 4 kb were purified using the *MinElute Gel Extraction Kit* (QIAGEN) according to the manufacturer's instructions. Buffer recipes were not provided with the kit. The method is based on the adsorption of nucleic acids to silica-gel surfaces in the presence of high chaotropic salt concentrations (Hamaguchi and Geiduschek, 1962; Vogelstein and Gillespie, 1979). All centrifugation steps were completed at $15,700 \times g$ and purified DNA was stored at -20°C . DNA fragments were visualised using UV light and excised from agarose gels using clean scalpel blades. *Buffer QG* (3 gel volumes) was added to solubilise each gel slice and to provide the appropriate conditions for binding of DNA to the silica-gel membrane. Samples were incubated at 55°C until the agarose had dissolved (up to 10 minutes) with regular mixing. If the mixture

was orange or violet, 10 μ l of 3 M sodium acetate (pH 5.0) were added to adjust the solution to the optimal pH for DNA binding. Following this, 100% (v/v) isopropanol was added to each sample (1 gel volume). Samples were then mixed and decanted into either a *QIAquick Spin* or a *MinElute* column and centrifuged for 1 minute to bind the DNA to the membrane. Traces of agarose were removed by the addition of 0.5 ml of *Buffer QG* to each column followed by centrifugation for 1 minute. DNA was washed by the addition of 0.75 ml of *Buffer PE* (containing 80% (v/v) ethanol) to each column followed by centrifugation for 1 minute to remove salts. Residual wash buffer was removed by centrifugation for an additional minute. DNA was eluted under low-salt conditions by the addition of either 30 μ l (*QIAquick*) or 10 μ l (*MinElute*) of *Buffer EB* or dH₂O to the centre of each membrane, columns were left to stand for 1 minute followed by centrifugation for 1 minute.

2.8. Quantification of DNA

Purified chromosomal DNA was diluted in dH₂O and quantified using absorption spectroscopy (Genova spectrophotometer, Jenway, Sambrook and Russell, 2001). Concentrations were calculated by measuring sample absorbance at 260 nm (A_{260}). A single A_{260} unit of double-stranded DNA is ~50 μ g/ml in H₂O. Purity was measured using the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}); a ratio of 1.8 to 2.0 indicated pure DNA. Where the sample absorbance ratio was less than 1.8 contamination with protein or aromatic substances may have occurred, while a ratio of above 2.0 indicated possible RNA contamination. A visual estimate of plasmid or DNA fragment concentration was made against the molecular weight standards used during agarose gel electrophoresis (119.22, 48.53, 34.44, 22.48, 11.97, 10.45 and 2.90 ng of the λ DNA fragments present and 25.12, 20.01, 16.19, 11.19, 5.74, 5.22, 5.03, 4.34, 3.60, 2.19 and 1.34 ng of the Φ X174 DNA fragments present, Section 2.6, Gibco-BRL).

2.9. DNA Manipulation

All buffers were used to a final concentration of $\times 1$ unless otherwise stated.

2.9.1. Restriction of DNA

All restriction enzymes, appropriate buffers and bovine serum albumin (BSA, 10 mg/ml stock used at a final concentration of 100 μ g/ml) were purchased from New England Biolabs (NEB) and used in accordance with the manufacturer's instructions.

2.9.2. Dephosphorylation of DNA

Where necessary the removal of 5' phosphate groups from restricted DNA was achieved by the addition of Shrimp Alkaline Phosphatase (SAP, Roche Diagnostics) (Sambrook and Russell, 2001). Following restriction, vector DNA was incubated at 37°C with dephosphorylation buffer (Roche Diagnostics) and either 1 unit (U) of SAP per 1 pmol of 5'-terminal phosphorylated DNA fragments (either 5'-protruding or 5'-recessive) for 10 minutes, or 1 U of SAP per 0.2 pmol of 5'-terminal phosphorylated blunt-ended DNA fragments for 60 minutes. Heat treatment at 65°C for 15 minutes inactivated the enzyme.

2.9.3 Ligation of DNA

Ligation of DNA was achieved using T4 DNA ligase (NEB) to catalyse the formation of phosphodiester bonds between adjacent 3'-OH and 5'-P groups on the ends of double stranded DNA fragments (Sambrook and Russell, 2001). A typical reaction consisted of vector DNA and insert DNA in a molar ratio of either 1:3 (protruding-ended fragments) or 1:5 (blunt-ended fragments), with ligation buffer (NEB), 1-5 U of T4 DNA ligase (per 1 μ g of DNA) and dH₂O to a final volume of 20 μ l. Reactions between fragments with protruding ends were incubated at 4-16°C and those with blunt ends at 16-25°C overnight. Enzyme inactivation was achieved at 65°C for 10 minutes.

2.9.4. Simultaneous Fill-in and Removal of 3'-Overhangs with Ligation

For ligation of double-stranded DNA fragments with non-compatible ends, simultaneous fill-in and removal of overhangs with ligation was performed. A molar ratio of 1:3 vector to insert DNA (with at least 100 ng of vector DNA) was included in a reaction with fill-in and ligation buffer (200 mM Tris-HCl pH 7.6, 100 mM MgCl₂, 100 mM dithiothreitol (DTT), 6 mM adenosine triphosphate (ATP) and 1 mM of each dNTP (dCTP, dGTP, dATP and dTTP), 400 U of T4 DNA ligase (2000 U for blunt-ended ligations) per 1 µg of DNA, 1 U of T4 DNA polymerase (NEB, per 1 µg of DNA), 1 U of Klenow DNA polymerase (NEB, per 1 µg of DNA) and dH₂O to a final volume of 20 µl. Reactions were incubated overnight at 16°C and enzyme inactivation was achieved by heating to 75°C for 10 minutes. Alternatively, vector DNA was incubated with cloned *Pfu* buffer (Promega, supplied with *Pfu*), 1 mM of each dNTP, 1.25 U of *Pfu* DNA polymerase (Promega, per 1 µg of DNA) and dH₂O to a final volume of 20 µl. Reactions were incubated at 72°C for 30 minutes followed by dephosphorylation of vector ends (Section 2.9.2). Vector DNA was purified and concentrated by ethanol precipitation (Section 2.7.1) and then ligated. A molar ratio of 1:3 vector to insert DNA (with at least 500 ng of vector DNA) was included in a reaction mix with rapid ligation buffer (600 mM Tris-HCl pH 7.8, 20 mM MgCl₂, 20 mM DTT, 2 mM ATP and 10% (v/v) polyethylene glycol (PEG) 8000), 400 U of T4 DNA ligase (2000 U for blunt-ended ligations) per 1 µg of DNA and dH₂O to a final volume of 30 µl. Reactions were incubated for 20 minutes or more at 16-25°C and purified by ethanol precipitation (Section 2.7.1).

2.10. Amplification of DNA

2.10.1. Amplification of DNA using the Polymerase Chain Reaction

DNA fragments to be cloned were amplified *in vitro* using high-fidelity polymerase chain reaction (PCR, Sambrook and Russell, 2001). Oligonucleotide primers (Table 2.3) were designed to anneal to specific sites on the DNA template. Reactions (20 µl) were completed using the *TripleMaster* high-fidelity PCR System (Eppendorf) according to the manufacturer's instructions in 0.2 ml thin-walled domed cap tubes (ABgene) and cycled in a thermocycler (Eppendorf Mastercycler or Mastercycler Gradient). The standard reaction mix contained the appropriate concentration of pure template DNA: 10-50 ng of DNA for

0.1-3.0 kb genomic DNA templates, 50-100 ng of DNA for 3-10 kb genomic DNA templates or 0.1-1.0 ng of DNA for plasmid DNA templates. Forward and reverse oligonucleotide primers were used at a final concentration of 200-400 nM. *HighFidelity PCR Buffer* with Mg^{2+} ($10 \times$ PCR buffer with 25 mM Mg^{2+}) was used to a final concentration of $\times 1$, resulting in a standard final Mg^{2+} concentration of 2.5 mM. Deoxyribonucleotide triphosphates (dNTPs, 10 mM each) were added to each reaction to a final concentration of 200 μM . *TripleMaster Polymerase Mix* (5 U/ μl *Taq* DNA polymerase, no information was provided on the other enzymes in the mix, enzyme storage buffer composition: 20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, 0.5% (v/v) Tween[®] 20 and 0.5% (v/v) Igepal[®] CA-630) was added to a final concentration of 0.05-0.075 U/ μl . Molecular biology grade water was used to make the reactions up to 20 μl final volumes. The specific activity of *Taq* polymerase in combination with other enzymes is higher than the specific activity of pure *Taq* in the same concentration (enzyme mix = 1×10^{-5} to 1×10^{-6} mutation rate compared to 'normal' low fidelity mutation rate of 1×10^{-3} when using *Taq* alone).

Pfu DNA polymerase (Promega) was also used to achieve high fidelity PCR. Reactions were prepared as described for the *TripleMaster System*, but with the inclusion of *Pfu* DNA polymerase $10 \times$ reaction buffer with MgSO_4 (200 mM Tris-HCl pH 8.8, 100 mM KCl, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgSO_4 , 1.0% (v/v) Triton[®]-X-100 and 1 mg/ml nuclease-free BSA) to a final concentration of $\times 1$, and *Pfu* DNA polymerase (2-3 U/ μl) to a final concentration of 1.25 U/50 μl . All other reaction conditions were the same. Confirmatory PCR reactions using pure DNA templates were completed as described, but using low fidelity *Taq* polymerase (ABgene).

Screening of DNA directly from large numbers of either *E. coli* or *C. jejuni* colonies (Gussow and Clackson, 1989) was performed by first spreading individual colonies to patches on fresh agar plates in order to increase cell numbers. A toothpick was used to collect cells from each patch, which were then suspended in 20 μl of molecular biology grade water in 0.2 ml thin-walled domed cap tubes (Abgene). Cell suspensions were heated to 96°C for 5 minutes in a thermocycler (Eppendorf Mastercycler) to lyse the cells, cell debris was pelleted (at $15,700 \times g$ for 2 min at room temperature) and 2 μl of each supernatant were used as template DNA in the PCR reactions. Primer concentrations were doubled compared to standard and all other parameters remained the same. Where PCR reactions failed, appropriate optimisation of conditions occurred including altering the annealing and denaturation temperatures, altering the magnesium concentration to dNTP

ratio, changing the number of cycles and the amount of template DNA, enzyme, primer, buffer, or magnesium where appropriate. Reactions were cycled according to the programme detailed in Table 2.4.

Table 2.4. Standard PCR thermal cycling programme.

Stage of Reaction	Temperature (°C)	Time	Cycles
Initial template denaturation	94	2 min	1
Template denaturation	94	30 sec	30
Primer annealing	5-10°C below the melting temperature of the primer pair.	30 sec	
Primer extension/elongation	72/68	1 min extension per kb of product to be amplified.	
<i>Final extension</i>	72/68	5 min	1

2.11. Sequencing of DNA

Sequencing of DNA was completed using an ABI 377 DNA sequencer and the *BigDye[®] Terminator v3.1 Cycle Sequencing Kit* (Applied Biosystems). Sequencing primers (Table 2.2, Appendix 2) were designed to anneal to appropriate sites on the DNA template. *BigDye[®] Terminator v3.1* (1.58 µM A-DyeDeoxy, 94.74 µM T-DyeDeoxy, 0.42 µM C-DyeDeoxy, 47.37 µM G-DyeDeoxy, 78.95 µM dITP, 15.79 µM dATP, 15.79 µM dCTP, 15.79 µM dTTP, 168.42 mM Tris-HCl pH 9.0, 4.21 mM (NH₄)SO₄, 42.10 mM MgCl₂, 0.42 U/µl AmpliTaq DNA polymerase, ABI) was diluted (1/8) using sequencing buffer (5 × Tris-HCl, MgCl₂ Sequencing Buffer from ABI) and molecular biology grade water. Each reaction was set up with the appropriate amount of purified template DNA (Table 2.5), 3.2 pmol of sequencing primer, 8 µl of diluted *BigDye[®] Terminator v3.1* and molecular biology grade water to 20 µl.

Table 2.5. Appropriate DNA template amounts used in sequencing reactions.

Template Type	Template Quantity (per reaction)	Primer Quantity (per reaction)
Double stranded plasmid (up to 20 kb)	200-300 ng	3.2 pmol
Single stranded phage	50-100 ng	
Double stranded DNA (>20 kb, e. g. lambda)	0.5-1.0 µg	7.5 pmol
PCR Product		
100-200 bp	1-3 ng	3.2 pmol
200-500 bp	3-10 ng	
500-1000 bp	5-20 ng	
1000-2000 bp	10-40 ng	
>2000 bp	40-100 ng	

Reactions were cycled according to the programme detailed in Table 2.6.

Table 2.6. Standard sequencing thermal cycling programme.

Stage of Reaction	Temperature (°C)	Time	Cycles
Initial template denaturation	96	10 sec	1
Template denaturation	96	10 sec	30
Primer annealing	5-10°C below the melting temperature of the primer pair.	10 sec	
Elongation	60	4 min	1
<i>Final elongation</i>	60	5 min	

Sequencing reactions were purified to remove unincorporated dye terminators firstly by the addition of SDS (0.2% (v/v) final concentration) to the reactions, followed by heating to 98°C for 5 minutes. Reactions were then purified using the *DyeEx 2.0 Spin Kit* (QIAGEN) according to the manufacturer's instructions. The method is based on size-

exclusion chromatography with *DyeEx 2.0 spin columns* containing prehydrated gel filtration resin. All centrifugation steps were performed at $750 \times g$. Spin columns were gently vortexed and centrifuged for 3 minutes. Sequencing reactions were applied directly to the centre of the gel-bed and centrifuged for 3 minutes. The eluate containing the purified DNA was processed by the Protein and Nucleic Acid Chemistry Laboratory (PNACL, The University of Leicester).

2.12. Transformation of DNA into Bacterial Cells

Competent cells were prepared and transformation was completed following standard protocols (Ausubel *et al.*, 1992; Sambrook and Russell, 2001).

2.12.1. Preparation of Competent *E. coli* Cells

Chemically competent *E. coli* cells were produced from a single bacterial colony used to inoculate 1-ml LB liquid cultures which were incubated overnight (Sambrook and Russell, 2001). Broths were then diluted 1/100 into 100 ml of fresh LB broth and incubated until the optical density at 600 nm (OD_{600}) had reached 0.6. The optical density was measured by spectrophotometry at determined intervals (Ultrospec 10, Amersham Biosciences, Cell Density Meter with 1.6 ml disposable cuvettes, Sarstedt). Cells were harvested (at $3202 \times g$ for 15 minutes at 4°C), washed once in an equal volume of ice cold 50 mM CaCl_2 and resuspended to $1/10^{\text{th}}$ of the original volume in ice cold 50 mM CaCl_2 with 20% (v/v) glycerol. Cells were aliquotted and stored at -80°C . Electro-competent *E. coli* cells were prepared from a 10 ml LB broth overnight culture inoculated with cells derived from a single colony. Fresh LB (100 ml) was inoculated to an OD_{600} of 0.1 and incubated until the OD_{600} had reached 0.5-0.7. Cells were harvested (at $3202 \times g$ for 15 minutes at 4°C in two 50 ml aliquots) and resuspended in an equal volume of ice cold ddH₂O (50 ml). Following this, cells were harvested as before, resuspended in ice cold ddH₂O (25 ml), harvested again and resuspended in 10 ml of ice cold 10% (v/v) glycerol. Finally, cells were harvested as before and resuspended in 1 ml of ice cold 10% (v/v) glycerol, aliquotted and stored at -80°C .

2.12.2. Heat-Shock Transformation of *E. coli*

Plasmid construct DNA (from protruding-end ligations) or vector DNA (for example pUC19) were transformed into chemically competent *E. coli* cells using heat shock transformation (Sambrook and Russell, 2001). All tubes (1.5 ml, Eppendorf) were pre-chilled on ice and chemically competent cells were defrosted on ice for around 5 minutes. Purified DNA (1 to 25 ng), half of any purified ligation reaction or appropriate control, was placed in a pre-chilled tube, mixed gently with 50 µl of competent cells and incubated on ice for 20 minutes. Cells were heat-shocked at 42°C for 45-50 seconds and subsequently transferred to ice for 2 minutes. Room temperature SOC (450 µl) was mixed with the cells which were then incubated at 37°C for 1-2 hours. Transformants were grown by overnight incubation on LB agar plates supplemented with the appropriate antibiotics (Table 2.1).

2.12.3. Transformation of *E. coli* Cells by Electroporation

Plasmid construct DNA (from blunt ended or 3-way ligation reactions) was transformed into electro-competent *E. coli* cells using electroporation (Sambrook and Russell, 2001). Electroporation cuvettes (Geneflow, 2 mm gap) were pre-chilled for 30 minutes at -20°C and competent cells were thawed on ice for around 5 minutes. Purified DNA (10 pg to 25 ng), half of any ligation reaction or appropriate control, was pipetted into the cuvette. Competent cells (40 µl) were added to the cuvette and mixed with the DNA. The liquid covered the basal surface of the cuvette to avoid air bubbles. The cuvette was placed into the electroporator chamber (Bio-Rad Gene Pulser) and one pulse was applied at 2.5 kV, 25 µF and 200 Ohms, with the pulse (time constant) typically lasting 4.5-5.0 milliseconds. Cells were recovered in 1 ml of LB broth at 37°C for 1 hour, plated onto selective LB agar plates and incubated overnight for the growth of transformants.

2.12.4. Preparation of Competent *C. jejuni* Cells

Competent *C. jejuni* cells were produced following the standard procedure (Wassenaar *et al.*, 1993a; van Vliet *et al.*, 1998a). *C. jejuni* strains were passaged 3-4 times on MH agar followed by overnight growth on four MH agar plates. Cells were harvested from each plate using ice cold CEB (2 ml/plate), pooled and made up to 10 ml with ice cold CEB. Cells were harvested (at $3202 \times g$ for 15 minutes at 4°C) and washed twice with ice

cold CEB (10 ml each). Each pellet was resuspended in 0.3 ml of ice cold CEB and either used immediately or aliquotted and stored long-term at -80°C .

2.12.5. Transformation of *C. jejuni* Cells by Electroporation

Plasmid construct DNA (5-10 μg) was transformed into electro-competent *C. jejuni* cells by electroporation (Miller *et al.*, 1988; van Vliet *et al.*, 1998a). Electroporation cuvettes (Geneflow, 2 mm gap) were pre-chilled for 30 minutes at -20°C ; competent cells were thawed on ice for about 5 minutes. A 5 μl volume of DNA was mixed with a 50 μl aliquot of competent cells and incubated on ice for 10 minutes. The suspension was transferred to an electroporation cuvette which was placed into the electroporator chamber (Bio-Rad Gene Pulser). One pulse was applied at 2.5 kV, 25 μF and 200 Ohms, with the pulse (time constant) typically lasting 4.5-5.0 milliseconds. SOC (0.1 ml) was immediately added and cells were plated onto non-selective MH agar plates which were then incubated overnight for cell recovery. Following this, cells were washed from the plates (2 ml of SOC/plate) and plated onto MH agar containing the appropriate antibiotics to select for transformants. Plates were incubated for 3-7 days for the growth of colonies.

2.12.6. Natural Transformation of *C. jejuni*

C. jejuni cells were naturally transformed using the bi-phasic method (Wang and Taylor, 1990; van Vliet *et al.*, 1998a). *C. jejuni* strains were passaged 2-3 times on MH agar plates followed by overnight growth on 2 or 3 MH agar plates. Cells were harvested from each plate using MH broth (2 ml/plate). The OD_{600} of the cell suspension was measured and cells were diluted to an OD_{600} of 0.5 ($\sim 3 \times 10^9$ cfu/ml) using MH broth. The suspension (0.5 ml) was added to a Falcon tube (15 ml) containing 1 ml of set MH agar and incubated for 3 hours. DNA (1-5 μg) was mixed gently with the cells which were then incubated for a further 3-5 hours. Cells were subsequently plated onto MH agar containing the selective antibiotic and incubated for 3-5 days for the growth of transformant colonies.

2.13. Growth Assays

Agar plate growth assays and liquid culture growth assays were used to investigate to ability of *C. jejuni* to acquire iron from the ferri-glycoproteins Lf, Tf and ovo-Tf.

2.13.1. Solid Media Growth Assays

C. jejuni NCTC 11168 was tested for the ability to use human ferri-Lf, human ferri-Tf or ferri-ovo-Tf as a sole source of iron for growth (Field *et al.*, 1986; Pickett *et al.*, 1992). *C. jejuni* cells were growth for 24 hours on either Brucella or MH agar plates and harvested with 2 ml of MH broth per plate. Molten (55°C) Brucella or MH agar was supplemented with 50 μM DesferalTM and 5×10^7 cfu of *C. jejuni*. Plates were poured, dried and incubated microaerobically for 2 hours, followed by the application of 10 μl spots of sterile dH₂O (negative control), 10 μM FeSO₄ (positive control), 30 μM ferri-Lf, 30 μM ferri-Tf and 30 μM ferri-ovo-Tf. Plates were incubated for a further 12-16 hours after which the area surrounding each iron source was inspected for a halo of growth.

2.13.2. Liquid Media Growth Curves

C. jejuni strains were grown in MEM α in the presence of human Lf, human Tf or ovo-Tf in both ferri- and apo-forms (0.27 μM or 1.11 μM , Rock, 2003), or of porcine haem (25 μM , Ridley *et al.*, 2006), or of FeSO₄ (10 μM , iron-replete positive control, van Vliet *et al.*, 1998b) or in unsupplemented MEM α (iron-limited negative control, van Vliet *et al.*, 1998b). Receptor specificity was tested by competing an excess of apo-protein or bovine serum albumin (BSA, both 0.81 μM) against ferri-protein (0.27 μM) within the same culture. Proximity dependency was tested by separating the ferri-proteins from the *C. jejuni* cells in narrow bore dialysis tubing (6 kDa cut-off, GRI, Fig. 2.1) during growth. During the dialysis tubing partition assay, partitioned (the FeSO₄ was initially held within the dialysis tubing) and mixed cultures with FeSO₄ as the iron source were included as a positive control and to confirm that the dialysis tubing did not interfere with the movement of free iron. Growth of all strains over 24 hours was also tested using complex MH broth. Wild-type *C. jejuni* NCTC 11168 growth was tested with a range of FeSO₄ concentrations (500, 200, 100, 50, 20, 10, 8, 6, 4, 2, 1.6, 1.2, 1, 0.8, and 0.4 μM). In addition, the catecholamine stress hormone noradrenaline (NA) was added to *C. jejuni* NCTC 11168

unsupplemented MEM α cultures and MEM α cultures supplemented with either 10 μ M FeSO $_4$, human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (each at 0.111 μ M or 0.27 μ M) to a final concentration of 100 μ M. *C. jejuni* mutant strains were also grown in the presence of all ferri-proteins with or without the addition of NA (100 μ M).

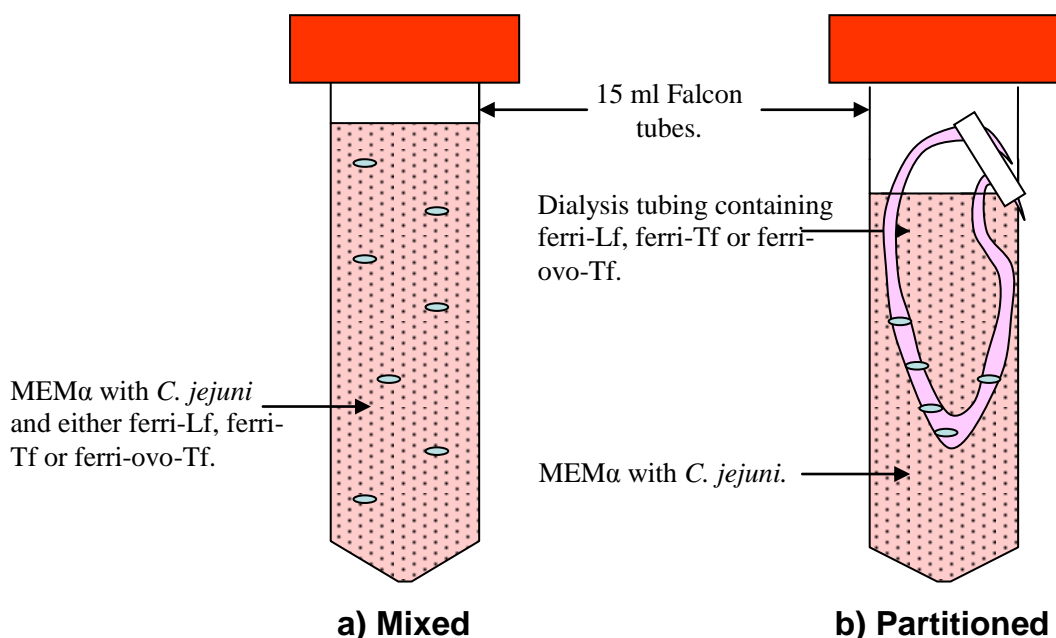


Figure 2.1. Dialysis tubing partition growth assay used to investigate the contact-dependency of iron uptake from ferri-proteins. *C. jejuni* NCTC 11168 cells were grown in MEM α microaerobically with agitation over a 24 hour period either **a)** mixed and in contact with 0.27 μ M human ferri-Lf, human ferri-Tf or ferri-ovo-Tf, or **b)** partitioned away from each ferri-protein, which was contained within low-molecular weight cut-off (6 kDa, GRI) dialysis tubing.

Strains were harvested with PBS (2 ml/plate) following 24 h growth on MH agar plates (4/strain). Cells were washed twice with MEM α , suspended in fresh MEM α and used to inoculate 5-ml MEM α cultures to an initial OD $_{600}$ of 0.1. Cultures were incubated overnight with agitation to achieve iron-depletion. Following this, cells were harvested and used to inoculate fresh 10-ml MEM α cultures to an initial OD $_{600}$ of 0.025 ($\sim 1.5 \times 10^8$ cfu/ml). Cultures were supplemented with the appropriate iron sources and grown as described previously (Rock, 2003; Ridley *et al.*, 2006). Optical density readings were taken at intervals (0, 4, 8, 12 and 24 hours) using an Ultrospec 10 cell density meter (Amersham Biosciences) and 1.6 ml disposable cuvettes (Sarstedt). Because of the dark coloration of haemin, when it was used as an iron source the appropriate amount was added to the medium used to calibrate the spectrophotometer to account for any change in optical density. All other samples were blanked against the appropriate medium. All conditions

were tested in triplicate and all growth assays were repeated on two separate days. Growth differences were analysed for significance using a paired or unpaired student's *t* test ($P = <0.05$; $n = 3$) where appropriate.

2.14. Protein Analysis

2.14.1. Fractionation of *C. jejuni* Cells

C. jejuni cells were fractionated into periplasmic, cytoplasmic, inner membrane and outer membrane fractions (van Vliet *et al.*, 1998b). *C. jejuni* cells were harvested using MEM α (2 ml/plate) from MH agar plates (8-10/fractionation), grown for 12-16 hours, washed twice in MEM α and used to inoculate 10 ml of fresh iron-limited or iron-replete MEM α to an initial OD₆₀₀ of 1.0. Cultures were incubated microaerobically for 12-16 hours with gentle agitation to induce iron-limited or iron-replete protein expression profiles. Sufficient cell numbers were then harvested to achieve an OD₆₀₀ of 30 in a volume of 1 ml. Cells were pelleted by centrifugation (at $15,700 \times g$ for 5 minutes at room temperature), washed once with PBS and suspended in 1 ml of TES buffer. Cells were pelleted, suspended in 1 ml of fresh TES and pelleted again as described above. Following this, ice-cold sterile dH₂O was used to suspend cells to an OD₆₀₀ of ~30 in 1 ml using the following calculation:

$$\text{Volume of dH}_2\text{O } (\mu\text{l}) = [\text{volume of cells } (\mu\text{l}) \times \text{OD}_{600}] / 30$$

The suspension was incubated on ice for 30 minutes. Centrifugation (at $15,700 \times g$ for 5 minutes at room temperature) pelleted the sphaeroplasts leaving the periplasmic fraction as the supernatant, which was then decanted. Samples were maintained on ice as much as possible during the preparation. The sphaeroplasts were suspended in an equal volume of 10 mM Tris-HCl (pH 7.5) and disrupted by sonication on ice. 8-10 pulses for 20 seconds each on low output with an amplitude of 1 were separated by 20 second interludes on ice; the small diameter (0.3 cm) probe was used (Soniprep sonicator, MSE). Unbroken cells were separated by centrifugation (at $15,700 \times g$ for 1 minute at room temperature). The total membranes were pelleted by centrifugation (at $15,700 \times g$ for 20 minutes at room temperature), leaving the crude membranes as a pellet and the cytoplasmic fraction as the supernatant, which was then decanted. The inner membranes were solubilised in an equal

volume of solubilisation buffer for 20-30 minutes at 37°C with mixing at intervals. Centrifugation (at $15,700 \times g$ for 20 minutes at room temperature) separated the inner membranes (supernatant) from the outer membranes, which formed a pellet. The pellet was washed three times in 10 mM Tris-HCl (pH 7.5). The outer membranes were suspended in an equal volume of the same buffer using sonication on ice as described previously. All fractions were stored long term at -20°C .

2.14.2. Sample Preparation for Protein Analysis

2.14.2.1. Fractionated Samples

Aliquots (10 μl) of each fraction were mixed with an equal volume of SDS-PAGE loading buffer (0.1 M Tris-HCl pH 6.8, 0.2 M 1,4-dithiothreitol (DTT), 4% (v/v) SDS, 0.2% (v/v) bromophenol blue and 2% (v/v) glycerol), heated, mixed and briefly centrifuged as described below, prior to analysis using SDS-polyacrylamide gel electrophoresis and immunoblotting (Sections 2.14.3 and 2.14.4).

2.14.2.2. Whole Cell Preparation

In order to demonstrate direct binding of Lf to the *C. jejuni* cell surface, samples were prepared as follows (Rock, 2003). *C. jejuni* cells were harvested using MEM α (2 ml/plate) from 8 MH agar plates grown for 24 hours, washed twice in MEM α and used to inoculate fresh iron-limited or iron-replete MEM α (5 ml) to an initial OD₆₀₀ of 0.5. Cultures were incubated overnight with gentle agitation to induce iron-limited or iron-replete protein expression profiles. Cultures were collected and the cells were suspended in 5 ml of fresh iron-limited or iron-replete medium to an initial OD₆₀₀ of 0.3 ($\sim 1.8 \times 10^9$ cfu/ml). Bacteria were then incubated in this medium microaerobically with or without the addition of ferri-Lf (50 μg) for 1 hour to allow binding of the glycoprotein. Cell suspensions were harvested by centrifugation (at $3202 \times g$ at room temperature for 15 minutes). Cells were washed 3 times in MEM α and pellet weight was used to equalise sample loading. Cells were suspended in 50 μl of MEM α , mixed with an equal volume of SDS-PAGE loading buffer, heated (to 99°C for 15 minutes), briefly centrifuged, electrophoresed (loading ~ 45 μl of each sample onto the gels) and electro-blotted as described below.

2.14.3. SDS-Polyacrylamide Gel Electrophoresis

Protein samples were analysed using SDS-polyacrylamide gel electrophoresis (PAGE, Laemmli, 1970; Sambrook and Russell, 2001). Polyacrylamide gels were prepared following standard protocols with a 5% (v/v) acrylamide (National Diagnostics, ProtoGel[®] (30%) 37.5:1 acrylamide to bisacrylamide stabilised solution) stacking layer overlaying the 10-12% (v/v) acrylamide resolving layer (Table 2.7).

Table 2.7. Composition of SDS-PAGE mini-gels.

Component	Stacking Layer (5%)	Resolving Layer (10%)	Resolving Layer (12%)
Gel buffer A (0.75 M Tris-HCl, 0.2% (w/v) SDS, pH 8.8)	-	2.70 ml	2.70 ml
Gel buffer B (0.25 M Tris-HCl, 0.2% (w/v) SDS, pH 6.8)	1.0 ml	-	-
30% (v/v) acrylamide mix	330 µl	1.83 ml	2.20 ml
dH ₂ O	616 µl	765 µl	395 µl
10 mg/ml (1% w/v) ammonium persulphate (APS)	50 µl	190 µl	190 µl
<i>N,N,N',N'</i> -tetramethylethylenediamine (TEMED)	4 µl	15 µl	15 µl

Protein samples were prepared by the addition of SDS-PAGE loading buffer followed by heating to 99°C for 15 minutes. Broad range biotinylated SDS-PAGE standards (Bio-Rad Laboratories, 6,500-200,000 daltons (Da): myosin, 200,000 Da; β -galactosidase, 116,250 Da; phosphorylase b, 97,400 Da; BSA, 66,200 Da; ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; soybean trypsin inhibitor, 21,500 Da; lysozyme, 14,400 Da; aprotinin, 6,500 Da) were prepared by diluting 5-10 µl (to 1:4 as per the manufacturer's instructions), mixing with an equal volume of SDS-PAGE loading buffer and heating to 99°C for 15 minutes. All samples were then mixed and briefly centrifuged (at $15,700 \times g$ for 5 seconds at room temperature) prior to loading into the SDS polyacrylamide mini-gel wells (up to 45 µl capacity). SDS-PAGE was performed using the *Mini-PROTEAN[®] II* (Bio-Rad Laboratories) electrophoresis cell apparatus. Mini-gels (7.2

cm × 10.2 cm) were electrophoresed in SDS-PAGE running buffer (0.025 M Tris, 0.192 M glycine and 0.19 % (w/v) SDS, pH 8.3-8.6) at a constant current of 15 mA for 30 minutes followed by 30 mA until the samples had run to the bottom of the gel. Gels were stained with SDS-PAGE gel stain solution (0.25% (w/v) Coomassie Brilliant Blue R-250, 40% (v/v) methanol and 10% (v/v) glacial acetic acid) for 12-16 hours, the background blue colouration was removed by soaking the gel in SDS-PAGE destain solution (25% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid and 65% (v/v) dH₂O) for 5-6 hours, followed by rehydration of the gel in two changes of dH₂O for 1 hour each. The gel was then soaked in gel drying solution (20% (v/v) ethanol, 10% (v/v) glycerol and 70% (v/v) dH₂O) for 1 hour and dried between *DryEase*[®] Mini cellophane sheets (Invitrogen) pre-soaked in gel drying solution, fixed together with two *DryEase*[®] mini-gel drying frames (Invitrogen) and four *Gel-Dry*[™] clamps (Invitrogen), and exposed to the air.

2.14.4. Immunoblotting of Proteins

The presence of Lf associated with *C. jejuni* cells or proteins was detected using immunoblotting (Towbin *et al.*, 1979; Sambrook and Russell, 2001). This was achieved by electro-blotting samples from SDS-PAGE gels onto activated polyvinylidene fluoride (PVDF) membranes (Immobilon-P transfer membrane, pore size 0.45 µm, Millipore). Rather than staining the SDS-PAGE gel with Coomassie Brilliant Blue, the gel was prepared for electro-blotting as shown in Fig. 2.2. Two scouring pads and 6 pieces of pre-cut 3MM filter paper (Whatman 3MM chromatography paper) were pre-soaked in transfer buffer (0.58% (w/v) Tris, 0.29% (w/v) glycine, 20% (v/v) methanol and 0.037% (w/v) SDS). PVDF membrane was pre-wetted in 100% (v/v) methanol for 5 minutes, washed in dH₂O for 5 minutes and soaked in transfer buffer for 10-20 minutes prior to blotting. The cassette was assembled with the gel adjacent to the PVDF membrane with 3 pieces of 3MM filter paper and a pre-wetted scouring pad on both sides (Fig. 2.2). Orientation was maintained by the removal of the lower left corner of all of the components. Proteins migrate towards the anode due to their net negative charge, so the anode cassette plate was placed on the side of the PVDF, and the cathode cassette plate was placed on the side of the gel to ensure protein migration from the gel onto the surface of the PVDF membrane (Fig. 2.2).



Figure 2.2. Apparatus assembly used for electro-blotting of proteins from SDS-PAGE gels to PVDF membranes.

Following assembly, the entire cassette was placed into the mini-immunoblotting tank (Geneflow) in the correct orientation; the tank was filled with chilled transfer buffer. Transfer was achieved at a constant current of 150 mA for 1 hour. Transfer of proteins was verified by staining the membrane for ~1 minute with Ponceau S. The membrane was subsequently destained by 2-3 rinses in dH₂O and molecular weight standard positions, once visualised, were marked on the membrane.

2.14.5. Lactoferrin-Specific Detection of Blots

Whole cell iron-replete and iron-restricted *C. jejuni* samples pre-incubated with human ferri-Lf (50 µg) were lysed, separated by SDS-PAGE and electro-blotted onto PVDF. The presence of Lf was determined as follows (Freestone *et al.*, 2000; Miller *et al.*, 2008). The membrane was placed in blocking buffer (5% (w/v) BSA in 0.1% (v/v) TBS-T); TBS-T was prepared beforehand (0.605% (w/v) Tris, 0.876% (w/v) NaCl, 0.1% (v/v) TWEEN[®]-20, pH 7.5) and shaken for 1 hour at room temperature. The membrane was then incubated overnight with shaking at 4°C, followed by 1 hour at room temperature the following day. The blocking buffer was removed and the membrane was incubated in primary antibody (rabbit anti-human Lf, 50 µg/µl diluted 1:5000 in blocking buffer) with shaking at room temperature for 1 hour, rinsed twice in TBS-T and washed three times in TBS-T for 10 minutes each with shaking. The membrane was then incubated in diluted conjugate specific to the sample (goat anti-rabbit IgG conjugated to HRP, 5 µg/µl diluted

1:20000 in blocking buffer) for 1 hour with shaking at room temperature and rinsed twice in TBS-T. Fresh TBS-T was then used to wash the membrane three times for 5 minutes, twice for 15 minutes and three times for 5 minutes. Detection was achieved using the *Enhanced Chemiluminescence (ECL) AdvanceTM Western Blotting Detection Kit* (Amersham Biosciences). *ECL Advance solution A* (containing Tris buffer in 3.2% (v/v) with ethanol) and *B* (proprietary substrate in Tris buffer) were mixed in equal volumes and used to cover the membrane surface. HRP reacted with the peroxide, the chemiluminescent substrate, producing a light signal that was then detected upon exposure of the membrane to autoradiography film (Fuji medical X-ray film, super RX, 13 × 18, Fujifilm) from 15 seconds to 1 hour as appropriate. Films were then developed using an autodeveloper (Xograph Imaging System, Compact × 4). Relative amounts of protein were determined by densitometry (Syngene Gene Genius Bioimaging System with Genesnap software).

2.14.6. Lactoferrin-Wash Immunoblotting

Fractionated samples were prepared (as described in Section 2.14.2.1), and the outer membrane proteins were transferred to PVDF membrane (Section 4.14.4). Membranes were blocked (as described in Section 2.14.5) and subsequently washed at room temperature in blocking buffer containing 10 µg/ml of ferri-Lf for 1 hour with shaking. Due to expected high background levels of binding, the membrane was rinsed vigorously with TBS-T four times and washed with fresh TBS-T three times for 5 minutes, twice for 15 minutes and three times for 5 minutes. Membranes were incubated with the primary antibody, washed, incubated with the secondary antibody, washed and detected using enhanced chemiluminescence as described above (Section 2.14.5).

2.14.7. Protein Identification

Lf-binding protein bands were analysed by matrix assisted laser desorption/ionisation-time of flight (MALDI-ToF) mass spectrometry and hybrid linear ion trap mass spectrometry (Q-TRAP) protein identification. Pure proteins can be identified by tryptic digestion and MALDI-ToF. Q-TRAP protein identification is used to identify components of a mixed sample. Protein identification was achieved by a database search using the Mascot search engine (Matrix Science, <http://www.matrixscience.com/>, completed by PNACL). *C. jejuni* outer membrane fractions were separated by SDS-PAGE,

gels were stained with Coomassie Brilliant Blue, destained as described above (Section 2.14.3) and maintained in destain solution. Protein identification was completed by PNACL (The University of Leicester).

2.14.8. Bradford Colorimetric Assay for Protein Quantification

The concentration of a given protein was established using the Bradford colorimetric assay (Bradford, 1976; Ausubel *et al.*, 1992). BSA protein standards of known concentration were prepared in the appropriate buffer from a 2 mg/ml stock solution by serial dilution. Room temperature Bradford Reagent (3 ml, 0.01% (w/v) Brilliant Blue G with 42.5% (v/v) phosphoric acid and 25% (v/v) methanol) was added to 0.1 ml samples of known protein standards, the blank and the unknown sample and vortexed. Samples were incubated at room temperature for 30 minutes and the absorbance of each sample was measured at OD₅₉₅ (Genova spectrophotometer, Jenway with 1.6 ml disposable cuvettes, Sarstedt). The formation of a dye-protein complex in each sample caused a dye absorption maximum shift proportional to the amount of protein present in the sample from 465 nm to 595 nm (Bradford, 1976). The protein concentration of the unknown sample was calculated from a calibration curve of the protein standard absorbances against known concentrations.

2.15. Detection of Promoter Activity using the β -Galactosidase Assay

C. jejuni promoter activity was measured using the β -Galactosidase assay.

2.15.1. Preparation of *C. jejuni* 480

Competent *C. jejuni* 480 cells (Section 2.14.4) were transformed by electroporation (as described in Section 2.12.5) with 1 μ g of each plasmid construct (Table 2.2, including appropriate controls). Constructs contained a promoter region (amplified by PCR (Section 2.10.1) using primers detailed in Table 2.3) cloned into pMW10 creating a promoter::*lacZ* transcriptional fusion (Wösten *et al.*, 1998; van Vliet *et al.*, 2000). Cells were grown for 12-16 hours on MH agar plates (3/promoter), harvested (2 ml PBS/plate), suspended in 1 ml of fresh PBS and used to inoculate 5 ml of iron-replete or iron-limited MH broth to an initial OD₆₀₀ of 0.1. Cultures were incubated at 37°C with gentle agitation for 5 hours,

followed by 20 minutes on ice. Cells were resuspended to an OD₆₀₀ of 0.4 in 1 ml of fresh PBS, and maintained on ice.

2.15.2. β -Galactosidase Assay

Z-Buffer (450 μ l, 60 mM Na₂HPO₄·2H₂O, 40 mM NaH₂PO₄·2H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, fresh β -mercaptoethanol to 50 mM), 8 μ l of 0.1% (v/v) SDS and 15 μ l of chloroform (Fisher Scientific) were added to 50 μ l of each cell sample. Samples were vortexed for 30 seconds and then incubated at 28°C for 5 minutes. The substrate *ortho*-nitrophenol- β -D-galactoside (ONPG, 4 mg/ml) was added to each sample (250 μ l). ONPG is normally colourless, but upon hydrolysis by β -galactosidase it is cleaved to galactose and *ortho*-nitrophenol producing a yellow colouration that can be measured by absorbance at OD₄₂₀ (Miller, 1972). The amount of colouration is an indication of promoter activity levels. Following the addition of ONPG, samples were mixed and incubated at 28°C for 1 hour. Na₂CO₃ (1 M, 250 μ l) was added to terminate each reaction. Cell debris was pelleted by centrifugation (at 15,700 $\times g$ for 1 minute at room temperature) and the top 0.7 ml of each reaction was removed and pipetted into a cuvette (1.6 ml disposable cuvettes, Sarstedt). The absorbance was measured at OD₄₂₀ (Jenova spectrophotometer, Jenway) and the β -galactosidase activity was calculated in Miller units (Miller, 1972) as follows:

$$\text{Miller units} = 1000 \times \frac{\text{OD}_{420}}{t \times v \times \text{OD}_{600}}$$

where t = time of the reaction in minutes (60), v = volume of culture in the assay (50 μ l), OD₆₀₀ = the cell density reading taken just before the assay began and OD₄₂₀ = the absorbance of the reaction mixture at the end of the assay.

2.16. Demonstration of Fur-Binding to Promoters by Electrophoretic Mobility Shift Assay (EMSA)

The binding of purified recombinant Fur protein to Fur-regulated promoter regions was determined by EMSA.

2.16.1. Preparation of Recombinant Fur Protein

The *Strep-Tag II*TM expression, purification and detection system (IBA) was used to produce recombinant *C. jejuni* Strep-tagged Fur protein. The *C. jejuni fur* gene was cloned into the pASK-IBA7 expression vector (Table 2.2) to make a gene fusion at the *Bsa*I site (Holmes *et al.*, 2005). Following transformation (Section 2.12.3) into the expression strain *E. coli* XL-1 Blue (Table 2.2), a single colony was grown for 12-16 hours, this culture was used to inoculate fresh LB broth to an initial OD₆₀₀ of 0.1 and grown until the OD₆₀₀ was 0.5. Expression was induced by the addition of anhydrotetracycline (AHT) to a final concentration of 0.2 µg/ml as the strep-tag::*fur* gene fusion was under the control of an inducible *tet*-promoter. The culture was incubated for 3 hours. Cells were subsequently pelleted by centrifugation (at 3202 × *g* for 12 minutes at 4°C), suspended in 2 ml of pre-cooled *buffer W* (100 mM Tris-HCl pH 8.0, 150 mM NaCl) and mixed with 10 µl of lysozyme (100 mg/ml). Bacterial cell wall breakdown was achieved by incubation at 37°C with shaking for 2 hours. Proteolytic degradation was avoided by the addition of 1 EDTA-free protease inhibitor cocktail tablet (Roche). The suspension was then disrupted by sonication on ice (as described in Section 2.14.1). Unbroken sphaeroplasts were separated by centrifugation (at 3202 × *g* for 20 minutes at 4°C). The soluble protein fraction was removed and stored at 4°C.

2.16.2. Purification of Recombinant Fur Protein

Recombinant Fur protein was purified by affinity chromatography using the *StrepTactin* sepharose column according to the manufacturer's instructions (IBA, Holmes *et al.*, 2005). Crude soluble protein fraction was allowed to pass through the column by gravity flow allowing the Fur:Strep-tag fusion protein to bind to the column. Associated *E. coli* host proteins were removed by washing 6 times with 10 ml of fresh *buffer W*. The fusion protein was eluted by the addition of 10 aliquots (1 ml each) of *buffer E* (*buffer W*

plus 2.5 mM desthiobiotin). *Buffer R* (*buffer E* plus 1 mM 2-(4'-hydroxyazobenzene) benzoic acid (HABA)) was added to the column to prepare it for another use. Pre-column, flow through, mid-wash, competitive-wash and the 10 elution fractions were collected during protein purification for analysis by SDS-PAGE (Section 2.14.3). The protein was concentrated and desalted using the *Microcon*[®] *Centrifugal Filter Devices* (Microcon YM-10, 10 kDa molecular weight cut-off, Millipore, as per the manufacturer's instructions). The concentration of the recombinant protein was determined using the Bradford Assay (Section 2.14.8).

2.16.3. Electrophoretic Mobility Shift Assays (EMSA)

Purified promoter fragments containing putative Fur binding sites (van Vliet *et al.*, 2002; Palyada *et al.*, 2004) were amplified by PCR (Section 2.10.1) using primers detailed in Table 2.2, labelled with digoxigenin (DIG)-ddUTP following the manufacturer's instructions (*DIG Gel Shift Kit, 2nd Generation*, Roche Diagnostics) and analysed by EMSA (Holmes *et al.*, 2005). DNA fragments (3.85 pmol/ μ l) were mixed with labelling buffer (Roche Diagnostics), 5 mM CoCl₂, 0.05 mM DIG-11-ddUTP and 1 U of terminal transferase and incubated at 37°C for 15 minutes. The addition of 3 μ l of dH₂O gave a final labelled DNA concentration of 155 fmol/ μ l. The appropriate amount of Fur was mixed with 1 \times binding buffer (20 mM Bis-Tris, pH 7.6), 2 mM DTT, 100 μ g/ μ l BSA, 100 μ l MnSO₄, 0.3 μ g/ μ l salmon sperm DNA, 1.55 fmol DIG-labelled DNA and dH₂O to 20 μ l. Competitive EMSAs were performed by the addition of unlabeled competitor DNA in excess to the labelled DNA. Following this, 5 μ l of colourless loading buffer (10 \times TBE buffer (1 M Tris-HCl pH 8, 1 M boric acid), 60% (v/v) glycerol) was added to each reaction. Native PAGE gels (8%) were prepared using 3.2 ml of *ProtoGel*[®] (Section 2.14.3), 8.2 ml of dH₂O, 0.6 ml of 10 \times TBE buffer, 0.2 ml of 10% (w/v) APS and 10 μ l of TEMED and electrophoresed using the *Mini-PROTEAN*[®] *II* (Bio-Rad Laboratories) gel electrophoresis cell apparatus. Gels were pre-run in 0.5 \times TBE buffer at 40 V/cm for 15 minutes, samples were loaded and the gels were then run at 80 V/cm for 2 hours. Following electrophoresis, samples were electro-blotted (Section 2.14.4) onto nylon membranes (Amersham) in 0.5 \times TBE buffer at 40 V/cm for 1 hour and DNA was fixed to the membrane by UV cross-linking (Amersham) at 700 KJ/cm². Blots were probed with blocking solution containing rabbit anti-digoxigenin antibodies conjugated to HRP (Roche Diagnostics, 1:10,000 dilution of antibody) according to the manufacturer's instructions.

Membranes were incubated with chemiluminescent substrate (CSPD) and exposed to autoradiography film (Fuji medical X-ray film, super RX, 13 × 18, Fujifilm) for 1 hour at room temperature. Work following DNA fragment preparation detailed in this section (2.16) was completed during this study by R. Ren (The University of Leicester).

2.17. Bioinformatics

Sequence trace files were viewed using Chromas v1.45 (Conor McCarthy, School of Health Science, Griffith University, Queensland, Australia) and analysed using Clone Manager Professional Suite (Sci Ed Central, version 9.0, copyright© 1994-2005, Scientific and Educational Software) and the Blast programmes on the NCBI website (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). *Campylobacter* genome sequence data were obtained from http://www.sanger.ac.uk/Projects/C_jejuni/ and <http://xbase.bham.ac.uk/campydb/>. Clone Manager Professional Suite was also used to analyse the GC content of regions of the NCTC 11168 genome. Predictions of the function of Cj0178 were made by searching Pfam (<http://pfam.sanger.ac.uk/>) using the Cj0178 amino acid sequence. The PredictProtein server (for sequence analysis, structure and function predictions, <http://www.predictprotein.org/>) was also used to investigate Cj0177 and Cj0178.

TbpA and TbpB protein sequences were obtained from the NCBI protein database at <http://www.ncbi.nlm.nih.gov>. Protein sequences from *C. jejuni* were obtained from CampyDB at <http://xbase.bham.ac.uk/campydb/>. Comparisons of protein sequences were made using either the protein-protein Blast tool from NCBI (<http://www.ncbi.nlm.nih.gov/>) or the CampyBlast tool from CampyDB (<http://xbase.bham.ac.uk/campdb/>). Similar protein sequences were aligned using Clustal W (EMBL-EBI, <http://www.ebi.ac.uk/Tools/clustalw/>) and shaded based on identity or similarity using Boxshade v3.21 (http://www.ch.embnet.org/software/BOX_form.html). Where necessary, gaps were introduced by the programme to improve alignments.

Chapter 3. Results: *Campylobacter jejuni* can use Lactoferrin- and Transferrin-Derived Iron for Growth

3.1. Introduction

Prior to preliminary work completed by J. D. Rock (2003), *Campylobacter jejuni* was thought to be unable to use ferri-lactoferrin (Lf) or ferri-transferrin (Tf) as a source of iron for growth (Pickett *et al.*, 1992). Lf is abundant in mucosal secretions, chelating iron and inhibiting microbial colonisation of the intestinal niche (Weinberg, 1978). It was not believed that *C. jejuni* had adapted to exploit this available iron source. To demonstrate that *C. jejuni* could not use iron derived from Lf or Tf for growth, the authors used iron-restricted Brucella agar plates containing 5×10^7 colony forming units (cfu) of strains 81-176, C31, 1376, and a number of strains isolated from human patients. Plates were incubated microaerobically and then spotted with the iron sources to be tested (Table 3.1). Due to the iron-restriction, *C. jejuni* cells were incapable of growth in the plates unless supplied with an external iron source.

Table 3.1. Growth of *C. jejuni* strains supplied with a range of iron sources in an agar plate assay (Pickett *et al.*, 1992).

Iron Source	Concentration	Growth
Ferri-Lf	30 μ M	X
Ferri-Tf	30 μ M	X
Ferritin	22 μ M	X
Haemin	10 μ M to 1 mM	√
Haemoglobin	4 to 600 μ M	√
Haemin-haemopexin	200 μ M	√
Haemoglobin-haptoglobin	100 μ M	√

Following microaerobic incubation overnight, growth stimulation was visible surrounding the spots of some of the iron sources, while others had failed to promote growth (Table 3.1). The authors concluded that, while the haem-containing compounds were important sources of iron for *C. jejuni*, ferri-Lf, ferri-Tf and ferritin were not significant iron sources and the cells were unable to exploit the iron associated with them

(Pickett *et al.*, 1992). Work completed immediately prior to this study, however, indicated that *C. jejuni* cells could in fact take iron up from $^{55}\text{Fe-Tf}$ or $^{55}\text{Fe-Lf}$, implying that the original conclusion that this source is unavailable to *C. jejuni* is incorrect (Rock, 2003).

In order to confirm that *C. jejuni* can acquire iron from host iron-binding glycoproteins, *C. jejuni* NCTC 11168 cells were grown with each ferri-glycoprotein as a sole iron source in both solid and liquid media growth assays. Following this, the manner of iron uptake was investigated to determine how this process may occur, confirming or refuting previous data and further characterising the process. Much of the content of this chapter has already been published (Miller *et al.*, 2008).

3.2. Results

3.2.1. Growth Promotion by Ferri-Glycoproteins in an Agar Plate Assay

Investigation of NCTC 11168 growth with iron supplied from the Tfs was initially completed using the same agar plate assay format used previously (Pickett *et al.*, 1992). Detailed descriptions of experimental procedures can be found in Section 2.13.1. The ability of *C. jejuni* to utilise iron derived from host glycoproteins was visualised by the amount of growth surrounding an iron source spotted directly onto an iron-restricted (50 μM deferoxamine mesylate salt, DesferalTM) agar plate containing bacterial cells (Pickett *et al.*, 1992). Iron-restricted Mueller-Hinton (MH) agar or Brucella (B) agar plates inoculated with *C. jejuni* (5×10^7 cfu) were spotted (10 μl) with 10 μM FeSO_4 (positive control), double distilled (dd) H_2O (negative control) and 30 μM human ferri-Lf, human ferri-Tf and ferri-ovo-transferrin (ovo-Tf), a member of the Tf protein family not previously tested (Pickett *et al.*, 1992). Following microaerophilic incubation, a halo of growth was visible surrounding the FeSO_4 (21 mm diameter), but no halo of growth was visible around the negative control or the other iron sources, indicating that *C. jejuni* can only exploit the iron provided in the form of FeSO_4 (Fig. 3.1). However, extremely faint, limited growth did appear to be present directly beneath each ferri-protein spot (~ 5 mm diameter, Fig. 3.1).

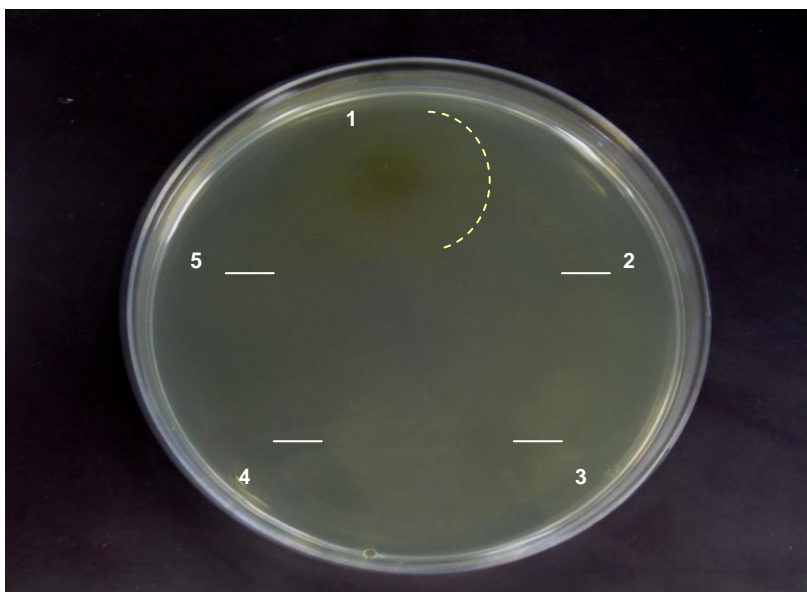


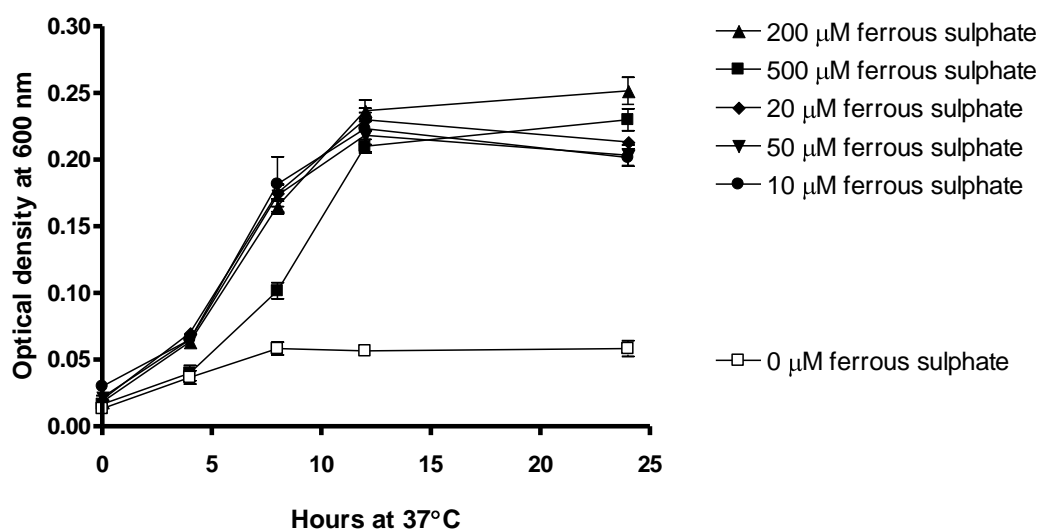
Figure 3.1. Solid medium iron utilisation assay. Brucella agar plate assay containing 50 μM DesferalTM, 5×10^7 cfu of *C. jejuni* NCTC 11168 and iron-containing compounds spotted directly onto the plate surface (10 μl) to test the ability of ferri-glycoproteins to stimulate growth. **1)** 10 μM FeSO_4 (positive control). **2)** 30 μM ferri-Tf. **3)** 30 μM ferri-Lf. **4)** 30 μM ferri-ovo-Tf. **5)** ddH₂O (negative control). A halo of growth is visible surrounding the FeSO_4 indicated by the curved dashed line, no growth promotion is visible surrounding the other sources (diameter of spots indicated by a white line).

The assay was repeated six times generating reproducible data (repeats not shown). The limited growth suggests that iron bound to proteins of the Tf family is not readily available to *C. jejuni* in solid agar-based assays; this is in agreement with Pickett *et al.* (1992). Growth testing was therefore repeated using a liquid medium assay.

3.2.2. Promotion of *C. jejuni* Growth by FeSO_4

Initial studies of *C. jejuni* NCTC 11168 growth over 24 h were carried out using FeSO_4 as the sole iron source to establish the expected growth patterns of wild-type cells with a range of iron concentrations in liquid culture. Minimal essential medium alpha (MEM α), a defined iron-restricted medium, was used (van Vliet *et al.*, 1998) and six replicates of each condition were tested. All raw data for liquid growth curves completed in this chapter and statistical testing of growth differences can be found in Appendix 3. Growth differences were analysed for significance using a paired student's *t* test where $P = <0.05$ and $n = 3$. NCTC 11168 grew poorly in unsupplemented MEM α alone (Fig. 3.2a, b; 0 μM FeSO_4), but the addition of a range of iron concentrations to induce iron-replete conditions promoted growth (Fig. 3.2a, b).

a)



b)

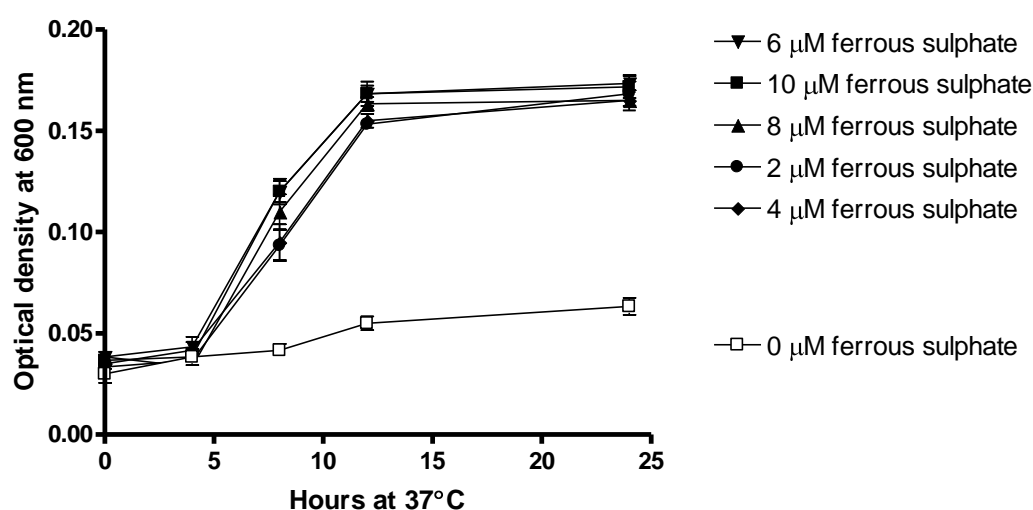


Figure 3.2. Growth assays of wild-type *C. jejuni* NCTC 11168 with a range of FeSO_4 concentrations. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. **a)** 10 μM to 500 μM FeSO_4 and **b)** 2 μM to 10 μM FeSO_4 , each with 0 μM FeSO_4 as a negative control (unsupplemented medium, iron-limited conditions).

Both iron-restricted and iron-replete conditions generated growth curves with trends similar to those seen before for a number of *C. jejuni* strains including NCTC 11168 (Field *et al.*, 1986; Pickett *et al.*, 1992; van Vliet *et al.*, 1998). The addition of FeSO_4 to a final concentration of 40 μM has been widely used as a standard for high-iron growth (Pickett *et al.*, 1992; van Vliet *et al.*, 1998; van Vliet *et al.*, 1999; van Vliet *et al.*, 2000; Palyada *et al.*,

2004; Holmes *et al.*, 2005; Ridley *et al.*, 2006). However, upon demonstrating that *C. jejuni* growth comparable to that seen with 20-50 μM FeSO_4 was achieved by the addition of 10 μM FeSO_4 (Fig. 3.2a), lower concentrations of iron were tested. A final concentration of 2 μM FeSO_4 promoted *C. jejuni* growth equivalent to 10 μM (Fig. 3.2b). Growth was promoted by levels of FeSO_4 as low as 0.4 μM (data not shown). This indicated that 40 μM may be unnecessarily high when inducing iron-replete conditions, but not apparently detrimental under the assay conditions used. Conversely, the addition of 500 μM FeSO_4 appeared to have a detrimental effect during early growth, but cells seemed to recover by 12 h (Fig. 3.2b). In addition, in contrast to previous reports (van Vliet *et al.*, 1998), the supplementation of MEM α with FeSO_4 promoted *C. jejuni* growth considerably when compared to growth in iron-restricted, unsupplemented medium. The addition of FeSO_4 to MEM α did in fact successfully stimulate growth in this medium and can be used as an iron-replete control. It is not clear why FeSO_4 failed to promote *C. jejuni* growth in the previous study (van Vliet *et al.*, 1998).

3.2.3. Liquid Medium Assays of *C. jejuni* Growth with Glycoproteins

3.2.3.1. Growth with Ferri- and Apo-Glycoproteins

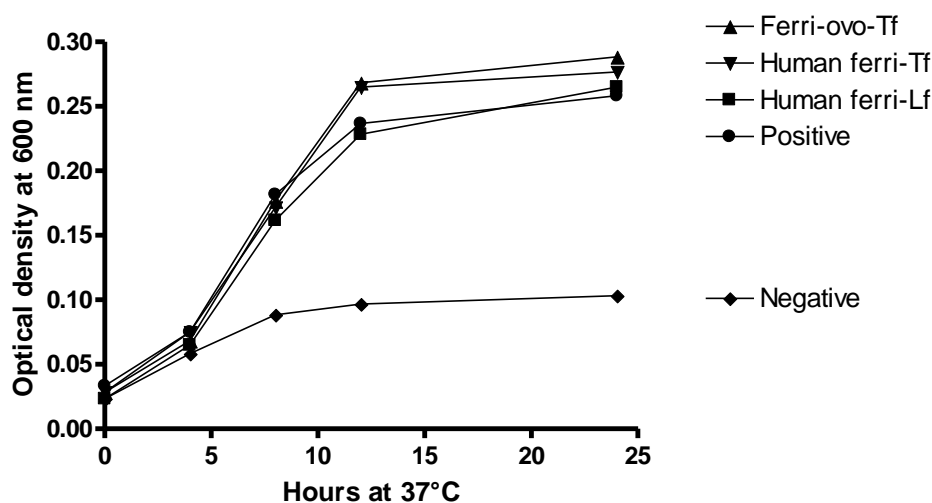
Previous work has shown that *C. jejuni* can acquire iron from ^{55}Fe -Lf or ^{55}Fe -Tf, demonstrating the uptake of iron by showing radioactivity to be predominantly associated with the periplasmic and cytoplasmic cellular fractions and showing a preference for Lf-derived iron (Rock, 2003). It is important, however, to demonstrate that the iron is biologically available and of use to *C. jejuni* for growth, otherwise uptake may be proposed to be irrelevant or an artefact. Again using MEM α as an iron-restricted culture medium, 24 h growth curve experiments with optical densities monitored at regular intervals were used to demonstrate whether *C. jejuni* could use iron supplied solely in the form of ferri-Lf, ferri-Tf or ferri-ovo-Tf for growth. The basic liquid assay format was originally used for *C. jejuni* in a previous study (van Vliet *et al.*, 1998). The use of MEM α is important as this avoids the need for the addition of an iron chelator and the liquid culture format is an alternative way of testing growth.

Following an initial overnight iron-depletion step, NCTC 11168 again grew poorly in unsupplemented MEM α (negative control, van Vliet *et al.*, 1998), but supplementation with human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (0.27 μM) resulted in growth to the

same levels as with 10 μM FeSO_4 (positive control, Fig. 3.3a). All trends seen were replicated (6 times) demonstrating consistency and reproducibility of the results. The OD_{600} of *C. jejuni* at 24 h in unsupplemented medium was approximately 0.10, comparable to that seen previously (van Vliet *et al.*, 1998). Under iron-replete conditions, a final OD_{600} of 0.25 to 0.30 was reached. The addition of the ferri-glycoproteins promoted growth similarly to, but slightly more than FeSO_4 ; growth with ferri-ovo-Tf was slightly higher than with ferri-Tf and ferri-Lf (Fig. 3.3a). This indicates that all host glycoproteins are available to *C. jejuni* as iron sources for growth.

By contrast, bacterial growth in the presence of equivalent concentrations of human apo-Tf or apo-ovo-Tf was lower than in unsupplemented medium alone (Fig. 3.3b). The phenotypes seen are probably due to the fact that the apo-proteins bind trace amounts of iron present in the MEM α , further limiting *C. jejuni* growth. The manufacturer's instructions state that there is no iron present in the medium, but MEM α is produced using distilled water which is likely to contain low levels of iron. There was limited growth in the presence of the human apo-Lf preparation (Fig. 3.3b), although significantly less than with the ferri-protein. This is most likely due to incomplete iron removal from the preparation during dialysis (Section 2.1).

a)



b)

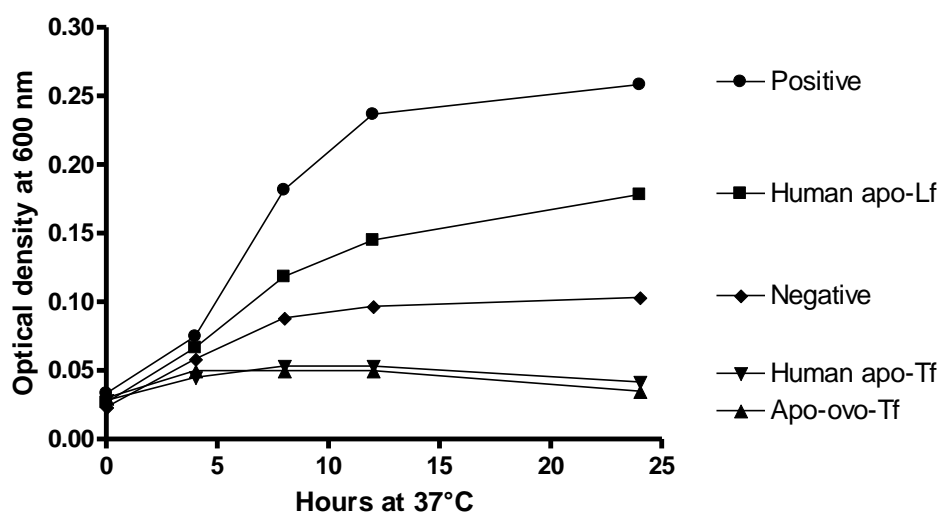


Figure 3.3. Growth assays of wild-type *C. jejuni* NCTC 11168 with iron supplied bound to human Lf, human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 with ferri-proteins (0.27 μ M). **b)** NCTC 11168 with apo-proteins (0.27 μ M).

Since *C. jejuni* strain 81-176 was reported to be unable to grow with Lf- or Tf-derived iron in the original study by Pickett *et al.* (1992), a liquid assay growth test of this strain was also performed. Again, in liquid culture, iron-replete conditions and iron-restriction resulted in comparable growth characteristics to strain NCTC 11168, and ferri-protein supplementation clearly promoted growth to a final OD $_{600}$ of around 0.35, slightly

higher than NCTC 11168 (Fig. 3.4). This indicates that strain 81-176 can also utilise iron from these proteins for growth, contradicting previous conclusions.

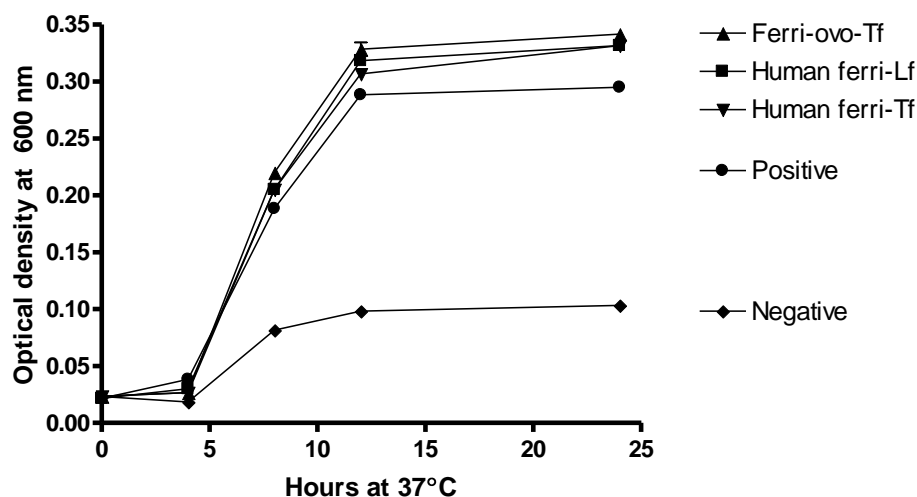


Figure 3.4. Growth assay of wild-type *C. jejuni* 81-176 with iron supplied bound to human Lf, human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative), ferri-proteins (0.27 μ M).

3.2.3.2. Contact Requirements for Growth with Ferri-Glycoproteins

The necessity for proximity between ferri-protein and the bacterial cell surface for successful iron acquisition was investigated in liquid culture. If the process is dependent upon an extracellular soluble compound, separating the ferri-protein from the *C. jejuni* cells using low molecular weight cut-off dialysis tubing should have no effect on iron transfer between protein and cells resulting in growth promotion as seen in Fig. 3.3a. If direct contact or close proximity between the cell surface and glycoprotein is required for successful iron acquisition via a receptor protein or action of a protease, growth promotion should be lost upon partitioning. Strain NCTC 11168 was grown in MEM α liquid culture with iron supplied in the form of each ferri-protein (0.27 μ M), either enclosed within dialysis membrane or in direct contact with the cells (Fig. 3.5a-c). For a diagram (Fig. 2.1) and detailed description of the set-up, see Section 2.13.2.

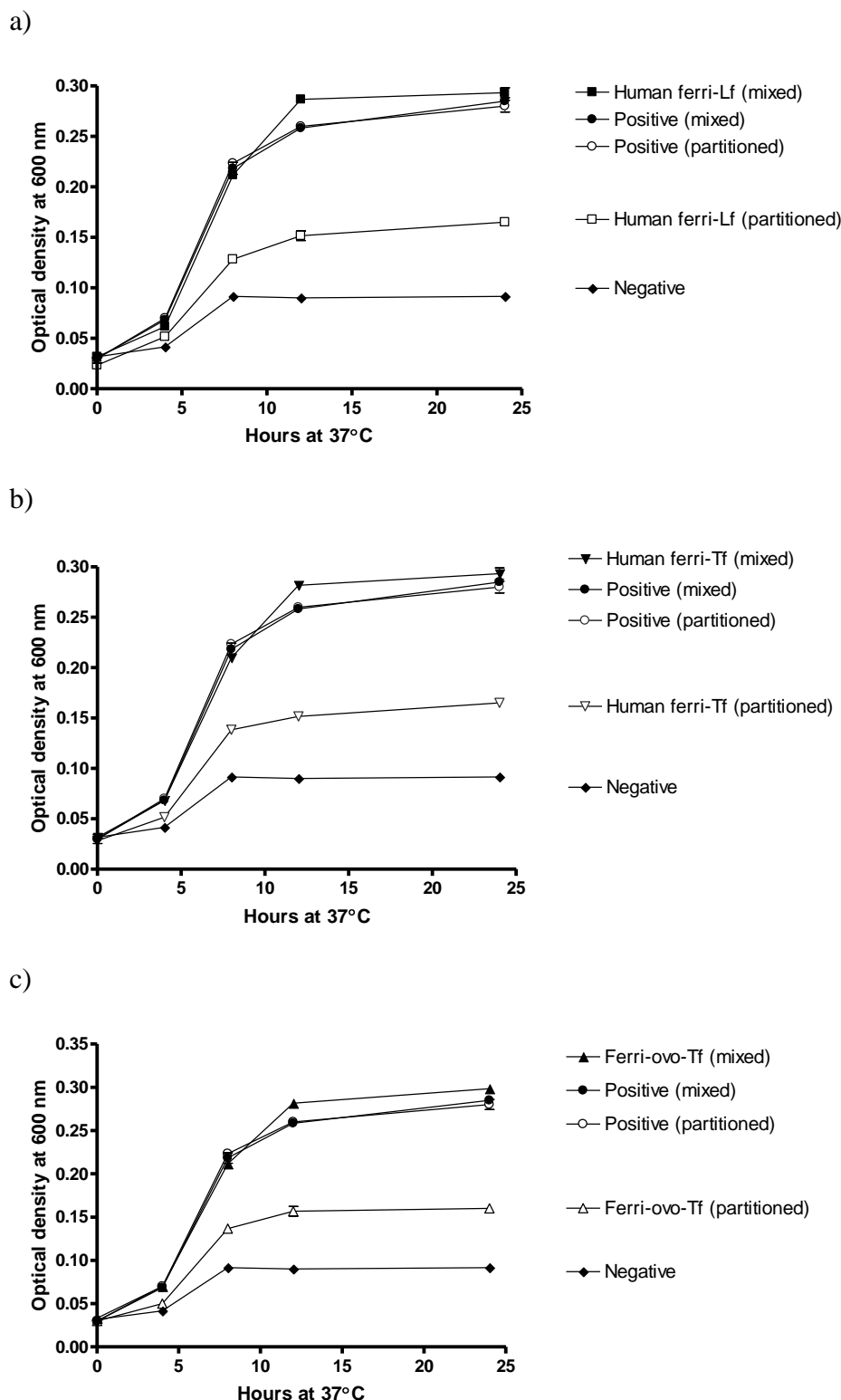


Figure 3.5. Growth assays of wild-type *C. jejuni* NCTC 11168 mixed with or partitioned from iron supplied bound to human Lf, human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. Glycoproteins were partitioned within dialysis tubing. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 with human ferri-Lf (250 μ g). **b)** NCTC 11168 with human ferri-Tf (250 μ g). **c)** NCTC 11168 with ferri-ovo-Tf (250 μ g).

When in contact with the iron source, *C. jejuni* grew to levels comparable with the iron-replete controls, but when the ferri-proteins were partitioned from the *C. jejuni* cells, growth was significantly reduced, with cultures reaching a final OD₆₀₀ of just above 0.15 with all host proteins (Fig. 3.5a-c). As expected, there was no significant difference between levels of *C. jejuni* growth when FeSO₄ was mixed with or partitioned from the cells, demonstrating that iron can move freely through the dialysis tubing. This partitioned iron-replete control also indicates that the dialysis membrane itself does not appear to inhibit or affect cell growth, so the differences seen between mixed and partitioned cultures are not likely to be a dialysis tubing artefact. These results support the conclusion that proximity is necessary for successful acquisition of iron from members of the Tf protein family and implies that iron uptake from these sources does not require the ability to utilise siderophores. This further indicates the presence of a specific ferri-Lf-, ferri-Tf-, ferri-ovo-Tf-derived iron uptake system, relying upon interaction of the ferri-protein with the cell surface, conceivably with an outer membrane receptor protein.

3.2.3.3. Growth with Competing Ferri- and Apo-Glycoproteins

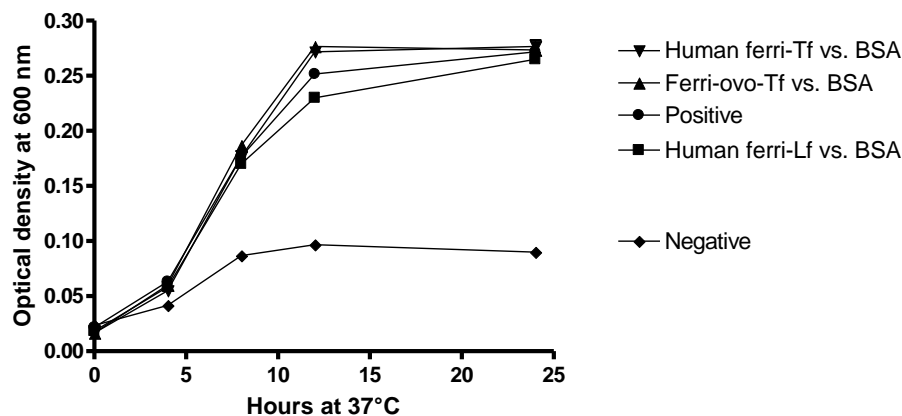
In order to further investigate the specificity of ferri-Lf, ferri-Tf or ferri-ovo-Tf interaction with *C. jejuni* cells, wild-type cells were grown in MEM α liquid culture with iron supplied solely as each ferri-protein (0.27 μ M) with the addition of a competitor protein (0.81 μ M, Fig. 3.6a, b). This method should allow differentiation between specific binding of the ferri-Tfs and non-specific association of protein with the bacterial cell surface. If the association is via a specific receptor, apo-Tfs may compete with ferri-Tfs for these sites on the cell surface causing a reduction in growth. If the cell surface association of ferri-Tfs is due to non-specific protein binding, another protein, bovine serum albumin (BSA) may block ferri-Tf association with the bacterial surface, also limiting growth. If the BSA does not interfere, *C. jejuni* growth promotion by ferri-Tfs would be expected, even in the presence of this protein.

Competition of each ferri-protein (0.27 μ M) against an excess of BSA (0.81 μ M; Fig. 3.6a) resulted in levels of growth comparable to ferri-protein alone (Fig. 3.3a). This indicated that BSA did not interfere with the process of iron uptake from ferri-proteins. Competition of ferri-protein (0.27 μ M) against an excess of the cognate apo-protein (0.81 μ M; Fig. 3.6b) limited growth to levels almost comparable with apo-protein alone, suggesting that there are specific receptor sites present on the *C. jejuni* cell surface for

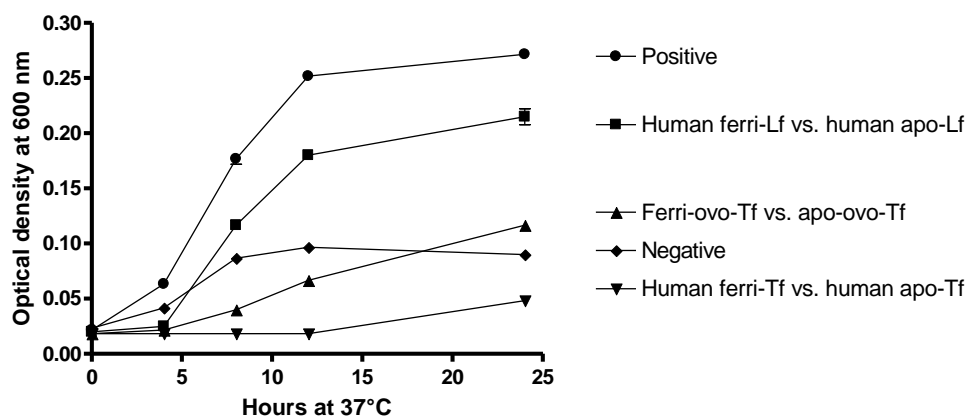
which apo- and ferri-forms of the Tfs can compete. If apo-protein was not interfering with ferri-protein association with the cell surface, no growth inhibition would be expected. In addition, from 12 h to 24 h, growth levels slightly increased, particularly highlighted by the Tf and ovo-Tf competition cultures (Fig. 3.6b). This implies that low levels of iron were being supplied to cells for growth (Fig 3.6b). This is different from non-competition cultures incubated with apo-protein alone, where growth levels had further diminished by 24 h (Fig. 3.3b). Overall growth levels were higher with human apo-Lf, again likely to be due to residual iron in the preparation as mentioned earlier.

All combinations of ferri- and apo-proteins (0.27 μ M and 0.81 μ M, respectively) were subsequently tested; growth limitation comparable to that illustrated in Fig. 3.6b was observed in all cases (Fig. 3.6c). Increased growth was again seen with the cultures containing ferri-Lf; the growth levels here were still less than when cells were grown with ferri-protein alone (Fig. 3.3a). This indicated that all combinations of ferri- and apo-proteins can compete to associate with the *C. jejuni* cell surface and supply iron for growth, possibly through the same receptor. This suggests a novel system used by *C. jejuni* to derive iron from the Tfs.

a)



b)



c)

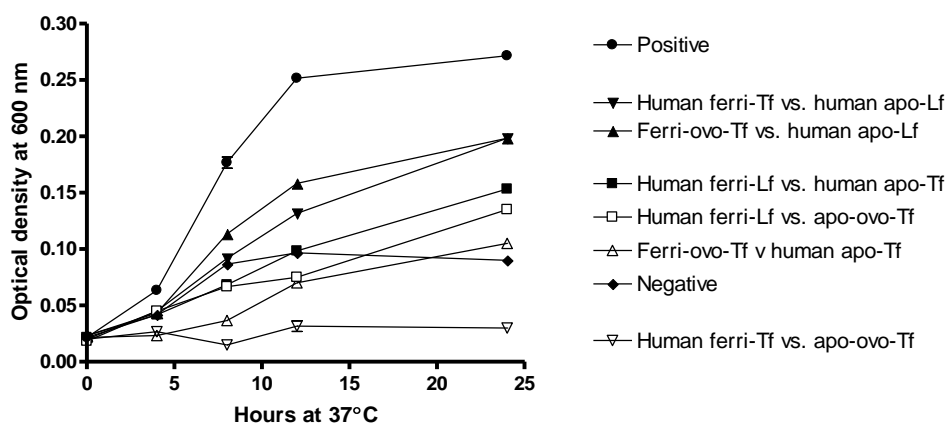


Figure 3.6. Competition growth assays of wild-type *C. jejuni* NCTC 11168. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 with competing ferri-protein (0.27 μ M) and BSA (0.81 μ M). **b)** NCTC 11168 with competing cognate ferri- (0.27 μ M) and apo-proteins (0.81 μ M). **c)** NCTC 11168 with competing opposite ferri- (0.27 μ M) and apo-proteins (0.81 μ M).

3.2.3.4. Binding of Lactoferrin to the *C. jejuni* Cell Surface

In order to demonstrate binding of human ferri-Lf to the *C. jejuni* cell surface, NCTC 11168 cells were incubated in the presence of ferri-Lf under iron-restricted and iron-replete conditions, pelleted, washed, lysed, and the proteins were separated by SDS-polyacrylamide gel electrophoresis (Section 2.14.3). Proteins were blotted to polyvinylidene difluoride (PVDF) membranes and probed with polyclonal anti-Lf antiserum (Fig. 3.7). The presence of an 80 kDa band in lanes 3 and 4 running to the same position as the mature pure Lf protein control (lane 5) indicated that Lf associated sufficiently strongly with the cell surface to avoid being removed by the washing step. Iron responsive regulation of this binding is shown by the decreased intensity of the band in lane 4 (band intensity is 42% of lane 3 band intensity). Each condition was repeated twice per blot and each blot was repeated twice; all results were consistent with those presented (Fig. 3.7). No protein bands were detected when cells were treated as described above, but without the Lf incubation step (lanes 1 and 2). These data not only confirm that Lf binds to the cell surface, but also indicate that the host cell component involved in binding is iron-responsive.

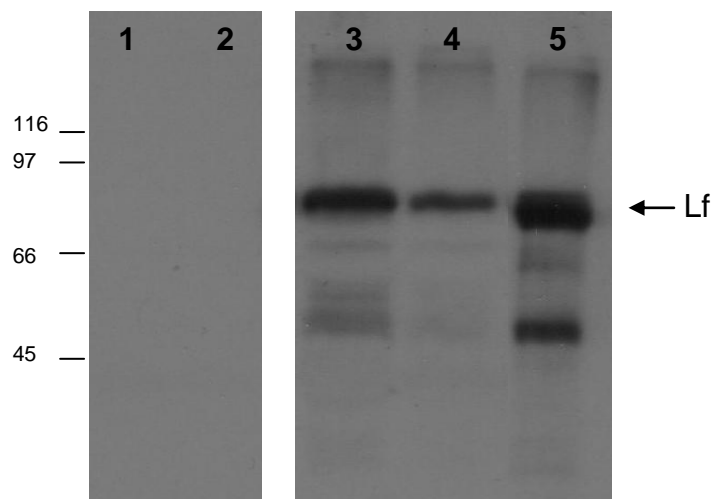


Figure 3.7. Lf binding to *C. jejuni* NCTC 11168 cells under iron-replete and iron-restricted conditions. Western blot analysis of solubilised *C. jejuni* proteins (approximately 25 µg of protein separated by SDS-PAGE) prepared from iron-restricted (lane 1) and iron-replete (lane 2) NCTC 11168 cells incubated without human ferri-Lf, and iron-restricted (lane 3) and iron-replete (lane 4) NCTC 11168 cells incubated in the presence of human ferri-Lf (50 µg). Lane 5 contained pure human ferri-Lf protein (1 µg, positive control). Blots were probed with anti-Lf antibodies. The arrow indicates the position of the mature Lf protein. Loading of protein was equalised by pellet weight to avoid differences in protein concentration due to experimental error. Protein marker sizes are indicated in kDa to the left of the blot (Miller *et al.*, 2008).

3.3. Discussion

The data presented in this chapter demonstrate that NCTC 11168 is capable of utilising iron bound to members of the Tf protein family for growth. The process appears to require proximity between the iron source and the bacterial cell surface (Fig. 3.5), and to be receptor specific (Fig. 3.6) possibly needing an initial binding to a *C. jejuni* cell surface-associated outer membrane protein. The necessity for a siderophore or other external low molecular weight iron-binding compound to complete the process of iron uptake from these sources is unlikely. This is different from the situation in *Escherichia coli*, where the production and subsequent uptake of the siderophore enterochelin is required for the successful acquisition of iron from Tf (Freestone *et al.*, 2003). The process in *E. coli* was also shown to be dependent upon the host catecholamine stress hormone noradrenaline (see Chapter 6; Freestone *et al.*, 2000).

The use of MEM α as a defined iron-limited medium for *C. jejuni* growth is now well established (van Vliet *et al.*, 1998; Rock, 2003; Palyada *et al.*, 2004; Naikare *et al.*, 2006) with other groups known to add an additional carbon source such as 20 mM sodium pyruvate (Naikare *et al.*, 2006) to aid growth. The use of MEM α here and in other studies

by this group (van Vliet *et al.*, 1998; Rock, 2003) does not include the addition of an extra carbon source, as this medium is being used to generate an iron model for growth studies, without the need for additional supplements. Furthermore, pyruvic acid is known to promote iron uptake by *Salmonella enterica* serovar Typhimurium (Reissbrodt *et al.*, 1997). The growth patterns of *C. jejuni* supplied with a range of FeSO₄ concentrations showed that the amounts of iron previously used (40 µM) to promote growth are above the concentrations needed; 10 µM FeSO₄ was chosen to give iron-replete control conditions in this study (Fig. 3.2). The addition of FeSO₄ to *C. jejuni* cultures did, however, promote growth above the unsupplemented MEMα negative controls (Fig. 3.2). A previous report showed no difference in the final OD₆₀₀ between these conditions, with all cultures reaching around 0.25 at 24 h with and without iron (van Vliet *et al.*, 1998). The differences between the iron-limited and iron-replete cultures are considerable and trends remain the same between all assays (Fig. 3.2). There may have been problems with iron contamination in the assays conducted in the original study, leading to higher background levels of iron, producing indistinguishable growth patterns. An initial overnight iron-depletion stage may not have been included, as was here, which may mean that growth patterns were influenced by intracellular iron stores producing misleading results.

Repetition of previous work (Pickett *et al.*, 1992) has confirmed that Lf-, Tf- and also ovo-Tf-bound iron is largely unavailable to *C. jejuni* for growth in an agar plate assay (Fig. 3.1). However, liquid culture assays demonstrated very clearly that iron bound to human ferri-Lf, human ferri-Tf and ferri-ovo-Tf is biologically available to *C. jejuni* for growth (Fig. 3.3). Supporting this, previous analysis of fractionated *C. jejuni* cells incubated in the presence of either ⁵⁵Fe-Tf or ⁵⁵Fe-Lf showed that iron obtained from these host proteins accumulated primarily within the soluble cell fraction (94%, Rock, 2003). There was far more ⁵⁵Fe from ⁵⁵Fe-Lf accumulated by cells than from ⁵⁵Fe-Tf, which was proposed to be due to an adaptation by *C. jejuni* to acquire iron from the source it is most likely to encounter while colonising and infecting the human host (Rock, 2003). However, this clear preference was not reflected in the growth curves completed during this study (Fig 3.3a), where there was very little difference between the final OD₆₀₀ reached with all ferri-proteins. Ferri-ovo-Tf, which was not tested before, appeared to promote growth comparably to the human proteins (Fig. 3.3a), which is likely to be due to an adaptation to commensal colonisation of the avian gut. Detection of radioactivity levels may be more sensitive than monitoring cellular growth over 24 h. *C. jejuni* 81-176 can also grow using glycoprotein-derived iron (Fig. 3.4). As the growth patterns with all proteins were

extremely similar, it is not likely that the kind of substrate specificity seen in other systems (Gray-Owen and Schryvers, 1996) is occurring here. Coupled with the radioactive iron uptake data (Rock, 2003), this implies that there may be a system present in *C. jejuni* capable of the specific uptake of iron derived from the Tfs. In addition, the 81-176 data suggests that these proteins could be an important iron source for other *C. jejuni* strains.

As suggested earlier, the use of unsupplemented MEM α to provide iron-limitation, rather than adding a chelator, was appropriate. When *C. jejuni* cells were grown with ferri-Lf in rich medium (MH broth) in which the iron had previously been chelated using 20 μ M DesferalTM (van Vliet *et al.*, 1998), cells failed to grow, reaching final optical densities of only 0.03-0.05 following 24 h of incubation (data not shown). The DesferalTM could be competing with the Lf for iron, limiting the availability to *C. jejuni*, which is known to be unable to use ferri-DesferalTM as an iron source for growth (Field *et al.*, 1986).

Partitioning of the ferri-glycoproteins away from the cells was shown to inhibit growth (Fig. 3.5). The growth differences are significant between partitioned and mixed cultures, but growth was not completely abolished. It was previously shown that significantly more ⁵⁵Fe was obtained from ⁵⁵Fe-Lf or ⁵⁵Fe-Tf when cells were mixed with, rather than partitioned away from, the iron source (Fig 3.8, only Tf shown, work performed by J. D. Rock). Those cells partitioned from the iron source still accumulated some iron, perhaps enough to allow the promotion of growth seen in the liquid culture experiments.

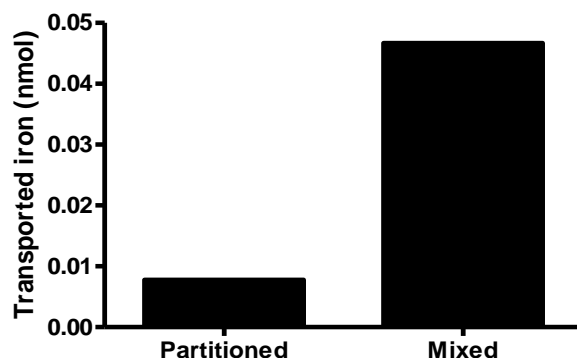


Figure 3.8. Radioactive ^{55}Fe -uptake from members of the Tf protein family by *C. jejuni*. The work presented in this figure was completed by J. D. Rock. All experiments were completed using 1 ml of culture at $2\text{--}3 \times 10^8$ cfu/ml. Radioactivity is represented as nmol of ^{55}Fe transported into the cell. Radioactivity associated with the *C. jejuni* cell pellet is presented from three dialysis partition experiments using ^{55}Fe -Tf as an iron source. ^{55}Fe -Tf was partitioned away from the bacteria using dialysis membrane (partitioned) and in contact with the bacteria (mixed), associated radioactivity was measured. Each column displays the mean values of three scintillation counts sampled from one experimental run (Miller *et al.*, 2008).

If uptake was solely reliant upon an outer membrane protein or proteins it would be expected that separation would result in limited growth, similar to the negative control cultures, due to iron deprivation. The small amount of growth and/or acquired radioactive iron observed with partitioned cultures may reflect some iron contamination and/or technical difficulties of establishing complete division between the compartments. The assay is complicated to assemble (see Fig. 2.1); however sterility was maintained throughout the growth curves, as streak plates of cultured cells from the 24 h samples were shown to be pure *Campylobacter* (data not shown). Clearly, if iron was released or associated with a stable diffusible siderophore, the difference noted in growth/uptake would not be seen, and the observed growth patterns would be identical to those seen with mixed cultures. There could be very low levels of secreted low molecular weight compounds or metabolic products allowing trace amounts of iron to be liberated from the proteins and taken up by *C. jejuni*. *S. Typhimurium* is known to excrete low molecular weight primary metabolites such as α -ketoacids when iron is restricted (Reissbrodt *et al.*, 1997). The α -keto- and α -hydroxyacids have been shown to promote the growth of some *Salmonella* serovars under iron-limitation when other siderophores are not present (Kingsley *et al.*, 1996). In addition, citrate is known to promote *E. coli* growth in a siderophore-like manner (Hancock *et al.*, 1976). Iron derived from citrate can be acquired by *E. coli*, but *Salmonella*

lack the transport system required (Payne and Mey, 2004). In contrast, *Salmonella* can use citrate as a carbon source, but *E. coli* cannot (Payne and Mey, 2004). However, there are no obvious candidate genes and also no evidence for the presence of siderophore biosynthesis genes in the NCTC 11168 or other *Campylobacter* genomes sequenced to date (Parkhill *et al.*, 2000; Fouts *et al.*, 2005). This implies that siderophores or other exogenous substrates are not essential for the uptake of iron from Lf or Tf by *C. jejuni*. *C. jejuni* appears to acquire iron in a proximity-dependent manner. In addition, there is a possibility that surface associated protease activity, as seen in other organisms (Okujo *et al.*, 1998), is involved in the use of iron from Lf and Tf by *C. jejuni*. The disruption caused may result in the release of iron due to breakdown of the protein, but proximity would be required if the protease was surface associated. It would be expected that this activity would also be abolished by partitioning. Even if the protease was secreted by *C. jejuni*, as the cells were partitioned, interaction of this protease with the Lf and Tf would also not be expected here. By mixing the Lf or Tf with culture supernatant and partitioning NCTC 11168 cells from the mixture, the release of iron that reached NCTC 11168 on the other side of the partition would indicate the presence of a secreted protease. Based on the data presented here, it is more likely that *C. jejuni* is secreting an as yet unidentified low molecular weight iron binding compound to low levels that can pass through the dialysis tubing and liberate some iron for use by NCTC 11168 for growth. Interestingly, in the plate growth assay, contact between the ferri-proteins and *C. jejuni* cell surface was limited to directly beneath the protein spot. Proximity dependency may explain the limited growth observed, as growth could only occur where the protein was in direct contact with the bacteria in the agar. No halo was observed due to poor diffusion of the protein into and across the agar surface (Fig. 3.1).

Bacterial growth was also reduced by competing excess apo-proteins against ferri-proteins in all combinations (Fig. 3.6a, c). Previous work by J. D. Rock directly investigating iron uptake is in agreement with this finding. Competition experiments using ^{55}Fe -Lf or ^{55}Fe -Tf competed against an excess of BSA, an excess of cold ferri-Lf or ferri-Tf, or incubated with cells alone demonstrated that significantly less ^{55}Fe was accumulated by cells incubated with both radioactively labelled and non-labelled iron loaded protein, as opposed to either ^{55}Fe -protein alone, or ^{55}Fe -protein and BSA (Fig. 3.9, only Tf shown, Rock, 2003).

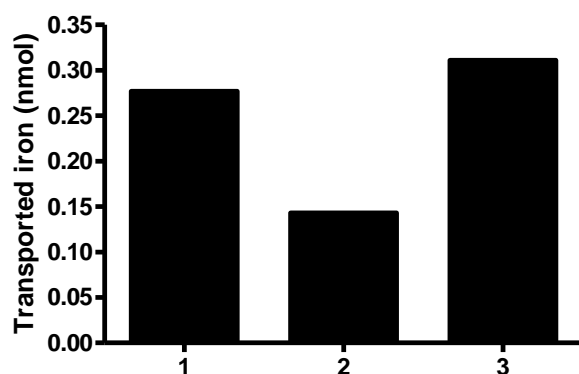


Figure 3.9. Radioactive ^{55}Fe -uptake from members of the Tf protein family by *C. jejuni*. The work presented in this figure was completed by J. D. Rock. All experiments were completed using 1 ml of culture at $2\text{--}3 \times 10^8$ cfu/ml. Radioactivity is represented as nmol of ^{55}Fe transported into the cell. *C. jejuni* incorporation of ^{55}Fe was measured when cells were incubated in direct contact with ^{55}Fe -Tf either alone (1), with an equivalent concentration of non-radioactive iron-saturated Tf (2), or an equivalent concentration of BSA (3). Each column displays the mean values of three scintillation counts sampled from one experimental run (Miller *et al.*, 2008).

Taken together, the data imply that either radioactive and non-radioactive or apo- and ferri-forms of the proteins compete for specific binding sites on the *C. jejuni* cell surface. As BSA causes no inhibition of radioactive iron uptake or growth in the presence of ferri-protein (Fig. 3.6a; Fig. 3.9), the sites involved are likely to be specific receptors. Because the work of J. D. Rock demonstrated only that iron uptake from the Tfs was occurring, it was important to establish as part of this study that the iron acquired is available for growth.

The presence of a non-essential accessory protein (TbpB) to the main receptor protein (TbpA) in *N. gonorrhoeae* has been proposed to aid iron uptake from Tf by allowing favoured binding of ferri-Tf, but TbpA itself is capable of binding all iron-states of Tf (Cornelissen and Sparling, 1996). Tf binding has also been shown in enteropathogenic *E. coli* (Freestone *et al.*, 2000). In *E. coli* 0157:H7, Tf binds to a non-iron regulated protein that can bind both apo- and ferri-Tf with equal affinity, unlike *Neisseria* spp. (Freestone, P. P. E., per. comm.). Whether a TbpB or TbpA homologue exists in *C. jejuni* is to be discovered, but based on the data presented, *C. jejuni* may be capable of recognising and binding all iron-forms of Tf. In *C. jejuni*, the necessity for proximity and possible involvement of a specific receptor is similar to the systems characterised in *Neisseria* spp. (Schryvers and Stojiljkovic, 1999; Perkins-Balding *et al.*, 2004). The

demonstration of competition favours the unusual and novel idea of a single receptor for all ferri-proteins. An explanation may be the structural and sequence similarities between members of the Tf protein family (Querineau *et al.*, 1971; Baker *et al.*, 1998; Baker *et al.*, 2002), but if this is the case, it is surprising that this mechanism has not been found before. Less inhibition of growth was seen when apo-Lf was added to cultures and the growth patterns of cells with apo-Lf alone are consistent with contamination of the preparation with bound iron that was not removed during dialysis. It could be conclusively resolved by the preparation or purchase of iron-free Lf. However, this protein preparation is no longer commercially available, and the low pH citrate dialysis performed resulted in the denaturation of around half of the original amount of protein; increasing the time of dialysis may be impractical, and could result in the loss of all of the protein.

In *C. jejuni*, iron is likely to be released from the ferri-proteins at the cell surface, transported across the outer membrane and then transported across the inner membrane by one of the known ABC transporter systems, as seen in *Neisseria* spp. (Perkins-Balding *et al.*, 2004). The ferri-glycoprotein could be taken up into the cell, but this has not been shown (Perkins-Balding *et al.*, 2004).

The demonstration of iron-responsive Lf binding to the *C. jejuni* cell supports the theory that the Lf binds to specific *C. jejuni* outer membrane proteins (Fig. 3.7). Tf binding to *C. jejuni* cells has also been shown previously (Fig. 3.10), but the cells were only incubated with and without Tf (Rock, 2003).

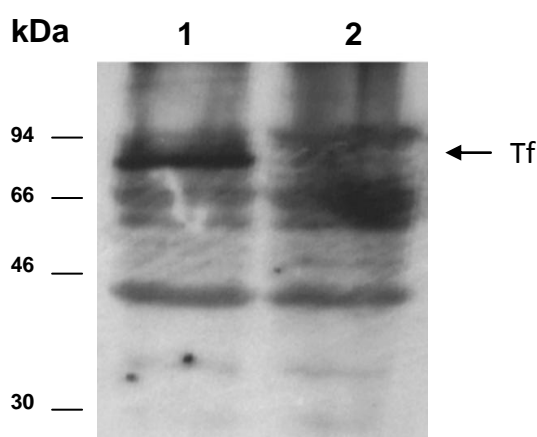


Figure 3.10. Tf binding to *C. jejuni*. The work presented in this figure was completed by J. D. Rock. Western blot analysis of solubilised *C. jejuni* proteins prepared from cells incubated in the presence (lane 1) and the absence (lane 2) of human ferri-Tf, probed with anti-Tf antibodies. The arrow indicates the position of the mature Tf protein. Protein marker sizes are indicated in kDa to the left of the blot (Rock, 2003).

This shows that Tf does appear to associate with the cell surface, but does not demonstrate iron-regulation. The incubation of both iron-replete and iron-restricted cells with ferri-Lf resulted in 42% less Lf associating with iron-replete cells than iron-restricted, clearly demonstrating iron-regulation of this binding, and pointing toward the binding of Lf to an iron-regulated protein (Fig. 3.7). The fact that some Lf still associated under iron-replete conditions may be due to contaminating Lf remaining in the samples even after thorough washing. There may be limited general association of the Lf with other cell surface structures or the iron-regulated protein that binds Lf may be still be expressed, but at a much lower level, under high-iron conditions. Lf may also bind to one or more proteins that may not be iron regulated or may be upregulated under high-iron conditions. Probing of iron-restricted and iron-replete cell samples not previously incubated with Lf using anti-Lf antibodies resulted in no detection of protein (Fig. 3.7, lanes 1 and 2). This confirms that the bands visualised when cells are incubated with Lf are not due to cross-reaction of the antibodies with *C. jejuni* proteins. The additional band present in lane 5 (Fig. 3.7) running to around 45 kDa may be a Lf breakdown product, although the majority of the protein is intact. There was less breakdown product associated with the cells following incubation with Lf than the mature Lf protein under either iron-restricted or iron-replete conditions (Fig. 3.7, lanes 3 and 4). In addition, less was detected in the test lanes (Fig. 3.7, lanes 3 and 4) than the pure Lf control lane (Fig. 3.7, lane 5). This implies that the mature form of Lf is required to successfully associate with the target *C. jejuni* binding site/sites. Taking into consideration the data detailed in the rest of this chapter, the iron-regulated adherence seen is most likely specific and due to direct binding of Lf to an outer membrane component/components of one or more of the *C. jejuni* iron uptake systems. *C. jejuni* is capable of binding both Lf and Tf, supporting the need for direct contact to acquire iron from either.

To conclude, these data demonstrate that *C. jejuni* can use iron derived from Lf and Tf, and also ovo-Tf for growth. Following this, further investigation of the process of iron uptake from host glycoproteins by *C. jejuni* was completed. Candidate genes whose protein products demonstrate similarity to Lf and/or Tf binding proteins were identified and those genes were mutated. Phenotypes were established to determine which iron uptake system or systems, if any, are involved in this process in *C. jejuni*.

Chapter 4. Results: Analysis and Inactivation of Iron Uptake Genes and Phenotyping of Mutant Strains

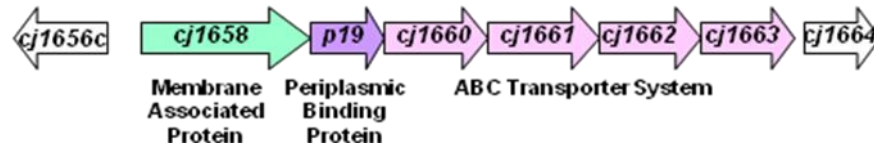
4.1. Introduction

The data detailed in Chapter 3 demonstrated that *Campylobacter jejuni* NCTC 11168 can acquire iron from human ferri-lactoferrin (Lf), human ferri-transferrin (Tf) and ferri-ovotransferrin (ovo-Tf) for growth. The process appeared to be receptor-dependent and require proximity, with the acquired iron being successfully transported across the outer membrane (Chapter 3; Rock, 2003; Miller *et al.*, 2008). The NCTC 11168 genome encodes a number of functional Fe^{3+} acquisition systems (Chapter 1; van Vliet *et al.*, 2002) capable of acquiring iron from ferri-enterochelin (*cfrA*, *ceuBCDE*; Fig. 4.1), ferri-rhodotorulic acid (*cj1658-cj1663*; Fig. 4.1), haem (*chuABCDZ*; Fig. 4.1.) and the uncharacterised *cj0173c-cj0178* system (Fig. 4.1). An Fe^{2+} uptake system encoded by *cj1397* and *cj1398*, the latter encoding a FeoB homologue, is also present (Chapter 1; Parkhill *et al.*, 2000). In addition, the gene *cj0444* shows homology to the other iron outer membrane receptor protein encoding genes. *cj0444* is functional in the *C. jejuni* strains 81-176 and 81116 (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006) and is a pseudogene in strain NCTC 11168 (Parkhill *et al.*, 2000).

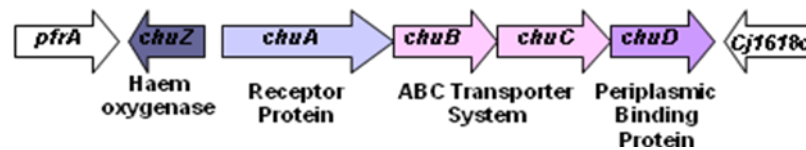
a) Ferri-enterochelin



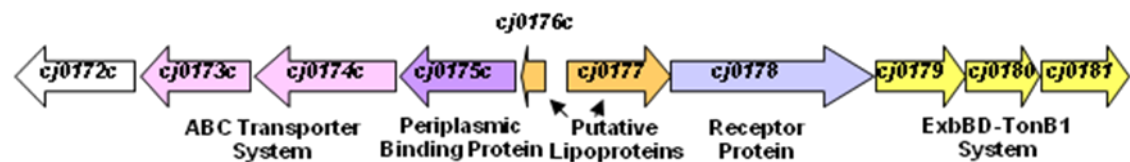
b) Ferri-rhodotorulic acid



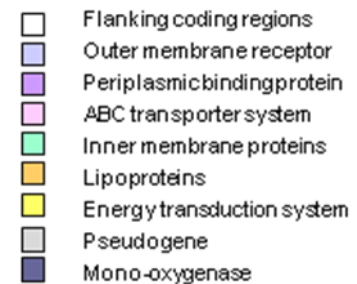
c) Haem



d) Uncharacterised system



e) Ferrous iron



f) *cj0444*

(putative TonB-dependent outer membrane receptor, pseudogene in NCTC 11168)

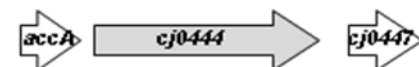


Figure 4.1. Genes involved in iron uptake in *C. jejuni* NCTC 11168. The genes involved in the uptake of iron from the sources **a)** ferri-enterochelin, **b)** ferri-rhodotorulic acid, **c)** haem, **d)** the uncharacterised system *cj0173c-cj0178* **e)** ferrous iron and **f)** the outer membrane receptor pseudogene *cj0444* are represented by diagrams indicating genomic organisation and functions of the components of each iron uptake system. (Diagram is based on the genomic structure taken from <http://xbase.bham.ac.uk/campydb/>).

The use of Lf- or Tf-derived iron is best characterised in *Neisseria* spp., principally the human pathogens *N. meningitidis* and *N. gonorrhoeae* (Perkins-Balding *et al.*, 2004). Both are dependent upon infecting mucous membranes, of either the meninges – the membranes lining the central nervous system – or the genitourinary tract, where Lf is abundant in secretions (Masson and Heremans, 1966). The classic two-part receptor structure for Tf or Lf in *Neisseria* spp. comprises either Tf binding protein (Tbp) A and TbpB or Lf binding protein (Lbp) A and LbpB, and has been reviewed in Section 1.4.5; of the two, the Tf receptors are better characterised in *Neisseria* spp.

In order to identify potential *C. jejuni* proteins involved in iron uptake from host ferri-Lf, ferri-Tf and ferri-ovo-Tf, the NCTC 11168 genome was searched for TbpAB homologues. Candidate genes were mutated and mutant strains were tested for growth with human ferri-Lf as a sole iron source to investigate if any mutants demonstrated altered phenotypes compared to wild-type cells.

4.2. Results

4.2.1. Search for Lactoferrin- or Transferrin-Binding Protein Homologues in the *C. jejuni* Genome

There are no obvious homologues of either TbpA/LbpA or TbpB/LbpB from *Neisseria* spp. encoded in the *C. jejuni* genomes (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007). When the *N. meningitidis* TbpA protein sequence was compared to all proteins from the sequenced *C. jejuni* genomes and those of related organisms, many putative iron regulated proteins and putative TonB-dependent proteins were identified. Many of the proteins did not have an assigned function, with several found in poorly characterised *C. jejuni* strains, such as strain 260.94, as well as the related *Helicobacter pylori* and *Wolinella succinogenes*. Similarly, when searching all known *Campylobacter* proteins using the *N. gonorrhoeae* TbpB protein sequence (used because of the availability of the full length sequence) there were no TbpB homologues encoded in the *C. jejuni* genome. Putative lipoproteins and hypothetical proteins from various organisms were identified as similar to TbpB, but no proteins were identified from NCTC 11168 with any similarity (data not shown).

The first protein from NCTC 11168 to be identified in the blast search of TbpA against proteins from campylobacters and related organisms was Cj0178, the putative

siderophore-dependent outer membrane receptor protein for which a definite iron source is yet to be assigned (Fig. 4.2). The next was CfrA, the ferri-enterochelin outer membrane receptor protein (Palyada *et al.*, 2004). Cj0178 and CfrA demonstrated 24% amino acid identity to TbpA, with the haem outer membrane receptor protein ChuA (Ridley *et al.*, 2006) displaying 20% identity (Fig. 4.2). In addition, the protein product of the functional copy of the NCTC 11168 pseudogene *cj0444* from *C. jejuni* strain 81-176 showed 26% sequence identity to TbpA (Fig. 4.2).

[illegible]

TbpA	529	PKTANPNGGKDQPYWVSIGRNVVTGQICRLGNNTYTDCTPRSINGKSYAAVRDNRVLG
Cj0178	440	-----
CfrA	409	-----
ChuA	409	-----
Cj0444	415	-----
consensus	541	-----
TbpA	589	RWADVGAELRYDNRSTHSDDGSVSTG-----THRTLSWNAGIVLKPTWLDITYRTSTG
Cj0178	440	-----FMCFPKAATDIDKNG-----LRNASVMSAAIHDLITPEVVFART
CfrA	409	-----FCARYNHHEIFGN-----NVSPRAYVYVYNPTNELTKGGVSTG
ChuA	409	-----GCARYEFSSYNTDRSYRNEMSMNGRPPSPTIDSTLFDTNKNANNFAFELTPN
Cj0444	415	-----TCLRYTYSDIFDS-----EFTPRVYLIVYHLNINIAKGGVSKG
consensus	601
TbpA	643	FRLESFAEMYGWRAG-----VQSKAVKIDPEKSFNKEAGIVFKGDFGNLEASWF
Cj0178	481	NRAENVQEMFESNNE-----GNGINSFLKPEQANTWQIGENSFKHGLKDD
CfrA	447	FRTEYANRLINGTYS-----YSGQGRFPTYGNPDLEKEETSINYEIAAIYNN-DLF
ChuA	463	FKYSDTGKLYIKYERGFVSPSPAQFVNKDKNQKYYSANLNFEIFDTFELGIDDFWDFY
Cj0444	453	YKTEAAKOLITNGYYN-----YANNN--AYFGNEDLKFEESINVELGVDFMVDFEA
consensus	661
TbpA	692	NNAYRDLVRGYLAQIQNGKETAKGDPAYLNAQSARITGINVLGKIDWNGVWDKLPEGW-
Cj0178	528	RGGFKAVVYHTKIKDYLYNEQFYLEDPKDDPPSQFYMHLSADDTIFKGVLELISYDLG
CfrA	496	YVSATGFLTNFKDKIKSSQSYNNSEPIPGIGTCADRCRRAINHGKVEYKGVLEAGAGISP-
ChuA	523	GENITLFLYILSKDEIS-----YLGNPHSTSGSWWKYYNIDOTRRILGVELSLQSNF-
Cj0444	501	HYSTIGFTTDFTNQISSEDLTG--MQNDINCSNGIVCKRPINLKGKTQTKGTFAFNTTKT-
consensus	721
TbpA	751	--YSTFAYNRVHVRDKKRAADRTDIQSHLFDAQPSRYVVLGLYDQPECKWNGMLTYS
Cj0178	588	FAYAKAVYSRQDTSSTISQTSGLPLGFSFSKIMEPKDYAN---VELGFRNDKISFG
CfrA	555	--LDN-LNVNFAYTYLDETEVKEAQDRSVIGKPEQDS-----LKHNLMLKTEYS
ChuA	573	--LDDDIFRESLTYLDAKISKGVN---DGMRTIPYV-----SKIKATAGLBYA
Cj0444	558	--YNG-FSLNSSYTFMDNRYKDCQKNWFGCDRIENL-----PHIAMLKINYE
consensus	781
TbpA	809	KAKEITELIG-SRALNENSENTK-----ATARRTRPWIYVDVSGYVTLIKKHFTRAG
Cj0178	644	GIAKMTGKAKRVNPNTDWNIDPNNPYYPKPTTQDLKPIPIVDLYWNIEWFKNLTRAE
CfrA	600	FYNKITPWLK-GEWQIDRYMGDTN-----INREYKDIPLASMCVRVDINKQWSINAA
ChuA	616	WNKNSNEID-LTYFSRAKDGGTIDENTGKMSKNSWIRDYFITDGMKYN--KKLQILAG
Cj0444	603	RG-KESSYIK-TRARLDAIAAKGGGNG--SLPWQKYKPFYIVDLGINYKINKQSSLSFA
consensus	841
TbpA	861	VYNLLNRYVVTWENVROTAGGAVNOHKNVGVYNRYA--APGFNYTFSLMKF
Cj0178	704	VQNLFDKNYMDALNAYNSLDNQLQNGAGDPTYLFSNSARGRTETVSEFYKY
CfrA	652	TYNLFDKSTETNGWESYASGSGST--NVNTYNRIE-----EGRRMYTISINGNF
ChuA	674	IRNLFDKRYTYTYQDS-----INDQYLVG-----NGRNYVVEKMAF
Cj0444	659	VQNLFDKNYFNPQVTRWDCANPAGMANRYQDYT-----EGRSEWLSYKYDF
consensus	901

Figure 4.2. Peptide alignment of the Tf binding protein A (TbpA) of *N. meningitidis* with the outer membrane receptor proteins of *C. jejuni* NCTC 11168 and Cj0444 from *C. jejuni* 81-176. Alignment (ClustalW, EMBL-EBI, <http://www.ebi.ac.uk/Tools/clustalw/>) of protein sequences (CampyDB, <http://xbase.bham.ac.uk/campydb/>) from NCTC 11168 in order of similarity to TbpA: Cj0178 (24% identity, putative TonB-dependent outer membrane receptor protein), CfrA (24% identity, *C. jejuni* ferri-enterochelin outer membrane receptor protein; Palyada *et al.*, 2004), ChuA (20% identity, *C. jejuni* haem outer membrane receptor protein; Ridley *et al.*, 2006) and finally Cj0444 from strain 81-176 (26% identity, *C. jejuni* putative TonB-dependent outer membrane receptor protein, CampyDB). Identity or similarity between aligned protein sequences is indicated by shading. Black denotes identical amino acids, grey shows similar amino acids and no shading indicates that amino acids are different. A dot highlights similar amino acids and an asterisk indicates complete identity. Gaps were introduced by the programme where necessary to improve alignment.

As the search highlighted the major NCTC 11168 outer membrane receptor proteins, it was decided that all systems would be targeted for investigation. It was also important to test all receptor proteins for involvement to confirm or refute the prediction of

a single receptor protein for all iron sources. Conceivably there could be two proteins, one principally for binding Tf and one principally for binding Lf. The *cj1658-cj1663* system was also included, even though the search did not identify proteins from this system. As there is no obvious outer membrane receptor protein for the *cj1658-cj1663* system, the known periplasmic binding protein P19 was targeted and a strain carrying a mutation in *cj1659* was also tested alongside the other mutants for growth defects. A mutant strain of *C. jejuni* 81-176 produced by another member of the group (R. D. Haigh) carrying a deletion in *cj0444* was also tested for growth with human ferri-Lf.

4.2.2. Cloning and Mutagenesis of Iron Uptake Genes

4.2.2.1. Cloning of Genes

A number of important Fe^{3+} uptake genes from strain NCTC 11168 (*chuA*, *p19*, *cj0178*, many of the ABC transporter system components and *cj0444* from strain 81-176) had already been cloned and mutated by our group (see Table 2.2 for mutant strains used in this study; Parkhill *et al.*, 2000; Rock, 2003; Ridley *et al.*, 2006; Miller *et al.*, 2008) prior to or during this study. However, there were still a number of key genes that had not been cloned or inactivated. In order to assess all known iron uptake systems for their role in the transport of Lf-, Tf- or ovo-Tf-derived iron, genes of interest were cloned including: *cfrA*, the ferri-enterochelin outer membrane receptor protein (Palyada *et al.*, 2004), *ceuE* encoding the periplasmic binding protein of the ABC transporter system for ferri-enterochelin (Park and Richardson, 1995; Richardson and Park, 1995; Parkhill *et al.*, 2000; Palyada *et al.*, 2004), *cj0172c*, *cj0173c*, *cj0174c* and *cj0177* (of the uncharacterised system that encode a protein of unknown function, an ATPase, a permease protein and a putative lipoprotein of unknown function, respectively; Parkhill *et al.*, 2000; Tom-Yew *et al.*, 2005). In addition, the Fe^{2+} transport gene *feoB* (Naikare *et al.*, 2006) was cloned and mutated during the course of this study by another member of the group (R. D. Haigh). Each gene was cloned using a similar strategy to allow functional analysis by subsequent mutagenesis and phenotypic testing. For a detailed description of each technique used during this process, see Chapter 2. The cloning strategy is outlined in Fig. 4.3.

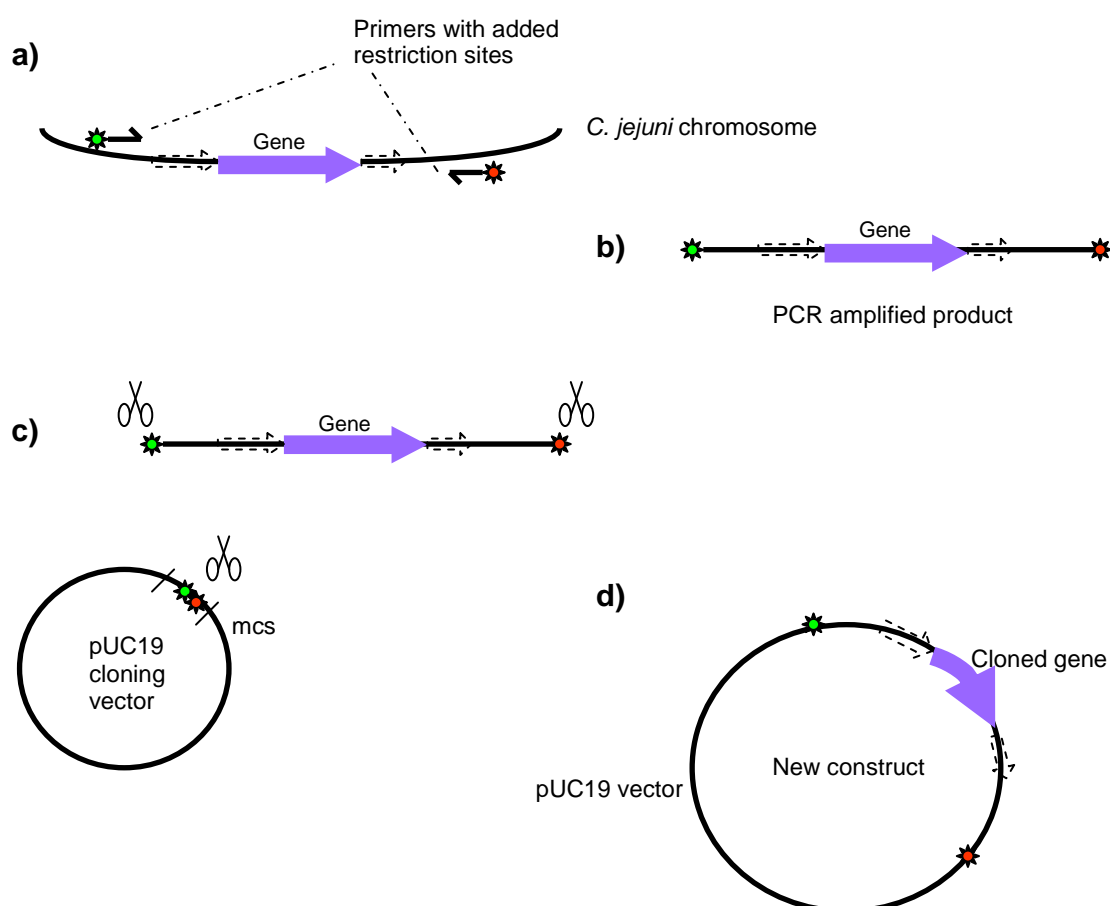


Figure 4.3. Cloning strategy. Diagram of the cloning of *C. jejuni* genes. **a)** The gene of interest was amplified from the chromosome using PCR and a primer pair with restriction enzyme sites incorporated at the 5' end (flanking genes are indicated by dotted arrows). The restriction sites *KpnI* (forward primer, green ☆) and *PstI* (reverse primer, red ☆) were chosen to allow directional cloning of the amplified product into the multiple cloning site (mcs) of the cloning vector pUC19. Primers were designed to anneal 500 bp upstream and downstream of the gene of interest **b)** generating a PCR product containing the gene, DNA flanking regions and restriction enzyme sites. **c)** Both the PCR product and the cloning vector were restricted (indicated by scissors), which allowed **d)** ligation to produce the initial construct containing the cloned gene. The mcs is within a *lacZ* gene allowing blue/white screening of *E. coli* colonies transformed with the construct. See Table 2.2 for a list of constructs and Appendix 1 for all maps of plasmid constructs produced during this study.

Each gene of interest was amplified from the NCTC 11168 chromosome using PCR (Section 2.10.1) and the oligonucleotide primer pairs detailed in Table 2.3 and Table 4.1. The production of the mutant strain CEM6 ($\Delta ceuE::aphA-3$, Table 2.2) is used as an example; PCR amplification of *ceuE* using the primer pair *ceuEF* and *ceuER* resulted in the generation of a 2009 bp product (Fig. 4.4, lane 2).

Table 4.1. Cloned genes, cloning primers used, primer binding positions on the *C. jejuni* chromosome and the sizes of products produced by PCR amplification.

Target gene and size (bp)	Cloning primer pairs	Positions primers anneal to on chromosome (bp)	Product size (bp)
<i>cj0172c</i> 1206	<i>cj0172cF</i> and <i>cj0172cR</i> .	169571 (forward) 167306 (reverse)	2265
<i>cj0173c</i> 909	<i>cj0173cF</i> and <i>cj0173cR</i> .	170551 (forward) 168481 (reverse)	2070
<i>cj0174c</i> 1617	<i>cj0174cF</i> and <i>cj0174cR</i> .	171984 (forward) 169436 (reverse)	2548
<i>cj0177</i> 852	<i>cj0177F1</i> , <i>cj0177F2</i> , <i>cj0177R1</i> and <i>cj0177R2</i> .	172625 (forward 1) 173291 (reverse 1) 173690 (forward 2) 174389 (reverse 2)	666 699
<i>cfrA</i> 2091	<i>cfrAF</i> and <i>cfrAR</i> .	704900 (forward) 708177 (reverse)	3277
<i>ceuE</i> 993	<i>ceuEF</i> and <i>ceuER</i> .	1286177 (forward) 1288186 (reverse)	2009

Primers were designed to anneal approximately 500 bp upstream or downstream of each target gene to incorporate DNA flanks either side of the coding region (Fig. 4.3). This was important for the final stage of the mutagenesis process, facilitating homologous recombination to replace the wild-type allele with the mutant allele in the *C. jejuni* chromosome. Primer design included the addition of a 5' restriction enzyme site to allow directional cloning of the PCR product. *KpnI* was included on the forward primer and *PstI* on the reverse primer. The PCR reaction produced a DNA product including the coding region of interest flanked by additional DNA with a restriction site at each end (product sizes are listed in Table 4.1). The products were purified, restricted and purified again (Sections 2.7 and 2.9.1). The success of each stage was visualised by agarose gel electrophoresis (Section 2.6), allowing confirmation of product size. The cloning vector used was pUC19 (Table 2.2), which contained a multiple cloning site (mcs) including single sites for both *KpnI* and *PstI*.

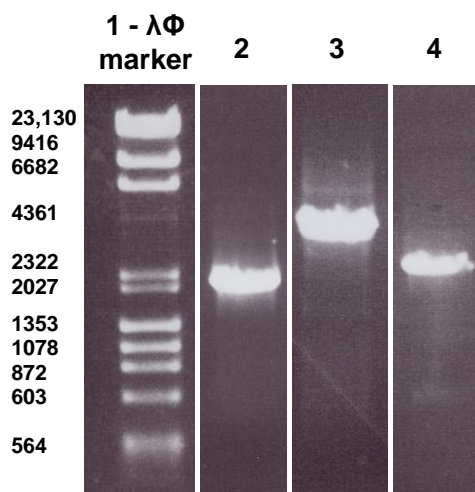


Figure 4.4. PCR amplified products from the stages of construction of the *ceuE* mutant strain CEM6. Lane 1 contained $\lambda\Phi$ marker DNA, the sizes of which are indicated in bp to the left (250 ng of λ DNA restricted with *Hind*III and 100 ng of Φ X174 DNA restricted with *Hae*III), lane 2 contained PCR amplified insert, amplified using primer pair *ceuEF* and *ceuER* (Table 4.1), generating a product that covered the *ceuE* coding region and the upstream and downstream flanking regions, 2009 bp (Table 4.1; 5 μ l). Lane 3 contained PCR amplified inverse PCR product, amplified using primer pair *ceuEInvF* and *ceuEInvR* (Table 4.2), generating a product including the pUC19 vector and flanking regions, but missing the *ceuE* coding region, 3887 bp (Table 4.2; 5 μ l). Lane 4 contained the *ceuE* region, amplified by PCR from the chromosome of the mutant strain (CEM6, Δ *ceuE::aphA-3*) using primer pair *ceuEF* and *ceuER* (2507 bp; Section 4.2.2.4). For agarose gel electrophoresis method see Section 2.6.

Purified pUC19 was restricted with *Kpn*I and *Pst*I, purified again and restriction was verified by agarose gel electrophoresis. Directional cloning of the inserts avoided the need to screen for insert orientation. The insert and vector were ligated (Section 2.9.3), purified by ethanol precipitation (Section 2.7.1) and transformed into *Escherichia coli* DH5 α cells by either heat shock transformation or electroporation (Sections 2.12.2 and 2.12.3). Cells were also transformed with a positive control of pUC19 vector alone; distilled water was included with the cells during transformation as a negative control. The pUC19 vector contained an ampicillin resistance gene, conferring resistance to the transformed *E. coli* cells (Table 2.1), and a *lacZ* gene containing the mcs. Transformed *E. coli* strains were grown on medium containing X-gal and IPTG to allow blue/white screening (Section 2.1). White colonies were re-plated and then screened by colony PCR (Section 2.10.1). Recombinant plasmid constructs were purified from overnight cultures (Section 2.5.3) and verified by PCR using either the appropriate cloning primer pair (Table 2.3, Table 4.1) or pUC19 specific M13F and M13R primers together or paired with an appropriate cloning primer (Table 2.3, Appendix 2). M13 primers bind to pUC19 either

side of the mcs and were also used to sequence (Section 2.11) across the inserts to verify that the constructs were correct and to check for PCR incorporation errors. Constructs were named pCEM1 (*cj0172c*), pCEM2 (*cj0173c*), pCEM3 (*cj0174c*), pCEM5 (*cfrA*) and pCEM6 (*ceuE*). Diagrams of all of the constructs produced during the course of this study are found in Appendix 1. All constructs are fully detailed in Table 2.2.

4.2.2.2. Inverse PCR Mutagenesis of Genes

Following successful production of the initial constructs containing cloned *C. jejuni* genes, the majority of coding regions were then deleted using an *inverse PCR* mutagenesis strategy (IPCRM, Fig. 4.5; Wren *et al.*, 1994; Dorrell *et al.*, 1996).

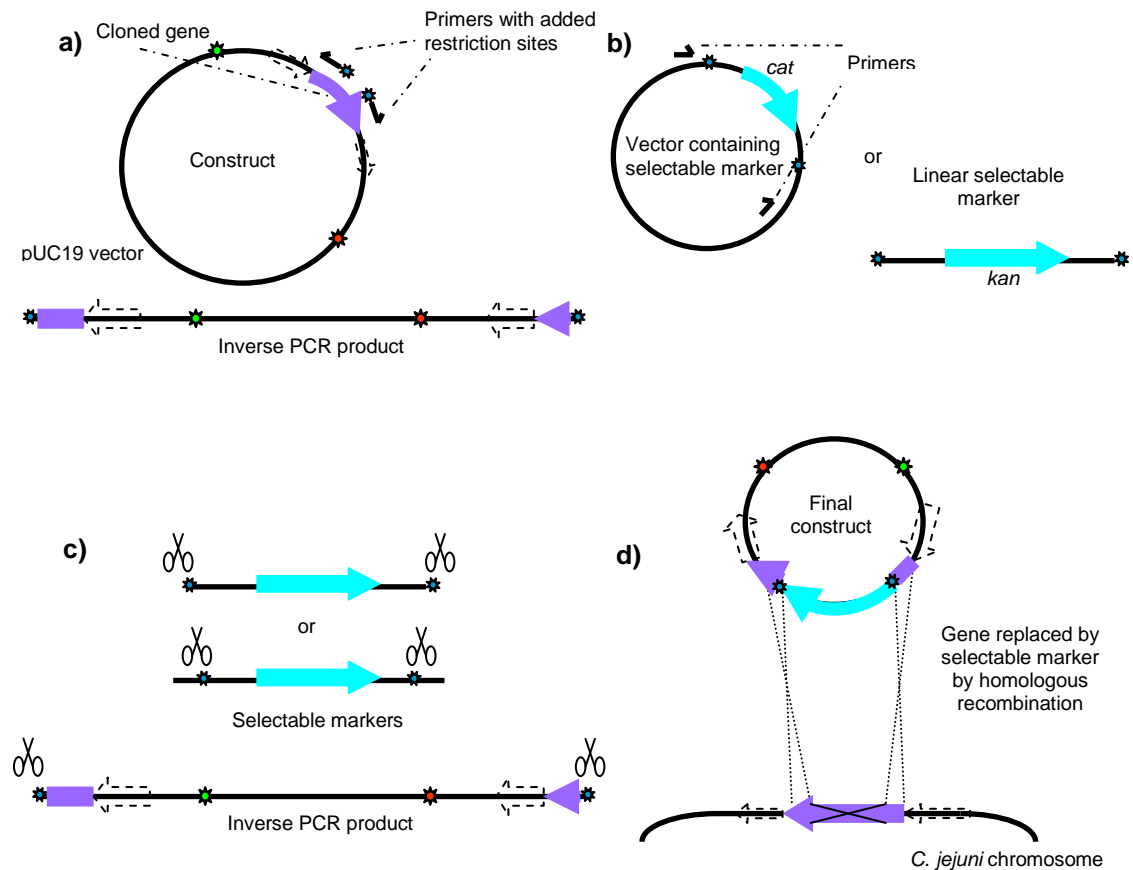


Figure 4.5. Inverse PCR mutagenesis strategy. Diagram of the inverse PCR mutagenesis strategy used to delete genes of interest in the *C. jejuni* chromosome. **a)** Inverse PCR primers with 5' restriction enzyme sites were designed to anneal within the coding region in the initial construct and amplify outwards, producing a linear inverse PCR product containing the flanking regions and vector DNA (flanking genes are indicated by dotted arrows). The same restriction enzyme site was used on each primer, either *Bgl*II or *Sma*I (forward and reverse primer, blue ✱), to allow cloning of the selectable marker. **b)** The selectable marker was amplified from the vector containing it with vector specific primers (Appendix 2). Alternatively, linear selectable markers which had been previously restricted and purified were used. **c)** The inverse PCR product and selectable marker were then restricted (indicated by scissors) with the appropriate enzyme and **d)** ligated to produce the final construct. The final constructs were transformed into *E. coli* cells and colonies were selected using blue/white screening and resistance to the antibiotic that the newly introduced selectable marker conferred. Following preparation and verification, the constructs were **d)** transformed into wild-type *C. jejuni* cells. Homologous recombination between the flanking regions in the construct and the corresponding flanks in the genome resulted in allelic replacement of the wild-type gene with the selectable marker in the *C. jejuni* chromosome. Deletion mutant strains were identified using the selective antibiotic, and confirmed by PCR screening and sequencing. Constructs are listed in Table 2.2 and maps of all final constructs can be found in Appendix 1.

Inverse PCR oligonucleotide primer pairs (Table 2.3, Table 4.2) were designed to anneal just within the coding region facing outwards. Amplification around each construct resulted in the production of a linear inverse PCR product containing the regions flanking

the gene of interest and the vector, but missing the majority of the gene itself (Fig. 4.5, Table 4.2). Inverse PCR amplification using pCEM6 as the template and the primer pair *ceuE*InvF and *ceuE*InvR resulted in the generation of a 3887 bp inverse PCR product (Fig. 4.4, lane 3).

Table 4.2. Initial construct templates used for inverse PCR mutagenesis, inverse PCR primer pairs, sizes of deletions created within the ORFs and the size of the final inverse PCR products.

Construct template	Inverse PCR primer pair	Deletion in ORF in construct (bp)	Product size (bp)
pCEM1	<i>cj0172c</i> InvF and <i>cj0172c</i> InvR.	1097	3839
pCEM2	<i>cj0173c</i> InvF and <i>cj0173c</i> InvR.	661	4080
pCEM3	<i>cj0174c</i> InvF and <i>cj0174c</i> InvR.	1477	3742
pCEM4	N/A	399	4038
pCEM5	<i>cfrA</i> InvF and <i>cfrA</i> InvR.	1956	3987
pCEM6	<i>ceuE</i> InvF and <i>ceuE</i> InvR.	800	3887

Oligonucleotide primers included a 5' restriction enzyme site to allow subsequent ligation to a selectable marker to replace the deleted gene. *Bgl*III restriction enzyme sites were included on all primers except *ceuE*InvF and *ceuE*InvR, which included 5' *Sma*I sites due to *Bgl*III cutting within the cloned region of pCEM6. No sites existed for either enzyme within the vector DNA. *Sma*I was not included on all primers due to the presence of *Sma*I sites within the cloned DNA. Initial construct DNA was purified (Section 2.5.3), and from this the inverse PCR product was generated (Section 2.10.1), purified, restricted, purified again and each stage was verified by agarose gel electrophoresis to allow confirmation of product size. Selectable markers were used that had been generated for mutant construction as part of a previous study. The markers carried a double tag, *Sma*I-restricted ends and kanamycin resistance (derived from pJMK30, Table 2.2, including the *aphA-3* gene; van Vliet *et al.*, 1998). Cassettes were ligated to the inverse PCR products for genes *cj0172c*, *cj0173c*, *cj0174c*, *cfrA* and *ceuE* (Table 4.2). As the previously prepared cassettes ended in *Sma*I sites, the *ceuE* inverse PCR product was ligated to the cassette using blunt ligation (Section 2.9.3). For the other genes a ligation reaction including filling in and/or removal of 3'-overhangs was carried out to join the *Bgl*III and *Sma*I restricted ends (Section 2.9.4).

Following ligation, the final constructs were purified by ethanol precipitation and transformed into *E. coli* DH5 α cells by either heat shock transformation or electroporation (Sections 2.12.2 and 2.12.3), with appropriate controls. As each construct still contained the pUC19 vector, ampicillin was used in the recovery medium for selection of recombinant colonies, as well as the antibiotic to which the selectable marker conferred resistance (Table 2.1). Blue/white screening was used as described in the previous section. White colonies were re-plated and screened by colony PCR; final plasmid constructs purified from overnight cultures were verified by PCR. M13 and cloning primers (Table 2.3, Table 4.1, Appendix 2) were combined to cover the entire cloned region. The presence of the antibiotic resistance cassette in forward orientation with respect to the interrupted gene in each construct was verified using cassette-specific primers with either the appropriate cloning or M13 primer (Table 2.3, Table 4.1, Appendix 2). M13 or cloning primers in combination with cassette specific primers were also used to sequence across the flanking regions to confirm that the construct was correct and to screen for PCR incorporation errors. Constructs were named pCEM7 ($\Delta cj0172c::aphA-3$), pCEM8 ($\Delta cj0173c::aphA-3$), pCEM9 ($\Delta cj0174c::aphA-3$), pCEM11 ($\Delta cfrA::aphA-3$) and pCEM12 ($\Delta ceuE::aphA-3$; Appendix 1; Table 2.2).

4.2.2.3. Mutagenesis of *cj0177*

The gene *cj0177* was cloned in two parts by a three-way ligation method, using the same basic strategy as detailed above, but with two inserts included in the ligation reaction instead of one (see Fig 4.6, the primers used and the sizes of each are listed in Table 4.1).

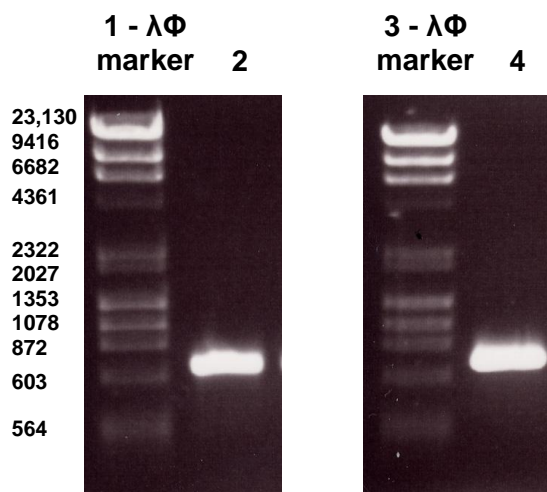


Figure 4.6. PCR amplified DNA products. Agarose gel electrophoresis of PCR amplified inserts 1 and 2 for the construction of a *cj0177* mutant. Lanes 1 and 3 contained $\lambda\Phi$ marker DNA, the sizes of which are indicated in bp to the left (250 ng of λ DNA restricted with *Hind*III and 100 ng of Φ X174 DNA restricted with *Hae*III), lane 2 contained PCR amplified insert 1, amplified using primer pair *cj0177F1* and *cj0177R1* (Table 4.1), generating a product that covers the upstream flank of *cj0177*, 666 bp (Table 4.1; 5 μ l). Lane 4 contained PCR amplified insert 2, amplified using primer pair *cj0177F2* and *cj0177R2* (Table 4.1), generating a product that covers the downstream flank of *cj0177*, 699 bp (Table 4.1; 5 μ l). For agarose gel electrophoresis method see Section 2.6.

The two fragments were amplified by PCR and covered the flanking regions of the gene at either side. *Kpn*I and *Pst*I restriction enzyme sites were included at the 5' ends of the external primers and *Sma*I sites were included on the 5' ends of the internal primers (Fig. 4.7, Table 2.3, Table 4.1). Following restriction and purification of the DNA, a ligation reaction included both inserts and the vector DNA (Fig. 4.7).

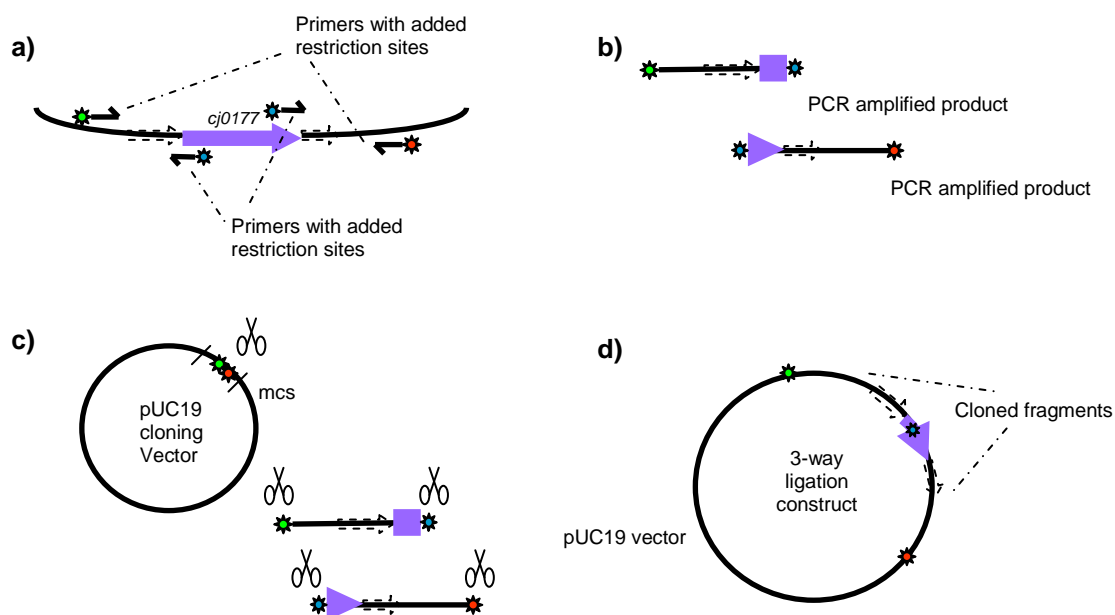


Figure 4.7. Cloning strategy used for gene *cj0177*. Diagram of the three-way cloning of *cj0177* flanks to produce a circularised inverse PCR product. **a)** The regions flanking *cj0177* were amplified from the *C. jejuni* chromosome using PCR and two pairs of primers with 5' restriction enzyme sites incorporated (flanking genes are indicated by dotted arrows). The upstream flank was amplified including the restriction sites *KpnI* (forward primer, green ☼) and *SmaI* (reverse primer, blue ☼); the downstream flank was amplified including the restriction sites *SmaI* (forward primer, blue ☼) and *PstI* (reverse primer, red ☼). **b)** The amplified fragments were **c)** restricted (indicated by scissors) and cloned into the restricted (indicated by scissors) mcs of the cloning vector pUC19. **d)** Ligation generated the 3-way ligation construct containing each fragment, in known orientation with a ready-made deletion in the open reading frame and the creation of an internal *SmaI* site for the cloning of a selectable marker. Interruption of the *lacZ* gene in the vector allowed blue/white screening of the *E. coli* colonies transformed with the construct. Table 2.2 contains a list of constructs and all plasmid construct maps produced during this study are contained in Appendix 1.

Ligation resulted in the production of a central *SmaI* restriction site for the insertion of a selectable marker at a later stage and also in the deletion of most of the coding region (Table 4.2). An inverse PCR stage was avoided, resulting in the production of a circularised inverse PCR product upon ligation of both restricted and purified inserts to restricted, clean pUC19 (Fig. 4.7). Ligated DNA was purified and transformed into *E. coli* DH5 α ; the presence of a construct was selected for and colonies were screened. Purified constructs from overnight cultures were PCR screened again as described above.

A selectable marker conferring erythromycin resistance (a cassette obtained from pRRE including the *ermC'* gene, Table 2.2) was prepared. The cassette was amplified from pRRE using vector specific primers (EryF and EryR, Appendix 2) containing 5' *SmaI*

restriction sites. The product was purified, restricted and purified again. Each stage was verified by agarose gel electrophoresis (Fig. 4.8).

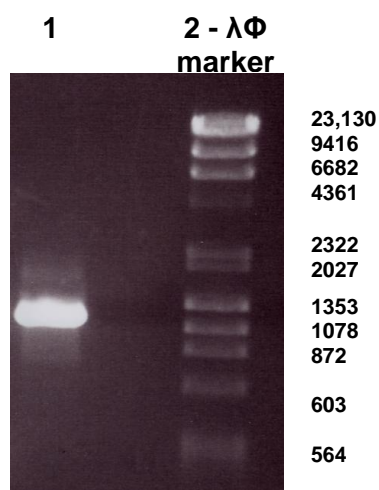


Figure 4.8. PCR amplified selectable marker. Agarose gel electrophoresis of PCR amplified erythromycin resistance cassette (containing *ermC'*) from vector pRRE (Table 2.2). Lane 1 contained PCR amplified erythromycin resistance cassette, amplified using primer pair eryF and eryR (Appendix 2) each carrying 5' *Sma*I sites, generating a product that covers the resistance gene, 1197 bp (5 µl). Lane 2 contained λΦ marker DNA, the sizes of which are indicated in bp to the right (250 ng of λ DNA restricted with *Hind*III and 100 ng of ΦX174 DNA restricted with *Hae*III). For agarose gel electrophoresis method see Section 2.6.

Construct preparations were made of the *cj0177* circularised inverse PCR product (pCEM4, Table 2.2, Section 2.5.3; Appendix 1). A digestion with *Sma*I was performed, cleaving pCEM4 at the internal *Sma*I site yielding a linear product. This was followed by purification and agarose gel electrophoresis verification of size (Fig. 4.9).

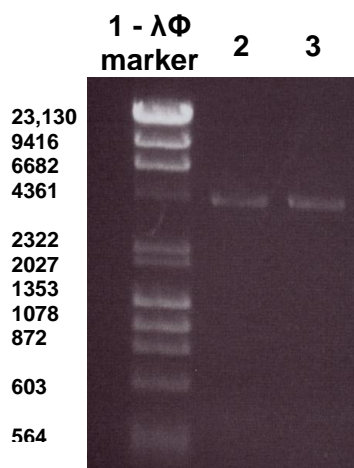


Figure 4.9. Restricted pCEM4 generating the *cj0177* inverse PCR product for mutagenesis of the gene. Agarose gel electrophoresis of the *cj0177* inverse PCR product generated by *Sma*I restriction of pCEM4. pCEM4 contained an internal deletion within the *cj0177* coding region of 399 bp and a *Sma*I was created at the point of deletion. Restriction generated a linear piece of DNA of 4038 bp (Table 4.3) which was purified, ready to be ligated to the erythromycin resistance selectable marker. Lane 1 contained $\lambda\Phi$ marker DNA, the sizes of which are indicated in bp to the left (250 ng of λ DNA restricted with *Hind*III and 100 ng of Φ X174 DNA restricted with *Hae*III). Lanes 2 and 3 contained the *Sma*I restricted *cj0177* inverse PCR product (1 μ l). For agarose gel electrophoresis method see Section 2.6.

Linear pCEM4 was then ligated to the *Sma*I-restricted and purified erythromycin resistance cassette from pRRE, purified and transformed into *E. coli* DH5 α ; constructs were selected for and colonies were screened. Purified constructs from overnight cultures were PCR screened again and sequenced as described above. The construct was named pCEM10 (Δ *cj0177::ermC*), Table 2.2; Appendix 1). Erythromycin resistance was used here to permit later construction of double mutants using strains already carrying single mutations with inserted cassettes conferring resistance to kanamycin.

4.2.2.4. Construction of Mutant *C. jejuni* Strains

Following confirmation that each construct was correct, purified constructs were transformed into wild-type *C. jejuni* NCTC 11168 by electroporation (Section 2.12.5). *C. jejuni* cells were recovered overnight on non-selective medium and subsequently transferred to medium containing the selective antibiotic. The regions of DNA flanking the gene of interest in the final constructs allowed allelic replacement of the wild-type copy of the gene in the *C. jejuni* chromosome with the selectable marker by homologous recombination (Fig. 4.5d). The plasmid acts as a suicide vector as strain NCTC 11168

cannot support pUC19. Colonies containing integrated antibiotic resistance cassettes grew on selective medium and were isolated. Colonies were re-plated, screened by colony PCR to check the presence of the cassette in the correct location in the genome, and the mutant DNA was prepared by either large- or small-scale chromosomal DNA preparation (Sections 2.5.1 and 2.5.2). Chromosomal preparations were verified by agarose gel electrophoresis (Section 2.6) to confirm that the DNA was not sheared. DNA was quantified by absorption spectroscopy before screening (Section 2.8).

Incorporation of the marker was verified by PCR from the mutant *C. jejuni* chromosomal DNA using either the original cloning primer pair or a combination of appropriate cloning primer and cassette specific primer (Table 2.3, Table 4.1, Appendix 2). The final primer combination also confirmed cassette orientation in the chromosome. PCR amplification of the *ceuE* mutant region using the primer pair *ceuEF* and *ceuER* resulted in the generation of a 2507 bp product including the flanking regions and the selectable marker, which had replaced the coding region in the chromosome (Fig. 4.4, lane 4). PCR products were purified and sequenced to verify cassette orientation and establish that there were no errors in the region. Single mutant strains constructed during this project were named CEM1 ($\Delta cj0172c::aphA-3$), CEM2 ($\Delta cj0173c::aphA-3$), CEM3 ($\Delta cj0174c::aphA-3$), CEM4 ($\Delta cj0177::ermC'$), CEM5 ($\Delta cfrA::aphA-3$) and CEM6 ($\Delta ceuE::aphA-3$). Strains are fully detailed in Table 2.2. The single mutant strains produced by other members of the lab (Table 2.2) were also confirmed to be correct by PCR and sequencing across the altered region of the *C. jejuni* chromosome using the appropriate primers (Table 2.3 and Appendix 2) prior to use in this study.

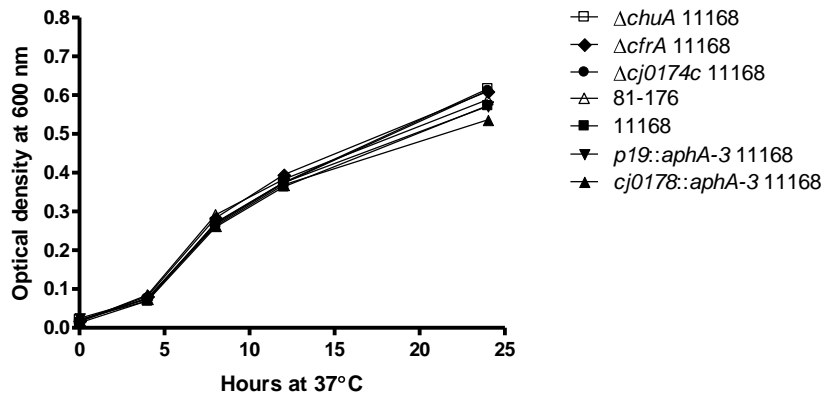
4.2.3. Phenotypic Analysis of Mutant Strains

Phenotypic analysis of *C. jejuni* mutant strains was carried out using the liquid medium growth curve strategy detailed in Chapter 3, the method for which can be found in Section 2.13. Strains carrying mutations in components of the iron uptake systems were grown with iron supplied solely in the form of human ferri-Lf. As the growth of wild-type NCTC 11168 cells was promoted equally by the Tfs (Fig. 3.3), human ferri-Lf was chosen to test mutant strains for altered phenotypes. If mutant phenotypes were altered, the other proteins were also tested to establish whether a particular receptor was involved in iron uptake from all proteins or if separate receptors exist for the different iron sources.

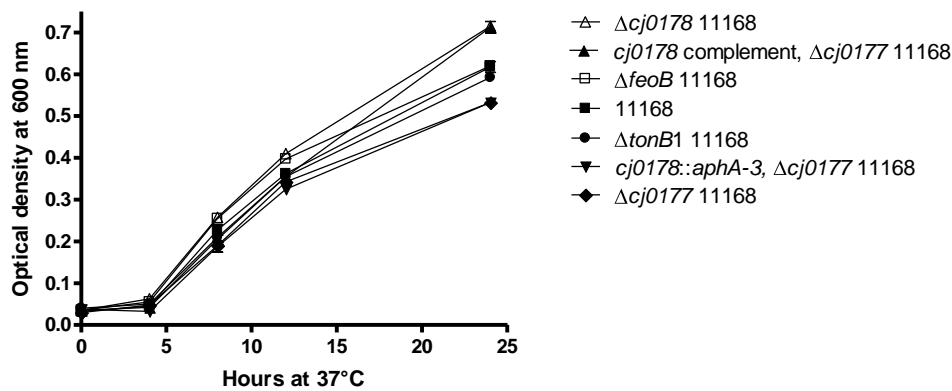
4.2.3.1. Mutant Strain Growth Patterns

Initial studies of all mutant strains produced and used during the course of this work were completed by testing growth over 24 h in rich medium (Mueller-Hinton broth, MHB). MHB was used to establish that no growth defects occurred in iron-replete rich growth media. Mutation of the genes resulting in differences during later phenotypic testing could then be attributed to the test conditions used. MHB was not supplemented and each strain was tested six times; three technical replicates per assay and two biological replicates of each assay. All raw data for the liquid growth assays found in this chapter and statistical testing of the differences seen are listed in Appendix 3. Wild-type NCTC 11168 and 81-176 grew well in MHB to a final optical density at 600 nm of around 0.6 (Fig 4.10a-c; 11168, 81-176). Growth patterns of all strains apart from $\Delta cj0444::aphA-3$ 81-176 (Fig. 4.10c) were similar to wild-type cells, with some variation in the final OD₆₀₀ reached at 24 h. This implies that these mutations do not have a serious effect on the normal growth of the strains. Any notable differences seen during subsequent growth assays can be attributed to a phenotype associated with the gene of interest and not a more general growth problem due to either the loss of the gene or disruption of the expression of a downstream gene by the introduction of the selectable marker into the chromosome. The 81-176 strain carrying a mutated *cj0444* was constructed by another member of the group during this study (R. D. Haigh) and demonstrated a different growth pattern to wild-type 81-176 (Fig. 4.10c). Data generated from this mutant strain could therefore be affected by this defect so conclusions drawn are likely to require further confirmation.

a)



b)



c)

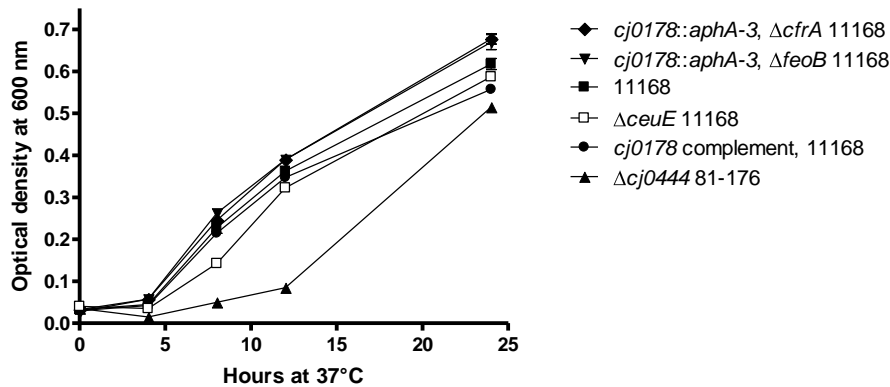


Figure 4.10. Growth of wild-type and mutant *C. jejuni* strains in rich medium (Mueller-Hinton broth, MHB). Assays were conducted using MHB with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. **a)** Wild-type 11168, KAR2 ($cj0178::aphA-3$), KAR3 ($p19::aphA-3$), CEM5 ($\Delta cfrA::aphA-3$), CEM3 ($\Delta cj0174c::aphA-3$), JDR5 ($\Delta chuA::cat$), wild-type 81-176. **b)** Wild-type 11168, CEM10 (CEM8, $\Delta cj0177::ermC'$), CEM9 (KAR2, $\Delta cj0177::ermC'$), CEM4 ($\Delta cj0177::ermC'$), JDR20 ($\Delta tonB1::cat$), 11168 $\Delta feoB::ermC'$, JDR21 ($\Delta cj0178::cat$). **c)** Wild-type 11168, 81-176 $\Delta cj0444::aphA-3$, CEM12 (KAR2, $\Delta feoB::ermC'$), CEM11 (KAR2, $\Delta cfrA::aphA-3$), CEM8 (complemented KAR2 strain), CEM6 ($\Delta ceuE::aphA-3$).

4.2.3.2. Outer Membrane Receptor Protein Mutants

In order to determine whether one or more of the NCTC 11168 outer membrane receptor proteins are involved in iron acquisition from the Tf family, mutant strains CEM5 ($\Delta cfrA::aphA-3$), KAR2 ($cj0178::aphA-3$), KAR3 ($p19::aphA-3$) and JDR5 ($\Delta chuA::cat$; Table 2.2) were tested for growth in liquid medium when iron was supplied solely in the form of human ferri-Lf (0.27 μ M; Fig. 4.11a-d). MEM α was used as a defined iron-restricted medium over a 24 h period with samples taken at intervals.

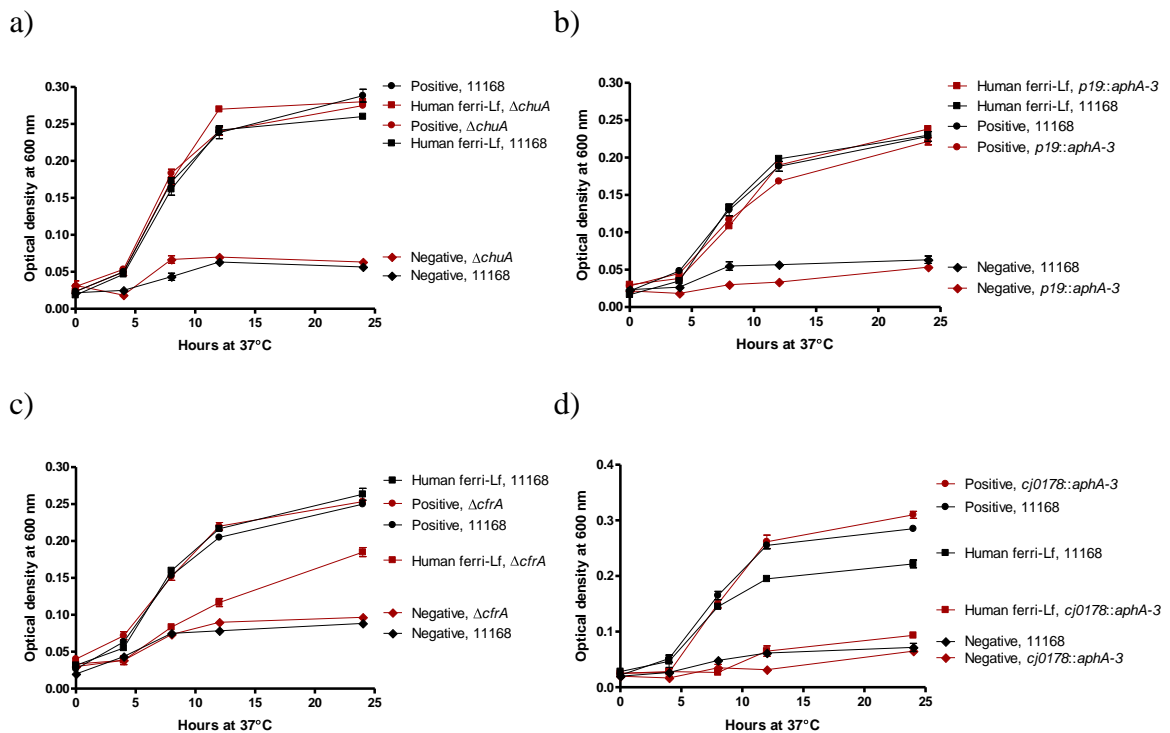
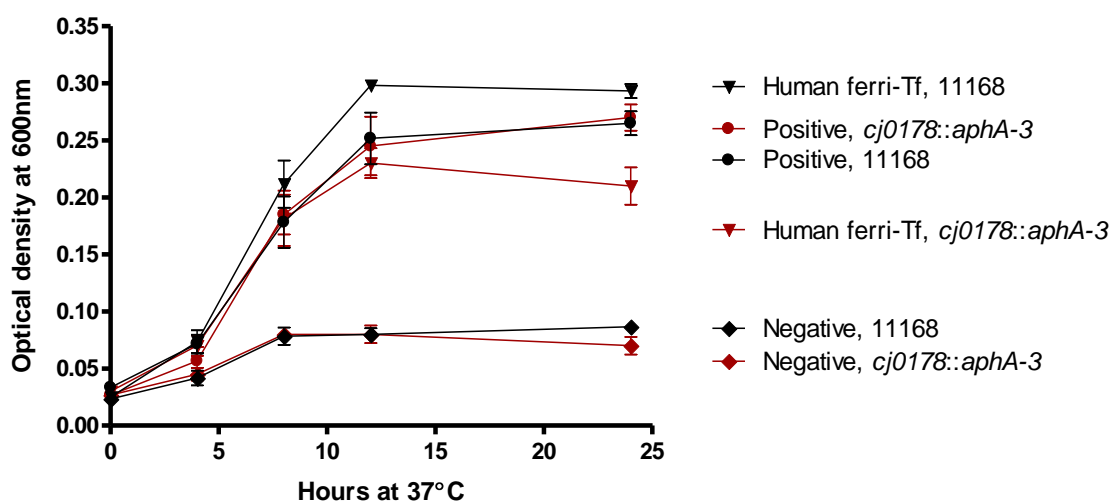


Figure 4.11. Growth assays of *C. jejuni* NCTC 11168 strains with mutated iron outer membrane receptor proteins supplied with iron bound to human Lf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative), human ferri-Lf (0.27 μ M). **a)** NCTC 11168 and JDR5 ($\Delta chuA::cat$). **b)** NCTC 11168 and KAR3 ($p19::aphA-3$). **c)** NCTC 11168 and CEM5 ($\Delta cfrA::aphA-3$). **d)** NCTC 11168 and KAR2 ($cj0178::aphA-3$).

All strains showed comparable growth promotion under iron-replete conditions (10 μM FeSO_4) and growth restriction under iron-limited conditions (unsupplemented MEM α ; Fig. 4.11a-d) over the 24 h period. Optical densities of around 0.25 to 0.30 were reached by all strains when supplied with iron; growth of all strains was limited to optical densities of between 0.05 and 0.10 in unsupplemented medium in the absence of iron. The growth patterns of the mutant strains in response to high iron or iron-restriction in MEM α are comparable to NCTC 11168.

In the presence of human ferri-Lf (0.27 μM), strains KAR3 (*p19::aphA-3*) and JDR5 ($\Delta\text{chuA}::\text{cat}$) showed no significant difference in growth from wild-type NCTC 11168 (Fig. 4.11a, b). NCTC 11168 cells grew well when supplied only with human ferri-Lf as a source of iron, reaching final optical densities of around 0.25 to 0.30, as seen previously in Chapter 3 (Fig. 3.3a). Strain CEM5 ($\Delta\text{cfrA}::\text{aphA-3}$) showed significantly less growth than wild-type cells in the presence of 0.27 μM human ferri-Lf (Fig. 4.11c), reaching a final optical density of almost 0.20, rather than 0.25 as seen with wild-type cells. KAR2 (*cj0178::aphA-3*), however, showed the most significantly decreased growth when compared to wild-type cells using 0.27 μM human ferri-Lf as a sole iron source (Fig. 4.11d). At 24 h KAR2 mutant cells had failed to reach an optical density of 0.10 (Fig. 4.11d), indicating that very little growth had been achieved resulting in a similar growth pattern to the negative control. As this mutant strain showed the most notable phenotype, growth of KAR2 in the presence of human ferri-Tf or ferri-ovo-Tf was also investigated (Fig. 4.12a-b).

a)



b)

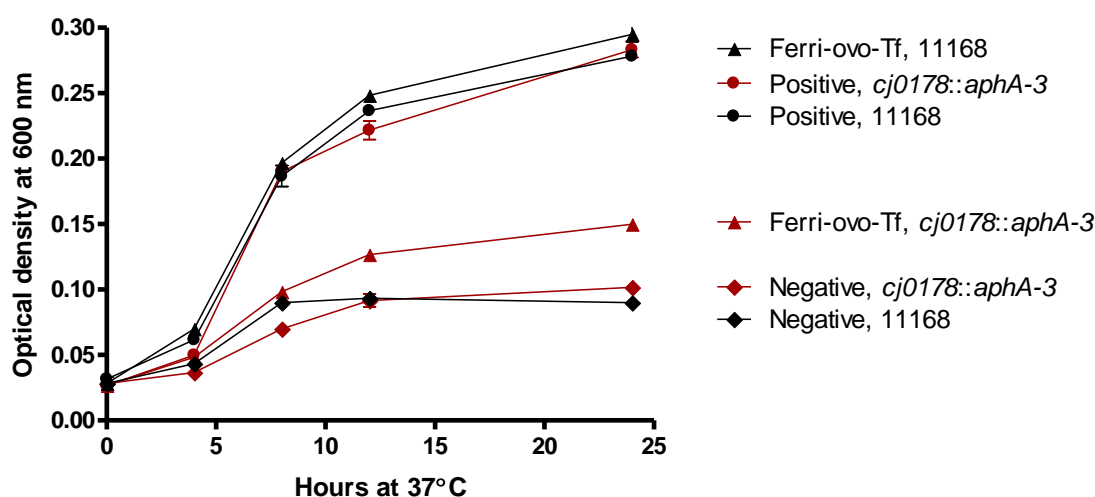


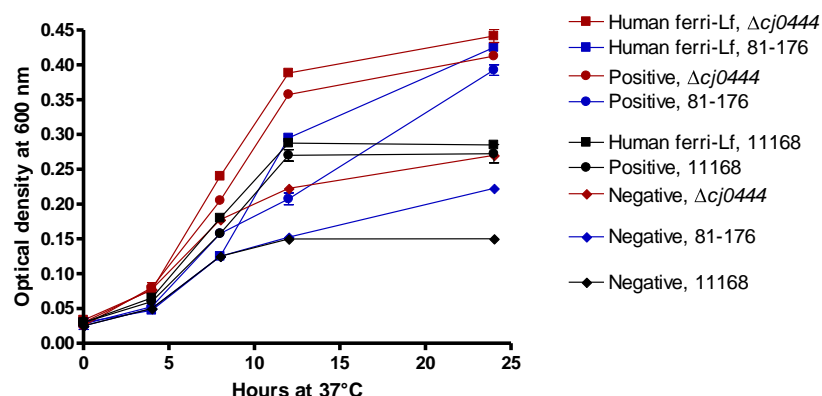
Figure 4.12. Growth assays of *C. jejuni* strain KAR2 (*cj0178::aphA-3* 11168) with iron supplied bound to human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative. **a)** NCTC 11168 and KAR2 with human ferri-Tf (0.27 μ M). **b)** NCTC 11168 and KAR2 with ferri-ovo-Tf (0.27 μ M).

In the presence of equivalent concentrations of human ferri-Tf or ferri-ovo-Tf (0.27 μ M; Fig. 4.12a-b), KAR2 grew less than wild-type, as seen with human ferri-Lf (Fig. 4.11d). Slightly more growth was achieved in the presence of human ferri-Tf than either human ferri-Lf or ferri-ovo-Tf, with a final optical density of around 0.20 reached. Ferri-ovo-Tf allowed only limited growth, similar to human ferri-Lf, with final optical densities

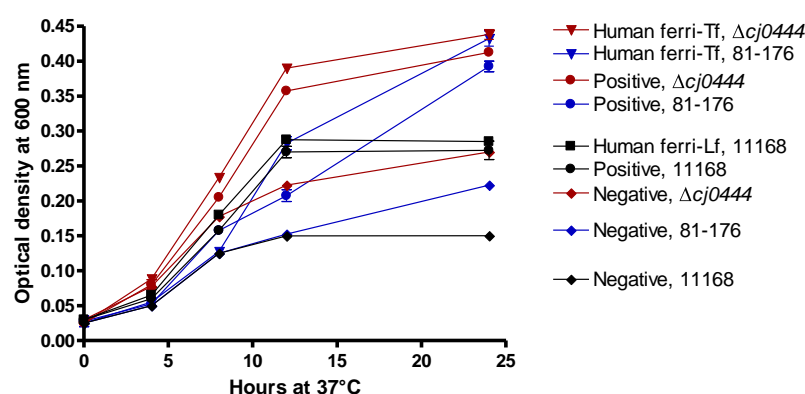
of between 0.10 and 0.15 achieved. The differences between the wild-type and mutant growth patterns with all ferri-proteins were statistically significant at 24 h.

Finally, the mutant 81-176 strain carrying a deletion in *cj0444* was tested for growth in MEM α with iron supplied solely in the form of human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (Fig. 4.13a-c). Unsupplemented MEM α was used to induce iron-restriction, the addition of 10 μ M FeSO₄ induced iron-replete conditions and 0.27 μ M ferri-Lf, ferri-Tf or ferri-ovo-Tf was included in the test cultures. Control cultures of both wild-type NCTC 11168 and wild-type 81-176 were included, grown under the same conditions as the mutant strain. All cultures were initially iron depleted by overnight growth in unsupplemented MEM α . Growth of strain NCTC 11168 (Fig. 4.13a-c) was comparable to the patterns seen before in all assays (for example, Fig. 3.3a). Strain 81-176 grew as seen in Chapter 3 (Fig. 3.4) reaching a higher final OD₆₀₀ with human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (0.27 μ M) than strain NCTC 11168 (Fig. 4.13a-c). The 81-176 strain carrying a mutation in *cj0444* reached the highest final optical densities under all conditions (Fig. 4.13a-c). Although growth was shown to be impaired in unsupplemented rich medium (Fig. 4.10a, c) when compared to either wild-type strain, growth does not appear to be limited here. As this strain grows well with ferri-protein derived iron, it would appear that Cj0444 does not play a role in the uptake process.

a)



b)



c)

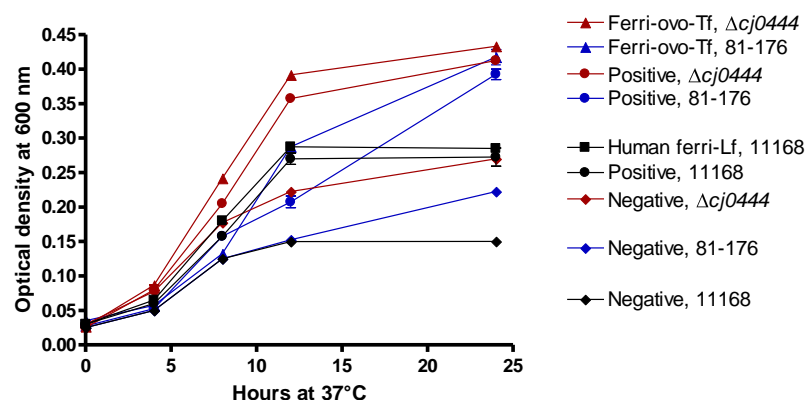


Figure 4.13. Growth assays of *C. jejuni* strain $\Delta cj0444::aphA-3$ 81-176 with iron supplied bound to human Lf, human Tf or ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168, 81-176 and $\Delta cj0444::aphA-3$ 81-176 with human ferri-Lf (0.27 μ M). **b)** NCTC 11168, 81-176 and $\Delta cj0444::aphA-3$ 81-176 with human ferri-Tf (0.27 μ M). **c)** NCTC 11168, 81-176 and $\Delta cj0444::aphA-3$ 81-176 with ferri-ovo-Tf (0.27 μ M).

4.2.3.3. ABC Transporter and Other Mutants

The results presented in the previous section indicate a primary role for Cj0178 in iron utilisation from members of the Tf protein family for growth. The genes surrounding *cj0178* in the NCTC 11168 genome appear to encode an iron uptake system that has not been thoroughly characterised, and prior to this study, the iron source that this system is involved in acquiring was unknown.



Figure 4.14. Genomic context of genes *cj0173c-tonB1* in the *C. jejuni* NCTC 11168 genome. Distances are indicated in base pairs, with *cj0173c-tonB1* in black and unrelated flanking genes in grey (Parkhill *et al.*, 2000; Miller *et al.*, 2008) (<http://xbase.bham.ac.uk/campydb>).

Since the publication of the NCTC 11168 genome sequence (Parkhill *et al.*, 2000), many genes have been identified and their functions predicted based on amino acid similarity. The genes *cj0175c-cj0173c* are proposed to encode an ABC transporter system, with *cj0176c* and *cj0177* encoding putative lipoproteins of unknown function. Genes encoding an ExbBD-TonB energy transduction system are found downstream of *cj0177* and *cj0178* (Fig. 4.14, Section 1.5.1.4). Genes *cj0176c-cj0173c* appear to be arranged in an operon (Fig. 4.14). Encoded on the opposite strand are *cj0177* to *tonB1* (*cj0181*; Fig. 4.14). The end of *cj0177* overlaps the beginning of *cj0178*; there is an 11 bp gap from the *cj0178* stop codon to the *exbB1* (*cj0179*) start codon. The genes *exbB1* to *tonB1* (*cj0179* to *cj0181*) overlap and have recently been shown by RT-PCR to be co-transcribed, confirming their organisation and expression as an operon (Palyada *et al.*, 2004). The region between *cj0176c* and *cj0177*, the first genes in each of the putative divergently transcribed operons is 221 bp. Both iron-responsive expression and Fur regulation of *cj0176c-cj0173c* and *cj0177-cj0178* have been shown (van Vliet *et al.*, 1998; Palyada *et al.*, 2004; Holmes *et al.*, 2005). The region is flanked by *cj0172c* (encoding a gene of unknown function) and *cj0182* (encoding a putative transmembrane transport protein; Fig. 4.14). The NCTC 11168 genome has an average GC content of 30.6% (Parkhill *et al.*, 2000). The *cj0176c-cj0173c* region has a GC content slightly below average, *cj0177* and *cj0178* are slightly

higher (Table 4.3); *exbB1*, *exbD1* and *tonB1* have 30%, 28% and 29% GC contents, respectively. For information on genes *cj0173c-cj0178* see Table 4.3.

Table 4.3. *cj0173c-cj0178* information.

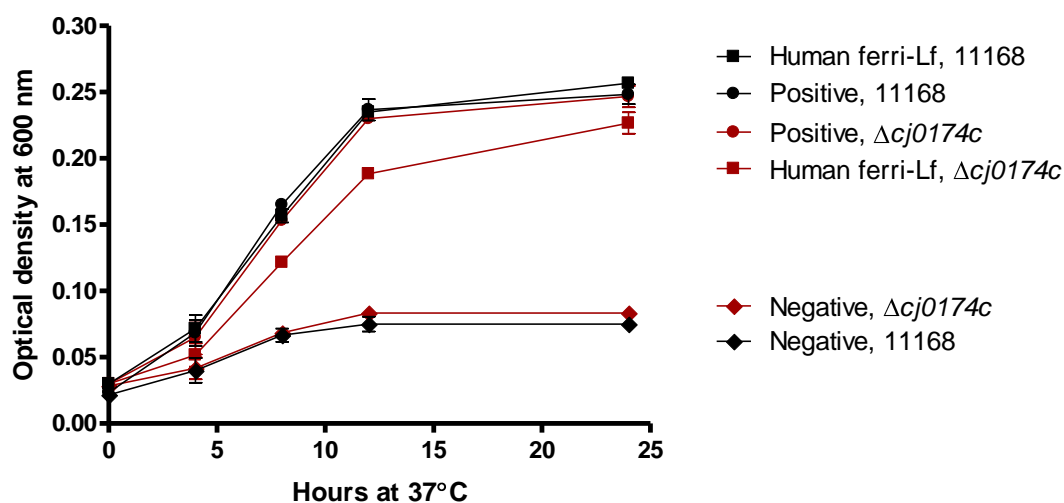
Gene	Function	Gene size (bp)	Protein size (aa)	GC content (%)
<i>cj0173c</i>	ABC transporter system ATP-binding protein.	909	302	26
<i>cj0174c</i>	ABC transporter system permease protein.	1617	538	26
<i>cj0175c</i>	ABC transporter system periplasmic binding protein.	1005	334	30
<i>cj0176c</i>	Putative lipoprotein.	132	43	26
<i>cj0177</i>	Putative lipoprotein.	852	283	29
<i>cj0178</i>	TonB-dependent outer membrane receptor protein.	2268	755	34

There is a 41 bp gap between the stop codon of *cj0173c* and the start codon of *cj0172c* (Fig. 4.14). *cj0172c* was mutated as part of the work completed during this project, resulting in strain CEM1 (Table 2.2; Section 4.2.2). Following transformation of NCTC 11168 with pCEM7 (Table 2.2) the resulting strain CEM1 could not be recovered. Because of sequence similarities, *cj0172c* was predicted to encode a saccharopine dehydrogenase or shikimate reductase, both of which are involved in the biosynthesis of amino acids (lysine or aromatic amino acids respectively, D. Kelly, per. comm.). CEM1 cells were recovered on medium containing either a mixture of the aromatic amino acids phenylalanine, tyrosine and tryptophan, or lysine (500 µg/ml final concentration); the addition of lysine to the medium allowed recovery of the transformants. Lysine is synthesised via the diaminopimelic acid pathway in bacteria and is an essential amino acid (Vogel, 1960; Umbarger, 1978; Garrad and Bhattacharjee, 1992). The data are consistent with *cj0172c* encoding a saccharopine dehydrogenase.

Strains carrying a mutation in either *cj0174c* (CEM3), the permease of the cognate ABC transporter system, or the putative lipoprotein *cj0177* (CEM4; Table 2.2) encoded immediately upstream of *cj0178* were tested for the ability to use iron derived from ferri-Lf

for growth. CEM3 ($\Delta cj0174c::aphA-3$) and CEM4 ($\Delta cj0177::ermC$) were grown in MEM α liquid medium under either iron-restricted (unsupplemented) or iron-replete (10 μ M FeSO₄) conditions, and with iron supplied solely in the form of human ferri-Lf (0.27 μ M) for 24 h with growth monitored at intervals (Fig. 4.15a, b). Strain CEM3 grew comparably to wild-type cells under iron-restricted conditions reaching a final OD₆₀₀ of between 0.05 and 0.10 (Fig. 4.15a). The addition of 10 μ M FeSO₄ allowed CEM3 and wild-type NCTC 11168 growth to be promoted to an OD₆₀₀ of around 0.25 after 24 h. When grown in the presence of 0.27 μ M human ferri-Lf, CEM3 showed a small, but significant decrease in growth compared to wild-type cells (Fig. 4.15a). This may indicate the involvement of, but not essential requirement for, this ABC transporter system in the transfer of iron derived from Lf across the inner cytoplasmic membrane.

a)



b)

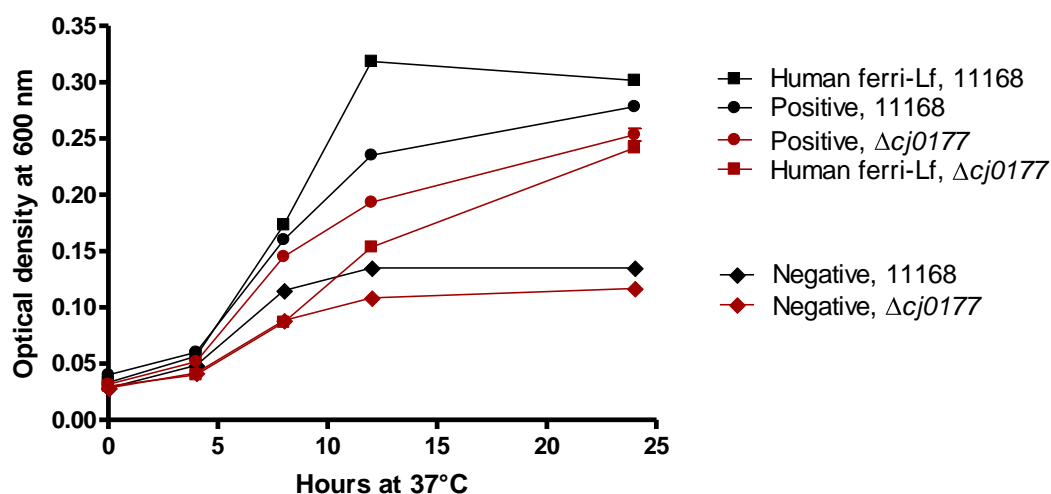


Figure 4.15. Growth assays of *C. jejuni* strains with mutations in components of the *cj0173c-cj0178* system supplied with iron bound to human Lf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and CEM3 ($\Delta cj0174c::aphA-3$ 11168) with human ferri-Lf (0.27 μ M). **b)** NCTC 11168 and CEM4 ($\Delta cj0177::ermC'$ 11168) with human ferri-Lf (0.27 μ M).

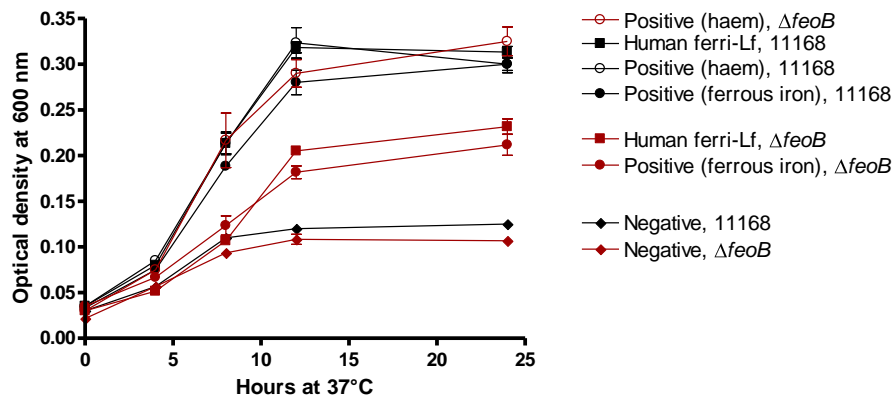
Strain CEM4 grew similarly to wild-type cells under low iron conditions with both strains achieving a final optical density of just above 0.10 (Fig. 4.15b). The addition of ferrous sulphate (10 μ M) or human ferri-Lf (0.27 μ M) promoted the growth of both strains, although at 12 h and 24 h, the growth of CEM4 was significantly less than wild-type with both iron sources (Fig. 4.15b). The difference in growth between the strains was most

significant when the iron source was human ferri-Lf; however, because of the difference noted with the positive control, part of the response seen may be a growth defect. CEM4 grew slightly less than wild-type cells in the MHB control (Fig. 4.10b), and this difference is comparable to the difference seen with the positive control in this experiment (Fig. 4.15b). The lower growth of CEM4 compared to wild-type when human ferri-Lf was provided as an iron source was considerably more marked at 12 h than 24 h (Fig. 4.15b). By 24 h, the difference between CEM4 and NCTC 11168 growth with ferri-Lf was, however, still about double that of the difference seen under positive control conditions (Fig. 4.15b). This may imply that Cj0177 does in fact play a role in the successful acquisition of iron from human ferri-Lf for growth, but is likely to be non-essential. The phenotype requires confirmation by further investigation.

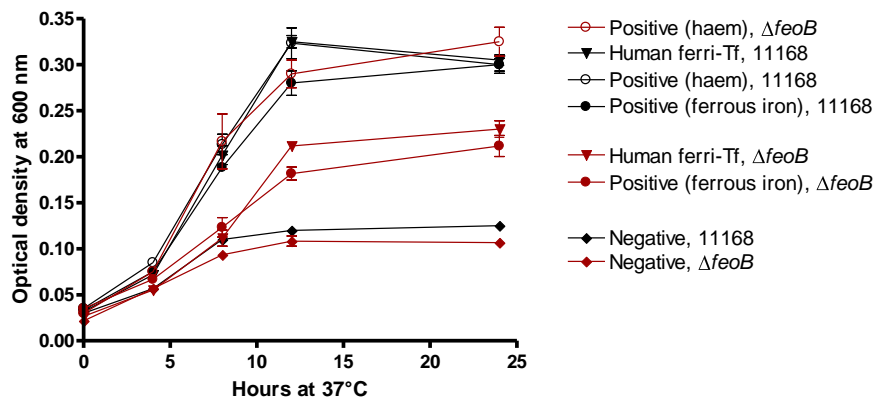
4.2.3.4. Requirement for a Ferrous Iron Uptake System

The Fe^{2+} transporter FeoB was inactivated by mutating *feoB* ($\Delta feoB::ermC'$; Table 2.2) in NCTC 11168 (mutation completed by R. D. Haigh) and the mutant strain was tested for growth in the absence of iron, and in the presence of ferrous sulphate (10 μM), porcine haem (25 μM) or human ferri-Lf (0.27 μM) in MEM α over a period of 24 h with samples taken at intervals. Similar patterns of growth promotion were seen when cells were supplied with haem as a sole source of iron, with both the wild-type and mutant strain reaching a final OD₆₀₀ of 0.30 to 0.35 (Fig. 4.16a). A final optical density of just above 0.10 was reached by both strains under iron-restriction (Fig. 4.16a). When supplied with ferrous sulphate (10 μM), the wild-type strain grew similarly to when provided with porcine haem (25 μM), but the *feoB* mutant strain grew less well than the wild-type strain, reaching a significantly lower final OD₆₀₀ of around 0.20 (Fig. 4.16a). This implies that a mutation in *feoB* causes a defect in the cell's ability to acquire Fe^{2+} . In addition to this phenotype, there was a statistically significant difference in the growth of the *feoB* mutant strain and the wild-type strain when human ferri-Lf (0.27 μM) was the iron source (Fig. 4.16a). The mutant strain reached a final OD₆₀₀ of just above 0.20 compared to the wild-type strain, which achieved just above 0.30 (Fig. 4.16a). This implies that some of the iron derived from ferri-Lf may be transported across the inner membrane in the reduced form. As there was a growth difference, the mutant strain was assessed for the ability to grow with either human ferri-Tf or ferri-ovo-Tf (Fig. 4.16b, c).

a)



b)



c)

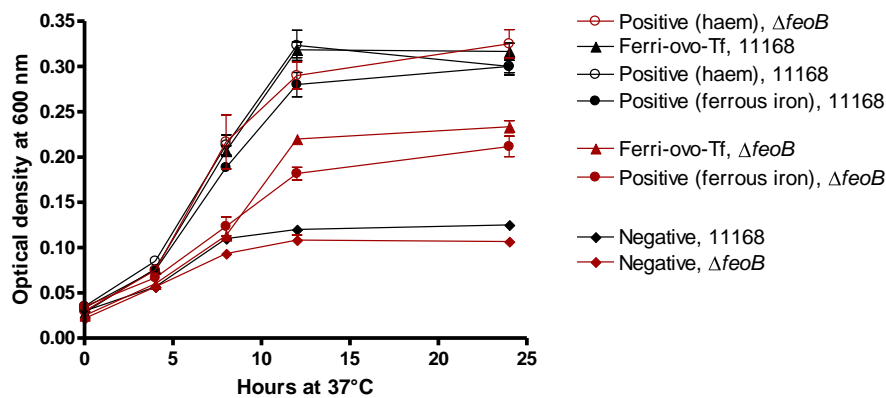


Figure 4.16. Growth assays of *C. jejuni* strain $\Delta\text{feoB}::\text{ermC}'$ 11168 with iron supplied bound to human Lf, human Tf or ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μM FeSO_4 (iron-replete conditions, positive), 25 μM porcine haem (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and $\Delta\text{feoB}::\text{ermC}'$ 11168 with human ferri-Lf (0.27 μM). **b)** NCTC 11168 and $\Delta\text{feoB}::\text{ermC}'$ 11168 with human ferri-Tf (0.27 μM). **c)** NCTC 11168 and $\Delta\text{feoB}::\text{ermC}'$ 11168 with ferri-ovo-Tf (0.27 μM).

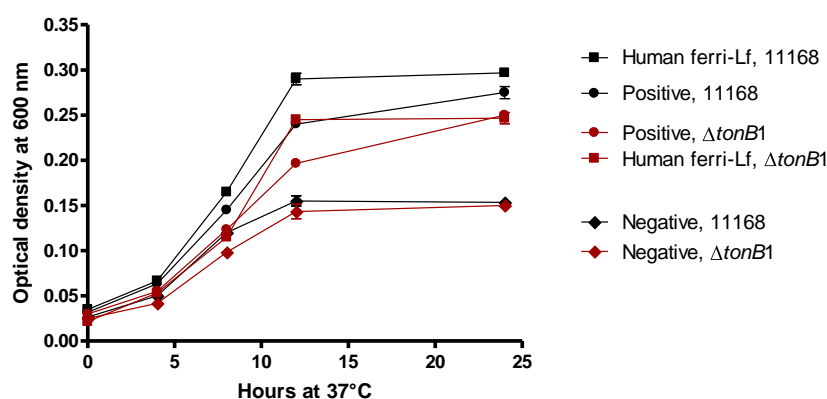
When supplied with either human ferri-Tf or ferri-ovo-Tf, the *feoB* mutant strain grew comparably to when it was supplied with human ferri-Lf (Fig. 4.16b, c). Growth differences in the presence of human ferri-Lf, human ferri-Tf and ferri-ovo-Tf at 24 h were significantly different from wild-type. All controls were repeated as part of the human ferri-Tf and ferri-ovo-Tf growth experiments and were as described for the human ferri-Lf assay (Fig. 4.16a-c).

4.2.3.5. TonB Dependency

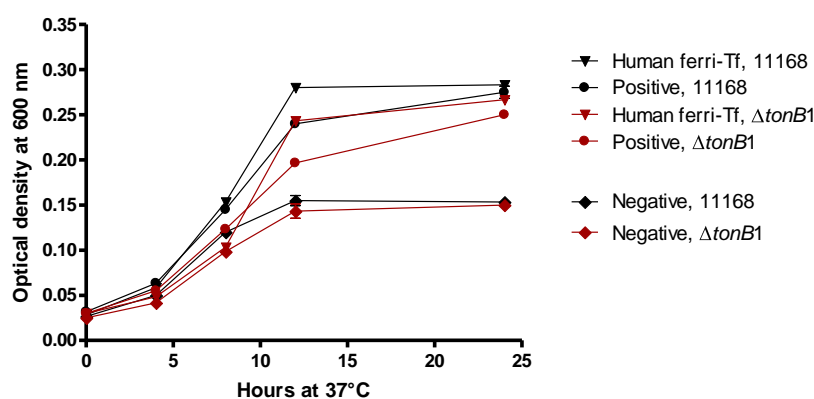
The dependency of the process of iron uptake from human ferri-Lf, human ferri-Tf and ferri-ovo-Tf on the ExbBD-TonB system encoded downstream of *cj0178* was tested. TonB1 was chosen as *cj0181* it is encoded near to *cj0178* in the genome, the product of which appears to be involved in iron uptake from the Tfs. Involvement of the other ExbBD-TonB systems was not investigated as part of this study. Growth of a strain carrying a mutation in *tonB1* (JDR20, $\Delta tonB1::cat$, Table 2.2) was monitored in MEM α liquid medium over a 24 h period with samples taken at intervals.

Following initial iron-depletion overnight, strain JDR20 grew comparably to wild-type under low iron conditions, reaching a final OD₆₀₀ of around 0.15 (Fig. 4.17a-c). The presence of iron supplied in the form of ferrous sulphate (10 μ M), human ferri-Lf (0.27 μ M), human ferri-Tf (0.27 μ M) or ferri-ovo-Tf (0.27 μ M) promoted the growth of JDR20 and wild-type NCTC 11168 cells (Fig. 4.17a-c). However, there was a small, but significant difference in growth between JDR20 and NCTC 11168 with ferrous sulphate at 12 h and 24 h (Fig. 4.17a-c). No difference was seen between JDR20 growth and wild-type growth during the MHB control assay (Fig. 4.10b). The difference in growth observed between the two strains is again significant by 24 h in the presence of human ferri-Lf and ferri-ovo-Tf (Fig. 4.17a-c). This difference is comparable to the difference seen between the strains under positive control conditions. This result is inconclusive and may reflect the loss of an energy transduction system and the effect this has on cellular growth, rather than a direct ferri-protein-dependent phenotype.

a)



b)



c)

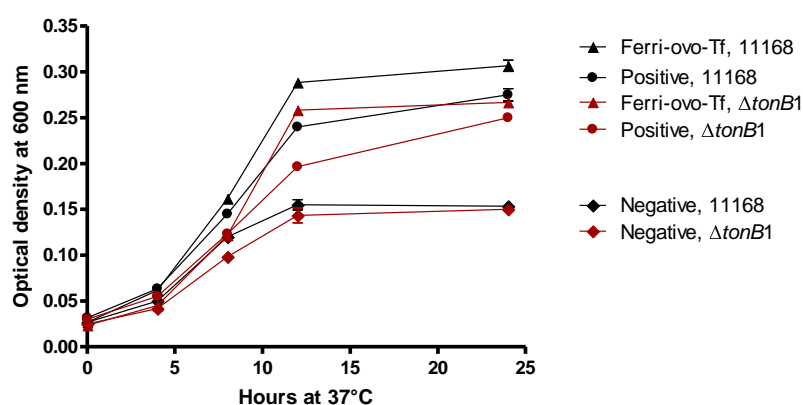


Figure 4.17. Growth assays of *C. jejuni* strain JDR20 ($\Delta tonB1::cat$ 11168) supplied with iron bound to human Lf, human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and JDR20 with human ferri-Lf (0.27 μ M). **b)** NCTC 11168 and JDR20 with human ferri-Tf (0.27 μ M). **c)** NCTC 11168 and JDR20 with ferri-ovo-Tf (0.27 μ M).

4.2.4. Production of Double Mutant Strains

4.2.4.1. Mutation Strategy

The inactivation of *cj0178*, and also of *cfrA* and *feoB* limited, but did not fully prevent, growth in the presence of human ferri-Lf. By inactivating two genes in a single strain, growth may be inhibited if both systems are simultaneously required for iron uptake from this source.

Double mutations were generated using strain KAR2 (*cj0178::aphA-3*) and inactivating either *feoB* or *cfrA* in this mutant background. KAR2 was used as the disruption of *cj0178* caused the most marked phenotypic effect (Fig. 4.11, Fig. 4.12, Fig. 4.16). By naturally transforming (Section 2.12.6) KAR2 cells with CEM5 ($\Delta cfrA::aphA-3$) DNA, a strain carrying mutations in both *cj0178* and *cfrA* was produced, both of which were interrupted with a cassette conferring resistance to kanamycin. Because of this, strains carrying the mutant copies of both *cfrA* and *cj0178* were hard to distinguish from a *cj0178* single mutant strain. Cells were therefore recovered on selective medium containing a higher concentration of kanamycin than standard. KAR2 was also naturally transformed with DNA prepared from $\Delta feoB$ 11168 ($\Delta feoB::ermC'$). Medium containing the appropriate antibiotics was used to select cells in which allelic replacement of the gene with the selectable marker had occurred. The resulting strains were named CEM11 (KAR2, $\Delta cfrA::aphA-3$) and CEM12 (KAR2, $\Delta feoB::ermC'$; Table 2.2). Mutation of both loci in CEM11 and CEM12 were verified by PCR screening and sequencing using the original cloning primers and cassette specific primers (Table 2.3, Appendix 2).

Wild-type cells cannot grow on the standard working concentration of kanamycin (50 µg/ml, Table 2.1) used to select cells carrying a single kanamycin resistance cassette. Prior to selection of CEM11, a kanamycin titration with KAR2 was performed to determine the level of kanamycin that cells carrying a single resistance cassette could survive on. Following initial growth on standard kanamycin-supplemented plates, cells were swabbed onto fresh plates containing varying amounts of kanamycin and incubated for up to 5 days, the length of time normally used to allow transformants to grow on selective medium following natural transformation. Cells survived on up to 17 times the standard amount of antibiotic. Cells did not survive when grown on plates containing 19 times more kanamycin than standard. This concentration (19 times more) was therefore used as a selection medium supplement to favour cells carrying a double mutation. As single mutant

cells should be prohibited from growth, the presence of colonies should indicate the presence of two resistance cassettes in the chromosome. These concentrations also reduced the likelihood of false positive large single mutant colonies, as seen on concentrations above 19 times the standard, which may be due to spontaneous kanamycin resistant cells selected by the increased concentration. Both strains were then tested for growth with iron supplied in the form of human ferri-Lf.

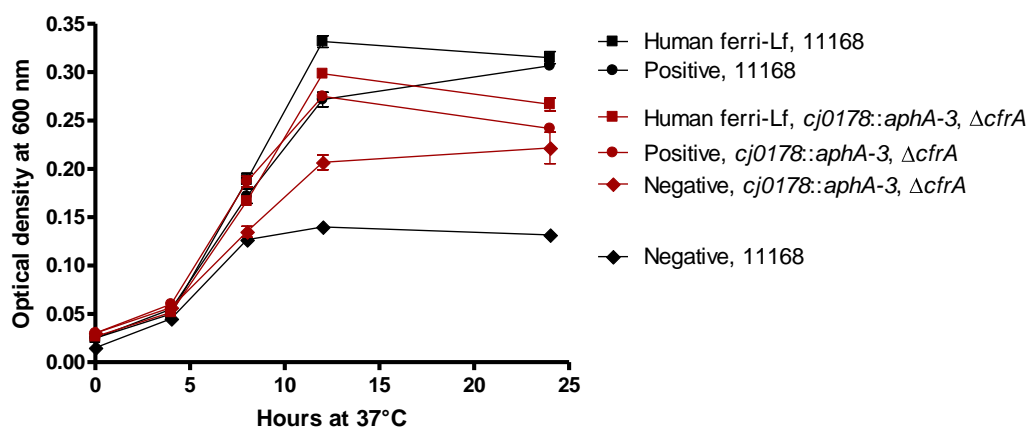
4.2.4.2. Phenotypic Analysis of Double Mutant Strains

In order to test the effect of the double mutations, strains CEM11 (KAR2, $\Delta cfrA::aphA-3$) and CEM12 (KAR2, $\Delta feoB::ermC'$; Table 2.2) were grown in liquid medium over 24 h supplied solely with human ferri-Lf as an iron source (Fig. 4.18a, b). MEM α was again used as a defined iron-limited growth medium and samples were taken at intervals. Following initial overnight iron-depletion, NCTC 11168 cells grew as expected with iron-restriction, high iron conditions (either 10 μ M FeSO₄ or 25 μ M haem) or human ferri-Lf (0.27 μ M; Fig 4.18a, b). Strain CEM12 grew similarly to the strains carrying a single mutation in either *cj0178* or *feoB*, demonstrating good growth with haem as an iron source reaching a final optical density of around 0.35 (Fig. 4.18b). Poor growth was achieved in the absence of iron with CEM12 reaching an OD₆₀₀ comparable to NCTC 11168 cells at 24 h of between 0.10 and 0.15 (Fig. 4.18b). Again, the loss of *feoB* resulted in significantly less growth with FeSO₄ than wild-type cells by 24 h (see Fig. 4.16); the loss of *cj0178* seemed to have no effect on this growth pattern. Also, significantly less growth was achieved by CEM12 with 0.27 μ M human ferri-Lf when compared to wild-type, reaching a final OD₆₀₀ of between 0.20 and 0.25, rather than 0.30 at 24 h, similar to CEM12 growth with FeSO₄ (Fig. 4.18b).

Strain CEM11 did not show growth patterns similar to wild-type cells, and these growth differences were significant under high-iron conditions, low-iron conditions and with human ferri-Lf (Fig. 4.18a). CEM11 grew consistently and significantly more than NCTC 11168 under iron restriction reaching a final optical density of above 0.20 rather than between 0.05 and 0.15 as seen with the other strains (Fig. 4.18a). Strain CEM11 grew significantly less than wild-type cells with 10 μ M FeSO₄ (Fig. 4.18a). This did not occur when either *cfrA* or *cj0178* were mutated individually (Fig. 4.11c, d). The single mutants grew to an OD₆₀₀ of between 0.25 and 0.30 by 24 h with ferrous sulphate (Fig. 4.11c, d), similar to the OD₆₀₀ reached by CEM11 (Fig. 4.18a). Wild-type cells had grown slightly

more with ferrous sulphate by 24 h; however growth patterns of both wild-type and CEM11 cells were comparable up to 12 h (Fig. 4.18a). In the presence of human ferri-Lf, strain CEM11 grew significantly less than wild-type cells reaching a final optical density of just above 0.25 rather than between 0.30 and 0.35 (Fig. 4.18a). This difference is statistically significant at 24 h, but surprisingly less than when either gene is mutated alone. If two genes are involved in a process and both are knocked out, it would be expected that there would be a more marked phenotype in the absence of both. This, taken with the negative control results may indicate a problem with mutant strain growth. Cells were streaked onto fresh agar plates following the assay from 24 hour cultures and identified as *C. jejuni* from colony morphology compared to wild-type cells. There were no contaminants and the maintenance of both mutations within the chromosome of CEM11 was confirmed following 24 h growth by colony PCR screening.

a)



b)

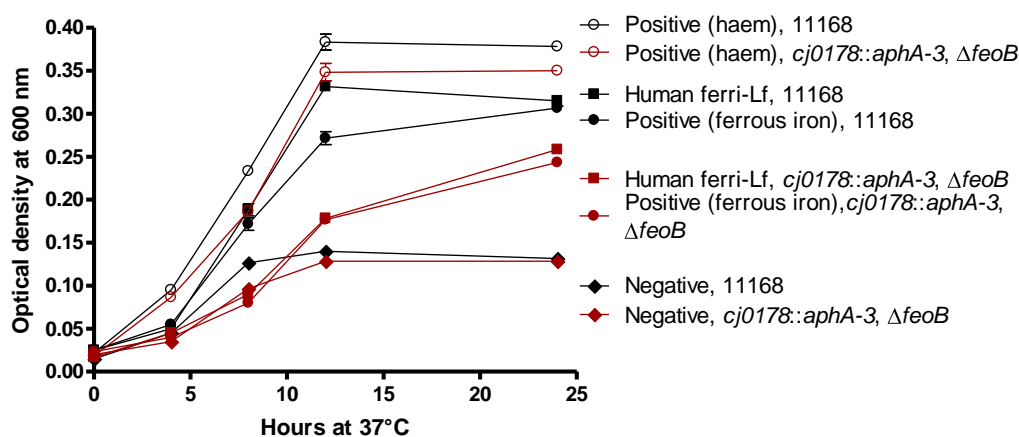


Figure 4.18. Growth assays of *C. jejuni* strains CEM11 (KAR2, $\Delta cfrA::aphA-3$ 11168) and CEM12 (KAR2, $\Delta feoB::ermC'$ 11168) supplied with iron bound to human Lf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), 25 μ M porcine haem (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and CEM11 with human ferri-Lf (0.27 μ M). **b)** NCTC 11168 and CEM12 with human ferri-Lf (0.27 μ M).

4.3. Discussion

4.3.1. Mutagenesis of Iron Uptake Genes

Investigation of the *C. jejuni* genomes for homologues of the Tf-binding protein A of *N. meningitidis* revealed the presence of no clear homologues, but the known major outer membrane receptor proteins Cj0178, CfrA and ChuA of strain NCTC 11168 and Cj0444 of strain 81-176 showed the highest levels of identity of all *Campylobacter* iron-associated proteins (Fig. 4.2). As a result, it was decided that all known iron uptake systems would be tested for a role in the uptake of human ferri-Lf-, human ferri-Tf- or ferri-ovo-Tf-bound iron. In order to complete this, a number of strains carrying mutations in key genes from all systems were tested for growth in the presence of human ferri-Lf. PCR amplification was successfully achieved for all genes. Where optimisation was required (Section 2.10.1), parameters were altered to improve product yield and reduce misprimed products. Cloning of *C. jejuni* genes is difficult due to the AT rich genome (Parkhill *et al.*, 2000). Constructs containing *C. jejuni* DNA transformed into *E. coli* can be recognised as ‘promoter’ regions or cause problems due to different codon usage or methylation patterns (Ketley, 1997). Some initial constructs, mostly containing the larger inserts, had deletions or rearrangements within the cloned DNA following transformation into *E. coli*. It is generally assumed that plasmid constructs transformed into *E. coli* are stable. Different sized plasmids or plasmids with different copy numbers can affect bacterial fitness measured as growth rate (Smith and Bidochka, 1998). In both rich and minimal selective media, maintaining a large plasmid has a detrimental effect on fitness, causing a significantly longer lag phase. In nonselective medium, subcultured cells have been shown to lose plasmids containing the largest inserts at a greater rate. In addition, the lowest plasmid copy number was seen when cells contained the construct with the largest insert (Smith and Bidochka, 1998). The same study found that plasmid loss was positively correlated with size when *E. coli* cells were grown on minimal medium; maintenance of large plasmids and/or high copy number plasmids was proposed to be detrimental to cell fitness under nutrient stress (Smith and Bidochka, 1998). This proposal contradicts the hypothesis that starved cells do not lose their plasmids, but in fact do not express the enzymes needed to inactivate selective antibiotics (Griffiths *et al.*, 1990). In order to limit the problems encountered, a reduction in plasmid size was recommended (Smith and Bidochka, 1998). This is difficult for constructs containing *C. jejuni* DNA due to the

necessity for flanking regions, and so screening of a large number of transformants to identify correct constructs was necessary during this study. Inverse PCR mutagenesis was used to inactivate genes in the *C. jejuni* chromosome by deletion of the majority of the gene and insertion of a selectable marker. Insertion of an antibiotic resistance cassette into the genome may have an effect on the downstream genes, particularly if the genes are found in an operon. The kanamycin resistance gene used had a promoter but no recognisable terminator signal sequence. The erythromycin resistance gene had a promoter and terminator sequence, possibly leading to problems of polarity.

4.3.2. Phenotypic Testing of Single Mutant Strains

Initially, all mutant strains produced during the course of this study and those made by other members of the group used during this work were assessed for growth under rich conditions (MHB) compared to the wild-type strains (Fig. 4.10a-c). Only the 81-176 strain with a mutated copy of *cj0444* showed different growth patterns from wild-type cells at 4, 8, 12 and 24 h (Fig 4.10a, c). This caused a problem for phenotypic testing of the mutant strain as any growth differences seen during growth using Tf-derived iron may be a general defect caused by the loss of *cj0444*. The mutant strain was constructed using a kanamycin resistance cassette. Reconstruction of the mutant using a selectable marker without a promoter or terminator, leaving the expression of the antibiotic resistance under the control of the native promoter may alleviate this problem. Complementation of this mutation and subsequent growth in MHB would determine whether the effect seen is due to the loss of *cj0444*.

Mutant strains of interest were then tested for the involvement of each mutated gene in the process of iron uptake from human ferri-Lf. There appears to be a primary role for the outer membrane receptor protein Cj0178 and a potential role for the associated ABC transport system Cj0173c-Cj0175c in this process. No significant difference was seen between the growth of mutant strains JDR5 ($\Delta chuA::cat$) and KAR3 (*p19::aphA-3*, periplasmic binding protein mutant, no known outer membrane receptor protein for this system at the time of this study) when compared to wild-type in the presence of human ferri-Lf (Fig. 4.11a, b). The ChuABCDZ system has been previously characterised as the haem-uptake system in *C. jejuni* NCTC 11168 (Ridley *et al.*, 2006) and the Cj1658-Cj1663 system is thought to be involved in the uptake of iron derived from ferri-rhodotorulic acid

(Stintzi *et al.*, 2008). These systems do not appear to be involved in the transport of iron from ferri-Lf.

Significantly less growth than wild-type was observed by 24 h when both strains CEM5 ($\Delta cfrA::aphA-3$) and KAR2 ($cj0178::aphA-3$) were grown with human ferri-Lf; the most marked difference was seen with KAR2 (Fig. 4.11c, d). CfrA has previously been identified as the ferri-enterochelin outer membrane receptor protein (Palyada *et al.*, 2004). An agar plate assay consisting of iron-restricted medium containing a known number of *C. jejuni* cells with sterile discs containing enterochelin laid on the agar surface was used. The zone of growth surrounding the iron source was measured following 24 h of microaerophilic incubation. A large halo was seen when NCTC 11168 and a $cj0178$ mutant strain were tested. There was no growth when a $cfrA$ mutant strain was tested; a $ceuE$ mutant strain was slightly impaired. The results support the hypothesis that $ceuE$ encodes a protein that is involved in the process of iron uptake from ferri-enterochelin, but is not essential. The authors concluded that CfrA is the outer membrane receptor protein responsible for ferri-enterochelin uptake, and that Cj0178 is not involved in this process and is therefore a receptor for another iron source (Palyada *et al.*, 2004). The role of CfrA in the process of iron uptake from human ferri-Lf may be direct, or could possibly involve the transport of iron while the Lf is bound by another receptor. To confirm this phenotype, the mutation in $cfrA$ should be complemented; however due to time constraints, this mutation was not complemented as part of the work for the current study. The complemented strain has subsequently been produced by another member of the group, but was not available in time for testing (R. D. Haigh).

The most marked phenotype was seen when $cj0178$ was mutated (Fig 4.11d). The most significant difference of all mutants from wild-type growth was observed when KAR2 was grown with human ferri-Lf, but growth was not completely abolished (Fig. 4.11d). When human ferri-Tf or ferri-ovo-Tf were tested, the mutant strain was again shown to grow significantly less than wild-type cells under these conditions (Fig. 4.11a, b). It is unclear why human ferri-Tf allowed more growth than either human ferri-Lf or ferri-ovo-Tf, but the difference in growth between KAR2 and NCTC 11168 in the presence of ferri-Tf was significant (Fig. 4.11d, Fig. 4.12a, b). NCTC 11168 is most likely to associate with the mucosal secretions in the chicken and human host where ferri-ovo-Tf and ferri-Lf, respectively, are found. The ferri-Tf assays may also have been contaminated with trace amounts of iron during set-up or in the medium. Mutation of $cj0178$ has also been shown to result in reduced colonisation ability in a chick caecum model and rabbit ileal loop

model (Palyada *et al.*, 2004; Stintzi *et al.*, 2005) indicating the necessity for Cj0178 *in vivo*. In addition, work completed prior to this study (Rock, 2003) demonstrated by ^{55}Fe -uptake experiments that the mutation of *cj0178* reduced the uptake of iron from ferri-Lf into the cell to approximately half that seen with the wild-type strain (Fig. 4.19).

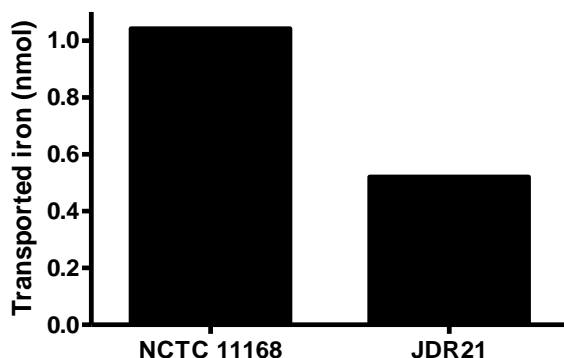


Figure 4.19. Radioactive ^{55}Fe -uptake from ferri-Lf by *C. jejuni*. All experiments were completed using 1 ml of culture at $2\text{--}3 \times 10^8$ cfu/ml. Radioactivity is represented as nmol of ^{55}Fe , taking into account the specific activity of the ^{55}Fe (5 Ci/g), converted to disintegrations per minute, assuming 4% efficiency. Cell-associated radioactivity of *C. jejuni* NCTC 11168 and the *cj0178* (JDR21, Chapter 2, Table 2.2) mutant strain measured from cells incubated in SAPI medium containing ^{55}Fe -Lf as an iron source. The radioactivity associated with the mutant strain was around half (49.93%) that of the wild-type strain (Miller *et al.*, 2008).

The growth data, taken with the ^{55}Fe data, demonstrate that *cj0178* is required for the uptake and use of iron from Lf, and confirms that the loss of *cj0178* reduces, but does not totally abolish, the uptake of Lf-derived iron into cells. Repetition of the ^{55}Fe work using ^{55}Fe -Tf and ^{55}Fe -ovo-Tf would confirm a role for Cj0178 in the uptake of iron from all of these proteins. Cj0178 was also found first in the search for proteins most similar to the *N. meningitidis* TbpA homologue in strain NCTC 11168 (Fig. 4.2). The observation that KAR2 cannot grow well using iron derived from any of the proteins tested implies that Cj0178 is involved in iron uptake from Lf, Tf and ovo-Tf. This is different from *Neisseria* spp., which instead have separate receptors for ferri-Lf and ferri-Tf (Gray-Owen and Schryvers, 1996). In the *C. jejuni* genome, there are also no obvious accessory proteins equivalent to TbpB/LbpB, which are thought to assist TbpA/LbpA in distinguishing between iron-bound and iron-free Tf or Lf in *Neisseria* (Cornelissen and Sparling, 1996). The potential involvement of two outer membrane receptor proteins, where the loss of either does not fully abolish growth, is different from the phenotypes seen when *chuA* or

cfrA were mutated and supplied with haem or ferri-enterochelin respectively (Palyada *et al.*, 2004; Ridley *et al.*, 2006). Neither mutant strain could grow at all, indicating the essential involvement of both in the transport of their specific iron source. The uptake mechanism for iron from the Tfs in *C. jejuni* appears to be novel. As the loss of Cj0178 caused a major phenotypic difference genetic complementation was attempted and further investigation of the *cj0173c-cj0178* region was completed (Chapter 5).

As *C. jejuni* strain 81-176 lacks *cj0178* and *cfrA* (Hofreuter *et al.*, 2006), it was surprising that the strain grew slightly better than NCTC 11168 when supplied with iron from human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (Fig. 3.4). As strains that lack *cj0178* and *cfrA* carry a functional copy of the NCTC 11168 pseudogene *cj0444* (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007), Cj0444 was a potential candidate for a Lf or Tf receptor protein in 81-176. This was supported by the highest identity (26%) to TbpA of all proteins found during the initial search (Fig. 4.2). There were, however, growth defects as described above with this strain under control conditions (Fig 4.10a, c). The 81-176 strain carrying a mutation in *cj0444* showed patterns of growth different from wild-type cells under iron-replete and iron-restricted conditions (Fig. 4.13a-c). In addition, as the strain reached a similar final optical density to wild-type cells when supplied with iron from the Tfs (Fig. 4.13a-c) it would appear that Cj0444 is not involved in this process, but clear conclusions cannot be drawn due to the differences in growth patterns between the mutant strain and wild-type 81-176 under control conditions (Fig. 4.10a, c; Fig. 4.13a-c). If Cj0444 is not involved in the uptake of Tf-derived iron in 81-176, it is not clear at present what could act as the receptor in this strain (Hofreuter *et al.*, 2006).

The small, but significant decrease seen in CEM3 ($\Delta cj0174c::kan$) growth when supplied with human ferri-Lf suggests a minor role for the ABC transport system Cj0173c-Cj0175c (Fig. 4.15a). Further ABC transporter systems in the NCTC 11168 genome (Palyada *et al.*, 2004; Holmes *et al.*, 2005; Ridley *et al.*, 2006) may complement Cj0173c-Cj0175c, resulting in the limited phenotype observed. Some redundancy of inner membrane ABC transporter systems has been observed for haem uptake in a range of bacteria (Ridley *et al.*, 2006), and ABC transporter system double mutants grew significantly less well than single mutant strains when supplied with haem as the sole source of iron. A small, but significant decrease in growth in the presence of haem was seen when mutant strain JDR6 ($\Delta chuB::cat$) was compared to wild-type NCTC 11168 (Ridley *et al.*, 2006). Growth assays performed by an undergraduate project student supervised as part of this work

showed that all ABC transporter systems may be involved in the transport of haem, since the same small but significant difference in growth was seen with strains KAR3 (*p19::aphA-3*), CEM3 (Δ *cj0174c::aphA-3*) and CEM6 (Δ *ceuE::aphA-3*). The student constructed double mutant strains using natural transformation of KAR3, CEM3 or CEM6 DNA into the JDR6 background producing strains CEM15 (JDR6, Δ *cj0174c::aphA-3*), CEM16 (JDR6, Δ *ceuE::aphA-3*) and CEM17 (JDR6, *p19::aphA-3*; Table 2.2). All strains were verified by PCR and sequencing across the mutant regions. When grown in the presence of haem as a sole iron source, the double mutant strains showed a significant decrease in growth that was twice that of the single mutant strains. This implied that all ABC transporter systems were involved in the transfer of haem across the inner membrane, with a loss of growth proportional to the loss of the transporters; this may also be the case for iron derived from the Tfs. Alternatively, the limited phenotype of the *cj0174c* mutant when grown in the presence of human ferri-Lf may be due to an as yet uncharacterised mechanism for uptake. In order to establish whether the *cj0174c* effect is also seen with any of the other ABC transporter mutants, single and double mutants in all known ABC transporter systems could be tested for growth with human ferri-Lf as a sole source of iron. Because of the apparent redundancy of the ABC transporters, the small decrease in growth seen when a single system is lost may also occur when NCTC 11168 cells lacking that system are supplied with any of the other iron sources. To confirm the use of ATP to drive inner membrane transport of the iron derived from any of the Tfs, ATP dependent transport systems and cytoplasmic membrane proton motive force could be disrupted. If the acquisition of radioactive ^{55}Fe from ^{55}Fe -loaded protein was dependent on ATP, uptake should be impaired in the presence of sodium azide and carbonylcyanide *m*-chlorophenylhydrazone (Rohde *et al.*, 2002)

Strain CEM4 (Δ *cj0177::ermC'*) appeared to grow less than wild-type cells in MHB culture (Fig. 4.10a, b), and under iron-replete and iron-restricted conditions in MEM α liquid culture (Fig. 4.15b); the differences were significant indicating a problem with the controls. When supplied with human ferri-Lf, growth of CEM4 was also significantly less than wild-type with the difference around double that of the other differences seen at 24 h; the difference was even greater at 8 h and 12 h, implying that there may be a ferri-Lf effect (Fig. 4.15b). *Cj0177* may, therefore, play a role in the acquisition of iron from human ferri-Lf for growth, but is likely to be non-essential as growth was not abolished. There may also be a problem with the type of cassette used to inactivate *cj0177*. The erythromycin cassette carries a terminator which could prevent the expression of downstream genes;

cj0177 is upstream of, and encoded in the same direction as, *cj0178*. If the expression of *cj0178* had been reduced by the mutation, the phenotype seen when KAR2 was grown with human ferri-Lf would be expected (Fig. 4.11d); however the phenotype was not as great as when *cj0178* was mutated (Fig. 4.15b). To confirm the phenotype, the *cj0177* mutation could be complemented, and expression of the transcript from *cj0177* in the mutant strain could be compared to the wild-type expression patterns. However, due to time constraints, this mutation was not complemented and transcripts were not analysed. The role of Cj0177 in this process requires further investigation and characterisation. The mutant strain could be re-constructed using a cassette lacking a terminator or promoter to establish whether the differences seen are due to the way the mutant was produced. If the differences are real, there may be a non-essential role for Cj0177 in the process of iron-uptake from human ferri-Lf. However, as Cj0177 does not demonstrate similarity to TbpA or TbpB, Cj0177 may have a different function in *C. jejuni*.

As a mutation in the *feoB* gene had been constructed by another group member (R. D. Haigh) during the course of this study, growth patterns of the mutant strain were compared to wild-type in the presence of human ferri-Lf, human ferri-Tf, ferri-ovo-Tf, haem and ferrous sulphate (Fig. 4.16a-c). MHB, iron-restricted and iron-replete control conditions were comparable between mutant and wild-type cells indicating that this mutation does not appear to have a detrimental effect on normal growth (Fig. 4.10a-c, Fig. 4.16a-c). A previous report concluded that the Fe^{2+} transporter of *C. jejuni*, FeoB, does not play a role in the transport of Fe^{2+} (Raphael and Joens, 2003), but subsequent to this a second study established a role for FeoB in the transport of Fe^{2+} across the inner membrane (Naikare *et al.*, 2006). The data presented in the second study showed that the mutant strain internalised about half as much Fe^{2+} as the wild-type strain and was compromised when colonising the chick caecum (Naikare *et al.*, 2006). The data presented here are in agreement with the second study; the mutant strain reached a final optical density of around half that reached by the wild-type strain; the difference is statistically significant. This confirms a role for FeoB in Fe^{2+} transport and demonstrates that the iron is used for growth. In addition, there appeared to be a phenotype caused by the mutation of *feoB* when cells were grown with human ferri-Lf; this was reproduced when iron was alternatively supplied as human ferri-Tf or ferri-ovo-Tf (Fig. 4.16a-c). Again, around half the growth of the wild-type strain was achieved by the mutant strain. This implies that FeoB plays a role in the transport of iron derived from the ferri-proteins. Iron may be liberated from the ferri-protein at the cell surface as Fe^{3+} and then transported through any of the inner membrane

ABC-transporter systems. Some Fe^{3+} may be reduced to Fe^{2+} at the cell surface, or within the periplasm following transport across the outer membrane. Fe^{2+} either having diffused through the outer membrane porins or having been produced by reduction would then be transported across the inner cytoplasmic membrane by the FeoB transporter. Alternatively, some Fe^{2+} may be transported across the inner membrane via the Cj0175c-Cj0173c ABC transporter system as Cj0175c has been shown to unusually and preferentially bind free Fe^{2+} over free Fe^{3+} (Tom-Yew *et al.*, 2005). As seen with KAR2, this phenotype applies to all ferri-proteins, indicating a lack of specificity which is not seen in other organisms. If iron is reduced at the cell surface, this would imply that NCTC 11168 possesses a cell surface-associated ferric reductase, candidate genes for which do not appear to be present in the genome (Parkhill *et al.*, 2000). Reductase activity has been shown using a ferrozine-based assay (R. D. Haigh, per. comm.) and the detection of *C. jejuni* ferric reductase activity has also recently been published (Crossley *et al.*, 2007). The phenotype seen with the mutant strain in the presence of human ferri-Lf is not a general property as haem utilisation is unaffected (Fig. 4.16a-c). The mutation was not complemented as part of this study or by another member of the group during this study. It is very unusual to have both a Fe^{2+} iron transport system and Fe^{3+} outer membrane receptor protein system involved in the same process.

The energy transduction system located immediately downstream of *cj0178* was tested for involvement in the process of iron uptake from human ferri-Lf, human ferri-Tf or ferri-ovo-Tf. Genes *cj0179-cj0181* encode the ExbBD-TonB1 system responsible for energy transduction to the outer membrane (Parkhill *et al.*, 2000). The differences between the growth of a mutant in *tonB1* (strain JDR20, $\Delta\text{tonB1}::\text{cat}$) and wild-type cells were consistent and significant under iron-replete control conditions and when provided with ferri-Lf and ferri-ovo-Tf (Fig. 4.17a-c). There were no growth differences between JDR20 and wild-type NCTC 11168 cells when grown in MHB (Fig. 4.10). The differences may imply a role for TonB in the uptake of Lf-bound iron or may be due to growth defects in the mutant strain due to the loss of an energy transduction system. Without the loss of the other two ExbBD-TonB systems encoded in the NCTC 11168 genome (Parkhill *et al.*, 2000), complementation may be occurring. The differences are too small to conclude that there is a definite Lf effect. In addition, disruption of any of the three *tonB* genes in NCTC 11168 has been shown to reduce growth under iron-restriction. TonB3 was associated with the use of ferri-enterochelin as an iron source. No *tonB* mutant strains had a problem using haemin as a sole source of iron (Naikare and Stintzi, 2006). Mutation of two or more

energy transduction systems in the same strain may lead to non-viable cells, meaning that this method may not be successful to determine whether Lf-derived iron uptake is TonB dependent. TonB1 was chosen due to its proximity to *Cj0178* (Parkhill *et al.*, 2000), but any or none of the systems may be involved in the energy transduction for Lf-derived iron uptake. The use of the test detailed above for the involvement of ABC transporter systems in the process should also disrupt energy transduction to the outer membrane due to the loss of proton motive force at the inner membrane (Rohde *et al.*, 2002). This may not allow distinction between the loss of the ATP-dependent inner membrane ABC transporter and the ExbBD-TonB energy transduction systems, but it would confirm energy dependency. The process of iron uptake from Tf in *H. influenzae* has been shown to be dependent upon an ExbBD-TonB system (Gray-Owen and Schryvers, 1996). A strain carrying *cj0178* with mutations in the TonB-box could be tested for the use of ferri-Lf, ferri-Tf or ferri-ovo-Tf as an iron source. If growth of the strain was impaired, this would imply a direct need for energy transduction by TonB to drive the process of iron uptake from these proteins.

4.3.3. Phenotypic Testing of Double Mutant Strains

The production of strains CEM11 (KAR2, $\Delta cfrA::aphA-3$) and CEM12 (KAR2, $\Delta feoB::ermC'$) carrying double mutations in either *cj0178* and *cfrA* or *cj0178* and *feoB*, allowed the involvement of multiple genes in the process of iron uptake from ferri-Lf to be assessed (Fig. 4.18a, b). As a partial phenotype was seen when the individual genes were inactivated, if two genes were involved in the process, removing them both in the same strain should result in a more marked phenotype. The loss of both *cj0178* and *feoB* in strain CEM12 resulted in a growth pattern similar to that seen when either of the genes were mutated individually (Fig. 4.11d, Fig. 4.16b). This implies that the loss of both *cj0178* and *feoB* is not enough to prevent iron from ferri-Lf entering the cell and being used for growth, indicating that there may be another route by which iron is acquired. Growth patterns of CEM11 were inconclusive due to problems with the controls, with CEM11 cells growing significantly more than wild-type under iron-restriction (Fig. 4.16a). The process that was used to construct CEM11, maintaining two mutations with the same selectable marker, was successful at the molecular level; however resulted in growth problems. The use of different selectable markers to remake the strain may alleviate these problems.

Chapter 5. Results: Further Investigation of the *cj0173c* to *cj0178* Region

5.1. Introduction

Campylobacter jejuni NCTC 11168 can use human ferri-lactoferrin (Lf), human ferri-transferrin (Tf) and ferri-ovotransferrin (ovo-Tf) as sources of iron for growth. Both CfrA, the ferri-enterochelin outer membrane receptor protein (Palyada *et al.*, 2004) and Cj0178, the previously uncharacterised putative TonB-dependent outer membrane receptor protein were associated with this process (Chapter 4). The most marked phenotype was observed when strain KAR2, carrying a mutated copy of *cj0178*, was grown in the presence of human ferri-Lf or ferri-ovo-Tf as the sole iron sources. Cj0178 demonstrated 24% identity to the Tf-binding protein A (TbpA) of *Neisseria meningitidis*. The loss of *cj0174c*, which encodes a permease protein and is part of an ABC transporter system encoded adjacent to *cj0178*, caused a small, but significant decrease in growth in the presence of Lf-bound iron.

Cj0178 was chosen to focus on as the phenotype seen when KAR2 was grown with ferri-Lf was greater than that seen when CfrA was lost. In addition, the function of Cj0178 and associated proteins in *C. jejuni* was not characterised prior to this study. Mutation of the cognate ABC transporter system also appeared to affect growth in the presence of ferri-Lf, possibly implying a role for the systems encoded adjacent to *cj0178* and further indicating the importance of this region. Although it is not known whether this phenotype may occur if any of the ABC transporter systems are lost due to redundancy. An initial search was completed to find proteins that demonstrate similarity to Cj0178. Genetic complementation of the *cj0178* mutation was also attempted, followed by growth of the complemented strain in the presence of human ferri-Lf as a sole source of iron. In addition, KAR2 was tested for growth using haem as an iron source to establish if Cj0178 plays a role in the uptake of haem as previously predicted (Chan *et al.*, 2006). KAR2 cells were tested for the ability to bind human Lf; any *C. jejuni* proteins found to bind Lf were also identified. Finally, an investigation of whether *cj0173c-cj0178* are iron and Fur regulated was completed.

5.2. Results

5.2.1. Search for Proteins with a Similarity to Cj0178

Cj0178 showed significant homology to TbpA of *N. meningitidis* (Section 4.2.1), and when mutated, the mutant strain (KAR2; Table 2.2) showed the most marked phenotype when grown in the presence of human ferri-Lf or ferri-ovo-Tf as a sole iron source (Section 4.2.3.2). A blast search of bacterial proteins was completed using the NCTC 11168 Cj0178 amino acid sequence, identifying a number of similar proteins. Proteins showing high similarity were the Cj0178 orthologues CjrC, a colicin Js receptor of *C. jejuni* HB93-13, and a TonB-dependent colicin receptor protein of *C. jejuni* strain RM1221 (99% identity). Tf-associated iron uptake proteins of *Haemophilus influenzae* Rd KW20 (31% identity) and *N. meningitidis* Z2491 (24% identity) were also highlighted, as well as a haem-utilisation protein of *H. influenzae* (30% identity).

A search of Pfam using the Cj0178 amino acid sequence indicated that there is a TonB-dependent plug domain at the N-terminal end of the protein from amino acids 40-151. At the C-terminal end from amino acids 458-755, there is a conserved part of the β -barrel structure assigned to the TonB-dependent receptor family. A conserved region assigned to the lipoprotein family was identified from amino acid 459-476. PredictProtein identified N-glycosylation sites at amino acids 222 (NYTV), 382 (NTSI), 458 (NASV) and 697 (NLTM). The protein is known to be glycosylated (Young *et al.*, 2002), although this has not yet been directly linked to function.

As *cj0177* is upstream and adjacent to *cj0178* in the NCTC 11168 genome, a blast search of bacterial proteins was also completed using the NCTC 11168 Cj0177 amino acid sequence. No obvious TbpB homologues were identified in the *C. jejuni* genomes searched (Section 4.2.1). As *cj0177* is encoded adjacent to *cj0178*, Cj0177 could still act as a TbpB-like protein, although conclusive results were not obtained from the growth assays (Fig. 4.15b). Many of the proteins identified in the search had not yet been assigned a function. A TonB-dependent lipoprotein from *C. jejuni* 260.94 demonstrated the highest level of similarity (99% identity). The same protein from *C. jejuni* strain RM1221 showed 98% identity and a putative lipoprotein from strain RM2228 showed 92% identity. Many other putative proteins or iron regulated proteins of unknown function were also highlighted from a range of *Campylobacter* species and strains (data not shown). PredictProtein identified a prokaryotic membrane lipoprotein lipid attachment site starting at amino acid 9

(ILFISFMLSAC) confirming the prediction that Cj0177 is a lipoprotein. N-linked glycosylation sites were also identified at amino acid positions 85 (NLSS), 156 (NLSR) and 262 (NLSY).

5.2.2. Complementation of KAR2 with a Functional Copy of *cj0178*

5.2.2.1. Complementation Strategy

As strain KAR2 (*cj0178::aphA-3*; Table 2.2) showed the most marked phenotype when grown in the presence of human ferri-Lf compared to wild-type NCTC 11168 growth, the mutation was complemented. A complementation system developed and successfully used previous to this study (Elvers *et al.*, 2005) involves the insertion of a functional copy of the gene of interest into the putative NCTC 11168 insertion sequence element transposase pseudogene *cj0752* in the mutant strain. If the phenotypic effect observed is solely due to the mutation of the gene of interest, replacing a wild-type allele back into the chromosome should allow restoration of the wild-type phenotype. If wild-type phenotype is not restored the mutation may be polar indicating a problem with the construction of the mutant strain.

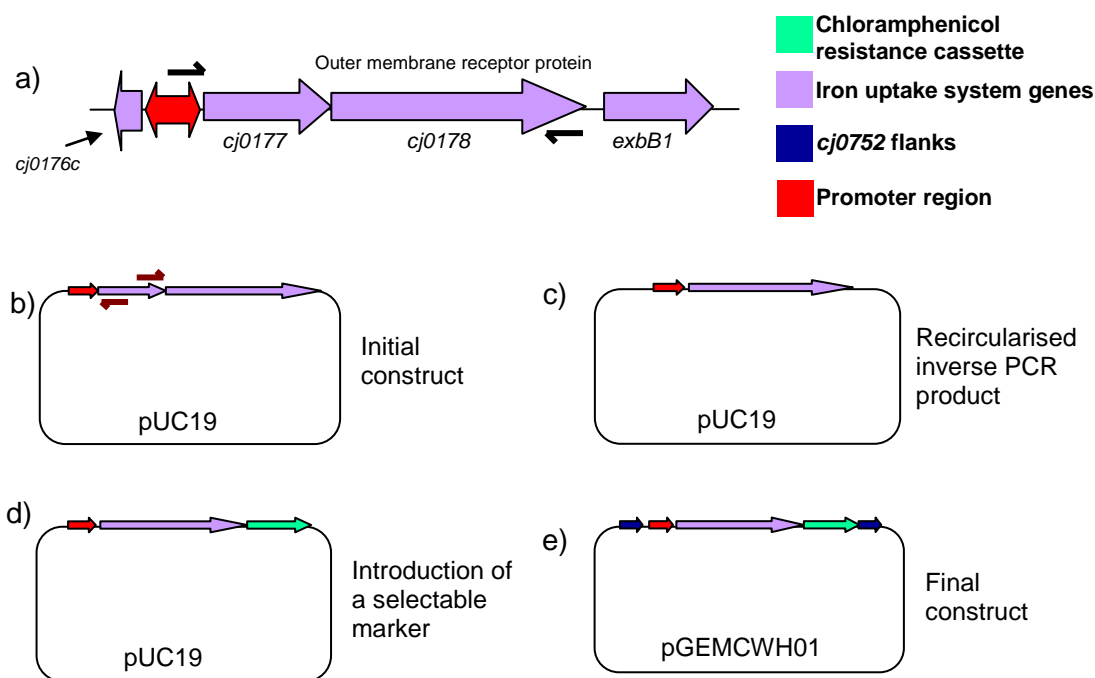


Figure 5.1. Complementation strategy. Diagram of the strategy used to produce the complementation construct. **a)** The region of interest was amplified from the NCTC 11168 chromosome by PCR using primers with restriction enzyme sites incorporated at the 5' end. The insert was directionally cloned into pUC19 producing the initial construct. The insert contained *cj0177*, *cj0178* and the upstream promoter region. **b)** Inverse PCR primers with 5' restriction enzyme sites were designed to anneal at either end of the *cj0177* gene in the initial construct and amplify outwards. Inverse PCR resulted in the deletion of *cj0177*. **c)** Self-ligation of the linear inverse PCR product resulted in a construct containing the native promoter region attached to *cj0178*. **d)** A selectable marker was prepared and introduced downstream of *cj0178* in the construct. **e)** The entire region was amplified by PCR from pUC19 and cloned into the mcs of the complementation plasmid pGEMCWH01 (Table 2.2) between the *cj0752* flanks in forward orientation to produce the final construct. The final construct was then prepared for transformation into KAR2 (Table 2.2). All constructs are listed in Table 2.2 and maps of each can be found in Appendix 1.

As *cj0178* is the second gene in the operon, with both *cj0177* and a potential promoter region upstream, both genes and the associated promoter were amplified from the NCTC 11168 chromosome using PCR (Section 2.10.1) with the oligonucleotide primer pair *cj0178compF* and *cj0178compR* (Fig. 5.1a, Table 5.1, Table 2.3). The primers were designed to amplify from just before the start of the promoter region to just after *cj0178*. It is optimal to include the wild-type promoter region so that expression of the newly incorporated gene is as close to the 'normal' level as possible and under the same regulation as it would have been originally. Primers were designed to incorporate 5' restriction sites; *KpnI* on the forward primer and *PstI* on the reverse primer (Table 5.1, Table 2.3).

Table 5.1. PCR reactions completed as part of the complementation of the *cj0178* mutation. Target regions, primer pairs used, positions that the primers anneal to on the *C. jejuni* chromosome and product sizes.

Target	Primer pair	Primer annealing positions on chromosome (bp)	Product size (bp)	Brief description
<i>cj0177</i> , <i>cj0178</i> and upstream promoter region in <i>C. jejuni</i> chromosome.	<i>cj0178</i> compF and <i>cj0178</i> compR.	172686 (forward) 176113 (reverse)	3440	Amplification of initial region to clone into pUC19.
<i>cj0177</i> ORF in pCEM18.	<i>cj0177</i> compInvF and <i>cj0177</i> compInvR.	172943 (inverse reverse) 173706 (inverse forward)	5337	Deletion of <i>cj0177</i> ORF from pCEM18.
Cloned region in pCEM20.	M13F and CatR_KitpI.	-	3543	Amplification of entire cloned region from pCEM20 to clone into pGEMCWH01.

The PCR amplification generated a product (Table 5.1) that included restriction sites at each end. The product was purified (Section 2.7), restricted (Section 2.9.1), purified again and cloned into pUC19 (Fig. 5.1b). pUC19 had been previously restricted with the same enzymes and prepared as described in Section 4.2.2.1. Each stage of the construction was verified by agarose gel electrophoresis (Section 2.6) to confirm DNA product sizes. Following ligation (Section 2.9.3) of the insert and vector, purification (Section 2.7.1) and transformation (Section 2.12) into *Escherichia coli* DH5 α , cells carrying the recombinant plasmid were selected for using appropriate antibiotics (Table 2.1) and screened by blue/white selection (Section 4.2.2.1). The construct was confirmed to be correct by colony PCR (Section 2.10), purified from overnight cultures (Section 2.5.3) and screened again using PCR with the cloning primer or M13 primer pairs (Table 2.3, Table 5.1, Appendix 2). All products generated from constructs produced as part of the complementation were

sequenced to check for incorporation errors (Sections 2.11 and 4.2.2.1). All constructs were named and descriptions can be found in Table 2.2. Construct maps are presented in Appendix 1. The initial construct was named pCEM18 (Table 2.2, Figure 5.1b).

Following cloning of the region of interest, the *cj0177* ORF was deleted using an inverse PCR mutagenesis strategy (Wren *et al.*, 1994). The oligonucleotide primer pair *cj0177*compInvF and *cj0177*compInvR (Table 2.3, Table 5.1 and Fig. 5.1) were used with clean pCEM18 template DNA. The product contained the *cj0178* ORF, the promoter upstream of *cj0177* and the pUC19 vector, but not *cj0177* (Fig. 5.1, Table 5.2). *Bgl*III sites were included at the 5' ends of the inverse PCR primers (Tables 2.3 and 5.2). The product was purified, restricted with *Bgl*III to allow re-circularisation of the inverse PCR product following restriction and purified again. Self-ligation (Fig. 5.1c) resulted in fusion of the promoter region to *cj0178* (for a description of the IPCRM strategy see Section 4.2.2.2 and Fig. 4.7) generating pCEM19 (Table 2.2). A selectable marker was then introduced (Fig 5.1d) by restriction of pCEM19 with *Pst*I for which there was a single site downstream of *cj0178*. The selectable marker conferred resistance to chloramphenicol as the *cj0178* mutation in KAR2 involved the use of a kanamycin resistance cassette. The chloramphenicol acetyl transferase (*cat*) gene was carried on a cassette derived from pAV35 (Table 2.2; van Vliet *et al.*, 1998).

The *cat* cassette was restricted from purified pAV35 using *Pst*I, generating a cassette of 824 bp carrying the desired resistance gene. The cassette was purified from the restriction mix using the gel extraction method detailed in Section 2.7.3. The 824-bp cassette band was excised from the agarose gel and purified generating a clean, restricted cassette. The cassette was ligated to the *Pst*I-restricted pCEM19 producing pCEM20 (Table 2.2, Fig. 5.1d). The ligation mix was purified, transformed into *E. coli*; colonies containing the appropriate constructs were identified using blue/white screening (Section 4.2.2.1) and selected for using the appropriate antibiotics (Table 2.1). The constructs were confirmed to be correct and the *cat* cassette was confirmed to be in forward orientation with respect to *cj0178* by colony PCR. Ligation of the cassette was non-directional; those constructs with cassettes in reverse orientation with respect to *cj0178* were discarded to avoid the potential production of an antisense transcript for the complementing gene. Correct constructs were purified from overnight cultures and screened again using the cloning primer or M13 primer pairs and combinations of these primers with cassette specific primers to confirm cassette orientation (Table 2.3, Table 5.1, Appendix 2).

The complementation system involved the insertion of the gene of interest into

cj0752 (Elvers *et al.*, 2005). A plasmid containing *cj0752* flanks was produced by C. Holmes (The University of Birmingham) including both upstream and downstream flanks of the gene with a central multiple cloning site (mcs) in a pGEM-T easy vector (Fig. 5.2).

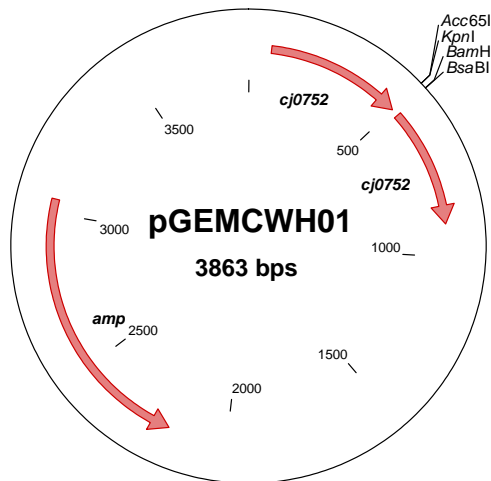


Figure 5.2. Plasmid map of pGEM-T easy vector containing the *cj0752* flanks used for the complementation of mutant genes. pGEMCWH01 contained the *cj0752* upstream and downstream gene flanks either side of a multiple cloning site (mcs) and an ampicillin resistance gene (*amp*), indicated by red arrows. Size is indicated in bp in increments; the total plasmid size is shown in the centre of the map. Restriction sites contained within the mcs are shown.

The entire cloned region from pCEM20 was amplified using the oligonucleotide primer pair M13F and CatR_ *KpnI* (Table 2.2, Table 5.2, Appendix 2). CatR_ *KpnI* allowed incorporation of a second *KpnI* restriction enzyme site at the opposite end of the product to the original site. M13F binds outside the original *KpnI* site. The PCR product covering the cloned region was generated from pCEM20. The product was purified following amplification, restricted with *KpnI*, and purified again.

pGEMCHW01 was transformed into *E. coli* DH5ae cells, which were recovered, screened and sequenced using combinations of the oligonucleotide primers *cj0752*upF, *cj0752*upR, *cj0752*downF and *cj0752*downR (Table 2.3) to verify that the *cj0752* flanks in the vector were correct. Following this, purified pGEMCWH01 was restricted using *KpnI*, purified and ligated to the prepared cloned region from pCEM20 producing the final construct pCEM21 (Fig. 5.1e; Table 2.2). Following purification, ligated constructs were transformed into *E. coli* cells, which were recovered and the presence of the correct construct was selected for by resistance to ampicillin and chloramphenicol (Table 2.1).

Cells containing the final construct were checked by colony PCR using combinations of vector, insert and cassette specific primers (Table 2.3, Appendix 2). This confirmed that the cloned region was correct by product size, and that the whole region was present in forward orientation with respect to the *cj0752* flanks in the vector. Correct constructs were purified from overnight cultures and screened again (Table 2.2, Table 5.1, Appendix 2).

Purified suicide vector pCEM21 was transformed into a KAR2 background by electroporation. Integration of the wild-type copy of *cj0178* with the promoter region and a downstream selectable marker into the chromosome occurred by homologous recombination between the chromosomal *cj0752* allele and the *cj0752* sequences in the complementation construct. Transformant cells containing the integrated region were selected for with chloramphenicol (Table 2.1). Resistant cells were re-plated and screened by colony PCR using the oligonucleotide primer pair *cj0178compR* and *cj0177compInvF*. PCR resulted in amplification of both the wild-type (replaced into *cj0752*, 2424 bp) and mutant (3691 bp) alleles from the chromosome of successfully constructed strains (Fig. 5.3).

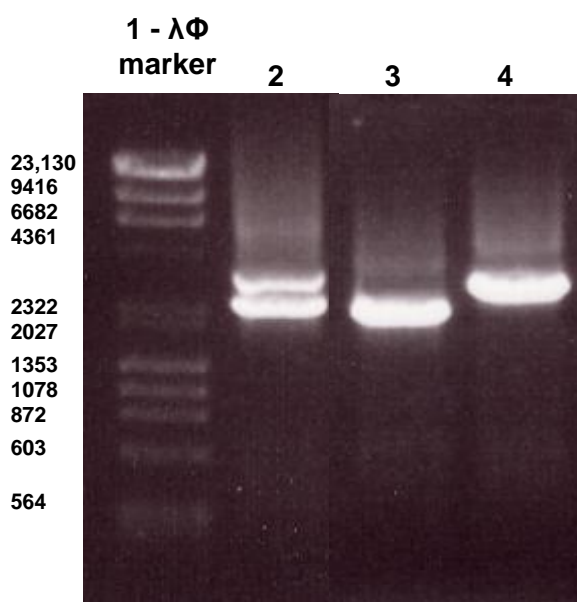


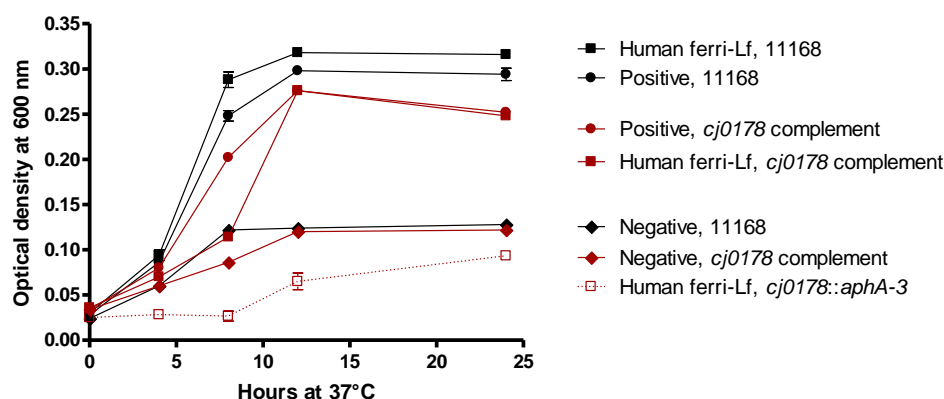
Figure 5.3. PCR amplified DNA products. Agarose gel electrophoresis of DNA from a strain carrying a *cj0178* mutation and a wild-type copy of the gene inserted into the pseudogene *cj0752*. Lane 1 contained $\lambda\Phi$ marker DNA, the sizes of which are indicated in bp to the left (250 ng of λ DNA restricted with *Hind*III and 100 ng of Φ X174 DNA restricted with *Hae*III), lane 2 contained both *cj0178* alleles from strain CEM8 (Table 2.2), amplified by PCR using the primer pair *cj0178compR* and *cj0177compInvF* (5 μ l; Table 2.3 and Table 5.1); the wild-type allele was 2424 bp replaced into *cj0752*, the mutant allele was 3691 bp. Lane 3 contained the wild-type *cj0178* allele amplified from the NCTC 11168 genome using the same primer pair, (5 μ l; 2424 bp). Lane 4 contained the mutant *cj0178* allele amplified from the KAR2 (Table 2.2) chromosome using the same primer pair, (5 μ l; 3691 bp).

Mutant DNA was isolated by large- or small-scale chromosomal DNA preparation (Sections 2.5.1 and 2.5.2) and quantified by absorption spectroscopy (Section 2.8). Incorporation of the wild-type gene into *cj0752* in the KAR2 genome was then confirmed by PCR screening of the purified DNA using combinations of *cj0752* specific, cassette specific and the original cloning primers (Table 2.3, Table 5.1, Appendix 2). The strain was designated CEM8 and is fully detailed in Table 2.2.

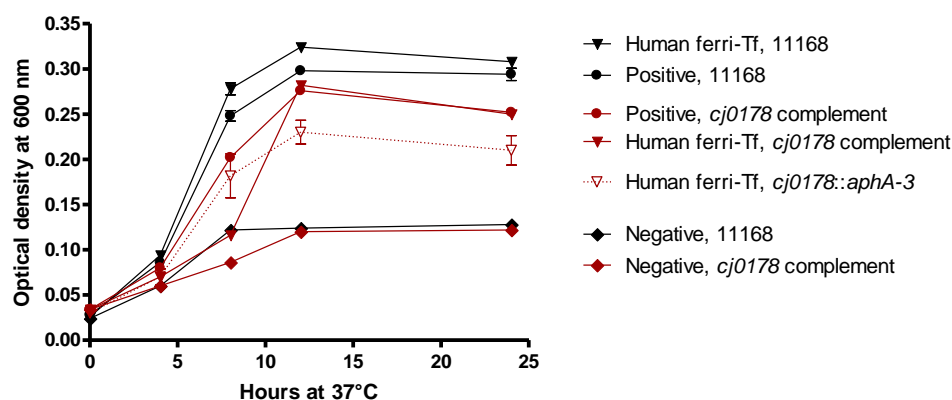
5.2.2.2. Phenotypic Analysis of CEM8

Following insertion of a wild-type copy of *cj0178* into *cj0752* in KAR2 (*cj0178::aphA-3*), growth of the resulting strain CEM8 (Table 2.2) was tested over 24 h to determine whether the *cj0178* mutation had been complemented. All raw data for the liquid growth assays found in this chapter and statistical testing of the differences in growth can be found in Appendix 3. The growth of CEM8 was assessed in comparison to wild-type and KAR2 cells in MEM α using the liquid medium growth curve strategy detailed in Chapter 3; the method for which is found in Section 2.13. CEM8 growth was tested under iron-replete conditions (10 μ M FeSO₄), iron-restricted conditions (unsupplemented MEM α) and in the presence of human ferri-Lf, of human ferri-Tf and of ferri-ovo-Tf (all at 0.27 μ M, Fig. 5.4a-c). Following overnight iron-depletion by growth in unsupplemented medium, samples were taken at intervals over the 24 h growth period.

a)



b)



c)

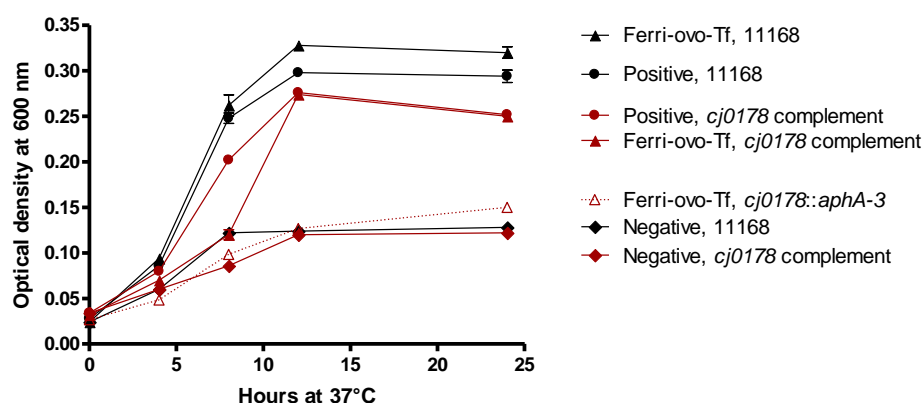


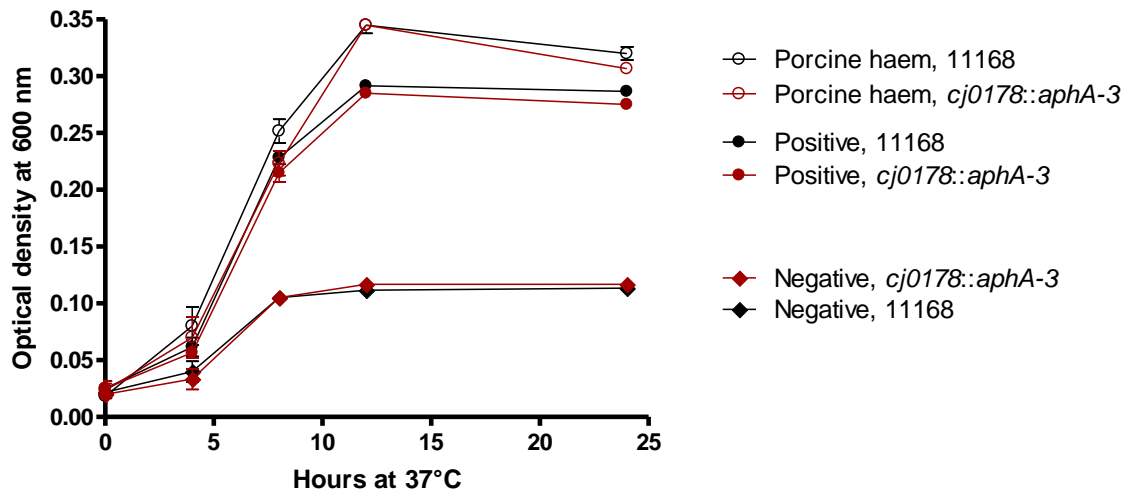
Figure 5.4. Growth assays of *C. jejuni* strain CEM8 (strain KAR2: *cj0178::aphA-3* 11168, complemented with a wild-type copy of *cj0178* inserted into the pseudogene *cj0752*) with iron supplied bound to human Lf, human Tf and ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and CEM8 with human ferri-Lf (0.27 μ M). **b)** NCTC 11168 and CEM8 with human ferri-Tf (0.27 μ M). **c)** NCTC 11168 and CEM8 with ferri-ovo-Tf (0.27 μ M).

Wild-type NCTC 11168 cells grew as previously shown (Fig. 3.3a) under all conditions reaching a final OD₆₀₀ of between 0.30 to 0.35 with FeSO₄, human ferri-Lf, human ferri-Tf and ferri ovo-Tf and between 0.10 to 0.15 with no iron (Fig. 5.4a-c). Strain CEM8 showed comparable growth to wild-type cells under iron restriction (Fig. 5.4a-c). Considerably more growth was achieved by CEM8 than KAR2 when supplied only with human ferri-Lf, human ferri-Tf or human ferri-ovo-Tf (Fig. 5.4a-c). However, slightly less growth was achieved by CEM8 than wild-type cells under both iron-replete conditions and with each of the test proteins (Fig. 5.4a-c). The final optical density reached by CEM8 with FeSO₄ and each test protein was just above 0.25; the final OD₆₀₀ reached by KAR2 with human ferri-Lf was just below 0.10 (Fig. 5.4a-c). Insertion of a wild-type copy of *cj0178* into the KAR2 mutant background complemented the mutant phenotype almost completely, restoring the ability to grow when supplied with human ferri-Lf, human ferri-Tf or human ferri-ovo-Tf (Fig. 5.4a-c). CEM8 had a slower growth response to Tf-derived iron up to 8 h, but growth had increased by 12 h. Identical regulation and expression of the complementing allele would not be expected, possibly explaining the growth patterns observed. In light of the data presented, the phenotype seen when *cj0178* is lost is likely to be due to the loss of the gene and not to polar effects of the mutation.

5.2.3. Growth of Strains using Haem as an Iron Source

In addition to Tf-binding proteins, Cj0178 also demonstrated sequence similarity to a haem-utilisation protein of *H. influenzae* (30% identity, Section 5.2.1) and, along with Cj0177, has been proposed to have a role in the uptake of haem by *C. jejuni* NCTC 11168 (Chan *et al.*, 2006). Although no direct role was demonstrated for Cj0178 in the uptake of haem, Cj0177 was shown to bind haem *in vitro* (Chan *et al.*, 2006). In order to ascertain whether Cj0178 was necessary for the acquisition of haem for growth, the mutant strain KAR2 (*cj0178::aphA-3*) was grown with iron supplied solely in the form of haem (25 µM) in liquid media growth assays (Fig. 5.5a). There was no significant difference between KAR2 (*cj0178::aphA-3*) and wild-type NCTC 11168 growth levels in the presence of haem (Fig. 5.5a). Both wild-type and KAR2 cells demonstrated comparable growth patterns to those seen in previous assays under all other conditions (Fig. 4.11d and Fig. 4.16a-c).

a)



b)

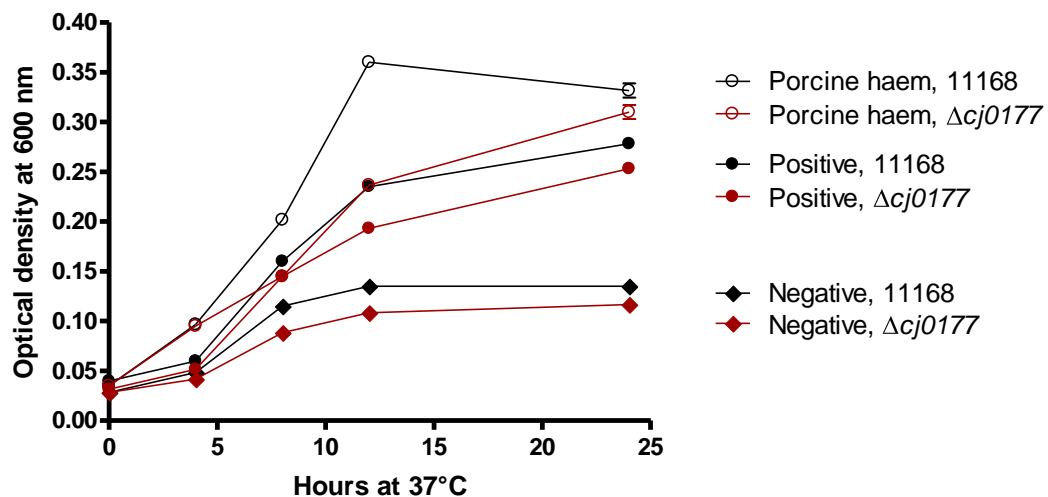


Figure 5.5. Growth assays of *C. jejuni* strains KAR2 (*cj0178::aphA-3* 11168) and CEM4 ($\Delta cj0177::ermC'$ 11168) with iron supplied solely in the form of haem. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative). **a)** NCTC 11168 and KAR2 with porcine haem (25 μ M). **b)** NCTC 11168 and CEM4 with porcine haem (25 μ M).

Strain CEM4, carrying a mutated copy of *cj0177*, was tested for a role in the uptake of haem for growth. CEM4 ($\Delta cj0177::ermC'$) was grown in the presence of haem (25 μ M) in liquid media growth assays (Fig. 5.5b), and growth was compared to wild-type. Wild-type cells grew comparably to the KAR2 growth assay (Fig. 5.5a) under all conditions. Under iron-replete and iron-restricted conditions, CEM4 showed the same small decrease in

growth seen in Chapter 4 (Section 4.2.3.3, Fig. 4.15b, also see Section 4.3 for discussion). Although the overall growth patterns of CEM4 and wild-type cells varied when supplied with haem and were significantly different at 12 h, by 24 h there was no significant difference between the strains. As CEM4 achieved a final optical density that was not statistically different from wild-type cells, this implies that Cj0177 is not essential for the uptake of haem in *C. jejuni* NCTC 11168.

5.2.4. Detection of Lactoferrin Binding

5.2.4.1. Binding of Lactoferrin to a *cj0178* Mutant Strain

To investigate whether there was a difference in the amount of human ferri-Lf that bound to the surface of KAR2 (*cj0178::aphA-3*) compared to wild-type NCTC 11168 under iron-restricted conditions, wild-type and mutant cells were incubated in the presence of human ferri-Lf, pelleted, washed, and lysed; the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Section 2.14.3). Proteins were blotted to polyvinylidene difluoride membranes (PVDF) and probed with polyclonal anti-Lf antiserum (Fig. 5.6). The absence of clear bands in lanes 1 and 2 (Fig. 5.6) indicates that under iron-restricted conditions, neither NCTC 11168 nor KAR2 proteins cross-react with the anti-Lf antibodies in the absence of human ferri-Lf. In the presence of human ferri-Lf, an 80 kDa band is visible running to the same position as the mature Lf protein control (lane 5, Fig. 5.6) for both the NCTC 11168 cells (lane 3, Fig. 5.6) and the KAR2 cells (lane 4, Fig. 5.6). The human ferri-Lf was not removed from the surface of either strain by the wash step. Less Lf was associated with the KAR2 cells (band intensity is 73.2% of lane 3 band intensity) than the NCTC 11168 cells, supporting a role for Cj0178 in the process of Lf binding. All blots were repeated twice; each condition was repeated twice per blot.

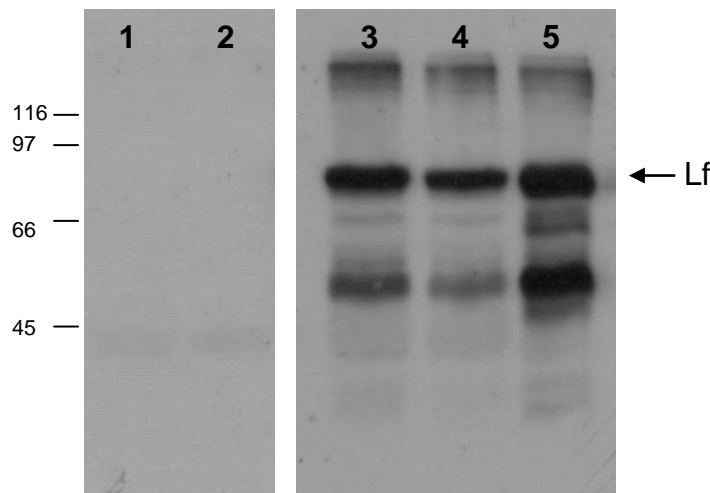


Figure 5.6. Lf binding to iron-restricted *C. jejuni* NCTC 11168 and mutant strain KAR2 (*cj0178::aphA-3*) cells. Western blot analysis of solubilised *C. jejuni* proteins (approximately 25 µg of protein separated by SDS-PAGE) prepared from NCTC 11168 cells (lane 1) and *cj0178* mutant strain cells (KAR2, lane 2) incubated without human ferri-Lf, and NCTC 11168 cells (lane 3) and *cj0178* mutant strain cells (KAR2, lane 4) incubated in the presence of human ferri-Lf (50 µg). Lane 5 contained pure human ferri-Lf protein (1 µg, positive control). Blots were probed with anti-Lf antibodies. The arrow indicates the position of the mature Lf protein. Loading of protein was equalised by pellet weight to avoid differences in protein concentration due to experimental error. Protein marker sizes are indicated in kDa to the left of the blot (Miller *et al.*, 2008).

5.2.4.2. Fractionation of *C. jejuni* Cells to Confirm the Binding of Lactoferrin to an Outer Membrane Receptor Protein

Although the mutant strain KAR2 was shown to bind less Lf than wild-type cells, this did not demonstrate direct binding of Lf to the outer membrane receptor proteins of *C. jejuni*. Wild-type NCTC 11168 cells were incubated under iron-restricted and iron-replete conditions, pelleted, washed, lysed and the proteins were subjected to SDS-PAGE (Fig. 5.7). Differences in the protein profiles were visible between iron-restricted and iron-replete cells indicating that the conditions used successfully induced changes in protein expression. The expression of 6 proteins was clearly different between lanes 1 and 2 (Fig. 5.7).

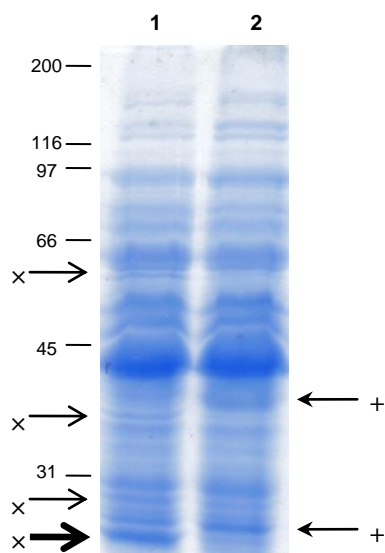


Figure 5.7. Comparison of *C. jejuni* NCTC 11168 protein profiles under iron-restricted and iron-replete conditions. SDS-PAGE analysis of solubilised *C. jejuni* proteins (approximately 25 µg of protein, with loading equalised by pellet weight) prepared from NCTC 11168 cells incubated without (lane 1) and with (lane 2) iron. Protein marker sizes are indicated in kDa to the left of the figure. Arrows indicate proteins that are expressed to a higher level under ×) iron-restricted or +) iron-replete conditions. The bold arrow indicates the position of periplasmic binding protein P19.

In order to demonstrate specific binding of human ferri-Lf to the outer membrane fraction or a particular protein within the fraction, *C. jejuni* cells were fractionated (Section 2.14.1) after iron-limited or iron-replete incubation. Initially, aliquots of all fractions were separated by SDS-polyacrylamide gel electrophoresis and proteins were visualised by staining. Following this, aliquots of the iron-restricted outer membrane fractions were separated by SDS-PAGE and blotted to PVDF membranes. The membrane was washed with human ferri-Lf (Section 2.14.6) prior to being probed with polyclonal anti-Lf antiserum. During the initial blot, there was no detection of the pure Lf positive control, possibly indicating a problem with the antibodies used. However, the Lf appeared to bind to an unknown protein of just less than 45 kDa. The experiment was therefore repeated to test whether the binding seen was an artefact. Two identical sets of samples (protein standards, iron-restricted NCTC 11168 outer membrane fractions and pure Lf positive control) containing equivalent amounts of protein were loaded onto the same SDS-PAGE gel, electrophoresed and transferred to PVDF membrane as described above. Following transfer, the membrane was split so that each half carried one set of samples. One half of the membrane was washed with human ferri-Lf as before (Section 2.14.6); one half was washed with blocking solution. From this point, both halves of the membrane were treated

the same with equal washes and then probed with the same polyclonal anti-Lf antiserum (Fig. 5.8a, b).

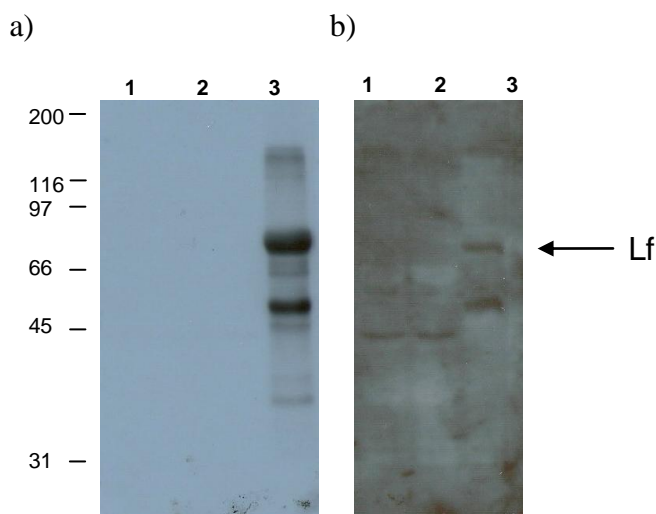


Figure 5.8. Lf binding to iron-restricted *C. jejuni* NCTC 11168 outer membrane proteins. Western blot analysis of solubilised *C. jejuni* outer membrane protein fractions (20 μ l each). Lanes 1 and 2 of both **a)** and **b)** contained iron-restricted outer membrane protein fractions, lane 3 of both **a)** and **b)** contained pure human ferri-Lf protein (1 μ g, positive control). The arrow indicates the position of the mature Lf protein. Following transfer membrane **a)** was washed with blocking solution alone, membrane **b)** was washed with human ferri-Lf; both were then probed with anti-Lf antibodies. Protein marker sizes are indicated in kDa to the left of the blot. Blots had equivalent exposure times and amounts of protein loaded were equal.

There was no apparent association of human ferri-Lf with proteins of 70 to 80 kDa, which would be expected if the Lf was binding to outer membrane receptor proteins, for example Cj0178 or CfrA (80 kDa and 75 kDa respectively). There was a higher overall background level of detection observed when Lf was used during the wash (Fig. 5.8b). There also appeared to be a negative effect of lower Lf association with the membrane where strong bands of protein were transferred from the gel. The position of the Lf positive control is indicated by an arrow (Fig. 5.8). The Lf again appeared to associate with the unknown protein of just below 45 kDa implying that this binding may not be an artefact (Fig. 5.8b). Where no Lf was included in the wash, there were no clear bands in the outer membrane fractions (lanes 1 and 2, Fig. 5.8a); however a faint band appeared to be present at the same position as the bands detected on the membrane washed with Lf (Fig. 5.8a, b). Upon longer exposure this band became stronger, but increased exposure resulted in an inability to observe any bands on the blot washed with Lf due to the very high background

detection (data not shown). Densitometry could not be completed on these blots due to this.

5.2.4.3. Mass Spectrometry Identification of Lactoferrin-Binding Protein

The *C. jejuni* protein that appeared to bind human ferri-Lf was identified by mass spectrometry (Fig. 5.8b). *C. jejuni* outer membrane fractions were subjected to SDS-PAGE analysis, stained and the band of interest was extracted. Peptide mass fingerprinting successfully identifies single proteins; the protein band was found to be mixed and so Q-Trap protein identification was used (Section 2.14.7). The greatest match was to PorA, a major outer membrane protein of *C. jejuni* (17 peptide matches). Weaker matches were found to a *C. jejuni* argininosuccinate synthase (3 matches) and a flagellar biosynthesis protein, FlhF (6 matches). These results suggest that PorA may bind Lf under these conditions.

5.2.5. Analysis of Promoter Activity and Regulation

5.2.5.1 Preparation of Promoter Regions

In order to verify the prediction of the *cj0176c-cj0177* promoter position, confirm that the promoter is iron-responsive and demonstrate that this is direct by the binding of Fur, the intergenic spacer region between genes *cj0176c* and *cj0177* was cloned into the *E. coli/C. jejuni* shuttle vector pMW10 (Fig. 5.9; Wösten *et al.*, 1998). The vector pMW10 can replicate in *E. coli* and *C. jejuni* and carries a *lacZ* gene lacking a promoter region preceded by a multiple cloning site (Fig. 5.9). The region of interest was cloned in both orientations to establish whether there are two independent promoters found within the intergenic spacer and to allow an assessment of promoter activity levels. The possible presence of additional promoter regions directly upstream of *cj0178* and *exbB1* was also investigated.

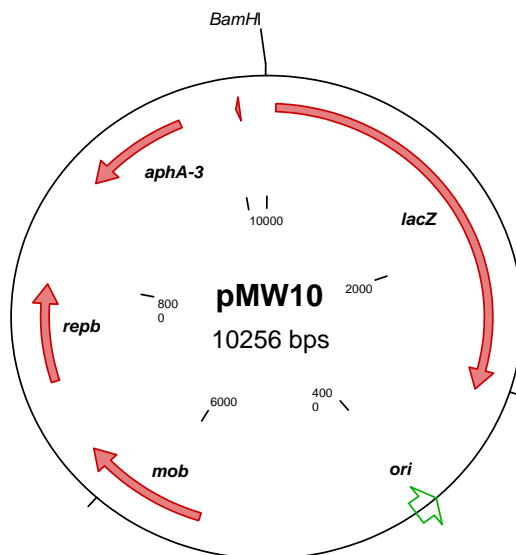


Figure 5.9. Plasmid map of pMW10 shuttle promoter probe vector. pMW10 contained a promoterless *lacZ* gene preceded by a multiple cloning site (mcs) and the *aphA-3* gene, derived from *Campylobacter* conferring kanamycin resistance. Genes are indicated by red arrows. The *ori* allows replication in *E. coli*; *mob* and *repB* allow replication in *C. jejuni*. Sizes are indicated in bp in increments, the total plasmid size is found in the centre of the map. The *Bam*HI restriction site, found within the mcs is indicated (Wösten *et al.*, 1998).

The *cj0176c-cj0177* promoter region and the regions (~200 bp) immediately upstream of *cj0178* and *exbB1* were amplified by PCR using the oligonucleotide primer pairs *cj0176c-77promF* and *cj0176c-77promR*, *cj0178promF* and *cj0178promR* and *exbB1promF* and *exbB1promR*, respectively (Fig. 5.10, Table 5.2 and Table 2.3). The primers *cj0176c-77promF* and *cj0176c-77promR* were designed to generate a product spanning from the base immediately upstream of each start codon, allowing a transcriptional fusion between each promoter region and the promoterless *lacZ* present in pMW10 upon cloning of the fragment in either orientation (Fig. 5.10). Primers were also designed to include a *Bam*HI site at the 5' end (Table 5.2 and Table 2.3), allowing cloning of the fragments into the *Bam*HI site of the mcs of pMW10 (Fig. 5.9).

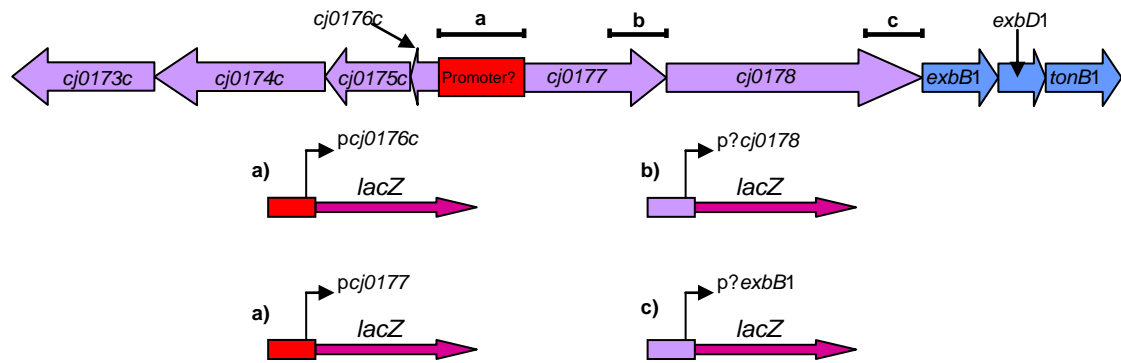


Figure 5.10. Production of reporter constructs. Diagram of the promoter regions amplified and cloned into pMW10 to assess promoter activity levels. **a)** The intergenic spacer region between *cj0176c* and *cj0177* was amplified from the NCTC 11168 chromosome and cloned into the multiple cloning site (mcs) of the vector pMW10 (Fig. 5.9; Wösten *et al.*, 1998) in both orientations. The 200 bp regions immediately upstream of **b)** *cj0178* and **c)** *exbB1* were also amplified and cloned into pMW10. This created a transcriptional fusion between the region of interest and the *lacZ* gene in the vector. See Table 2.2 for a list of constructs and Appendix 1 for all plasmid construct maps produced during this study.

Table 5.2. PCR reactions completed as part of the promoter activity investigations for the *cj0173c-tonB1* region of the NCTC 11168 chromosome. Target regions, primer pairs used, positions that primers anneal to on the *C. jejuni* NCTC 11168 chromosome and product sizes.

Target	Primer pair	Primer annealing positions on chromosome (bp)	Product size (bp)	Brief description
<i>cj0176c/cj0177</i> intergenic spacer region.	<i>cj0176-77promF</i> and <i>cj0176c-77promR</i> .	172695 (forward) 172915 (reverse)	237	Amplification of promoter region to clone into pMW10.
Putative <i>cj0178</i> promoter region.	<i>cj0178promF</i> and <i>cj0178promR</i> .	173477 (forward) 173763 (reverse)	303	Amplification of possible promoter region to clone into pMW10.
Putative <i>exbB1</i> promoter region.	<i>exbB1promF</i> and <i>exbB1promR</i> .	175801 (forward) 176042 (reverse)	257	Amplification of possible promoter region to clone into pMW10.

PCR amplified product sizes are listed in Table 5.2. Verification of success by confirmation of product sizes was achieved by agarose gel electrophoresis at each stage. Products were purified, restricted with *Bam*HI, purified again and ligated to the previously *Bam*HI-restricted and purified pMW10 (Fig. 5.9) immediately upstream of the *lacZ* gene. Each ligation mix was purified by ethanol precipitation and transformed into *E. coli*. Cells carrying recombinant plasmids were selected using the antibiotic kanamycin (Table 2.1). Each construct was confirmed to be correct by colony PCR using the appropriate cloning primers (Table 5.2, Table 2.3) to check the insert size. Primers specific to the kanamycin resistance gene (Appendix 2) in combination with the appropriate cloning primers were used to determine the orientation of each insert in pMW10. Following purification of the constructs from overnight culture, PCR screening was repeated and the resulting products were sequenced to confirm there were no errors. Constructs produced as part of this work were named and are listed in Table 2.2. Construct maps can be found in Appendix 1. Introduction of the *cj0176c-cj0177* promoter region in the *cj0176c* orientation generated pCEM14 (*pcj0176c::lacZ*) and in the *cj0177* orientation generated pCEM15 (*pcj0177::lacZ*; Table 2.2). Cloning of the possible *cj0178* and *exbB1* promoter regions into pMW10 generated constructs pCEM16 (*p?cj0178::lacZ*) and pCEM17 (*p?exbB1::lacZ*; Table 2.2), respectively.

Previously generated constructs were also used as controls including the constitutively expressed promoter region of the housekeeping gene *metK* (van Vliet *et al.*, 2000; p23E5, Table 2.2, *pmetK::lacZ*, positive control) and the iron-responsive promoter region of *chuA* (Ridley *et al.*, 2006; pJDR13, Table 2.2, *pchuA::lacZ*, iron-responsive control), each cloned into pMW10, and pMW10 alone (Fig. 5.9, Table 2.2, negative control). All constructs were transformed into *C. jejuni* strain 480 (Table 2.2) as described in Section 2.15.1. Constructs were screened again following transformation as described above to confirm that the inserts were free from errors.

5.2.5.2. β -Galactosidase Assay of Promoter Activity

Promoter activity levels were determined from β -galactosidase measurements of iron-restricted or iron-replete *C. jejuni* 480 cultures containing reporter constructs grown in MH broth over a 5 h period at 37°C (Miller, 1972; Wösten *et al.*, 1998). Each condition was tested 3 or 4 times and each assay was completed twice. Strain 480 showed identical growth patterns to NCTC 11168 in MHB, MEM α and MEM α with iron (data not shown).

The promoter regions of both *cj0176c* and *cj0177* demonstrated significantly increased promoter activity under iron-limitation (Fig. 5.11). However, based on the promoter activity levels it appears that *cj0177* is expressed to a lower level than *cj0176c-cj0173c*. The regions immediately upstream of *cj0178* and *exbB1* demonstrated no promoter activity (compared to the negative control, Fig. 5.11) suggesting that *cj0177-tonB1* are expressed as an operon.

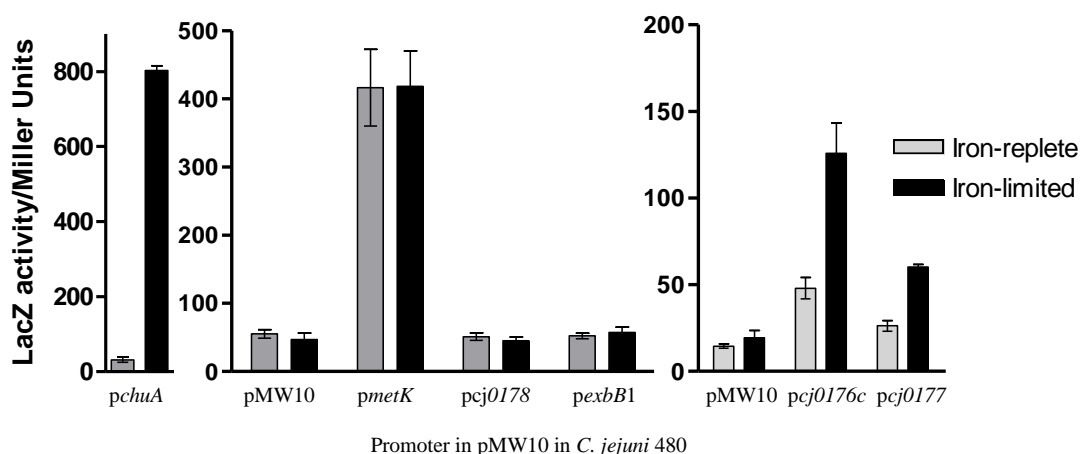


Figure 5.11. β -galactosidase assay to determine activity levels of promoter regions across the *cj0173c-tonB1* region of the *C. jejuni* NCTC 11168 genome. Reporter gene assay of pMW10 with no promoter region (negative control) and the promoter regions upstream of housekeeping gene *metK* (positive control; van Vliet *et al.*, 1998), *chuA* (iron responsive control; Ridley *et al.*, 2006), *cj0176c*, *cj0177*, *cj0178* and *exbB1*. Promoter activity levels were determined from β -galactosidase activity of iron-limited (20 μ M Desferal) or iron-replete (40 μ M FeSO_4) *C. jejuni* 480 cultures containing reporter constructs (Table 2.2), grown in MH broth for 5 h at 37 $^{\circ}\text{C}$. All conditions were tested in triplicate in two independent assays. Each data point is the mean of 6 replicates with the standard error of the mean shown (Miller *et al.*, 2008).

5.2.5.3. Fur-Regulation of the *cj0173c-cj0178* Region

To demonstrate Fur binding to the *cj0176c-cj0177* intergenic region and confirm the presence of two separate Fur boxes, the *cj0176c-cj0177* intergenic region was divided (using oligonucleotide primers listed in Table 2.3). PCR using the oligonucleotide primer pair *cj0176c-77promF* and *cj0176c-p-split* generated a 113 bp fragment. The primer pair *cj0176c-77promR* and *cj0177-p-split* generated a 140 bp fragment containing the putative *cj0177* Fur-box predicted previously (van Vliet *et al.*, 2002). Preparation of the promoter fragments was completed during this study by C. Miller; the EMSAs were performed as part of this study by another member of the group (R. Ren, The University of Leicester).

Electrophoretic mobility shift assays (EMSA; Section 2.16.3) were performed on the total promoter region and each half using recombinant purified Fur^{Cj} protein (Section 2.16.2). As the amount of Fur protein was increased, a concentration-dependent shift in the mobility of all DNA fragments was observed (Fig. 5.12a-c), demonstrating the presence of two separate functional Fur-boxes in this intergenic region. Competitive EMSAs (Section 2.16.3) were performed as described previously (Holmes *et al.*, 2005), in which unlabelled competitor DNA (to a 1500- or 2000-fold excess) was added to the reaction just before the Fur and labelled DNA. A decrease in the DNA shift was visible with an increase of unlabelled self-competitors for the total intergenic region and each half of the promoter indicating specificity of binding (Fig. 5.12d-f).

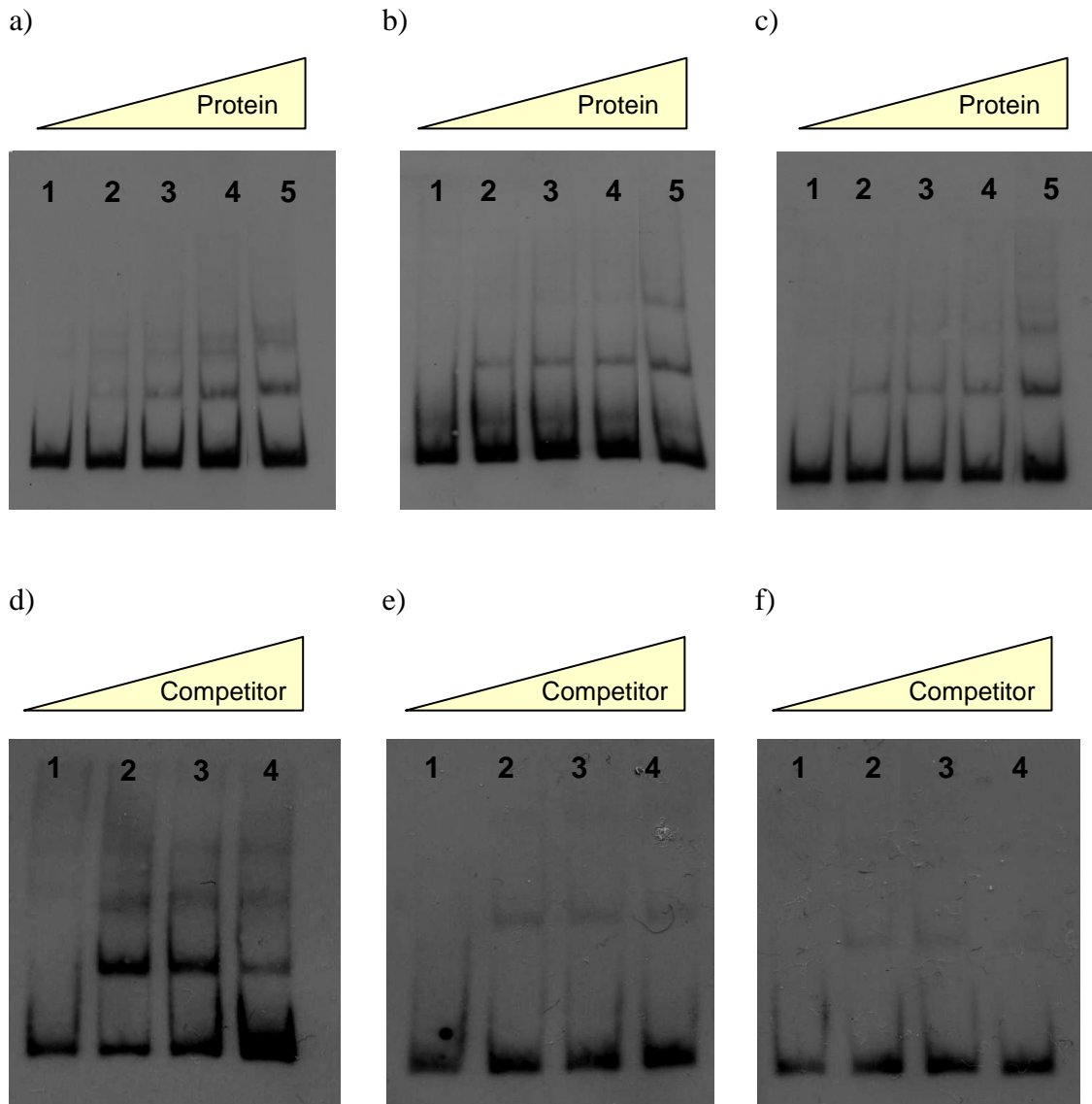


Figure 5.12. EMSA of the *cj0176c-cj0177* intergenic region using Fur, a-c). Digoxigenin-labelled DNA was present at 0.0775 nM. Lane 1, no protein, lanes 2 to 5, labelled DNA fragments incubated with 30, 60, 90, and 210 ng/μl *C. jejuni* Fur, respectively. **Competitive EMSA, d-f)** Digoxigenin-labelled DNA was present at 0.0775 nM. Lane 1, no protein, lanes 2 to 4, labelled DNA fragments incubated with 210 ng/μl *C. jejuni* Fur, Fur (210 ng/μl) with a 1,500-fold increase of competitors (unlabelled DNA fragments) and Fur (210 ng/μl) with a 2,000-fold increase of competitors (unlabelled DNA fragments), respectively. **a) and d)** Labelled probe of total intergenic region (possibly carrying two Fur-boxes, 237 bp fragment), **b) and e)** labelled probe of *cj0176c* side of intergenic region split by PCR to contain *cj0176c* Fur-box (113 bp fragment), **c) and f)** labelled probe of *cj0177* side of intergenic region split by PCR to contain *cj0177* Fur-box (140 bp fragment). The direction of increase of either Fur protein or unlabelled competitor is indicated above each film (Miller *et al.*, 2008).

5.3. Discussion

A blast search of bacterial proteins highlighted colicin receptors as orthologues of Cj0178. Colicins are antibacterial proteins produced by certain *E. coli* strains and other *Enterobacteriaceae* that kill members of the same family or species, conferring a selective advantage. Organisms are protected from their own colicins by producing proteins conferring ‘immunity’ (Braun *et al.*, 1994; Šmarda and Šmajs, 1998; Šmajs and Weinstock, 2001). Cj0178 has not been predicted to be a colicin receptor in NCTC 11168. The effect of colicin Js or other colicins on NCTC 11168 and the role of Cj0178 in this process have not been tested as part of this project. Tf-associated and haem-associated uptake proteins were also identified, which is unsurprising as Cj0178 has been predicted to be a TonB-dependent iron outer membrane receptor protein in NCTC 11168. This prediction is confirmed by the domains identified by the Pfam search using the Cj0178 protein sequence. A plug domain, which is an independently folding subunit of TonB dependent receptors (Oke *et al.*, 2004), was identified at the N-terminal end of the protein. The plug is a channel gate, which blocks the pore of the receptor until the substrate binds resulting in the induction of conformational changes that open the channel. Part of the C-terminal β -barrel structure was also identified as common to TonB-dependent receptor proteins (Buchanan *et al.*, 1999). In *E. coli*, TonB interacts with a number of receptors including FepA (ferri-enterochelin), FecA (ferri-citrate) and FhuA (ferrichrome), whose structures have been solved (Ferguson *et al.*, 1998; Locher *et al.*, 1998; Buchanan *et al.*, 1999; Ferguson *et al.*, 2002). These receptors were identified as examples of the same type of protein as Cj0178; the pyochelin outer membrane receptor FptA from *Pseudomonas aeruginosa* was also identified. This strongly suggests that Cj0178 is indeed a TonB-dependent ligand-gated porin with a β -barrel structure. Determining the three dimensional structure of Cj0178 by X-ray crystallography would confirm this prediction.

Insertion of a functional copy of *cj0178* in the insertion sequence element transposase pseudogene *cj0752* virtually restored wild-type growth in the presence of human ferri-Lf, human ferri-Tf and ferri-ovo-Tf. The phenotypic response of KAR2 is therefore unlikely to be due to polar effects of the kanamycin resistance cassette on downstream gene expression. The growth patterns of CEM8 are slightly different from wild-type, possibly due to the different genomic context of the complementing allele. Had the wild-type phenotype failed to be restored by complementation, *cj0178* expression could have been assessed in the wild-type NCTC 11168, KAR2 and CEM8 cells under iron-

replete and iron-restricted conditions. Cultures of each strain could be grown and samples taken at intervals, subjected to SDS-PAGE, transferred to PVDF membranes and probed with anti-Cj0178 antibodies. Cj0178 would be expected to be expressed in the wild-type and CEM8 cells, but not in the KAR2 cells. The expression of Cj0178 has not been directly demonstrated as part of this study. Strain CEM8 could also be tested for the ability to take up ^{55}Fe from either ^{55}Fe -loaded-Lf or -Tf; the amount acquired could then be compared to that acquired by KAR2 and NCTC 11168 cells.

A previous study demonstrated the ability of a Cj0177 homodimer to bind two cofacial haem groups (Chan *et al.*, 2006). In addition, when a homologue of *cj0177* in *P. aeruginosa*, *phuW*, was mutated and the strain was supplied solely with haem, growth was impaired (Ochsner *et al.*, 2000). It was therefore important to establish whether there was any connection between Cj0178 and haem uptake in NCTC 11168 as the authors proposed this but did not demonstrate it experimentally. Comparison of wild-type NCTC 11168 and KAR2 growth profiles when only porcine haem was supplied showed no significant difference. Reliance upon sequence similarity and an *in vitro* demonstration of haem binding to Cj0177 may not be sufficient to conclusively demonstrate the role, if any, that Cj0178 plays in haem uptake. Moreover, *chuABCD* has been identified as the major haem uptake cluster in NCTC 11168 (Ridley *et al.*, 2006). The authors proposed that as *chuA* and *cj0178* showed comparable expression patterns under iron-restriction, immobilised growth (on agar with increased population density to mimic the early stages of biofilm formation) or in the intestinal niche (Palyada *et al.*, 2004; Holmes *et al.*, 2005; Stintzi *et al.*, 2005; Sampathkumar *et al.*, 2006), they may function synergistically (Chan *et al.*, 2006). The assumption was made that two haem uptake systems are required by NCTC 11168 due to the range of haem sources available (Chan *et al.*, 2006). A role for Cj0178 as the haemopexin-haem receptor protein was proposed (Chan *et al.*, 2006). The role of the haem bound to Cj0177 is not known, but it may be transported via Cj0177 to either a periplasmic binding protein or to periplasmic proteins such as cytochromes (Chan *et al.*, 2006) or function as a Cj0177 co-factor. Although the growth of CEM4 is slightly less than wild-type NCTC 11168 with and without iron and with haem, these differences are comparable to the slight growth defect seen in Chapter 4 (Section 4.2.3.3, Fig. 4.15, discussed in Section 4.3). The final optical density reached by CEM4 was not significantly different from NCTC 11168 when haem was supplied as the sole iron source. Therefore, a role for Cj0177 in the uptake of haem for growth does not seem to be indicated by the data presented.

Less human ferri-Lf was bound by KAR2 whole cells than NCTC 11168. Under iron-restriction, 73.2% of the ferri-Lf bound by NCTC 11168 cells was bound by mutant cells, implying a role for Cj0178 in the binding of human ferri-Lf. To attempt to block ferri-Lf binding to Cj0178, anti-Cj0178 antibody could be included and the amount of Lf associated with NCTC 11168 cells compared to the amount associated in the absence of antibody. To determine whether *C. jejuni* exhibits specificity for ferri-Lf over apo-Lf, binding experiments could be repeated with wild-type and KAR2 cells incubated with apo-Lf. Following SDS-PAGE and Western blotting, apo-Lf could be detected using anti-Lf antibodies. The band intensities seen when using either apo-Lf or ferri-Lf may be compared. If less apo-Lf bound to wild-type cells than ferri-Lf, this may indicate a preference for ferri-Lf. If more apo-Lf than ferri-Lf bound to KAR2 cells, this may indicate a role for Cj0178 in the preferential binding of ferri-protein.

Previous expression level profiling of the *C. jejuni* iron regulon indicated that the *cj0173c-tonB1* region of the *C. jejuni* genome was expressed at high levels under iron-restriction and at low levels when iron was in excess (Holmes *et al.*, 2005). The transcript levels of genes *cj0173c*, *cj0175c* and *cj0176c* increased under iron-restriction (4.1-, 30.5- and 13-fold respectively). A transcript level increase was also seen with *cj0177* and *cj0178* (25.4- and 16-fold respectively). By inducing iron-replete and iron-restricted conditions as part of this study, differences in the protein profiles of NCTC 11168 wild-type cells were visible, indicating that the conditions used were appropriate. An iron-restricted protein expression profile was necessary for successful cell fractionation, generating an outer membrane fraction possibly containing proteins necessary for iron uptake from ferri-Lf. Because of the size and increased expression under iron-restriction of a 19 kDa protein (indicated by the bold arrow in Fig. 5.7), this is likely to be the periplasmic binding protein P19, encoded by *cj1659*. P19 has previously been demonstrated to have increased expression in wild-type cells under low iron conditions (van Vliet *et al.*, 1998). Under iron-restriction, *p19* transcript levels were also shown to increase the most (110-fold) out of all of the iron transport and storage genes tested; proteomics data demonstrated it was one of the major proteins induced under iron-restriction (Holmes *et al.*, 2005). Other proteins were also identified by a previous study to have increased expression under iron-restriction including KatA (55 kDa), CeuE (36 kDa), ChuD (29 kDa) and AhpC (26 kDa), possibly also visible on Fig. 5.7 (indicated by arrows with a ×; van Vliet *et al.*, 1998).

Following the transfer of the NCTC 11168 outer membrane fraction to PVDF, membranes were washed with Lf and a protein of just below 45 kDa was detected binding

the Lf. There was also a high level of background Lf binding to the membrane. All membranes were blocked with BSA; however a high concentration of Lf was used and it is therefore unlikely that the BSA would be able to prevent all non-specific Lf binding. There is a negative effect where large protein bands were already present on the gel, presumably non-specific Lf binding was not possible here due to the presence of another protein. In the absence of Lf, the 45 kDa protein was not as easily detected; however, upon longer exposure a visible, but weak band of the same size was detected. Q-Trap protein identification indicated that this protein was highly likely to be PorA, a 45 kDa major outer membrane protein (Khan *et al.*, 2005). A prediction that *C. jejuni* produces a protein that cross-reacts with cholera toxin (CT) antibodies was recently confirmed (Albert *et al.*, 2007). A protein was detected by rabbit CT antibody in Western blots of all *C. jejuni* strains tested, including NCTC 11168 (which lacks a CT gene homologue). The protein was identified as PorA. One-way cross-reaction was proposed to rely on differential accessibility of an epitope common to both antigens. The authors concluded that no functional cholera-like toxin was being produced by *C. jejuni*, but rather that the CT antibody detected PorA (Albert *et al.*, 2007). The detection of PorA by rabbit anti-Lf antibodies in the absence of any Lf may also be due to cross-reaction. The addition of Lf increased the detection, possibly indicating that some Lf is actually binding to a protein of 45 kDa, which could be PorA. Other than PorA, mass spectrometry identified argininosuccinate synthase and a flagellar biosynthesis protein, FlhF. Mass spectrometry identifies the most abundant proteins in the band, but as the protein band was not pure there may be yet another 45 kDa protein present that is capable of binding Lf, but expressed to a sufficiently low level that it was not detected. Human Lf has been shown to bind to purified *E. coli* porin OmpC and PhoE trimers and preferentially to strains expressing OmpC or PhoE (Sallmann *et al.*, 1999). Lf was not shown to bind to OmpF. Ferri- and apo-Lf bind porins comparably (Sallmann *et al.*, 1999). The favoured binding of human Lf to OmpC and PhoE was investigated for an effect on growth. Only the growth of a strain with PhoE was prevented by human Lf (above 2.4 mg/ml) (Sallmann *et al.*, 1999). Human Lf and Tf have been previously shown to cause the release of lipopolysaccharide under physiological conditions from the bacterial outer membrane, causing damage and altering permeability (Ellison III *et al.*, 1988). LPS release was inhibited by saturating Lf with iron (Ellison III *et al.*, 1988). OmpC and PhoE have been proposed to anchor Lf to the *E. coli* cell surface, aiding access to the outer membrane resulting in damage (Sallmann *et al.*, 1999). More recently, human Lf was shown to bind to OmpA of *E. coli*; however, upon

mutation of *ompA*, Lf still bound to cells indicating a partial, but non-essential role for OmpA in Lf binding (R. D. Haigh, per. comm.).

The removal of PorA from the *C. jejuni* cell surface would allow further investigation of the role it plays and possibly aid the identification of other proteins that can bind Lf. However, so far no mutations of *porA* have been maintained. PorA is an important outer membrane porin responsible for regulation of outer membrane solute flow so mutation could be lethal (Albert *et al.*, 2007). In addition, proteins were initially separated on a denaturing SDS polyacrylamide gel. Following transfer of the proteins to PVDF membranes, limited renaturation may be possible on the membrane, but normal folding of the proteins is unlikely to occur. Because of this, proteins that would ordinarily bind Lf may not, and proteins unlikely to bind Lf *in vivo* may do so here. The use of a non-denaturing gel for separating NCTC 11168 protein fractions prior to transfer to the membranes may result in different proteins binding Lf due to the need for native NCTC 11168 protein folding. This may be more representative of what is occurring *in vivo* and lead to the identification of iron-uptake proteins directly involved in the process.

Work completed prior to this study involved the application of *C. jejuni* outer membrane proteins to a Lf-sepharose column; proteins that bound were eluted and from this a number of potential Lf-binding proteins were identified. Of the proteins found to be present in the outer membrane preparation, absent from the column run-through, and present in the column eluate, the most obvious was a 65 kDa protein that was identified as flagellin A by N-terminal sequencing (Rock, 2003). Repetition of this work with a *C. jejuni* flagellin mutant resulted in the loss of the 65 kDa protein; however due to inferior gel resolution, time constraints and the fact that iron-restricted conditions were not used, no other potential candidates for Lf-binding were identified (Rock, 2003). Incubation of the outer membrane proteins with Lf reduced the FlaA retention in the column, but binding was not lost (Rock, 2003). It was proposed that this may not be due to specificity of Lf binding, but rather to retention of flagellar material in the column. This work could be repeated using the flagellin mutant strain and iron-restricted conditions to attempt to identify other Lf-binding proteins. Once binding was shown for NCTC 11168, outer membrane receptor mutant strains could also be tested to determine if binding was lost or reduced by the loss of a known receptor protein. All experiments could be repeated using either ferri-Tf or ferri-ovo-Tf, or both, to support the theory that *C. jejuni* can use all these proteins as iron sources. In addition, the binding of ferri-Lf or ferri-Tf to proteins in NCTC 11168 whole cell preparations or outer membrane fractions may be determined by cross-linking of the

iron source to the bound *C. jejuni* protein followed by purification. If Cj0178 or another receptor were shown to directly bind Lf or Tf, deletions may be made in the extracellular protein loops using IPCRM. Constructs containing versions of *cj0178* carrying different mutations could be transformed into *E. coli* and the proteins expressed and purified. Expression of mutated Cj0178 in *C. jejuni* may also be achieved following insertion into *cj0752* in KAR2. Specific regions of Cj0178 necessary for Lf or Tf binding may then be identified by the ability of each mutant Cj0178 or *C. jejuni* *cj0178* mutant strain cells to bind ferri-Lf or ferri-Tf. If Cj0178 does not directly bind Lf or Tf, there may be a possibility that cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is involved. GAPDH may conceivably be involved in binding as well as Cj0178 in *C. jejuni*. The cell wall-associated staphylococcal GAPDH has been associated with Tf binding (Modun *et al.*, 1994; Modun *et al.*, 1998; Modun and Williams, 1999). *C. jejuni* possesses a single GAPDH, encoded by *gapA* (Parkhill *et al.*, 2000) which could be investigated for a role in the binding of Lf or Tf.

In a *fur* mutant strain, derepression of the *cj0173c-tonB1* region was observed under iron replete conditions, indicating the involvement of Fur in the regulation of this region (Palyada *et al.*, 2004; Holmes *et al.*, 2005). The genes are also known to be induced under iron-limitation in the wild-type strain NCTC 11168 (Palyada *et al.*, 2004; Holmes *et al.*, 2005). The intergenic region between *cj0176c* and *cj0177* was proposed to contain a Fur-box consensus sequence upstream of *cj0177* (van Vliet *et al.*, 2002). Co-transcription of the region was proposed to be from the promoter upstream of *cj0177*; derepression of the downstream *cj0176c-cj0173c* operon in the *fur* mutant was proposed to be due to regulation of this region by the same Fur-box (Holmes *et al.*, 2005). Using computational analysis, another study proposed that potential Fur-boxes were present upstream of both *cj0177* and *cj0176c*, implying that both operons are Fur-regulated via different operator sequences (Palyada *et al.*, 2004).

Reporter gene studies of the intergenic spacer region between *cj0176c* and *cj0177*, and the regions upstream of *cj0178* and *exbB1* carried out as part of this project confirmed the iron responsiveness of the *cj0176c* and *cj0177* promoter region. The regions immediately upstream of *cj0178* and *exbB1* demonstrated no promoter activity when iron was restricted, which is consistent with the prediction that *cj0177-tonB1* are co-transcribed from the promoter region located upstream of *cj0177* (Fig. 5.11; Holmes *et al.*, 2005). Another study proved by RT-PCR that *exbB1-tonB1* are co-transcribed but did not state that they are an independent transcriptional unit (Palyada *et al.*, 2004). Therefore *cj0177-tonB1*

may be transcribed together; the region upstream of *exbB1* was not investigated (Palyada *et al.*, 2004). The results presented here also indicated that *cj0176c-cj0173c* may be regulated by their own Fur-box as promoter activity levels under iron-restriction were different from those seen with the *cj0177* promoter (Fig. 5.11). Promoter activity was significantly greater under iron-restriction than iron-replete conditions; the promoter upstream of *cj0176c* had about twice the activity of the *cj0177* promoter, indicating a higher expression level of *cj0176c-cj0173c* than *cj0177-tonB1* in the cells under iron-restriction. Promoter activity of this region under iron-restriction appears to be much lower than the promoter region of *chuA* (Ridley *et al.*, 2006). However, comparisons between strains containing the different pMW10::promoter constructs cannot be conclusively relied upon as the system used was plasmid-based. Activity of the *cj0177* promoter did not appear to be completely lost under iron-replete conditions indicating that *cj0178* expression may not be completely off in the presence of iron. Because of this, binding of Lf to NCTC 11168 cells under iron-replete conditions may not be expected to be completely absent, concurring with the data presented in Fig. 3.7. In order to define transcriptional start sites, each reverse transcribed full length mRNA could be mapped to the genomic sequence. Preliminary transcriptional start mapping of the *cj0176c* and *cj0177* transcripts using 5'-rapid amplification of cDNA ends (RACE) was inconclusive (K. A. Ridley, unpublished data).

EMSAs (R. Ren, The University of Leicester) showed that Fur binds directly to both halves of the intergenic spacer region between *cj0176c* and *cj0177*, indicating the presence of two separate Fur boxes and confirming classical Fur-dependent, iron-repressible regulation of both *cj0176c-cj0173c* and *cj0177-tonB1*. The double-banding pattern seen in Figure 5.12a may be due to the presence of the two Fur-boxes within the total promoter fragment. The polymerised Fur could also be progressively wrapping around the binding sequence and along the DNA as the concentration increases (Fréchon and Le Cam, 1994; Le Cam *et al.*, 1994). A confirmation of the exact locations of each Fur-box could be completed using DNase I footprinting. Following this, site-directed mutagenesis of the Fur-boxes to sequences more or less like the consensus, followed by EMSAs could be completed.

The data presented up to this point enhance our understanding of the *cj0173c-cj0178* region in terms of the regulation and potential role in *C. jejuni* iron uptake. Further characterisation of this system and the process of Lf- and Tf-bound iron uptake in NCTC 11168 are still required as many questions have been raised by the results presented here.

Chapter 6. Results: The Role of Noradrenaline in Growth Promotion of Wild-type and Mutant *Campylobacter jejuni* Strains

6.1. Introduction

6.1.1. Noradrenaline and the Host Nervous System

Noradrenaline (NA, or norepinephrine), a neuroendocrine stress-related hormone and catecholamine, possesses a benzene ring structure with two hydroxyl groups and an amino side chain (Fig. 6.1). Catecholamine synthesis begins with phenylalanine (often dietary), which is converted to tyrosine by phenylalanine hydroxylase. The conversion of tyrosine to *L*-dopa is catalysed by tyrosine hydroxylase. *L*-dopa is then converted to dopamine, NA and finally adrenaline (epinephrine) by aromatic *L*-amino acid decarboxylase, dopamine β -hydroxylase and phenylethanolamine-N-methyltransferase, respectively (Freestone *et al.*, 2007a; Freestone *et al.*, 2007b; Freestone *et al.*, 2008). Catecholamines are involved in neuroendocrine signalling throughout the human host and in mediating host stress responses (Freestone *et al.*, 2008).

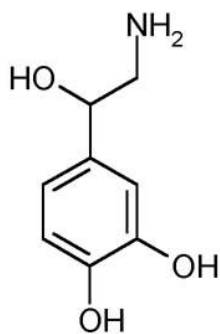


Figure 6.1. The structure of noradrenaline, the catecholamine stress hormone and neurotransmitter.

The host nervous system consists of the central, peripheral and enteric sections. The enteric nervous system (ENS) is innervated with at least 500 million neurones throughout the entire length of the tract. The nerve endings of the sympathetic system (forming part of the ENS) contain and release NA and dopamine. Adrenaline is principally released from the adrenal glands and not synthesised in the ENS because of a lack of neurones containing phenylethanolamine-N-methyltransferase (Costa *et al.*, 2000). Half of

the NA in the body is produced and released within the gut by the ENS (Costa *et al.*, 2000; Freestone *et al.*, 2008). Following release at the gut surface, NA can enter the circulatory systems, resulting in transport through the surrounding organs and potential leakage into the gut lumen (Åneman *et al.*, 1996; Lyte and Bailey, 1997; Lundgren, 2000). NA modulates contractions of the gut smooth muscle, blood flow in the submucosa and active ion transport across the epithelium. NA may also interact with and regulate the adaptive immune responses of the gut (McIntyre and Thompson, 1992; Gonzalez-Ariki and Husband, 1998). In addition to their native roles in the host, catecholamine hormones have also been suggested to influence the progression and final outcome of bacterial infections.

6.1.2. Stress and the Progression of Infection

Catecholamines such as NA were first investigated for a direct effect on bacterial growth in the early 1990s, but the influence of stress on infection progression had been implicated before this (Lyte and Ernst, 1992; Lyte, 2004). Stress or trauma can result in damage to the noradrenergic nerve cells within human tissue leading to NA secretion and higher than normal systemic levels of NA (Woolf *et al.*, 1992; Lyte and Bailey, 1997). Tyrosine hydroxylase intestinal expression is also upregulated in a stressed host adding to the NA levels in the gut as this enzyme catalyses the rate limiting step in catecholamine biosynthesis (Zhou *et al.*, 2004). Commensal gastrointestinal micro-organisms may become capable of causing infection following trauma, even when the gastrointestinal tract has not been specifically damaged (Deitch *et al.*, 1985; Marshall *et al.*, 1988; Nieuwen Huijzen *et al.*, 1996). NA release into the mouse gastrointestinal tract following treatment with a neurotoxin caused an increase (>100,000-fold) in gut *Escherichia coli* numbers within 24 h and associated invasion of tissues (Lyte and Bailey, 1997). The time taken for nerves containing catecholamines to repair was equivalent to the time taken for normal levels of gut bacteria to be re-established (Lyte and Bailey, 1997). Increased infections seen in trauma patients are proposed to be due to the massive release of NA systemically, which also enters the gut, leaving patients open to infection from opportunistic pathogens as well as overgrowth and infection by natural gastrointestinal flora (Deitch *et al.*, 1985; Marshall *et al.*, 1988; Woolf *et al.*, 1992; Nieuwen Huijzen *et al.*, 1996). Increased infections were also seen in heart patients treated with intravenous catecholamines (Smythe *et al.*, 1993). Consequently, there appears to be a direct link between increased

presentation of sepsis in critically ill patients and increased NA levels (Freestone *et al.*, 2000).

6.1.3. Noradrenaline-Specific Changes to Pathogenicity

The host cell-associated factors allowing human colonisation by pathogens are not fully characterised. Pathogens must out-compete the natural gut flora and avoid host immune responses to successfully infect the human host. Stress-associated NA release is known to influence the virulence of bacteria, including the pathogens *E. coli* (Green *et al.*, 2003; Green *et al.*, 2004) and *Salmonella* (Green *et al.*, 2003), as well as commensal organisms naturally found in the gut (Freestone *et al.*, 2002; Zhou *et al.*, 2004).

In a serum-containing medium, NA promotion of *E. coli* O157:H7 growth is accompanied by the production of a heat-stable growth autoinducer (Lyte and Ernst, 1992; Lyte *et al.*, 1996a). K99 pilus expression, necessary for adhesion and host infection, was also increased in a bovine enterotoxigenic *E. coli* (ETEC) strain during culture in the presence of NA (Lyte *et al.*, 1997). The exposure of attenuated *Salmonella enterica* serovar Typhimurium to NA following shedding from juvenile rhesus monkeys significantly increased bacterial growth. The NA growth effect was not seen in bacteria before infection of the animals (Bailey *et al.*, 1999). In addition, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, wild-type *S. Typhimurium* and commensal human *E. coli* strains have also been shown to have enhanced *in vitro* growth in the presence of NA under conditions designed to mimic the mammalian host (Lyte and Ernst, 1992; Lyte, 1997; Freestone *et al.*, 2002). Commensal and environmental *E. coli* strains were grown from low inocula in nutrient-limited minimal medium supplemented with serum (serum-SAPI minimal medium; Lyte and Ernst, 1992; Freestone *et al.*, 1999) in an attempt to mimic *in vivo* conditions (Freestone *et al.*, 2002). The addition of catecholamines to cultures caused a significant increase in growth (3 logs) during 24-h incubation compared to bacterial growth without catecholamine (Freestone *et al.*, 2002). NA metabolites lacking the catechol (3,4-dihydroxybenzoyl) group could not stimulate growth, showing the importance of this structure for growth promotion (Freestone *et al.*, 2002). In addition, enterohaemorrhagic *E. coli* (EHEC) strain NCTC 12900 (O157:H7) had increased adherence to NA-treated porcine colonic epithelium; avirulent *E. coli* and porcine-adapted non-O157 *E. coli* did not show NA-increased adherence. This indicates that in times of stress, infection of the host by pathogens is likely to be augmented (Green *et al.*, 2004). The adherence by O157:H7 was

enhanced further by the addition of a PKA inhibitor or sympathomimetic agents. NA-enhanced EHEC adherence was prevented by the addition of yohimbine, an α -adrenergic antagonist, and Sp-8-bromoadenosine-3',5'-cyclic monophosphorothioate, a PKA activator. Increased NA-dependent EHEC adherence seemed to be due to cells interacting with α_2 -adrenergic receptors (Green *et al.*, 2004). NA supplementation of Gram-positive cultures in serum-SAPI minimal medium resulted in a growth increase of 10-fold or greater for bacteria including *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes* and *Staphylococcus epidermidis* (Freestone *et al.*, 1999). In general, NA-induced growth of Gram-positive bacteria was less marked than that of most Gram-negative bacteria tested; however *S. epidermidis* showed one of the most notable increases (Freestone *et al.*, 1999). *Enterobacter aerogenes*, *Serratia marcescens*, *S. aureus* and *S. dysgalactiae* did not show NA-enhanced growth, achieving similar growth levels to the control cultures (Freestone *et al.*, 1999).

Listeria spp. are incapable of producing their own siderophores, but can use exogenous siderophores and catecholamines to grow in the presence of the iron-chelator tropolone (Coulanges *et al.*, 1998). Catecholamine-dependent growth promotion was independent of the enantiomeric form of NA leading the authors to suggest that specific eukaryotic-like NA receptors are not required for iron delivery to the bacteria (Coulanges *et al.*, 1998). Normetanephine and 4-hydroxy-3-methoxyphenylglycol-piperazine salt (MHPG) did not reverse the tropolone iron-limitation-dependent growth inhibition. NA differs from normetanephine and MHPG by a catechol-*o*-methylation, and oxidative deamination of NA for MHPG; the *o*-diphenol (catechol) function must therefore be present to facilitate iron-dependent recovery from growth inhibition. This may imply that the catechols act as phenolate siderophores to allow iron uptake by bacteria for growth (Coulanges *et al.*, 1998).

6.1.4. The Role of Iron

6.1.4.1. Host Glycoprotein-Derived Iron

Iron plays a key role in colonisation of the host by bacteria, with the iron-limited host environment acting as a non-specific defence mechanism against the invading micro-organism (Sections 1.3.5 and 1.4). Iron is also vital in the process of bacterial growth enhancement by catecholamines. Iron bound to the host glycoproteins lactoferrin (Lf) and

transferrin (Tf; discussed in Section 1.4.5) has been shown to be important in this process, as iron removal from these proteins can occur via the action of NA, adrenaline and dopamine (Freestone *et al.*, 2000; Freestone *et al.*, 2002; Freestone *et al.*, 2003). The transfer of iron from Tf to bacteria mediated by NA stimulates growth under conditions of iron restriction similar to those found in the host (Freestone *et al.*, 2000).

In enteropathogenic *E. coli* (EPEC) strain E2348/69, serotype O127:H6, Tf was shown to be necessary for NA-induced growth enhancement (Freestone *et al.*, 2000). NA concentrations that stimulated growth in serum-SAPI minimal medium, used to mimic the nutritionally poor *in vivo* conditions encountered by bacteria, caused iron-saturated Tf to lose some bound iron; the same effect was observed with Lf (Freestone *et al.*, 2000). The effects were not seen when norepinephrine 3-*O*-sulphate, the sulphated, inactive form was used (Coughtrie, 1996; Freestone *et al.*, 2000). NA-Tf and NA-Lf complexes also enhanced bacterial growth in serum-SAPI minimal medium. Both the uptake and internalisation of radiolabelled iron from Tf or Lf, and the uptake and internalisation of radiolabelled NA were demonstrated following NA-enhanced growth of cells. The addition of non-radioactive iron reduced the amount of cell-associated radioactivity, but not growth levels (Freestone *et al.*, 2000). Complexing of the NA with either Tf or Lf was proposed to enhance iron availability *in vivo* allowing increased bacterial growth.

The process of NA-dependent growth enhancement in EHEC strain O157:H7 was shown to be dependent upon enterochelin synthesis and uptake, the absence of which could not be overcome by the growth autoinducer known to be synthesised by *E. coli* in the presence of NA (Lyte *et al.*, 1996b; Freestone *et al.*, 2003). NA was shown to bring about redistribution, referred to as shuttling, of the iron bound to Tf. Iron was transferred between available iron binding sites on Tf molecules either side of a dialysis partition. Apo-Tf was partitioned within dialysis tubing away from ferri-Tf, which was present in the buffer. Mobility shifts indicating that some changes between the iron-loading status of Tf molecules in the dialysis tubing occurred in the absence of NA. Following the addition of NA, the majority of apo-Tf partitioned away from the ferri-Tf was found to be in various states of iron-saturation; much of the previously iron-saturated Tf in the buffer was now monoferric or iron free (Freestone *et al.*, 2003). The use of enterochelin as the acceptor of iron liberated from Tf by NA was demonstrated. Strains carrying mutations in *entA* and *tonB* acquired much less radiolabelled iron from Tf partitioned away from the cells in the presence of NA than the wild-type strain. A plasmid-borne wild-type *entA* allele was introduced into the *entA* mutant strain. Complementation allowed restoration of the cell's

capability to acquire radiolabelled iron from partitioned Tf in the presence of NA (Freestone *et al.*, 2003). An increase in ferri-enterochelin allowed increased iron supply to the cells (Freestone *et al.*, 2003). The enterochelin allows bacterial internalisation of the iron released from Tf or Lf by NA (Freestone *et al.*, 2003); however the induction of increased iron uptake from Tf or Lf by NA is not yet fully characterised. NA (Fig. 6.1) demonstrates similarity to the catecholate structure of enterochelin (Fig. 6.2) that is known to be important for iron binding (Raymond and Dertz, 2004).

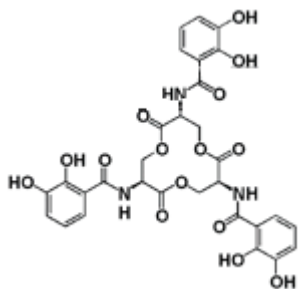


Figure 6.2. The structure of the ferric iron binding catecholate siderophore enterochelin (enterobactin).

Promotion of wild-type *S. Typhimurium* growth in serum-SAPI minimal medium occurred in the presence of NA (Methner *et al.*, 2008). Increased colonisation and systemic spread of *S. Typhimurium* also occurred in chicken and mouse infection models after NA pre-treatment of the animals (Methner *et al.*, 2008). In contrast to the results presented for *E. coli*, studies with *S. Typhimurium* indicated that growth stimulation by NA is not dependent upon the uptake of enterochelin or salmochelin S4. Enterochelin secretion or conversion to salmochelin S4 are also not vital as strains defective for either trait grew comparably to wild-type (Methner *et al.*, 2008). Growth of strains carrying mutations in *fepA* and *iroN* was also promoted by NA in serum-SAPI minimal medium *in vitro* and was not altered in models of infection when compared to wild-type cells (Rabsch *et al.*, 2003; Williams *et al.*, 2006). Conversely, an *ent* mutant strain TA2700 did not show NA-dependent growth promotion in serum supplemented liquid medium or on hens' egg-white supplemented agar plates. A strain carrying a mutation in *tonB* demonstrated the same phenotype. Therefore, the loss of enterochelin synthesis and TonB-dependent iron uptake by *S. enterica* resulted in the loss of NA-induced growth promotion (Rabsch *et al.*, 2003; Williams *et al.*, 2006; Methner *et al.*, 2008). The loss of enzymes involved in the breakdown of enterochelin or salmochelin S4 also resulted in the loss of the NA effect (Methner *et al.*, 2008). In addition, a strain carrying mutations in all three outer membrane receptor genes *iroN* (salmochelins), *fepA* (enterochelin) and *cir* (precursors/breakdown

products of enterochelin) did not demonstrate NA-dependent growth promotion in serum-SAPI minimal medium (Rabsch *et al.*, 2003; Williams *et al.*, 2006). Taken together, the synthesis of enterochelin and excretion of breakdown products, such as DHBS, followed by TonB-dependent uptake via the major iron receptor proteins are vital in the acquisition of NA-liberated Tf-derived iron under iron-restriction, leading to the growth promotion observed (Rabsch *et al.*, 2003; Williams *et al.*, 2006; Methner *et al.*, 2008). *In vivo*, however, *tonB* mutants were comparable to wild-type in chick and mouse models of infection (Methner *et al.*, 2008). In addition, DHBA, the enterochelin precursor known to supply iron to *S. enterica* independently of TonB (Kingsley *et al.*, 1996), allowed growth promotion of a *tonB* mutant strain in the presence of NA in serum-SAPI minimal medium (Methner *et al.*, 2008). This indicates that NA-dependent growth promotion in *S. Typhimurium* may also proceed in a TonB-independent manner involving DHBA (Methner *et al.*, 2008).

Growth and biofilm formation by the commensal organism *S. epidermidis* was enhanced in a dose-dependent manner by incubation with catecholamine inotropes and human plasma (Lyte *et al.*, 2003). Low bacterial inocula were used to mimic bacterial infective doses and inotropic drugs were added at the concentration used in hospital intensive care units (Lyte *et al.*, 2003). *S. epidermidis* were incubated in SAPI minimal medium with plasma in which Tf loaded with radiolabelled iron was present. The addition of catecholamine inotropes was shown to stimulate bacterial growth. In addition, enhanced levels of radiolabelled iron from Tf were shown to be incorporated by the cells (Lyte *et al.*, 2003). Previous studies have shown that staphylococci growth in nutrient poor liquid culture with serum is supported by catecholamine supply of iron derived from Tf to cells (Freestone *et al.*, 2000; Neal *et al.*, 2001). The supply of catecholamines to patients in intensive care may therefore also induce *S. epidermidis* growth and potential biofilm formation leading to increased bloodstream infections (Lyte *et al.*, 2003).

NA stimulated delivery of iron from host Tf and Lf to bacteria allows increased growth. The exact mechanisms involved still require further investigation and the putative bacterial receptors through which this process acts are not fully characterised (Freestone *et al.*, 2008). The synthesis and breakdown of enterochelin, followed by TonB-dependent uptake of DHBS is important for NA-dependent growth promotion of *S. enterica* (Methner *et al.*, 2008). Enterochelin synthesis by *E. coli* has been shown to be essential for NA promotion of growth (Freestone *et al.*, 2003), a process which requires further investigation. Based on the evidence in *Salmonella*, the breakdown products or precursors

of enterochelin may also be supplying the iron to *E. coli*. The presence of NA widely enhances bacterial growth and virulence allowing survival and replication in the nutrient-poor host environment resulting in increased levels of infection.

6.1.5. Noradrenaline and *Campylobacter*

Recent work has demonstrated that in iron-limited serum-containing medium the addition of NA (100 μ M) results in a significant increase in the final optical density of *Campylobacter jejuni* cultures compared to when NA was absent (Cogan *et al.*, 2006). When adrenoreceptor antagonists were added, the NA effect was not reversed, indicating a lack of mammalian-type adrenoreceptors on the *C. jejuni* cell surface. The authors suggested that there may be a role for iron-uptake receptor proteins in this process based on previous work with *Salmonella* spp. (Williams *et al.*, 2006; Section 6.1.4.1). The addition of NA to iron-restricted culture medium also increased the number of motile *C. jejuni* cells. Pre-incubation of cells with NA increased bacterial invasion of Caco-2 cells compared to wild-type. Bacteria cultured with NA also demonstrated a greater ability to disrupt cultured epithelial cell monolayers and break down Caco-2 cellular tight junction barriers (Cogan *et al.*, 2006).

Growth of bacteria in the presence of radiolabelled NA resulted in low levels of radioactivity associated with the cell pellet (1 ± 1 count per minute). Due to this, the authors concluded that the NA was not internalised by the cells. Radiolabelled iron from serum was taken up more readily by cells in the presence of NA than in the absence of NA, indicating NA augmentation of the iron uptake process (Cogan *et al.*, 2006). The system in *C. jejuni* (Cogan *et al.*, 2006) appears to differ from that seen in *E. coli* where NA is internalised (Freestone *et al.*, 2000). Preliminary work with *C. jejuni* cells both in contact with and partitioned away from radioactively-labelled iron-loaded Tf and Lf showed increased iron acquisition in the presence of NA, but the NA was not essential for uptake (Rock, 2003).

Poultry meat contaminated with *C. jejuni* is known to be a key source of human infection (Hood *et al.*, 1988; Altekruuse *et al.*, 1999). As an increase in stress causes increases in the levels of secreted catecholamine hormones in the human host, this may also occur in the chicken. Processes encountered by chickens during food production, such as transport, flock thinning, over-crowding, and pain from hock- and pad-burn due to poor living conditions, are likely to induce NA production and secretion into the gastrointestinal

tract due to elevated stress levels (Slader *et al.*, 2002). The increase in NA in the avian gut, which is the niche of commensal *C. jejuni*, may induce the changes in phenotype described above *in vivo* (Cogan *et al.*, 2006). In combination with a stress-weakened immune capability, increased colonisation of the gut and invasion of surrounding tissues by *C. jejuni* may then ensue (Peterson *et al.*, 1991). Increased cell numbers may lead to a higher rate of *C. jejuni* infection of humans following inadequate cooking of poultry meat.

6.1.6. Noradrenaline, Host Transferrins and *Campylobacter*

Previous work to investigate the role of NA in bacterial growth promotion included the use of medium supplemented with 10% foetal bovine serum (FBS; Cogan *et al.*, 2006). Mueller-Hinton (MH) broth, a complex medium, chelex-treated MH broth (CMH), and Dulbecco's Modified Eagles Medium (DMEM), a minimal medium, were supplemented with FBS and used to grow *C. jejuni* in the presence and absence of NA (Cogan *et al.*, 2006). Iron limitation in this system is principally achieved by the iron-binding serum protein Tf (Weinberg, 1978). The belief that campylobacters lack a specific uptake system for Lf- or Tf-derived iron has probably resulted in the host glycoproteins being overlooked as key factors contributing to the NA effects seen. Moreover, the study described in Section 6.1.5 (Cogan *et al.*, 2006) is the first investigation of any specific effects of NA on *C. jejuni* biology and virulence. In light of the data presented in Chapters 3-5, investigation of Tf- or Lf-specific NA growth responses is necessary as part of this project. The growth of *C. jejuni* in the nutrient poor and iron restricted medium MEM α supplemented with human ferri-Lf, human ferri-Tf or human ferri-ovo-Tf, as used throughout the course of this study, should demonstrate whether growth promotion occurs in the absence of the other components of serum. MH broth and CMH broth used during the previous study (Cogan *et al.*, 2006) are rich, complex media and CMH was also chelex-treated. MEM α is a defined medium and is already iron-limited, avoiding the need for chelex-treatment. The iron-restricted and nutrient limited conditions induced by MEM α may act to mimic the host environment and by using MEM α , the components included in the assay can be controlled, with each of the Tfs added individually with and without NA. The exact composition of the serum cannot be defined as easily. Serum-based medium may mimic the host environment more closely, but within the controlled MEM α assay system any growth effects seen may be attributed to a particular Tf. Attempts to identify a system or systems involved in the uptake of iron liberated from host proteins by NA was completed by testing

of the mutant strains already constructed as part of this project. Growth of the mutant strains in the presence and absence of a particular Tf, with and without NA, was monitored for differences.

6.2. Results

6.2.1. Growth Promotion of *C. jejuni* by Noradrenaline in Liquid Culture

In a previous study *C. jejuni* demonstrated increased growth in the presence of NA (100 μ M) under iron-limited conditions (Cogan *et al.*, 2006). Initial phenotypic analysis of *C. jejuni* NCTC 11168 in the presence and absence of NA was carried out as part of the present study, to establish whether the same growth patterns could be produced using the defined MEMa growth system supplemented with a particular iron source. NCTC 11168 was initially grown in the presence and absence of iron, with and without NA (100 μ M) in MEMa over 24 h with samples taken at intervals (Fig. 6.3). Cells were inoculated to initial optical densities at 600 nm of either 0.025 or 0.0125 (corresponding to approximately 2×10^8 cfu/ml and 1×10^8 cfu/ml, respectively), the standard initial inoculum used during this study and half that normally used. The inoculum used in the previous study was lower (approximately 1×10^4 cfu/ml), resulting in an initial optical density that was not detectable by the Bioscreen plate reader. The different initial optical densities were used as part of the present study to investigate whether a more marked effect could be seen with slightly lower or higher initial cell numbers, or whether there was no notable difference. All raw data for the liquid growth assays found in this chapter and statistical testing of the differences seen are detailed in Appendix 3.

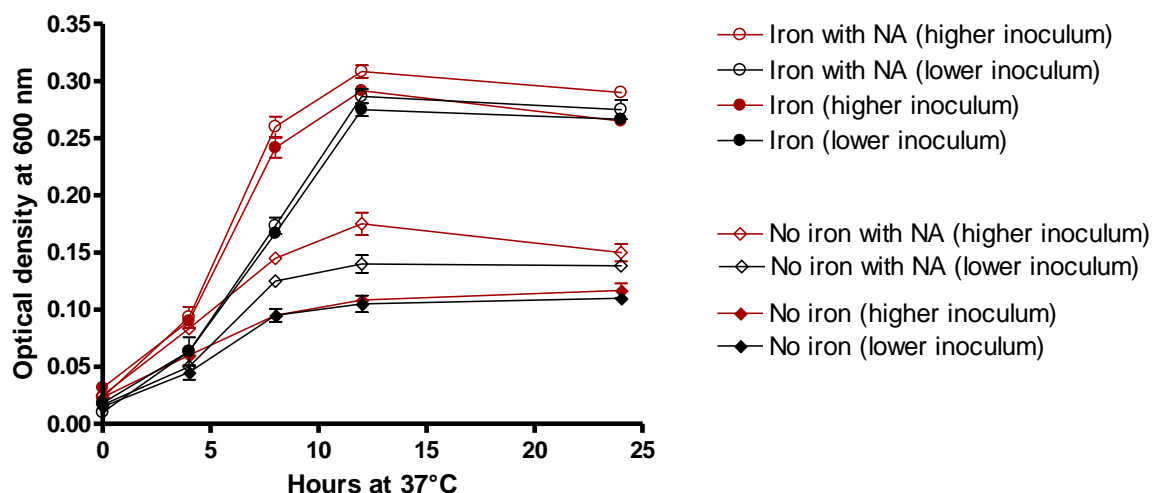


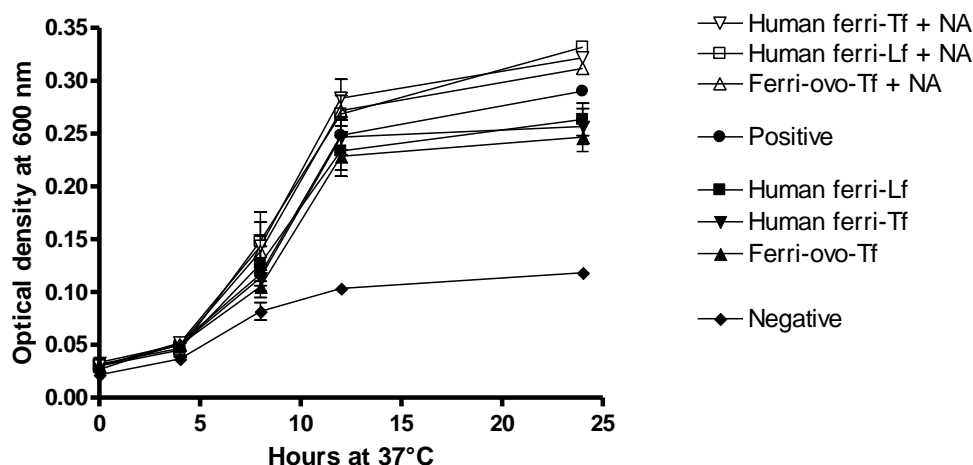
Figure 6.3. Growth assay of *C. jejuni* NCTC 11168 with and without NA in the presence and absence of iron. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO $_4$ (iron-replete conditions), 10 μ M FeSO $_4$ with NA (100 μ M), unsupplemented medium (iron-limited conditions), unsupplemented medium with NA (100 μ M). Cultures initially inoculated to an optical density at 600 nm (OD $_{600}$) of 0.025 (approximately 2×10^8 cfu/ml) at 0 h are labelled as higher inoculum. Those cultures inoculated to an initial OD $_{600}$ of 0.0125 (approximately 1×10^8 cfu/ml) at 0 h are labelled as lower inoculum.

Following overnight iron-depletion, NCTC 11168 cells reached optical densities at 600 nm of around 0.10 after 24 h growth in unsupplemented MEM α , and between 0.25 and 0.30 in the presence of 10 μ M FeSO $_4$ (Fig. 6.3). The final optical densities were comparable to those seen under control conditions in all other growth assays (Chapters 3 and 4). The addition of 100 μ M NA resulted in a small, but significant increase in growth under both iron-replete and iron-limited conditions (Fig. 6.3). The increase was greater under iron-restricted conditions with NA supplemented cultures growing more than the corresponding culture minus NA from 8 h onwards, particularly notable in exponential growth phase (Fig. 6.3; lower inoculum, $P = <0.0154$, higher inoculum, $P = <0.0083$, Appendix 3). The increase in growth under iron-rich conditions was significantly more when NA was added to cultures that had been inoculated to the higher initial optical density of 0.025 (Fig. 6.3; $P = <0.0352$, Appendix 3). When an initial OD $_{600}$ of 0.0125 was used, the difference in growth between NA and non-NA cultures was not significant (Fig. 6.3; $P = 0.0572$, Appendix 3).

6.2.2. Effect of Noradrenaline on *C. jejuni* Growth with Ferri-Glycoproteins

Following growth in the presence and absence of iron, the effect of NA supplementation on NCTC 11168 growth with human ferri-Lf, human ferri-Tf or ferri-ovo-Tf was tested. Growth was monitored over a 24 h period. Two concentrations of ferri-protein were tested to determine whether there were any ferri-protein concentration-dependent differences in growth promotion caused by the addition of NA. Iron-replete (10 μM FeSO_4) and iron-restricted (unsupplemented MEM α) conditions induced NCTC 11168 growth patterns comparable to those seen in Figure 6.3. High-iron conditions allowed cells to achieve a final OD₆₀₀ of between 0.25 and 0.30; low-iron limited cells to a final OD₆₀₀ of just above 0.10 (Fig. 6.4a, b). In the presence of 0.111 μM human ferri-Lf, human ferri-Tf or ferri-ovo-Tf, NCTC 11168 cells reached an OD₆₀₀ of just above 0.20 at 24 h (Fig. 6.4a). When 0.27 μM of each ferri-protein was added to cultures, cells reached a final OD₆₀₀ of between 0.25 and 0.30 (Fig. 6.4b). These values were comparable to the final OD₆₀₀ seen with all proteins in previous growth assays (for example Fig. 3.3a). The addition of NA to cultures containing each ferri-protein resulted in a significant increase in growth with concentrations of 0.111 μM (Fig 6.4a) and 0.27 μM (Fig. 6.4b) by 24 h. The greatest increase was seen when 0.111 μM ferri-protein was used (Fig. 6.4a); the difference was significant. The NA-induced growth increase was not observed to be specific for a particular ferri-protein, with NA augmenting NCTC 11168 growth with all proteins.

a)



b)

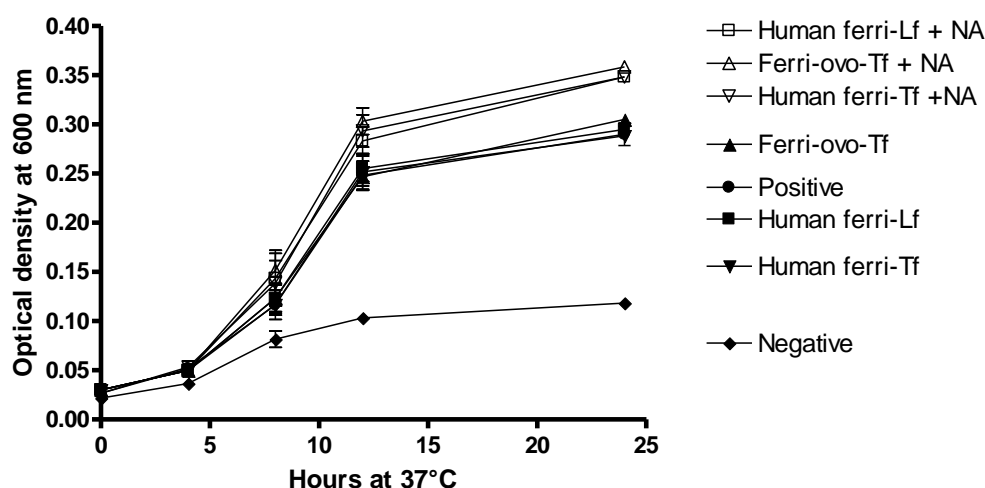


Figure 6.4. Growth assay of *C. jejuni* NCTC 11168 with and without NA in the presence of human ferri-Lf, human ferri-Tf or ferri-ovo-Tf. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μM FeSO_4 (iron-replete conditions), unsupplemented medium (iron-limited conditions). **a)** NCTC 11168 with 0.111 μM human ferri-Lf, human ferri-Tf or ferri-ovo-Tf in the presence and absence of NA (100 μM). **b)** NCTC 11168 with 0.27 μM human ferri-Lf, human ferri-Tf or ferri-ovo-Tf in the presence and absence of NA (100 μM).

6.2.3. Mutant Strain Growth with Noradrenaline

As NA-dependent growth enhancement was observed under iron-limitation, high-iron conditions and in the presence of human ferri-Lf, human ferri-Tf and ferri-ovo-Tf, strains carrying mutations in key iron uptake genes were tested for growth in the presence

of ferri-protein with NA. The growth increase seen may be due to NA increasing the availability of the iron and uptake of this iron may proceed via a known iron-uptake system. If a specific system is involved, the loss of the outer membrane receptor protein for that system should abolish the NA-dependent increase in growth. If a system is not involved, mutant strain growth patterns similar to wild-type cells would be expected. Cells were grown over 24 h, samples were taken at intervals and all growth assays were preceded by overnight iron-depletion. As little difference was seen between the growth patterns of cells supplied with iron bound to all ferri-proteins, a single protein, human ferri-Lf was chosen for use in the growth assays of mutant strains (Fig. 6.5a-e).

All strains demonstrated comparable growth to wild-type cells under iron-replete (10 μ M FeSO₄, 25 μ M haem) and iron-restricted (unsupplemented MEM α) conditions (Fig. 6.5a-e). Final optical densities of between 0.25 and 0.30 were reached in the presence of FeSO₄, between 0.30 and 0.40 in the presence of porcine haemin and between 0.10 and 0.15 in unsupplemented medium (Fig. 6.5a-e). All final optical densities were comparable to those observed in previous growth assays (Chapter 4).

In the presence of 0.27 μ M human ferri-Lf as a sole iron source, mutant strains JDR5 (Δ *chuA::cat*), KAR3 (*p19::aphA-3*, periplasmic binding protein mutant strain), KAR2 (*cj0178::aphA-3*), CEM5 (Δ *cfrA::aphA-3*), Δ *feoB::ermC'* 11168 and CEM12 (KAR2 Δ *feoB::ermC'*, Table 2.2) grew similarly to previous growth assays (Fig. 6.5a-e; Fig. 4.11a-d, 4.16a, 4.18b; NCTC 11168 also grew as expected when provided with iron in the form of human ferri-Lf, Fig. 6.5a-e compared to Fig. 3.3a).

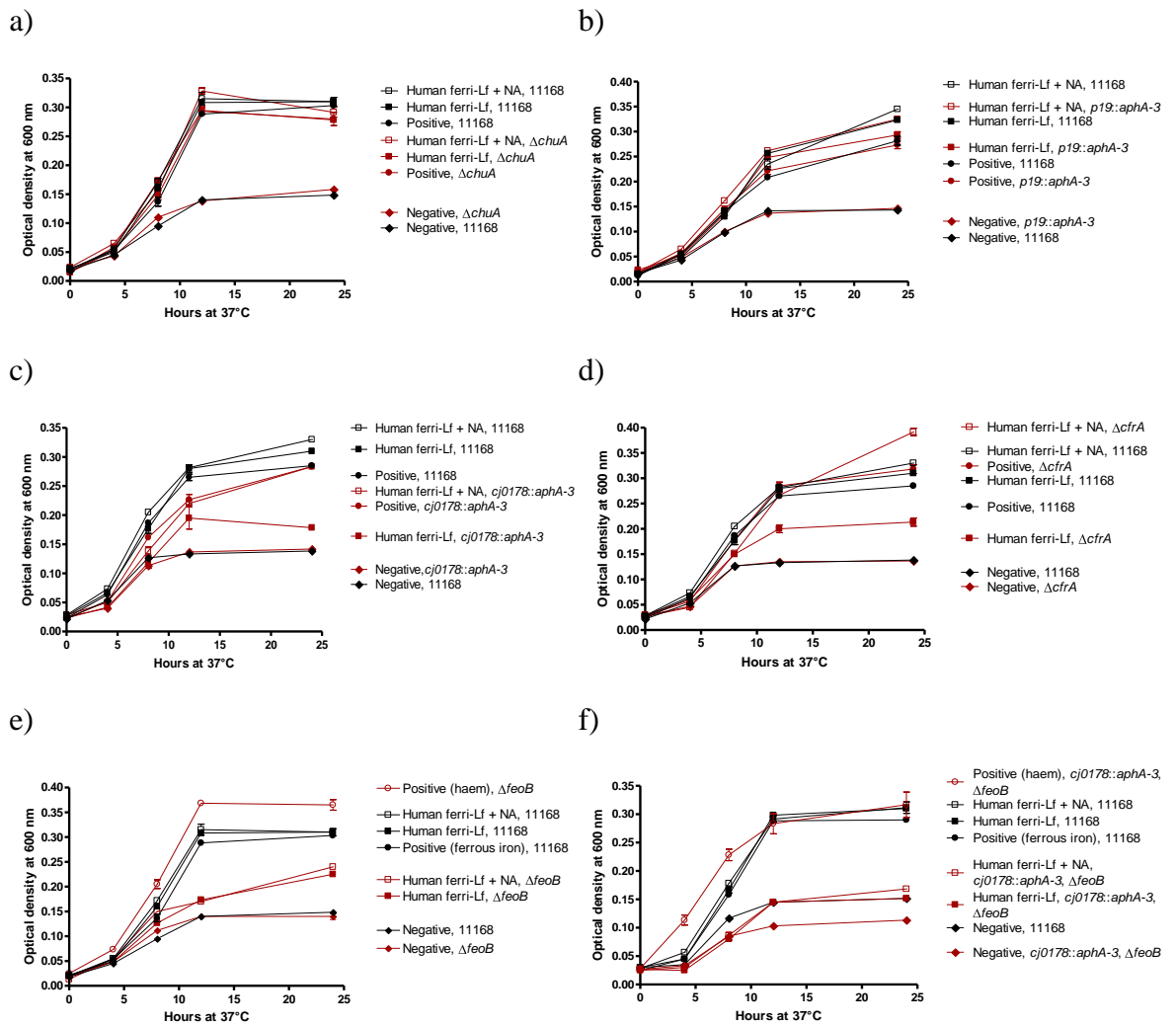


Figure 6.5. Growth assays of *C. jejuni* NCTC 11168 mutant strains with iron supplied bound to human Lf in the presence and absence of NA. All assays were conducted using MEM α with cultures incubated microaerobically with agitation. All conditions were tested in triplicate in two independent assays. Each data point is the mean of the 6 replicates with sample error shown. 10 μ M FeSO₄ (iron-replete conditions, positive), 25 μ M haem (iron-replete conditions, positive), unsupplemented medium (iron-limited conditions, negative), human ferri-Lf (0.27 μ M), human ferri-Lf (0.27 μ M) with NA (100 μ M). **a)** NCTC 11168 and JDR5 ($\Delta chuA::cat$). **b)** NCTC 11168 and KAR3 ($p19::aphA-3$). **c)** NCTC 11168 and KAR2 ($cj0178::aphA-3$). **d)** NCTC 11168 and CEM5 ($\Delta cfrA::aphA-3$). **e)** NCTC 11168 and $\Delta feoB::ermC'$ 11168. **f)** NCTC 11168 and CEM12 (KAR2, $\Delta feoB::ermC'$ 11168).

Strains JDR5 (Fig. 6.5a) and KAR3 (Fig. 6.5b) demonstrated similar growth patterns to wild-type cells when NA (100 μ M) was added to cultures with human ferri-Lf (0.27 μ M). By 24 h there was a small, but significant increase in growth observed when cultures contained NA compared to the cultures only containing human ferri-Lf (Fig. 6.5a, b; JDR5, $P = <0.0316$, KAR3, $P = <0.0203$, Appendix 3). Strains KAR2 (Fig. 6.5c) and CEM5 (Fig. 6.5d) grew less than wild-type when supplied with human ferri-Lf (0.27 μ M)

as seen previously (Fig.4.11). KAR2 and CEM5 also showed the most marked NA effect; by 24 h the difference was considerable between the KAR2 cultures, with and without NA ($P = <0.0052$, Appendix 3), and the CEM5 cultures, with and without NA ($P = <0.0023$, Appendix 3). Cultures containing NA grew far more than the cultures without NA (Fig. 6.5c, d). The addition of NA allowed KAR2 cells to achieve a final OD₆₀₀ of just above 0.25 in the presence of human ferri-Lf, compared to just above 0.10 without NA (Fig. 6.5c). NA supplementation augmented CEM5 growth to an optical density at 600 nm at 24 h of 0.40 in the presence of human ferri-Lf, compared to 0.20 when NA was not present (Fig. 6.5d). The loss of *feoB* (Fig. 6.5e, f) resulted in cells growing less than wild-type in the presence of ferri-Lf (0.27 μ M) as seen before (Fig. 4.16). The same small but significant increase in growth seen with strains JDR5 and KAR3 was seen with the *feoB* mutant strain and strain CEM12 which carried a double mutation in *cj0178* and *feoB* (Fig. 6.5e, f; *feoB* mutant, $P = <0.0352$, CEM12, $P = <0.0378$, Appendix 3). The addition of NA increased the growth of the strains in the presence of human ferri-Lf by 24 h (Fig. 6.5e, f), but did not result in the phenotype seen with strains KAR2 and CEM5 (Fig. 6.5c, d).

6.3. Discussion

The ability of pathogenic bacteria to acquire iron is vital for successful colonisation and infection of the human host (Andrews *et al.*, 2003). In times of stress, elevated levels of catecholamine hormones are produced and secreted by the host, a large proportion of which can be found in the gut (Costa *et al.*, 2000). Catecholamines such as NA affect a range of bacterial factors leading to increased pathogenicity (Freestone *et al.*, 2008). NA is proposed to liberate iron from members of the Tf family (Freestone *et al.*, 2000). The iron can be shuttled between Tf molecules and sites within a molecule allowing increased iron supply to bacteria. The process is dependent upon the ability to synthesise the bacterial siderophore enterochelin in *E. coli* (Freestone *et al.*, 2003).

The addition of NA to iron-restricted MEM α (unsupplemented) and MEM α with added iron (10 μ M FeSO₄) induced a significant increase in *C. jejuni* growth compared to cultures without NA (Fig. 6.3). The only exception was when cells initially inoculated to an OD₆₀₀ of 0.0125 (corresponding to approximately 1×10^8 cfu/ml) were grown in the presence of iron with and without NA; the difference in growth seen here was not significant. The increase in growth seen under iron-limitation in MEM α may be due to NA acting as a siderophore supplying the trace amounts of iron in the medium to cells more

readily than it can be acquired in the absence of the neurotransmitter. MEM α contains no added iron, but the manufacturers state that there may be traces of iron in the medium from the distilled water used to produce it (Invitrogen, per. comm.). NA may be capable of binding and supplying the iron to *C. jejuni* cells, causing the observed increase in growth. In the presence of iron, the NA may again increase iron availability and augment the uptake process, so explaining the slight increase in growth seen.

A significant increase in *C. jejuni* growth was also seen when NA was added to cultures in MEM α containing human ferri-Lf, human ferri-Tf or ferri-ovo-Tf (each at 0.111 μ M or 0.27 μ M) compared to those without added NA (Fig. 6.4). An increase in *C. jejuni* growth in the presence of NA was also shown in a previous study (Cogan *et al.*, 2006). The increases seen during the present study were not as marked as those seen in the previous study (Cogan *et al.*, 2006) where cells were grown in iron-restricted medium (DMEM) with 10% serum, with and without NA. The use of MEM α as part of the current work allowed iron-restriction in a defined system. The addition of a particular iron source was followed by growth under nutrient limited conditions; this allowed the generation of data using the same experimental system throughout the current project. The two systems (used here and by Cogan and co-workers) differ experimentally and technically so identical results would not be expected; however general trends do match, and NA augmentation of growth is reproducible. The initial inoculum used during the previous study (Cogan *et al.*, 2006) was reported to be 1×10^4 cfu/ml. The use of far fewer cells at the beginning of the assay generated a considerably greater growth difference than was seen in the present study when NA was added, perhaps due to the lower inoculum and use of serum better mimicking the conditions likely to occur *in vivo*. To maintain consistency between growth assays during the course of the present study, the same initial inoculum was used throughout. The experiments completed as part of this chapter should be repeated with an inoculum comparable to that used in the previous study (Cogan *et al.*, 2006) and any changes in growth patterns noted. The work presented here does, however, indicate that the NA-dependent growth effect is specific for iron bound by the Tfs. Growth increases were seen with all proteins tested, indicating that NA can augment the availability of Lf-, Tf- and ovo-Tf-bound iron for use by *C. jejuni*. Although Tf is a major serum iron-binding protein (Weinberg, 1978), the use of serum did not directly indicate the involvement ferri-Tf in the NA-dependent *C. jejuni* growth increases (Cogan *et al.*, 2006). Although growth promotion by NA occurred in the absence of each protein when cells were incubated in either MEM α or MEM α with iron in the present study (Fig. 6.3), the increase in growth

seen when NA was added to cultures containing the Tfs was greater (Fig. 6.4). The growth augmentation seen may be due to the NA promoting iron shuttling between Tf, Lf or ovo-Tf molecules (Freestone *et al.*, 2003) possibly altering iron-binding affinities and enabling NA-delivery of iron to the *C. jejuni* cells.

The use of NA-liberated iron by *C. jejuni* does not appear to be dependent upon enterochelin. The NA is likely to be acting as a siderophore, directly supplying the liberated iron to *C. jejuni*. Alternatively, it is possible that an unidentified low molecular weight, iron-binding compound produced by *C. jejuni* could be involved. There is also a possibility that a compound within the MEM α is acting in this way. A more complex process whereby NA interacts with the Tf and the *C. jejuni* cell surface, requiring a degree of contact for iron acquisition, may occur. A dialysis partition assay could be used where the *C. jejuni* cells are partitioned from each of the ferri-Tfs. The addition of NA may allow comparable growth to that seen when there is no partition. If this occurs, it would imply that iron is liberated from Tf and delivered to *C. jejuni* by NA or via NA in combination with another small compound. Were contact required, less growth promotion by NA may be expected in this system. As NA seems to augment growth in the presence of Tf-, Lf- and ovo-Tf-bound iron, NA may be capable of mobilising iron between different proteins. By separating apo-Lf within dialysis tubing from ferri-Tf in the surrounding buffer, the effect of adding NA to the system could be monitored as done previously with apo- and ferri-Tf (Freestone *et al.*, 2003). Changes in the iron-saturation of the proteins would indicate that NA is redistributing the bound iron between iron-free binding sites on proteins found on either side of the partition. This could be repeated with all combinations of apo- and ferri-proteins. Repetition of the work completed with radiolabelled iron loaded-Tf or -Lf would demonstrate increased iron uptake in the presence of NA (Rock, 2003). By incubating *C. jejuni* with ^{55}Fe -Lf, ^{55}Fe -Tf or ^{55}Fe -ovo-Tf in iron-limited medium with NA, uptake of iron from each protein could be shown and compared to iron uptake in the absence of NA. The partitioning of cells from the iron source and the addition of NA would directly associate NA with the uptake process if cells acquired more iron than those partitioned and incubated without NA. This would also demonstrate that contact was not essential. NA-mediated iron supply to *C. jejuni* may, however, proceed via a diffusible non-contact dependent process or a contact dependent process under different conditions.

The potential involvement of the major iron outer membrane receptor proteins in this process was investigated. *C. jejuni* iron outer membrane receptor mutants were grown in the presence of the Tfs, with and without NA in MEM α . The growth patterns were

compared to wild-type cells grown under the same conditions. NA-dependent delivery of iron to the *C. jejuni* cell surface does not appear to proceed through the ChuABCDZ system or the Cj1658-Cj1663 system, responsible for the transport of haem (Ridley *et al.*, 2006) and the fungal hydroxamate siderophore rhodotorulic acid (Ketley, J. M., unpublished data), respectively. Strains carrying mutations in either *chuA* or *p19* grew comparably to wild-type cells under all conditions (Fig. 6.5a, b). The addition of NA to cultures of KAR2 (*cj0178::aphA-3*) or CEM5 (Δ *cfrA::aphA-3*) containing human ferri-Lf resulted in restoration of the wild-type growth patterns (*cj0178*) or growth exceeding wild-type levels (*cfrA*; Fig. 6.5c, d). The loss of either gene impairs the ability of *C. jejuni* to grow with human ferri-Lf as a sole iron source (Fig. 4.11). Specific involvement of Cj0178 or CfrA as a receptor for NA supplied iron would be proposed if KAR2 or CEM5 had been incapable of the NA-induced growth increase. The growth increase is therefore unexpected, and may imply that the iron is being liberated from human ferri-Lf by NA and supplied to *C. jejuni* through a different system. The addition of NA to cultures of Δ *feoB::ermC* 11168 and CEM12, the double mutant strain lacking both *feoB* and *cj0178*, containing human ferri-Lf resulted in the same small but significant increase in growth seen with wild-type cells (Fig. 6.5e, f). The increase in growth seen when NA was added to cultures of KAR2 and CEM5 containing human ferri-Lf was not seen here. Therefore, the phenotype of strains lacking *feoB* in the presence of human ferri-Lf does not appear to be rescued by NA, even with the absence of *cj0178* (CEM12). The loss of *feoB* in a *cj0178* mutant strain background abolishes the growth increase seen when only *cj0178* was mutated. In light of this, the NA-liberated iron from human ferri-Lf could be entering the cell cytoplasm via the FeoB system. Cells lacking Cj0178, CfrA or FeoB are impaired in the uptake of iron for growth from the Tfs compared to wild-type cells. The addition of NA may liberate iron from the Tfs, allowing uptake to proceed independently of Cj0178 or CfrA and resulting in the increase in growth seen in Fig. 6.5c, d. If this liberated iron, either alone or complexed with NA were entering the cell via the outer membrane porins or an unidentified transporter, transport across the inner membrane may be dependent upon FeoB. This would explain the loss of the NA-dependent growth boost in the two strains lacking *feoB*. The requirement for FeoB would also indicate that Tf-derived iron is reduced during this process, possibly at the *C. jejuni* cell surface or within the periplasm by ferric reductases. Recent work completed by another member of the group (R. D. Haigh) has shown that under iron-restriction (using DMEM) in the presence of serum a *cfrA* mutant strain cannot grow in the presence of NA, whereas wild-type cells and other outer

membrane receptor mutant strains can. The mutant phenotype was complemented. The data indicate a primary role for CfrA in the process of accepting NA-liberated iron from serum iron-binding proteins. The NA may therefore supply iron directly to CfrA in a siderophore-like manner, and once transported across the outer membrane, the iron may be reduced within the periplasm. Transport of the Fe^{2+} may then proceed across the inner membrane via FeoB; however, no role was demonstrated for FeoB using the serum-based growth system (R. D. Haigh). There are differences between the results generated from the serum-based system and MEM α system for studying NA growth induction of *C. jejuni*. Technical and experimental inconsistencies may go some way to explaining this, as different media, growth conditions and supplements were used. Key differences are the use of serum and a different initial inoculum. As this process in *C. jejuni* is only just being characterised, very little is known about the mechanisms involved which require further investigation.

The process of iron uptake from human ferri-Lf, human ferri-Tf and ferri-ovo-Tf appears to require direct contact and proceed via the outer membrane receptor protein Cj0178, with the potential involvement of the ferri-enterochelin outer membrane receptor CfrA (Palyada *et al.*, 2004). Transport of the iron may then proceed across the inner membrane via the FeoB transport system or via the ABC transporter systems. The inclusion of NA appears to liberate Fe^{3+} from the host iron-binding proteins, which is transported through CfrA (based on work by R. D. Haigh), and possibly across the inner membrane via the ABC transporter systems. Evidence presented here suggests that under the conditions used, some iron may be reduced to the ferrous form in the presence of NA, requiring transport across the inner membrane by the FeoB system. The loss of CfrA did not affect the NA response in the system used during the present study.

Chapter 7. General Discussion

7.1. General Discussion

The aim of the present study was to advance the general understanding of the mechanisms of iron uptake from transferrins (Tfs) by *Campylobacter jejuni* NCTC 11168. Prior to this project, the uptake of iron from ^{55}Fe -loaded Lf and ^{55}Fe -loaded Tf by *C. jejuni* was shown (Rock, 2003). Involvement of the putative outer membrane receptor protein Cj0178 (Section 1.5.1.4) was indicated (Rock, 2003), but in depth characterisation of the process was not completed.

7.1.1. Receptor-Based Uptake

Much of the data presented in this study favours the involvement of an iron-regulated ligand-gated outer membrane porin in the uptake of iron from Tfs, favouring the idea of a receptor-based system in *C. jejuni* (Fig. 7.1).

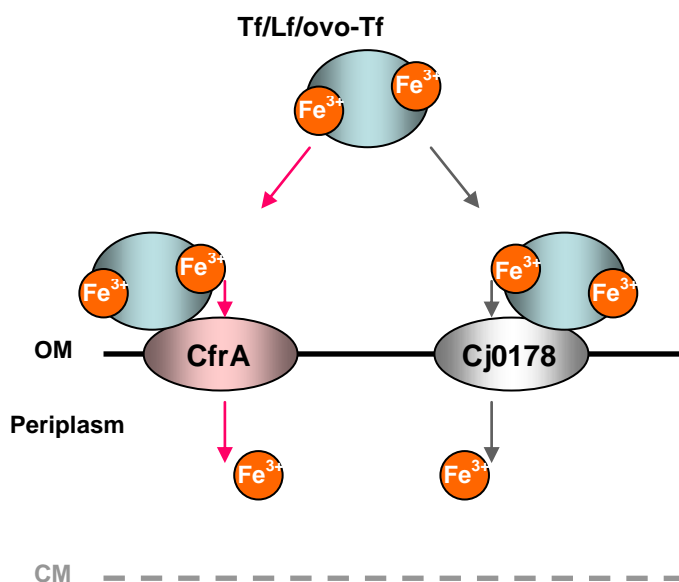


Figure 7.1. Proposed model involving an outer membrane receptor-based system of transferrin-derived iron uptake in *C. jejuni*. The direct binding of ferri-Tf, ferri-Lf or ferri-ovo-Tf to an outer membrane receptor protein would be followed by conformational changes in the ligand-gated porin resulting in active transport of the Fe^{3+} across the outer membrane. Based on the evidence, the most likely receptor appears to be Cj0178, but the receptor CfrA could also be involved.

Direct contact is necessary as partitioning of the cells away from Tfs reduced growth. Iron acquisition via a specific receptor also appears to be occurring. Separate receptor-based systems specific for the uptake of iron from individual Tfs are commonly found in other bacteria (Gray-Owen and Schryvers, 1995; Cornelissen and Sparling, 2004). The evidence presented here suggests the presence of a novel, single global system capable of iron uptake from all Tfs tested. The need for contact indicates that the process does not seem to rely upon a siderophore in *C. jejuni*. A lack of siderophore biosynthesis genes in the NCTC 11168 genome (Parkhill *et al.*, 2000) supports this. Siderophores are necessary for iron uptake from the Tfs by *Pseudomonas aeruginosa* (Sriyosachati and Cox, 1986), and also *Bordetella pertussis* and *B. bronchiseptica*, which use a siderophore-dependent or contact-dependent method (Redhead, 1987; Gorringer *et al.*, 1990; Menozzi *et al.*, 1991).

Iron-responsive binding of ferri-Lf to the *C. jejuni* cell surface favours the idea of binding via an iron-regulated outer membrane component. As Tf was also previously shown to bind to *C. jejuni* whole cells (Rock, 2003), binding of both Tf and Lf may occur via the same receptor. Confirmation of iron-responsive binding of Tf and ovo-Tf to *C. jejuni* whole cells is still required. The primary receptor for Tfs in *C. jejuni* appears to be Cj0178 (Fig. 7.1). However, Cj0178 does not appear to be essential, as growth of a *cj0178* mutant strain was not completely abolished in the presence of the Tfs. The *cj0178* mutation was complemented. Demonstration of the uptake of comparable amounts of ^{55}Fe to wild-type cells from Lf, Tf or ovo-Tf by CEM8 is still required. Less ferri-Lf bound to cells that lacked Cj0178 under iron-restriction which indicated a partial role for the OMRP in Lf binding. The simplest explanation would be that Cj0178 binds Tfs at the cell surface (Fig. 7.1). Acting as a TonB-dependent ligand-gated porin, Cj0178 then transports Fe^{3+} from Tfs into the periplasm (Fig. 7.1). Solving the crystal structure of Cj0178, with and without bound substrate, as a typical ligand-gated porin would further support a role for the protein in this capacity. Some bound ferri-protein may be transported into the cell as part of the uptake process, but this is unlikely based on the mechanisms characterised in other bacteria (Simonson *et al.*, 1982). Demonstration of a reduction in binding of ferri-Tf and ferri-ovo-Tf to cells lacking Cj0178 would further indicate a global role for Cj0178 and support the presence of a single transport system. If iron uptake from ^{55}Fe -Tf and ^{55}Fe -ovo-Tf was impaired in KAR2, this would also support this proposal. Binding may be blocked by the use of anti-Cj0178 antibodies to demonstrate specificity. Differences in the preference of KAR2 and NCTC 11168 cells for apo- and ferri-protein binding may indicate a role for Cj0178 in distinguishing between the two forms.

In addition to Cj0178, a possible role for the ferri-enterochelin outer membrane receptor protein CfrA (Palyada *et al.*, 2004) was also indicated by the data presented (Fig. 7.1). Sequence similarities between Cj0178 and CfrA, and comparable identities with TbpA from *N. meningitidis* may explain the effect seen. CfrA has been shown conclusively to be involved in ferri-enterochelin uptake (Palyada *et al.*, 2004); however, a role cannot be ruled out for this protein in iron uptake from Lf or Tf. As CfrA is also a TonB-dependent ligand-gated porin, the mechanism here may be similar to that seen with Cj0178. The ferri-protein may be bound by CfrA and the Fe^{3+} removed and transported across the outer membrane followed by release of the apo-protein without internalisation (Fig. 7.1). The mutation requires complementation and further investigation of the mutant phenotype is needed. Demonstration of less ferri-Lf, ferri-Tf or ferri-ovo-Tf binding to cells lacking CfrA would support a similar role to Cj0178 for CfrA in this process. Alternatively, Cj0178 and CfrA may be acting co-operatively to acquire the iron. In *Neisseria*, a second protein (TbpB or LbpB) is known to be involved in augmenting the uptake process; however TbpB and LbpB are lipoproteins, not ligand-gated porins (Legrain *et al.*, 1993; Anderson *et al.*, 1994; Pettersson *et al.*, 1998). Further investigation as to whether one receptor could be more specific for a particular Tf than the other is required. Successful construction and testing of a *cj0178*, *cfrA* double mutant strain would show whether the loss of both receptors in a single strain had a more marked effect on growth, indicating a co-operative role for the receptors in the uptake of iron bound to the Tfs. Conclusive identification of receptors may involve cross-linking of the iron source to *C. jejuni* proteins. Following purification, if the *C. jejuni* protein identified was a known iron outer membrane receptor protein, deletions may be made in the extracellular loops. Expression of the different deletion proteins and investigation of binding capability would demonstrate which parts of the protein are important.

7.1.2. Liberation of Iron from the Transferrins

Iron may be liberated from Tfs at the cell surface by binding to a ligand-gated outer membrane porin as described in the previous section. Alternatively, liberation of iron may occur by the action of an outer membrane or secreted protease. Iron can be liberated from Tfs by proteolytic degradation at key iron binding sites or sites needed for protein structure preservation; the liberated iron can then be acquired (Wolz *et al.*, 1994; Brochu *et al.*, 2001). There was no demonstration of protease activity completed during this project, but *C. jejuni* may employ this mechanism. Once liberated by proteolytic cleavage of the Tfs, Fe^{3+} may be transported by a known uptake system. In order to investigate this mechanism, culture supernatant and Tf partitioned away from *C. jejuni* would be expected to release iron from the Tf if a secreted protease was contained in the supernatant. The iron could then diffuse freely to *C. jejuni* cells on the opposite side of the barrier causing growth promotion. Sensitivity of the *C. jejuni* secreted protease to heat or to protease degradation and the loss of intact Tf following the assay would support this hypothesis.

C. jejuni may be capable of the production of a small metabolic product or other low molecular weight compound able to liberate iron from Tfs for uptake. The compound may be capable of supplying Tf-derived iron to an outer membrane receptor protein in a siderophore-like manner. The compound may directly interact with Tfs causing iron release. Alternatively, secretion of the compound may be required for successful iron uptake following binding of Tfs to an outer membrane receptor. The release of iron from Tfs by an uncharacterised small compound may explain the small amount of growth seen during partitioned assays. Alternatively, this growth may be due to iron contamination. Sterile *C. jejuni* cell culture supernatant supplied to growth-restricted *E. coli* in serum-SAPI medium may induce a growth stimulatory effect (Freestone *et al.*, 2000). If growth stimulation occurred, this would indicate a role for a compound produced by *C. jejuni* in liberating and possibly supplying iron from Tfs to bacteria for growth. If growth promotion did not occur here, this would support the proposal that iron contamination had arisen during dialysis partitioned assays due to the complicated set-up of the experimental system.

7.1.3 ABC Transporter Systems

Following transport across the outer membrane by a ligand-gated porin, iron liberated from the Tfs may be transported across the inner cytoplasmic membrane by an ABC transporter system (Fig. 7.2).

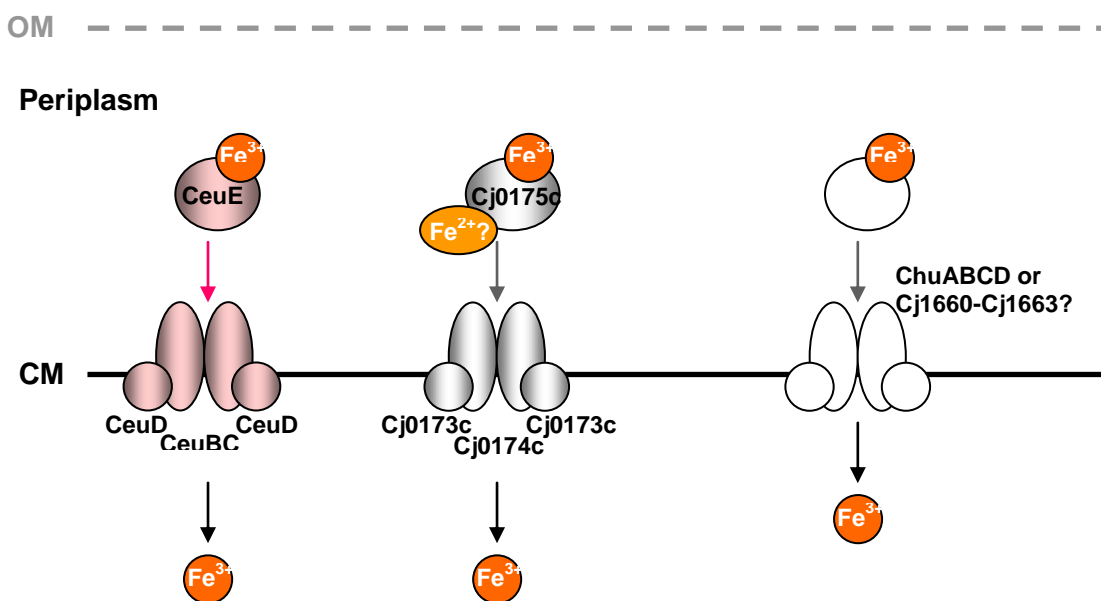


Figure 7.2. Proposed model of iron transport across the inner cytoplasmic membrane following acquisition from transferrins. Once in the periplasm, Fe³⁺ transport across the inner cytoplasmic membrane may proceed via the ABC transporter system associated with Cj0178, CfrA or any of the known *C. jejuni* ABC transporter systems due to redundancy. The periplasmic binding protein Cj0175c may also be capable of binding Fe²⁺.

Iron transport across the inner membrane may proceed via the ABC transporter system Cj0175c-Cj0173c encoded adjacent to Cj0178 in NCTC 11168 (Fig. 7.2; Parkhill *et al.*, 2000). A small growth defect was seen when a component (Cj0174c, Fig. 7.2) of the ABC transporter system was mutated, indicating a deficiency in Tf-derived iron transport across the inner membrane. Redundancy amongst the inner membrane transporters has been indicated by a loss of growth in the presence of haem proportional to the loss of transport systems (Chapter 4); this may also be occurring here. As CfrA also appeared to be involved in the uptake process, the associated ABC transporter system CeuBCDE may be involved in the transport of iron across the inner membrane (Fig. 7.2); however this requires investigation. Growth assays of single and double ABC transporter mutant strains would confirm whether the small defect occurs with the loss of all systems and whether the defect increases as more systems are lost. Confirmation of the involvement of any of the *C.*

jejuni ABC transporter systems may be achieved by the expression of a particular transport system in an organism that cannot utilise iron from the Tfs. Treatment of cells with sodium azide and carbonylcyanide *m*-chlorophenylhydrazone would disrupt ATP-dependent processes. A decrease in growth in the presence of Tf-derived iron under these conditions may indicate that the process is energy dependent.

Roles for the lipoprotein Cj0177 encoded upstream of Cj0178 and the ExbBD-TonB system encoded downstream of Cj0178 were not conclusively proven. Co-expression of the genes *cj0177-tonB1* (*cj0181*) was indicated by the demonstration of iron responsive promoter activity immediately upstream of *cj0177*, and the apparent lack of promoter activity upstream of *cj0178* or *exbB1* (*cj0179*). As the genes appear to be expressed as an operon, Cj0177 and the associated ExbBD-TonB system may still function in the uptake of Tf-derived iron even though the growth studies proved inconclusive. The loss of two or more ExbBD-TonB systems would be likely to demonstrate more clearly whether the process is TonB-dependent, but may lead to non-viable cells. Mutations of the Cj0178 N-terminal region, typically containing the TonB box in ligand-gated porins (Braun and Endriß, 2007), would allow specific investigation of TonB dependency. Repeat construction of a non-polar *cj0177* mutant strain which could be tested for growth defects in the presence of the Tfs, followed by complementation of the mutation, would allow further investigation of the role of Cj0177. Conceivably, the process may not be TonB dependent and may be dependent on an unidentified system. Cj0178 TonB-box mutants with a wild-type phenotype may indicate a lack of TonB dependency. Neisserial mutant strains lacking the characterised TonB have been shown to acquire iron from Tf or Lf via an independent energy transduction system coupled to outer membrane receptors capable of facilitating iron uptake from TonB-dependent ligands (Desai *et al.*, 2000). Searches for *N. meningitidis* and *N. gonorrhoeae* *tonB* homologues did not identify *tonB* genes additional to those already characterised (Desai *et al.*, 2000). The authors proposed that a lack of homology between *tonB* and the unidentified energy transduction system components may account for failing to find the unknown energy transduction system within the genome (Desai *et al.*, 2000).

7.1.4. Ferric Reduction

Evidence is also presented that does not support the role of a conventional OMRP coupled with an ABC transporter system in iron uptake from Tfs. Transport of iron derived from the Tfs may proceed across the inner membrane via the Fe^{2+} transport system, FeoB (Naikare *et al.*, 2006; Fig. 7.3).

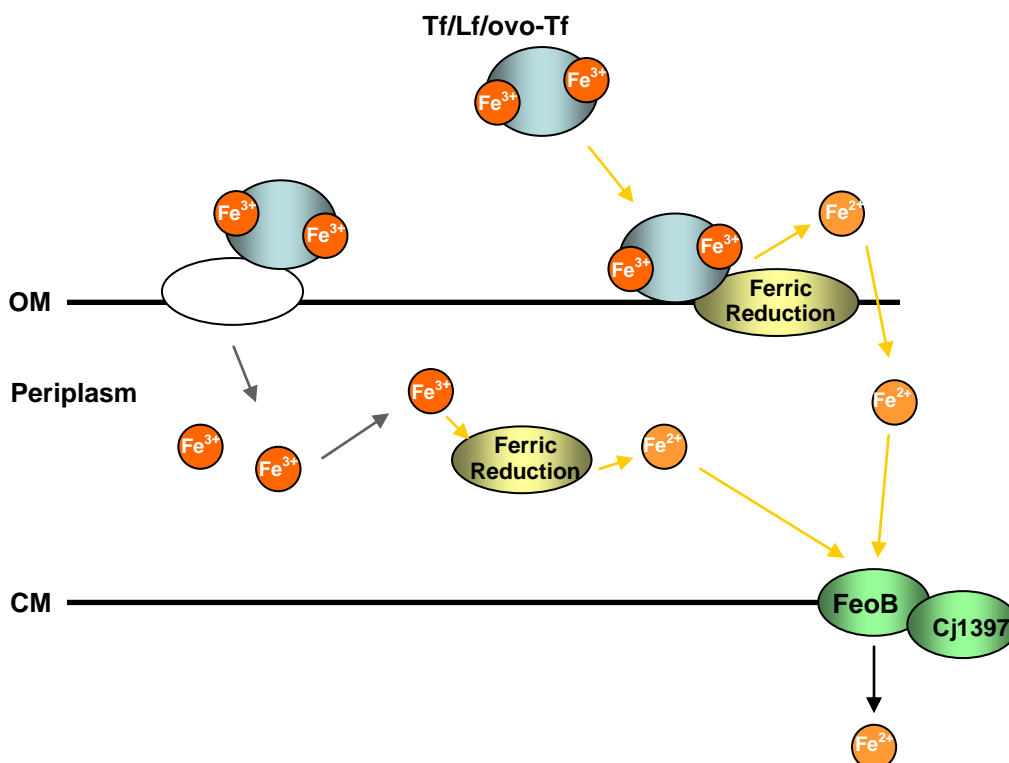


Figure 7.3. Proposed model of ferric reduction of transferrin-derived iron and subsequent transport of Fe^{2+} across the inner membrane by FeoB. The process may involve cell surface-associated or periplasmic ferric reduction.

The partial involvement of FeoB may support the requirement for external reduction of Tf-associated iron (Fig. 7.3). Direct reduction of Tf-associated Fe^{3+} may occur resulting in dissociation of the Fe^{2+} , followed by passive diffusion through the outer membrane porins (Fig. 7.3). Alternatively, once transported across the outer membrane by a ligand-gated porin, Fe^{3+} may be reduced to Fe^{2+} in the periplasm (Fig. 7.3). Fe^{2+} would then be transported across the inner membrane by the FeoB transporter into the cytoplasm (Fig. 7.3). As the Cj0175c periplasmic binding protein seemed to preferentially bind Fe^{2+} over Fe^{3+} in a previous study (Tom-Yew *et al.*, 2005; Fig. 7.2), this protein may be responsible for transport of Fe^{2+} in the periplasm. A direct involvement for Cj0175c in the transport of

Fe^{2+} derived from ferri-Tfs has not been shown by this study. If an outer membrane receptor and the FeoB transporter are both involved, binding to the receptor may first be required to allow cell surface-associated reduction. Ferric reductase activity has been detected (Crossley *et al.*, 2007; Ketley, J. M. and Haigh, R. D., unpublished data), but there are no obvious candidate genes for extracellular ferric reductases in the NCTC 11168 genome (Parkhill *et al.*, 2000). Ferric reductase activity was reduced in a strain carrying a mutated copy of *cj0572*, required for riboflavin biosynthesis, and increased in a *fur* mutant strain (Crossley *et al.*, 2007). The investigation of both *cj0572* and *fur* mutant strains for ferric reductase activity and the uptake of iron from the Tfs would demonstrate if there is a correlation. If riboflavin biosynthesis is directly linked with ferric reductase activity, and that activity results in an increase in iron uptake from the Tfs, ferric reduction could be another potential mechanism of acquiring iron from this source. Coupling of the need for ferric reduction of Tf-derived iron and receptor-mediated transport across the outer membrane implies periplasmic reduction of the iron. The need for ferric reduction without receptor-based transport implies that reduction is extracellular involving either direct ferric reductase-Tf interaction or cell surface associated reduction following liberation of iron from the protein. Reduction may instead proceed via the action of a compound produced by *C. jejuni* itself, but this has not yet been demonstrated. Conceivably, ferric reductase activity may not be involved.

7.1.5. Noradrenaline

The catecholamine and neurotransmitter noradrenaline (NA) was shown to augment, but was not essential for, the process of iron-uptake from Tfs. NA is known to supply iron for the growth of *E. coli* in the presence of Tf and Lf (Freestone *et al.*, 2000). The growth enhancement in the presence of the Tfs, proposed to be due to the uptake of NA-liberated iron, appeared to be impaired in strains carrying a mutated copy of *feoB* in the MEM α assay system. Fe^{3+} liberated by NA in a serum-based system requires CfrA for successful uptake by *C. jejuni* (R. D. Haigh). NA may be acting as a siderophore, liberating iron from the Tfs, which is then transported through CfrA. Once in the periplasm, some iron may be reduced and then transported across the inner membrane by FeoB (Fig. 7.3). Some CfrA transported Fe^{3+} may also be transported across the inner membrane by CeuBCDE (Fig. 7.2), but this remains to be characterised. It is not likely that NA-iron complexes are taken up by CfrA as radioactivity was not shown to be cell

associated following growth in the presence of radiolabelled NA (Cogan *et al.*, 2006). Further investigation of the process is required due to the apparent involvement of different uptake systems when using different experimental set-ups.

7.1.6 Proposed Model and the Importance of Iron Uptake from the Transferrins *in vivo*

Based on the evidence presented the most likely model would appear to involve a ligand-gated outer membrane porin in the binding of Tfs. The primary receptor would appear to be Cj0178. Transport is likely to proceed in a TonB-dependent manner, using energy transduced by the ExbBD-TonB system (Fig. 7.4).

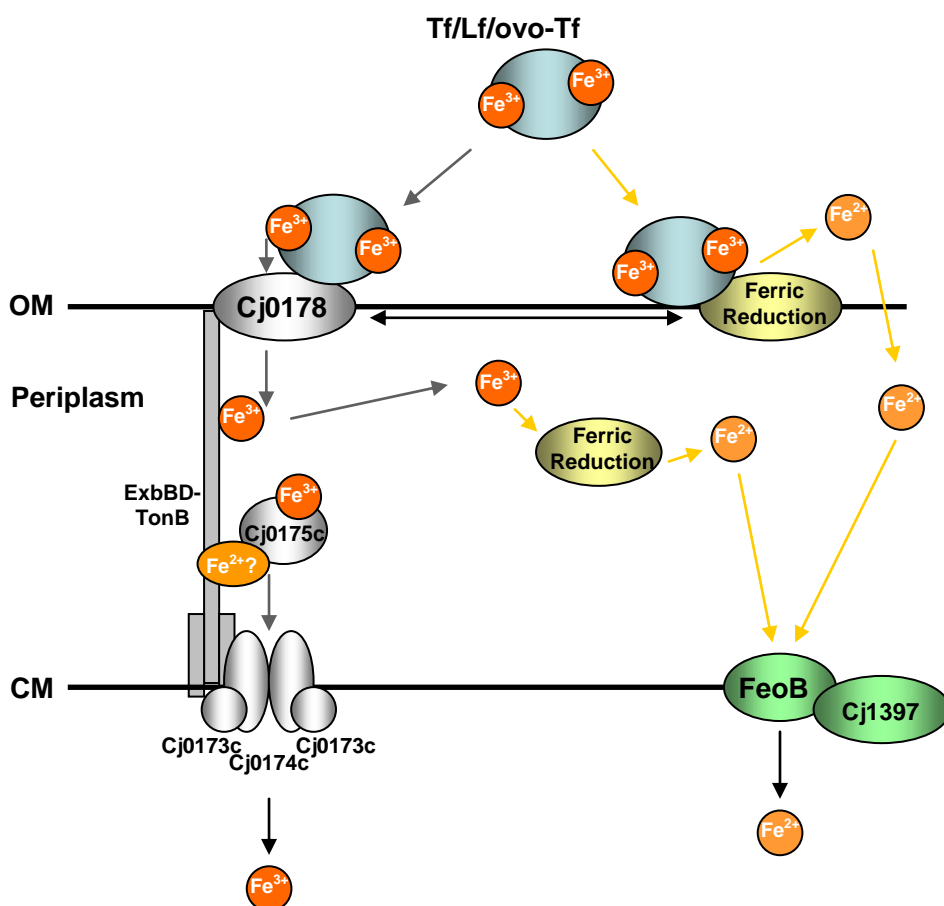


Figure 7.4. Proposed model of transferrin-derived iron uptake in *C. jejuni* NCTC 11168. Acquisition of iron from Tfs is proposed to require binding of Tfs to a TonB-dependent outer membrane ligand-gated porin and extracellular reduction. Once in the periplasm, Fe^{3+} is proposed to be transported across the inner membrane via an ABC transporter. Reduction of periplasmic Fe^{3+} may occur. Periplasmic Fe^{2+} is proposed to be transported across the inner membrane via the FeoB transporter. Transport of Fe^{2+} in the periplasm may involve Cj0175c.

Following removal and transport of the Tf-associated Fe^{3+} across the outer membrane by Cj0178, transport across the inner membrane is proposed to involve the Cj0175c-Cj0173c ABC transporter system (Fig. 7.4). Tfs are not proposed to be internalised. A requirement for ferric reduction is also proposed (Fig. 7.4). Extracellular reduction involving a direct interaction with Tfs, or Tfs and Cj0178, would be followed by diffusion of Fe^{2+} into the periplasm and transport across the inner membrane via FeoB (Fig. 7.4). Fe^{3+} transported across the outer membrane by Cj0178 may alternatively be reduced in the periplasm and then transported by FeoB (Fig. 7.4). There may be a role for Cj0175c in the transport of Fe^{2+} in the periplasm (Fig. 7.4). Once in the cytoplasm, the iron would be available for use in cellular and metabolic processes or storage.

In vivo, *C. jejuni* is likely to encounter a range of iron sources during colonisation, the major source being ferri-enterochelin, produced by members of the *Enterobacteriaceae* present in the intestinal niche of the human host (Payne and Mey, 2004). Iron is also present bound to Lf in the mucus secretions. Upon translocation or due to damage of the epithelium, iron may be encountered bound to Tf. Iron bound to Tfs would also be available during commensal colonisation of the chicken (Masson and Heremans, 1966; Masson *et al.*, 1966; Weinberg, 1978; Giansanti *et al.*, 2002; Section 1.4.5). Based on the data presented here, uptake of Tf-derived iron in the host may proceed via Cj0178, which has been shown to be required for colonisation *in vivo* using animal models (Palyada *et al.*, 2004; Stintzi *et al.*, 2005). The substrate for Cj0178 had not been identified prior to this study. CfrA, an outer membrane receptor that is also required for successful colonisation of animal models (Palyada *et al.*, 2004), may also be involved in Tf-bound iron transport in the host. Transport of Fe^{2+} derived from ferri-Tfs by FeoB may be required *in vivo*; requirement for the FeoB system for *in vivo* colonisation by *C. jejuni* has been indicated (Naikare *et al.*, 2006). The involvement of these systems in the uptake of iron from the Tfs suggests that this iron source may be crucial for colonisation and survival in human and avian hosts. The secretion of NA due to stress is likely to increase the availability of iron and in turn augment the growth of *C. jejuni in vivo*. Avian colonisation and increased growth due to the production of NA under times of stress lead to *Campylobacter* contamination of poultry food products. NA-supply of iron to *C. jejuni* appears to directly involve CfrA. The process of iron uptake from Tfs has not been characterised in *C. jejuni* strain 81-176. This strain can grow using the iron supplied by Tfs, but lacks both Cj0178 and CfrA (Hofreuter *et al.*, 2006) and the involvement of Cj0444 was not demonstrated.

The mechanism of uptake in this strain and other strains lacking Cj0178 and CfrA requires further investigation.

Further characterisation and identification of the components involved in this process are required before a conclusive model of the mechanism of Tf-derived iron uptake in *C. jejuni* can be proposed. The identification of an uptake system that appears to be capable of acquiring iron from Lf-, Tf- and ovo-Tf in *C. jejuni* is novel. The apparent involvement of a number of systems indicates that this process is not only novel, but also complex, and at the point of writing this study, incompletely understood. Conclusive identification of the *C. jejuni* components involved in the uptake of Tf-derived iron, and the development of therapeutic agents designed to block iron uptake during colonisation, would aid control of human infection. In addition, targeting of those components may prevent bacterial overgrowth in the avian host, so decreasing contamination during production. A reduction in the stress encountered by poultry during production processes may lead to a reduction in the NA-enhanced growth effect, which in turn, would also reduce food contamination. The development of measures to control colonisation would lead to a reduction in human infections and in turn reduce the economic burden of disease.

Bibliography

Abdallah, F. B. and El Hage Chahine, J.-M. (2000). Transferrins: Iron release from lactoferrin. *Journal of Molecular Biology* **303**, 255-266.

Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R. and Andrews, S. C. (1999). Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. *Journal of Bacteriology* **181**, 1415-1428.

Adak, G. K., Long, S. M. and O'Brien, S. J. (2002). Trends in indigenous food borne disease and deaths, England and Wales: 1992 to 2000. *Gut* **51**, 832-841.

Adhikari, P., Berish, S. A., Nowalk, A. J., Veraldi, K. L., Morde, S. A. and Mietzer, T. A. (1996). The *fbpABC* locus of *Neisseria gonorrhoeae* functions in the periplasm-to-cytosol transport of iron. *Journal of Bacteriology* **178**, 2145-2149.

Aisen, P. and Leibman, A. (1972). Lactoferrin and transferrin: A comparative study. *Biochimica et Biophysica Acta* **257**, 314-323.

Albert, M. J., Haridas, S., Steer, D., Dhaunsi, G. S., Smith, A. I. and Adler, B. (2007). Identification of a *Campylobacter jejuni* protein that cross-reacts with cholera toxin. *Infection and Immunity* **75**, 3070-3073.

Almiron, M., Link, A. J., Furlong, D. and Kolter, R. (1992). A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes and Development* **6**, 2646-2654.

Altekruse, S. F., Stern, N. J., Fields, P. I. and Swerdlow, D. W. (1999). *Campylobacter jejuni* - an emerging food borne pathogen. *Emerging Infectious Diseases* **5**, 28-35.

Althaus, E. W., Outten, C. E., Olson, K. E., Cao, H. and O'Halloran, T. V. (1999). The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. *Biochemistry* **38**, 6559-6569.

Altuvia, S., Almiron, M., Huisman, G., Kolter, R. and Storz, G. (1994). The *dps* promoter is activated by OxyR during growth and by IHF and σ^S in stationary phase. *Molecular Microbiology* **13**, 265-272.

Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L. and Storz, S. (1997). A small, stable RNA induced by oxidative stress: Role as a pleiotropic regulator and antimutator. *Cell* **90**, 43-53.

Amabile-Cuevas, C. F. and Demple, B. (1991). Molecular characterization of the *soxRS* genes of *Escherichia coli*: Two genes control a superoxide stress regulon. *Nucleic Acids Research* **19**, 4479-4484.

Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rum ball, S. V., Waters, J. M. and Baker, E. N. (1987). Structure of human lactoferrin at 3.2-Å resolution. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 1769-1773.

Anderson, J. E., Sparling, P. F. and Cornelissen, C. N. (1994). Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. *Journal of Bacteriology* **176**, 3162-3170.

Andrews, S. C., Le Brun, N. E., Barynin, V., Thomson, A. J., Moore, G. R., Guest, J. R. and Harrison, P. M. (1995). Site-directed replacement of the coaxial heme ligands of bacterioferritin generates heme-free variants. *Journal of Biological Chemistry* **270**, 23268-23274.

Andrews, S. C. (1998). Iron storage in bacteria. *Advances in Microbial Physiology* **40**, 281-351.

Andrews, S. C., Robinson, A. K. and Rodríguez-Quinones, F. (2003). Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**, 215-237.

Åneman, A., Eisenhofer, G., Olbe, L., Dalenbäck, J., Nitescu, P., Fändriks, L. and Friberg, P. (1996). Sympathetic discharge to mesenteric organs and the liver. *The Journal of Clinical Investigation* **97**, 1640-1646.

Archibald, F. (1983). *Lactobacillus plantarum*, an organism not requiring iron. *FEMS Microbiology Letters* **19**, 29-32.

Åslund, F., Zheng, M., Beckwith, J. and Storz, G. (1999). Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 6161-6165.

Attardo-Genco, C. and White-Dixon, D. (2001). Emerging strategies in microbial haem capture. *Molecular Microbiology* **39**, 1-11.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1992). *Short protocols in molecular biology.*, Second edn. New York: Greene Publishing Associates and John Wiley and Sons.

Autenreith, I. B., Schwarzkopf, A., Ewald, J. H., Karch, H. and Lissner, R. (1995). Bactericidal properties of *Campylobacter jejuni* specific immunoglobulin M antibodies in commercial immunoglobulin preparations. *Antimicrobial Agents and Chemotherapy* **39**, 1965-1969.

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

- Bacon, D. J., Alm, R. A., Hu, L., Hickey, T. E., Ewing, C. P., Batchelor, R. A., Trust, T. J. and Guerry, P. (2002).** DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. *Infection and Immunity* **70**, 6242-6250.
- Bacon, D. J., Alm, R. A., Burr, D. H., Hu, L., Kopecko, D. J., Ewing, C. P., Trust, T. J. & Guerry, P. (2000).** Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infection and Immunity* **68**, 4384-4390.
- Baichoo, N. and Helmann, J. D. (2002).** Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. *Journal of Bacteriology* **184**, 5826-5832.
- Bailey, M. T., Karaszewski, J. W., Lubach, G. R., Coe, C. L. and Lyte, M. (1999).** *In vivo* adaptation of attenuated *Salmonella typhimurium* results in increased growth upon exposure to norepinephrine. *Physiology and Behaviour* **67**, 359-364.
- Baillon, M.-L. A., van Vliet, A. H. M., Ketley, J. M., Constantinidou, C. and Penn, C. W. (1999).** An iron-regulated alkyl hydroperoxide reductase (AhpC) confers aerotolerance and oxidative stress resistance to the microaerophilic pathogen *Campylobacter jejuni*. *Journal of Bacteriology* **181**, 4798-4804.
- Baker, E. N., Anderson, B. F., Baker, H. M., MacGillivray, R. T. A., Moore, S. A., Peterson, N. A., Shewry, S. C. and Tweedie, J. W. (1998).** Three-dimensional structure of lactoferrin - implications for function, including comparisons with transferrin. *Advances in Experimental Medicine and Biology* **443**, 1-14.
- Baker, E. N., Baker, H. M. and Kidd, R. D. (2002).** Lactoferrin and transferrin: Functional variations on a common structural framework. *Biochemistry and Cell Biology* **80**, 27-34.
- Barkley, M. D. and Bourgeois, S. (1978).** *Repressor recognition of operators and effectors in The Operon*, pp.177-220.: Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Batchelor, R. A., Pearson, B. M., Friis, L. M., Guerry, P. and Wells, J. M. (2004).** Nucleotide sequences and comparison of two large conjugative plasmids from different *Campylobacter* species. *Microbiology* **150**, 3507-3517.
- Bauer, C. E., Elsen, S. and Bird, T. H. (1999).** Mechanisms for redox control of gene expression. *Annual Review of Microbiology* **53**, 495-523.
- Beery, J. T., Hugdahl, M. B. and Doyle, M. P. (1988).** Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Applied and Environmental Microbiology* **54**, 2365-2370.
- Beinert, H., Holm, R. H. and Munck, E. (1997).** Iron-sulfur clusters: Nature's modular, multipurpose structures. *Science* **277**, 653-659.
- Bereswill, S., Lichte, F., Vey, T., Fassbinder, F. and Kist, M. (1998).** Cloning and characterization of the *fur* gene from *Helicobacter pylori*. *FEMS Microbiology Letters* **159**, 193-200.

- Berish, S. A., Subbarao, S., Chen, C.-Y., Trees, D. L. and Morse, S. A. (1993).** Identification and cloning of a Fur homologue from *Neisseria gonorrhoeae*. *Infection and Immunity* **61**, 4599-4606.
- Bernheimer, A. W. and Schwartz, L. L. (1960).** Leucocidal agents of haemolytic streptococci. *Journal of Pathology and Bacteriology* **79**, 37-46.
- Bindereif, A. and Neilands, J. B. (1985).** Promoter mapping and transcriptional regulation of the iron assimilation system of plasmid ColV-K30 in *Escherichia coli* K-12. *Journal of Bacteriology* **162**, 1039-1046.
- Birnboim, H. C. and Doly, J. (1979).** A rapid alkaline lysis procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1522.
- Birnboim, H. C. (1983).** A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods in Enzymology* **100**, 243-255.
- Biswas, D., Itoh, K. and Sasakawa, C. (2003).** Role of microfilaments and microtubules in the invasion of INT407 cells by *Campylobacter jejuni*. *Microbiology and Immunology* **47**, 469-473.
- Biswas, G. D., Anderson, J. E., and Sparling, P. F. (1997).** Cloning and functional characterization of *Neisseria gonorrhoeae* *tonB*, *exbB* and *exbD* genes. *Molecular Microbiology* **24**, 169-179.
- Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. and Blaser, M. J. (1988).** Experimental *Campylobacter jejuni* infection in humans. *Journal of Infectious Diseases* **157**, 472-479.
- Blaser, M. J. and Duncan, D. (1984).** Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. *Infection and Immunity* **44**, 292-298.
- Bleuel, C., Grosse, C., Taudte, N., Scherer, J., Wesenberg, D., Krauss, G. J., Nies, D. H. and Grass, G. (2005).** TolC is involved in enterobactin efflux across the outer membrane of *Escherichia coli*. *Journal of Bacteriology* **187**, 6701-6707.
- Bolton, F. J., Dawkins, H. C. and Hutchinson, D. N. (1985).** Biotypes and serotypes of thermophilic campylobacters isolated from cattle, sheep and pig offal and other red meats. *Journal of Hygiene* **95**, 1-6.
- Bourke, B., al Rashid, S. T., Bingham, H. L. and Chan, V. L. (1996).** Characterization of *Campylobacter upsaliensis* *fur* and its localization in a highly conserved region of the *Campylobacter* genome. *Gene* **183**, 219-224.
- Bourke, B., Chan, V. L. and Sherman, P. (1998).** *Campylobacter upsaliensis*: waiting in the wings. *Clinical Microbiology Reviews* **11**, 440-449.

- Bozzi, M., Mignogna, G., Stefanini, S., Barra, D., Longhi, C., Valenti, P. and Chiancone, E. (1997).** A novel non-heme iron-binding ferritin related to the DNA-binding proteins of the Dps family in *Listeria innocua*. *Journal of Biological Chemistry* **272**, 3259-3265.
- Bradford, M. M. (1976).** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.
- Bradley, T. M., Hidalgo, E., Leautaud, V., Ding, H. and Demple, B. (1997).** Cysteine-to-alanine replacements in the *Escherichia coli* SoxR protein and the role of the [2Fe-2S] centers in transcriptional activation. *Nucleic Acids Research* **25**, 1469-1475.
- Brandonisio, O., Panaro, M. A., Marzio, R., Marangi, A., Faliero, S. M. and Jirillo, E. (1994).** Impairment of the human phagocyte oxidative responses caused by *Leishmania* lipophosphoglycan (LPG): *In vitro* studies. *FEMS Immunology and Medical Microbiology* **8**, 57-62.
- Brás, A. M. and Ketley, J. M. (1999).** Transcellular translocation of *Campylobacter jejuni* across human polarised epithelial monolayers. *FEMS Microbiology Letters* **179**, 209-215.
- Braun, V., Pils, H. and Gross, P. (1994).** Colicins: Structures, modes of action, transfer through membranes, and evolution. *Archives of Microbiology* **161**, 199-206.
- Braun, V. (1997).** Surface signalling: Novel transcription initiation mechanism starting from the cell surface *Archives of Microbiology* **167**, 325-331.
- Braun, V. and Braun, M. (2002).** Iron transport and signalling in *Escherichia coli*. *FEBS Letters* **529**, 78-85.
- Braun, V. and Endriß, F. (2007).** Energy-coupled outer membrane transport proteins and regulatory proteins. *Biometals* **20**, 219-231.
- Brochu, V. I., Grenier, D. I., Nakayama, K. and Maynard, D. (2001).** Acquisition of iron from human transferrin by *Porphyromonas gingivalis*: A role for Arg- and Lys-gingipain activities. *Oral Microbiology and Immunology* **16**, 79-87.
- Brown, J. S. and Holden, D. W. (2002).** Iron acquisition by Gram-positive bacterial pathogens. *Microbes and Infection* **4**, 1149-1156.
- Bruschi, M. and Guerlesquin, F. (1988).** Structure, function and evolution of bacterial ferredoxins. *FEMS Microbiology Reviews* **54**, 155-175.
- Bryans, J. T., Smith, A. G. and Baker, A. G. (1960).** Ovine vibronic abortion caused by a new variety of *Vibrio*. *Cornell Veterinarian* **50**, 54-59.
- Bsat, N., Herbig, A., Casillas-Martínez, L., Setlow, P. and Helmann, J. D. (1998).** *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. *Molecular Microbiology* **29**, 189-198.

Buchanan, S. K., Smith, B. S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D. and Deisenhofer, J. (1999). Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **6**, 56-63.

Buffone, G. J. and Darlington, G. J. (1985). Isolation of DNA from biological specimens without extraction with phenol. *Clinical Chemistry* **31**, 164-165.

Bullen, J. J., Rogers, H. J., Spalding, P. B. & Ward, C. G. (2006). Natural resistance, iron and infection: A challenge for clinical medicine. *Journal of Medical Microbiology* **55**, 251-258.

Burnens, A. P. and Nicolet, J. (1992). Detection of *Campylobacter upsaliensis* in diarrheic dogs and cats, using a selective medium with cefoperazone. *American Journal of Veterinary Research* **53**, 48-51.

Butzler, J. P., Dekeyser, P., Detrain, M. and Dehaen, F. (1973). Related vibrio in stools. *Journal of Paediatrics and Child Health* **82**, 493-495.

Cadieux, N. and Kadner, R. J. (1999). Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 10673-10678.

Calderwood, S. B. and Mekalanos, J. J. (1988). Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. *Journal of Bacteriology* **170**, 1015-1017.

Calva, J. J., Ruiz-Palacios, G. M., Lopez-Vidal, A. B., Ramos, A. and Bojalil, R. (1988). Cohort study of intestinal infection with *Campylobacter* in Mexican children. *Lancet* **i**, 503-506.

Canonne-Hergaux, F., Gruenheid, S., Govoni, G. and Gros, P. (1999). The Nramp1 protein and its role in resistance to infection and macrophage function. *Proceedings of the Association of American Physicians* **111**, 283-289.

Carlioz, A. and Touati, D. (1986). Isolation of superoxide dismutase mutants in *Escherichia coli*: Is superoxide dismutase necessary for aerobic life? *EMBO Journal* **5**, 623-630.

Carniel, E. (2001). The *Yersinia* high-pathogenicity island: An iron-uptake island. *Microbes and Infection* **3**, 561-569.

Cartron, M. L., Maddocks, S., Gillingham, P., Craven, C. J. and Andrews, S. C. (2006). Feo - Transport of ferrous iron into bacteria. *Biometals* **19**, 143-157.

Cawthraw, S. A., Lind, L., Kaijser, B. and Newell, D. G. (2000). Antibodies, directed towards *Campylobacter jejuni* antigens, in sera from poultry abattoir workers. *Clinical and Experimental Immunology* **122**, 55-60.

- Cellier, M., Beiouchi, A. and Gros, P. (1996).** Resistance to intracellular infections: Comparative genomic analysis of *NRAMP1*. *Trends in Genetics* **12**, 201-204.
- Chan, A. C. K., Lelj-Garolla, B., Rosell, F. I., Pedersen, K. A., Mauk, A. G. and Murphy, M. E. P. (2006).** Cofacial heme binding is linked to dimerization by a bacterial heme transport protein. *Journal of Molecular Biology* **362**, 1108-1119.
- Chan, E. and Weiss, B. (1987).** Endonuclease IV of *Escherichia coli* is induced by paraquat. *Journal of Bacteriology* **84**, 3189-3193.
- Chan, V. L. and Bingham, H. L. (1991).** Complete sequence of the *Campylobacter jejuni* *glyA* gene encoding serine hydroxymethyltransferase. *Gene* **101**, 51-58.
- Chan, V. L. and Bingham, H. L. (1992).** Lysyl-tRNA synthetase gene of *Campylobacter jejuni*. *Journal of Bacteriology* **174**, 695-701.
- Chan, V. L., Louie, H. and Bingham, H. L. (1995).** Cloning and transcription regulation of the Ferric Uptake Regulatory gene of *Campylobacter jejuni* TGH9011. *Gene* **164**, 25-31.
- Chen, C.-Y., Berish, S. A., Morse, S. A. and Mietzner, T. A. (1993).** The ferric iron-binding protein of pathogenic *Neisseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. *Molecular Microbiology* **10**, 311-318.
- Chen, L., Keramati, L. and Helmann, J. D. (1995).** Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8190-8194.
- Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G. and Ryu, S. (2001).** Structural basis of the redox switch in the OxyR transcription factor. *Cell* **105**, 103-113.
- Christman, M. F., Morgan, R. W., Jacobson, F. S. and Ames, B. N. (1985).** Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**, 753-762.
- Christman, M. F., Storz, G. and Ames, B. N. (1989).** OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 3484-3488.
- Clairborne, A. and Fricovich, I. (1979).** Purification of the *o*-Dianisidine peroxidase from *Escherichia coli* B. Physicochemical characterization and analysis of its dual catalatic and peroxidatic activities. *Journal of Biological Chemistry* **254**, 4245-4252.
- Clairborne, A., Malinowsky, D. P. and Fridovich, I. (1979).** Purification and characterization of hydroperoxidase II of *Escherichia coli* B. *Journal of Biological Chemistry* **254**, 11664-11668.
- Clarke, T. E., Ku, S. Y., Dougan, D. R., Vogel, H. J. and Tari, L. W. (2000).** The structure of the ferric siderophore binding protein FhuD complexed with gallichrome. *Nature Structural Biology* **7**, 287-291.

Clarke, T. E., Braun, V., Winkelmann, G., Tari, L. W. and Vogel, H. J. (2002). X-ray crystallographic structures of the *Escherichia coli* periplasmic protein FhuD bound to hydroxamate-type siderophores and the antibiotic albomycin. *The Journal of Biological Chemistry* **277**, 13966-13972.

Cogan, T. A., Thomas, A. O., Rees, L. E. N., Taylor, A. H., Jepson, M. A., Williams, P. H., Ketley, J. M. and Humphrey, T. J. (2006). Norepinephrine increases the pathogenic potential of *Campylobacter jejuni*. *Gut* **56**, 1060-1065.

Cope, L. D., Thomas, S. E., Hrkal, Z. and Hansen, E. J. (1998). Binding of heme-hemopexin complexes by soluble HxuA protein allows utilization of this complexed heme by *Haemophilus influenzae*. *Infection and Immunity* **66**, 4511-4516.

Cornelissen, C. N., Biswas, G. D., Tsai, J., Paruchuri, D. K., Thompson, S. A. and Sparling, P. F. (1992). Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. *Journal of Bacteriology* **174**, 5788-5797.

Cornelissen, C. N. and Sparling, P. F. (1996). Binding and surface exposure characteristics of the gonococcal transferrin receptor are dependent on both transferrin-binding proteins. *Journal of Bacteriology* **178**, 1437-1444.

Cornelissen, C. N., Kelley, M., Hobbs, M. M., Anderson, J. E., Cannon, J. G., Cohen, M. S. and Sparling, P. F. (1998). The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Molecular Microbiology* **27**, 611-616.

Cornelissen, C. N. and Sparling, P. F. (2004). *Neisseria*. In *Iron Transport in Bacteria*, pp. 256-272. Edited by J. H. Crosa, A. R. Mey and S. M. Payne. Washington DC: ASM Press.

Costa, M., Brookes, S. J. and Hennig, G. W. (2000). Anatomy and physiology of the enteric nervous system. *Gut* **47** (Suppl 4), iv15-19. discussion iv26.

Coughtrie, M. W. H. (1996). Sulphation catalysed by the human cytosolic sulphotransferases - chemical defence or molecular terrorism? *Human and Experimental Toxicology* **15**, 547-555.

Coulanges, V., Andre, P. and Vidon, D. J.-M. (1998). Effect of siderophores, catecholamines, and catechol compounds on *Listeria* spp. growth in iron-complexed medium. *Biochemical and Biophysical Research Communications* **249**, 526-530.

Cowart, R. E. (2002). Reduction of iron by extracellular iron reductases: Implications for microbial iron acquisition. *Archives of Biochemistry and Biophysics* **400**, 273-281.

Coy, M. and Neilands, J. B. (1991). Structural dynamics and functional domains of the Fur protein. *Biochemistry* **30**, 8201-8210.

- Crossley, R. A., Gaskin, D. J. H., Holmes, K., Mulholland, F., Wells, J. M., Kelly, D. J., van Vliet, A. H. M. and Walton, N. J. (2007).** Riboflavin biosynthesis is associated with assimilatory ferric reduction and iron acquisition by *Campylobacter jejuni*. *Applied and Environmental Microbiology* **73**, 7819-7825.
- Davidson, A. L. and Maloney, P. C. (2007).** ABC transporters: How small machines do a big job. *Trends in Microbiology* **15**, 448-455.
- Day, C. L., Stowell, K. M., Baker, E. N. and Tweedie, J. W. (1992).** Studies of the N-terminal half of human lactoferrin produced from the cloned cDNA demonstrate that interlobe interactions modulate iron release. *Journal of Biological Chemistry* **267**, 13857-13862.
- Day, J. W. A., Sajecki, J. L., Pitts, T. M. and Joens, L. A. (2000).** Role of catalase in *Campylobacter jejuni* intracellular survival *Infection and Immunity* **68**, 6337-6345.
- De Lorenzo, V., Bindereif, A., Paw, B. H. and Neilands, J. B. (1986).** Aerobactin biosynthesis and transport genes of plasmid ColV-K30 in *Escherichia coli* K-12. *Journal of Bacteriology* **165**, 570-578.
- De Lorenzo, V., Wee, S., Herrero, M. and Neilands, J. B. (1987).** Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. *Journal of Bacteriology* **169**, 2624-2630.
- De Lorenzo, V., Giovannini, F., Herrero, M. and Neilands, J. B. (1988a).** Metal ion regulation of gene expression: Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30. *Journal of Molecular Biology* **203**, 875-884.
- De Lorenzo, V., Herrero, M., Giovannini, F. and Neilands, J. B. (1988b).** Fur (ferric uptake regulation) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. *European Journal of Biochemistry* **173**, 537-546.
- Deitch, E. A., Maejima, K. and Berg, R. (1985).** Effect of oral antibiotics and bacterial overgrowth on the translocation of the GI tract microflora in burned rats. *Journal of Trauma* **25**, 385.
- Dekeyser, P., Gossuin-Detrain, M., Butzler, J. P. and Sternon, J. (1972).** Acute enteritis due to related vibrio: first positive stool cultures. *Journal of Infectious Diseases* **125**, 390-392.
- Delany, I., Spohn, G., Rappuoli, R. and Scarlato, V. (2001).** The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Molecular Microbiology* **42**, 1297-1309.
- Desai, P. J., Garges, E. and Genco, C. A. (2000).** Pathogenic *Neisseriae* can use hemoglobin, transferrin, and lactoferrin independently of the *tonB* locus. *Journal of Bacteriology* **182**, 5586-5591.

- Dhaenens, L., Szczebara, L. and Husson, M. O. (1997).** Identification, characterization and immunogenicity of the lactoferrin-binding protein from *Helicobacter pylori*. *Infection and Immunity* **65**, 514-518.
- Ding, H., Hidalgo, E. and Demple, B. (1996).** The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. *Journal of Biological Chemistry* **271**, 33173-33175.
- Ding, H. and Demple, B. (1997).** *In vivo* kinetics of a redox-regulated transcriptional switch. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 8445-8449.
- Doig, P., Yao, R. J., Burr, D. H., Guerry, P. and Trust, T. J. (1996).** An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. *Molecular Microbiology* **20**, 885-894.
- Dorrell, N., Gyselman, V. G., Foyes, S., Li, S. R. and Wren, B. W. (1996).** Improved efficiency of inverse PCR mutagenesis (IPCRM). *Biotechniques* **21**, 604-608.
- Doyle, L. P. (1948).** The aetiology of swine dysentery. *American Journal of Veterinary Research* **9**, 50-51.
- Drechel, H., Thieken, A., Reissbrodt, R., Jung, G. and Winkelmann, G. (1993).** α -Ketoacids are novel siderophores in the genera *Proteus*, *Providencia* and *Morganella* and are produced by amino acid deaminases. *Journal of Bacteriology* **175**, 2727-2733.
- Dubrac, S. and Touati, D. (2000).** Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: Functional analysis of the *sodB* promoter. *Journal of Bacteriology* **182**, 3802-3808.
- Dubrac, S. and Touati, D. (2002).** Fur-mediated transcriptional and post-transcriptional regulation of FeSOD expression in *Escherichia coli*. *Microbiology* **148**, 147-156.
- Dyer, D. W., West, E. P. and Sparling, P. F. (1987).** Effects of serum carrier proteins on the growth of pathogenic *Neisseriae* with heme-bound iron. *Infection and Immunity* **55**, 2171-2175.
- Earhart, C. F. (2004).** Iron uptake via the enterobactin system. In *Iron transport in bacteria*, pp. 133-146. Edited by J. H. Crosa, A. R. Mey and S. M. Payne. Washington, DC: ASM Press.
- Ekins, A., Khan, A. G., Shouldice, S. R. and Schryvers, A. B. (2004).** Lactoferrin receptors in Gram-negative bacteria: Insights into the iron acquisition process. *Biometals* **17**, 235-243.
- Ellison III, R. T., Giehl, T. J. and LaForce, F. M. (1988).** Damage to the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity* **56**, 2774-2781.

Elvers, K. T., Turner, S. M., Wainwright, L. M., Marsden, G., Hinds, J., Cole, J. A., Poole, R. K., Penn, C. W. and Park, S. F. (2005). NssR, a member of the Crp-Fnr superfamily from *Campylobacter jejuni*, regulates a nitrosative stress-responsive regulon that includes both a single-domain and a truncated haemoglobin. *Molecular Microbiology* **57**, 735-750.

Endtz, H. P., Vliegthart, J. S., Vandamme, P., Weverink, H. W., van den Braak, N., Verbrugh, H. A. and van Belkum, A. (1997). Genotypic diversity of *Campylobacter lari* isolated from mussels and oysters in The Netherlands. *International Journal of Food Microbiology* **34**, 79-88.

Ernst, J. F., Bennett, R. L. and Rothfield, L. I. (1978). Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. *Journal of Bacteriology* **135**, 928-934.

Escherich, T. (1886). Beiträge zur Kenntniss der Darmbakterien. III. Über das Vorkommen van Vibrionen im Darmcanal und den Stuhlgängen der Säuglinge. *Münchener Medizinische Wochenschrift* **33**, 815-817, 833-835.

Escolar, L., Pérez-Martín, J. and de Lorenzo, V. (1998). Binding of the Fur (ferric uptake regulator) repressor of *Escherichia coli* to arrays of the GATAAT sequence. *Journal of Molecular Biology* **283**, 537-547.

Escolar, L., Pérez-Martín, J. and De Lorenzo, V. (1999). Opening the iron box: Transcriptional metalloregulation by the Fur protein. *Journal of Bacteriology* **181**, 6223-6229.

Escolar, L., Pérez-Martín, J. and De Lorenzo, V. (2000). Evidence of an unusually long operator for the Fur repressor in the aerobactin promoter of *Escherichia coli*. *The Journal of Biological Chemistry* **275**, 24709-24714.

Everest, P., Goossens, H., Butzler, J. P., Lloyd, D., Knutton, S., Ketley, J. M. and Williams, P. H. (1992). Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *C. coli*. *Journal of Medical Microbiology* **37**, 319-325.

Falt, H., Hernell, O. and Blackberg, L. (2001). Do human bile salt stimulated lipase and colipase-dependent pancreatic lipase share a common heparin-containing receptor? *Archives of Biochemistry and Biophysics* **386**, 188-194.

Faraldo-Gómez, J. D. and Sansom, M. S. P. (2003). Acquisition of siderophores in Gram-negative bacteria. *Nature reviews* **4**, 105-116.

Farr, S. B., D'Ari, R. and Touati, D. (1986). Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8268-8272.

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

Fawcett, W. and Wolf, R. E. (1994). Purification of a MalE-SoxS fusion protein and identification of the control sites of *Escherichia coli* superoxide-inducible genes. *Molecular Microbiology* **14**, 669-679.

Ferguson, A. D., Hofmann, E., Coulton, J. W., Diederichs, K. and Welte, W. (1998). Siderophore-mediated iron transport: Crystal structure of FhuA with bound lipopolysaccharide. *Science* **282**, 2215-2220.

Ferguson, A. D., Chakraborty, R., Smith, B. S., Esser, L., van der Helm, D. and Deisenhofer, J. (2002). Structural basis of gating by the outer membrane transporter FecA. *Science* **295**, 1715-1719.

Ferguson, A. D. and Deisenhofer, J. (2002). TonB-dependent receptors - structural perspectives *Biochimica et Biophysica Acta* **1565**, 318-332.

Field, L. H., Headley, V. L., Payne, S. M. and Berry, L. J. (1986). Influence of iron on growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. *Infection and Immunity* **54**, 126-132.

Finlay, B. B. and Falkow, S. (1990). *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells *Journal of Infectious Diseases* **162**, 1096-1106.

Fisher, M. (1956). An unusual variant of acute idiopathic polyneuritis (syndrome of ophthalmoplegia ataxia and areflexia). *New England Journal of Medicine* **255**, 57-65.

Florent, A. (1953). Isolement d'un vibron saprophyte du sperme du Taureau et du vagin de la vache (*Vibrio bubulus*). *C R Acad Med* **147**, 2066-2069.

Fouts, D. E., Mongodin, E. F., Mandrell, R. E., Miller, W. G., Rasko, D. A., Ravel, J., Brinkac, L. M., DeBoy, R. T., Parker, C. T., Daugherty, S. C., Dodson, R. J., Durkin, A. S., Madupu, R., Sullivan, S. A., Shetty, J. U., Ayodeji, M. A., Shvartsbeyn, A., Schatz, M. C., Badger, J. H., Fraser, C. M. and Nelson, K. E. (2005). Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *PLoS Biology* **3**, e15.

Frankenburg, S., Leibovici, V., Mansbach, N., Turco, S. J. and Rosen, G. (1990). Effect of glycolipids of *Leishmania* parasites on human monocyte activity: Inhibition by lipophosphoglycan. *Journal of Immunology* **145**, 4284-4289.

Fréchon, D. and Le Cam, E. (1994). Fur (ferric uptake regulation) protein interaction with target DNA: Comparison of gel retardation, footprinting and electron microscopy analyses. *Biochemical and Biophysical Research Communications* **201**, 346-355.

Freestone, P. P. E., Haigh, R. D., Williams, P. H. and Lyte, M. (1999). Stimulation of bacterial growth by heat-stable, norepinephrine-induced autoinducers. *FEMS Microbiology Letters* **172**, 53-60.

Freestone, P. P. E., Lyte, M., Neal, C. P., Maggs, A. F., Haigh, R. D. and Williams, P. H. (2000). The mammalian neuroendocrine hormone norepinephrine supplies iron for bacterial growth in the presence of transferrin or lactoferrin. *Journal of Bacteriology* **182**, 6091-6098.

Freestone, P. P. E., Williams, P. H., Haigh, R. D., Maggs, A. F., Neal, C. P. and Lyte, M. (2002). Growth stimulation of intestinal commensal *Escherichia coli* by catecholamines: a possible contributory factor in trauma-induced sepsis. *Shock* **18**, 465-470.

Freestone, P. P. E., Haigh, R. D., Williams, P. H. and Lyte, M. (2003). Involvement of enterobactin in norepinephrine-mediated iron supply from transferrin to enterohaemorrhagic *Escherichia coli*. *FEMS Microbiology Letters* **222**, 39-43.

Freestone, P. P. E., Haigh, R. D. and Lyte, M. (2007a). Blockade of catecholamine-induced growth by adrenergic and dopaminergic receptor antagonists in *Escherichia coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica*. *BMC Microbiology* **7**, 8.

Freestone, P. P. E., Haigh, R. D. and Lyte, M. (2007b). Specificity of catecholamine-induced growth in *Escherichia coli* O157:H7, *Salmonella enterica* and *Yersinia enterocolitica*. *FEMS Microbiology Letters* **269**, 221-228.

Freestone, P. P. E., Sandrini, S. M., Haigh, R. D. and Lyte, M. (2008). Microbial endocrinology: How stress influences susceptibility to infection. *Trends in Microbiology* **16**, 55-64.

Friedman, C. R., Neimann, J., Wegener, H. C. and Tauxe, R. V. (2000). Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In *Campylobacter*, pp. 121-138. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.

Fuangthong, M., Herbig, A. F., Bsat, N. and Helmann, J. D. (2002). Regulation of the *Bacillus subtilis fur* and *perR* genes by PerR: Not all members of the PerR regulon are peroxide-inducible. *Journal of Bacteriology* **184**, 3276-3286.

Furrer, J. L., Sanders, D. N., Hook-Barnard, I. G. and McIntosh, M. A. (2002). Export of the siderophore enterobactin in *Escherichia coli*: Involvement of a 43 kDa membrane exporter. *Molecular Microbiology* **44**, 1225-1234.

Gaballa, A. and Helmann, J. D. (1998). Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. *Journal of Bacteriology* **180**, 5815-5821.

Galindo, M. A., Day, W. A., Raphael, B. H. and Joens, L. A. (2001). Cloning and characterization of a *Campylobacter jejuni* iron-uptake operon. *Current Microbiology* **42**, 139-143.

Ganz, T. (2003). Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* **102**, 783-788.

- Garrad, R. C. and Bhattacharjee, J. K. (1992).** Lysine biosynthesis in selected pathogenic fungi: Characterization of lysine auxotrophs and the cloned *LYSI* gene of *Candida albicans*. *Journal of Bacteriology* **174**, 7379-7384.
- Gaudu, P. and Weiss, B. (1996).** SoxR, a [2Fe-2S] transcription factor, is active only in its oxidized form. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 10094-10098.
- Giansanti, F., Rossi, P., Massucci, M. T., Botti, D., Antonini, G., Valenti, P. and Seganti, L. (2002).** Antiviral activity of ovotransferrin discloses an evolutionary strategy for the defensive activities of lactoferrin. *Biochemistry and Cell Biology* **80**, 125-130.
- Gonzalez-Ariki, S. and Husband, A. J. (1998).** The role of sympathetic innervation of the gut in regulating mucosal immune responses. *Brain, Behavior and Immunity* **12**, 53-63.
- Goossens, H., Pot, B., Vlaes, L., Van Den Boore, C., Van den Abbeele, R., Van Naelten, C., Levy, J., Cogniau, H., Marbehant, P., Verhoef, J., Kersters, K., Butzler, J. P. and Vandamme, P. (1990).** Characterization and description of "*Campylobacter upsaliensis*" isolated from human feces. *Journal of Clinical Microbiology* **28**, 1039-1046.
- Gorringe, A. R., Woods, G. and Robinson, A. (1990).** Growth and siderophore production by *Bordetella pertussis* under iron-restricted conditions. *FEMS Microbiology Letters* **54**, 101-105.
- Grant, C. C., Konkel, M. E., Cieplak, W. J. and Tompkins, L. S. (1993).** Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures *Infection and Immunity* **61**, 1764-1771.
- Grant, K. A. and Park, S. F. (1995).** Molecular characterization of *kataA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange *Microbiology* **141**, 1369-1376.
- Grau, F. H. (1988).** *Campylobacter jejuni* and *Campylobacter hyointestinalis* in the intestinal tract and on the carcasses of calves and cattle. *Journal of Food Protection* **51**, 857-861.
- Gray-Owen, S. D., Loosmore, S. and Schryvers, A. B. (1995).** Identification and characterisation of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae*. *Infection and Immunity* **63**, 1201-1210.
- Gray-Owen, S. D. and Schryvers, A. B. (1995).** Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of *Haemophilus influenzae*. *Infection and Immunity* **63**, 3809-3815.
- Gray-Owen, S. D. and Schryvers, A. B. (1996).** Bacterial transferrin and lactoferrin receptors. *Trends in Microbiology* **4**, 185-191.
- Green, B. T., Lyte, M., Kulkarni-Narla, A. and Brown, D. R. (2003).** Neuromodulation of enteropathogen internalization in Peyer's patches from porcine jejunum. *Journal of Neuroimmunology* **141**, 74-82.

- Green, B. T., Lyte, M., Chen, C., Xie, Y., Casey, M. A., Kulkarni-Narla, A., Vulchanova, L. and Brown, D. R. (2004).** Adrenergic modulation of *Escherichia coli* O157:H7 adherence to the colonic mucosa. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **287**, 1238-1246.
- Griffiths, R. P., Moyer, C. L., Caldwell, B. A., Ye, C. and Morita, R. Y. (1990).** Long term starvation-induced loss of antibiotic resistance in bacteria. *Microbial Ecology* **19**, 252-257.
- Griggs, D. W. and Konisky, J. (1989).** Mechanism for iron-regulated transcription of the *Escherichia coli* *cir* gene: Metal-dependent binding of Fur protein to the promoters. *Journal of Bacteriology* **171**, 1048-1054.
- Guerry, P., Logan, S. M., Thornton, S. and Trust, T. J. (1990).** Genomic organization and expression of *Campylobacter* flagellin genes. *Journal of Bacteriology* **172**, 1853-1860.
- Guerry, P., Perez-Casal, J., Yao, R., McVeigh, A. and Trust, T. J. (1997).** A genetic locus involved in iron utilization unique to some *Campylobacter* strains. *Journal of Bacteriology* **179**, 3997-4002.
- Gussow, D. and Clackson, T. (1989).** Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Research* **17**, 4000.
- Hackan, D. J., Rotstein, O. D., Zhang, W., Gruenheid, S., Gros, P. and Grinstein, S. (1998).** Host resistance to intracellular infection: Mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. *Journal of Experimental Medicine* **188**, 351-364.
- Hall, H. K. and Foster, J. W. (1996).** The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. *Journal of Bacteriology* **178**, 5683-5691.
- Hamaguchi, K. and Geiduschek, E. P. (1962).** The effect of electrolytes on the stability of deoxyribonucleate helix. *Journal of the American Chemical Society* **84**, 1329-1337.
- Hanahan, D. (1983).** Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557-580.
- Hancock, R. E. W., Hantke, K. and Braun, V. (1976).** Iron transport in *Escherichia coli* K-12: Involvement of the colicin B receptor and of a citrate-inducible protein. *Journal of Bacteriology* **127**, 1370-1375.
- Hantke, K. (1981).** Regulation of ferric iron transport in *Escherichia coli* K-12: Isolation of a constitutive mutant. *Molecular and General Genetics* **182**, 288-292.
- Hantke, K. (1987).** Ferrous iron transport mutants in *Escherichia coli* K-12. *FEMS Microbiology Letters* **44**, 53-57.
- Harris, L. A., Logan, S. M., Guerry, P. and Trust, T. J. (1987).** Antigenic variation of *Campylobacter* flagella. *Journal of Bacteriology* **169**, 5066-5071.

- Heidrich, C., Hantke, K., Bierbaum, G. and Sahl, H. G. (1996).** Identification and analysis of a gene encoding a Fur-like protein of *Staphylococcus epidermidis*. *FEMS Microbiology Letters* **140**, 253-259.
- Henderson, D. P., Wyckoff, E. E., Rashidi, C. E., Verlei, H. and Oldham, A. L. (2001).** Characterization of the *Plesiomonas shigelloides* genes encoding the heme iron utilization system. *Journal of Bacteriology* **183**, 2715-2723.
- Hendley, J. O., Powell, K. R., Rodewald, P. R., Holzgreffe, H. H. and Lyles, R. (1975).** Demonstration of a capsule on *Neisseria gonorrhoeae*. *New England Journal of Medicine* **296**, 608-611.
- Hentze, M. W., Muckenthaler, M. U. and Andrews, N. C. (2004).** Balancing acts: Molecular control of mammalian iron metabolism. *Cell* **117**, 285-297.
- Herbig, A. F. and Helmann, J. D. (2001).** Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Molecular Microbiology* **41**, 849-859.
- Herrington, D. A. and Sparling, P. F. (1985).** *Haemophilus influenzae* can use human transferrin as a sole source for required iron. *Infection and Immunity* **48**, 248-251.
- Heuck, D., Beer, W. and Reissbrodt, R. (1995).** Iron supply of staphylococci and of micrococci by α -ketoacids. *Journal of Medical Microbiology* **43**, 26-32.
- Higgs, P. I., Larsen, R. A. and Postle, K. (2002).** Quantification of known components of the *Escherichia coli* TonB energy transducing system: TonB, ExbB, ExbD and FepA. *Molecular Microbiology* **44**, 271-281.
- Hofreuter, D., Tsai, J., Watson, R. O., Novik, V., Altman, B., Benitez, M., Clark, C., Perbost, C., Jarvie, T., Du, L. and Galán, J. E. (2006).** Unique features of a highly pathogenic *Campylobacter jejuni* strain. *Infection and Immunity* **74**, 4694-4707.
- Holmes, K., Mulholland, F., Pearson, B. M., Pin, C., McNicholl-Kennedy, J., Ketley, J. M. and Wells, J. M. (2005).** *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. *Microbiology* **151**, 243-257.
- Hood, A. M., Pearson, A. D. and Shahamat, M. (1988).** The extent of surface contamination of retailed chickens with *Campylobacter jejuni* serogroups. *Epidemiology and Infection* **100**, 17-25.
- Horsburgh, M. J., Clements, M. O., Crossley, H., Ingham, E. and Foster, S. J. (2001).** PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infection and Immunity* **69**, 3744-3754.
- Humphrey, T. (2006).** Are happy chickens safer chickens? Poultry welfare and disease susceptibility. *British Poultry Science* **47**, 379-391.

- Hunt, M. D., Pettis, G. S. and McIntosh, M. A. (1994).** Promoter and operator determinants for Fur-mediated iron regulation in the bidirectional *fepA-fes* control region of the *Escherichia coli* enterobactin system. *Journal of Bacteriology* **176**, 3944-3955.
- Hussein, S., Hantke, K. and Braun, V. (1981).** Citrate-dependent iron transport system in *Escherichia coli* K-12. *European Journal of Biochemistry* **117**, 431-437.
- Husson, M. O., Legrand, D., Spik, G. and Leclerc, H. (1993).** Iron acquisition by *Helicobacter pylori*: Importance of human lactoferrin. *Infection and Immunity* **61**, 2694-2697.
- Imlay, J. A. and Linn, S. (1988).** Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. *Science* **240**, 640-642.
- Imlay, J. A. and Fridovich, I. (1991).** Assay of metabolic superoxide production in *Escherichia coli*. *Journal of Biological Science* **266**, 6957-6965.
- Irwin, S. W., Averil, N., Cheng, C. Y. and Schryvers, A. B. (1993).** Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. *Molecular Microbiology* **8**, 1125-1133.
- Ishikawa, T., Mizunoe, Y., Kawabata, S., Takade, A., Harada, M., Wai, S. N. and Yoshida, S. (2003).** The iron-binding protein Dps confers hydrogen peroxide stress resistance to *Campylobacter jejuni*. *Journal of Bacteriology* **185**, 1010-1017.
- Jacquamet, L., Aberdam, D., Adrait, A., Hazemann, J. L., Latour, J. M. and Michaud-Soret, I. (1998).** X-ray absorption spectroscopy of a new zinc site in the Fur protein from *Escherichia coli*. *Biochemistry* **37**, 2564-2571.
- Jacques, J.-F., Jang, S., Prévost, K., Desnoyers, G., Desmarais, M., Imlay, J. A. and Massé, E. (2006).** RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*. *Molecular Microbiology* **62**, 1181-1190.
- Janvier, B., Constantinidou, C., Aucher, P., Marshall, Z. V., Penn, C. W. and Fauchère, J. L. (1998).** Characterization and gene sequencing of a 19-kDa periplasmic protein of *Campylobacter jejuni/coli*. *Research in Microbiology* **149**, 95-107.
- Jin, S., Joe, A., Lynett, J., Hani, E. K., Sherman, P. and Chan, V. L. (2001).** JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. *Molecular Microbiology* **39**, 1225-1236.
- Johnson, W. M. and Lior, H. (1988).** A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microbial Pathogenesis* **4**, 115-126.
- Jones, A. S. and Walker, R. T. (1963).** Isolation and analysis of the deoxyribonucleic acid of *Mycoplasma mycoides* var. *capri*. *Nature* **198**, 588-589.
- Jones, F. S., Orcutt, M. and Little, R. B. (1931).** Vibrios (*Vibrio jejuni*, N. Sp.) associated with intestinal disorders of cows and calves *Journal of Experimental Medicine* **53**, 853-867.

- Kammler, M., Schön, C. and Hantke, K. (1993).** Characterization of the ferrous iron uptake system of *Escherichia coli*. *Journal of Bacteriology* **175**, 6212-6219.
- Kaplan, J. (2002).** Mechanisms of cellular iron acquisition: Another iron in the fire. *Cell* **111**, 603-606.
- Karjalainen, T. K., Evans, D. G., Evans, D. J., Graham, D. Y. and Lee, C. H. (1991).** Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. *Microbial Pathogenesis* **11**, 317-323.
- Karlyshev, A. V. and Wren, B. W. (2005).** Development and application of an insertional system for gene delivery and expression in *Campylobacter jejuni*. *Applied and Environmental Microbiology* **71**, 4004-4013.
- Ketley, J. M. (1997).** Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**, 5-21.
- Khan, I., Adler, B., Haridas, S. and Albert, M. J. (2005).** PorA protein of *Campylobacter jejuni* is not a cytotoxin mediating inflammatory diarrhoea. *Microbes and Infection* **7**, 853-859.
- Kikuchi, G., Yoshida, T. and Noguchi, M. (2005).** Heme oxygenase and heme degradation. *Biochemical and Biophysical Research Communications* **338**, 558-567.
- Kim, I., Stiefel, A., Plantor, S., Angerer, A. and Braun, V. (1997).** Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. *Molecular Microbiology* **23**, 333-344.
- King, E. O. (1957).** Human infections with *Vibrio fetus* and a closely related vibrio. *Journal of Infectious Diseases* **101**, 119-128.
- King, E. O. (1962).** The laboratory recognition of *Vibrio fetus* and a closely related *Vibrio* isolated from cases of human vibriosis. *Annals of the New York Academy of Sciences* **98**, 700-701.
- King, V., Wassenaar, T. M., van der Zeijst, B. A. M. and Newell, D. G. (1991).** Variations in *Campylobacter jejuni* flagellin, and flagellin genes, during *in vivo* and *in vitro* passage. *Micr Ecol Health Dis* **4**, 135-140.
- Kingsley, R., Rabsch, W., Roberts, M., Reissbrodt, R. and Williams, P. H. (1996).** TonB-dependent iron supply in *Salmonella* by α -ketoacids and α -hydroxyacids. *FEMS Microbiology Letters* **140**, 65-70.
- Kingsley, R. A., Reissbrodt, R., Rabsch, W., Ketley, J. M., Tsolis, R. M., Everest, P., Dougan, G., Bäumler, A. J., Roberts, M. and Williams, P. H. (1999).** Ferrioxamine-mediated iron (III) utilization by *Salmonella enterica*. *Applied and Environmental Microbiology* **65**, 1610-1618.

- Kist, M. (1986).** Who discovered *Campylobacter jejuni/coli*? A historical review. *Zentralbl Bakteriol [B]* **261**, 177-186.
- Knight, S. A. B., Vilaire, G., Lesuisse, E. and Dancis, A. (2005).** Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. *Infection and Immunity* **73**, 5482-5492.
- Konkel, M. E., Hayes, S. F., Joens, L. A. and Cieplak, W. J. (1992a).** Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cells. *Microbial Pathogenesis* **13**, 357-370.
- Konkel, M. E., Mead, D. J., Hayes, S. F. and Cieplak, W. J. (1992b).** Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. *Journal of Infectious Diseases* **166**, 308-315.
- Konkel, M. E., Mead, D. J. and Cieplak, W. J. (1993).** Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. *Journal of Infectious Diseases* **168**, 948-954.
- Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson, D. E. J. and Cieplak, W. J. (1997).** Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Molecular Microbiology* **24**, 953-963.
- Konkel, M. E., Grey, S. A., Kim, B. J., Garvis, S. G. and Yoon, J. (1999a).** Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cadF* virulence gene and its product. *Journal of Clinical Microbiology* **37**, 510-517.
- Konkel, M. E., Kim, B. J., Rivera-Amill, V. and Garvis, S. G. (1999b).** Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Molecular Microbiology* **32**, 691-701.
- Konkel, M. E., Joens, L. A. and Mixter, P. F. (2000).** Molecular characterization of *Campylobacter jejuni* virulence determinants. In *Campylobacter*, pp. 217-240. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.
- Korlath, J. A., Osterholm, M. T., Judy, A., Forgang, J. C. and Robinson, R. A. (1985).** A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *Journal of Infectious Diseases* **152**, 592-596.
- Köster, W. (2001).** ABC-transporter mediated uptake of iron, siderophores, heme and vitamin B₁₂. *Research in Microbiology* **152**, 291-301.
- Kramer, J. M., Frost, J. A., Bolton, F. J. and Wareing, D. R. A. (2000).** *Campylobacter* contamination of raw meat and poultry at retail sale: Identification of multiple types and comparison with isolates from human infection. *Journal of Food Protection* **63**, 1654-1659.
- Kuhn, D. E., Baker, B. D., Lafuse, W. P. and Zwilling, B. S. (1999).** Differential iron transport into phagosomes isolated from RAW264.7 macrophage cell lines transfected with Nramp1^{Gly169} or Nramp1^{Asp169}. *Journal of Leukocyte Biology* **66**, 113-119.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lastovica, A. J. and Skirrow, M. B. (2000). Clinical significance of *Campylobacter* and related species other than *Campylobacter jejuni* and *C. coli*. In *Campylobacter*, pp. 89-120. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.

Lavrrar, J. L., Christoffersen, C. A. and McIntosh, M. A. (2002). Fur-DNA interactions at the bi-directional *fepDGC-entS* promoter region in *Escherichia coli*. *Journal of Molecular Biology* **322**, 983-995.

Le Cam, E., Fréchon, D., Barray, M., Fourcade, A. and Delain, E. (1994). Observation of binding and polymerization of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11816-11820.

Learn, D. B., Brestel, E. P. and Seetharama, S. (1987). Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infection and Immunity* **55**, 1813-1818.

Lee, A., Smith, S. C. and Coloe, P. J. (1998). Survival and growth of *Campylobacter jejuni* after artificial inoculation onto chicken skin as a function of temperature and packaging conditions *Journal of Food Protection* **61**, 1609-1614.

Legrain, M., Mazarin, V., Irwin, S. W., Bouchon, B., Quentin-Millet, M.-J., Jacobs, E. and Schryvers, A. B. (1993). Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* **130**, 73-80.

Letoffe, S., Delepelaire, P. and Wandersman, C. (2004). Free and hemophore-bound heme acquisitions through the outer membrane receptor HasR have different requirements for the TonB-ExbB-ExbD complex. *Journal of Bacteriology* **186**, 4067-4074.

Lewin, A. C., Doughty, P. A., Flegg, L., Moore, G. R. and Spiro, S. (2002). The ferric uptake regulator of *Pseudomonas aeruginosa* has no essential cysteine residues and does not contain a structural zinc ion. *Microbiology* **148**, 2449-2456.

Lewis, L. A., Rohde, K., Gipson, M., Behrens, B., Gray, E., Toth, S. I., Roe, B. A. and Dyer, D. W. (1998a). Identification and molecular analysis of *lbpAB*, which encodes the two-component meningococcal lactoferrin receptor. *Infection and Immunity* **66**, 3017-3023.

Lewis, L. A., Sung, M. H., Gipson, M., Hartman, K. and Dyer, D. W. (1998b). Transport of intact porphyrin by HpuAB, the hemoglobin-haptoglobin utilization system of *Neisseria meningitidis*. *Journal of Bacteriology* **180**, 6043-6047.

Li, Z. and Demple, B. (1994). SoxS, an activator of superoxide stress genes in *Escherichia coli*. Purification and interaction with DNA. *Journal of Biological Chemistry* **269**, 18371-18377.

Li, Z. and Demple, B. (1996). Sequence specificity for DNA binding by *Escherichia coli* SoxS and Rob proteins. *Molecular Microbiology* **20**, 937-945.

- Linton, D., Gilbert, M., Hitchen, P. G., Dell, A., Morris, H. R., Wararchuk, W. W., Gregson, N. A. and Wren, B. W. (2000).** Phase variation of a β -1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of *Campylobacter jejuni*. *Molecular Microbiology* **37**, 501-514.
- Litwin, M., Boyko, S. A. and Calderwood, S. B. (1992).** Cloning, sequencing and transcriptional regulation of the *Vibrio cholerae fur* gene. *Journal of Bacteriology* **174**, 1897-1903.
- Litwin, M. and Calderwood, S. B. (1993).** Role of iron in regulation of virulence genes *Clinical Microbiology Reviews* **6**, 137-149.
- Locher, K. P., Rees, B., Koebnik, R., Mitschler, A., Moulinier, L., Rosenbusch, J. P. and Moras, D. (1998).** Transmembrane signaling across the ligand-gated FhuA receptor: Crystal structures of free and ferrichrome-bound states reveal allosteric changes. *Cell* **95**, 771-778.
- Loewen, P. C., Switala, J. and Triggs-Raine, B. L. (1985).** Catalases HPI and HPII in *Escherichia coli* are induced independently. *Archives of Biochemistry and Biophysics* **243**, 144-149.
- Loewen, P. C. and Switala, J. (1987).** Multiple catalases in *Bacillus subtilis*. *Journal of Bacteriology* **169**, 3601-3607.
- Loewen, P. C. (1989).** Genetic mapping of *katB*, a locus that affects catalase 2 levels in *Bacillus subtilis*. *Canadian Journal of Microbiology* **35**, 807-810.
- Luckey, M., Pollack, J. R., Wayne, R., Ames, B. N. and Neilands, J. B. (1972).** Iron uptake in *Salmonella typhimurium*: Utilization of exogenous siderophores as iron carriers. *Journal of Bacteriology* **111**, 731-738.
- Lundgren, O. (2000).** Sympathetic input into the enteric nervous system. *Gut* **47** (Suppl iv), iv33-iv35.
- Lyte, M. and Ernst, S. (1992).** Catecholamine induced growth of Gram-negative bacteria. *Life Sciences* **50**, 203-212.
- Lyte, M., Arulanandam, B. P. and Frank, C. D. (1996a).** Production of Shiga-like toxins by *Escherichia coli* O157:H7 can be influenced by the neuroendocrine hormone norepinephrine. *Journal of Laboratory and Clinical Medicine* **128**, 392-398.
- Lyte, M., Frank, C. D. and Green, B. T. (1996b).** Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. *FEMS Microbiology Letters* **139**, 155-159.
- Lyte, M. (1997).** Induction of Gram-negative bacterial growth by neurochemical containing banana (*Musa x paradisiaca*) extracts. *FEMS Microbiology Letters* **154**, 245-250.
- Lyte, M. and Bailey, M. T. (1997).** Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. *Journal of Surgical Research* **70**, 195-201.

- Lyte, M., Erickson, A. K., Arulanandam, B. P., Frank, C. D., Crawford, M. A. and Francis, D. H. (1997).** Norepinephrine-induced expression of the K99 pilus adhesion of enterotoxigenic *Escherichia coli*. *Biochemical and Biophysical Research Communications* **232**, 682-686.
- Lyte, M., Freestone, P. P. E., Neal, C. P., Olson, B. A., Haigh, R. D., Bayston, R. and Williams, P. H. (2003).** Stimulation of *Staphylococcus epidermidis* growth and biofilm formation by catecholamine inotropes. *The Lancet* **361**, 130-135.
- Lyte, M. (2004).** Microbial endocrinology and infectious disease in the 21st century. *Trends in Microbiology* **12**, 14-20.
- MacCallum, A., Hardy, S. P. and Everest, P. H. (2005).** *Campylobacter jejuni* inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions. *Microbiology* **151**, 2451-2458.
- Manning, G., Duim, B., Wassenaar, T. M., Wagenaar, J. A., Ridley, A. and Newell, D. G. (2001).** Evidence for a genetically stable strain of *Campylobacter jejuni*. *Applied and Environmental Microbiology* **67**, 1185-1189.
- Marlovits, T. C., Haase, W., Herrmann, C., Aller, S. G. and Unger, V. M. (2002).** The membrane protein FeoB contains an intramolecular G protein essential for Fe (II) uptake in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16243-16248.
- Marshall, J. C., Christou, N. V., Horn, R. and Meakins, J. L. (1988).** The microbiology of multiple organ failure. The proximal gastrointestinal tract as an occult reservoir of pathogen. *Archives of Surgery* **123**, 309.
- Massé, E. and Gottesman, S. (2002).** A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 4620-4625.
- Massé, E., Escorcia, F. E. and Gottesman, S. (2003).** Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes and Development* **17**, 2374-2383.
- Massé, E. and Arguin, M. (2005).** Ironing out the problem: New mechanisms of iron homeostasis. *Trends in Biochemical Sciences* **30**, 462-468.
- Massé, E., Vanderpool, C. K. and Gottesman, S. (2005).** Effect of RyhB small RNA on global iron use in *Escherichia coli*. *Journal of Bacteriology* **187**, 6962-6971.
- Masson, P. L. and Heremans, J. F. (1966).** Studies on lactoferrin, the iron binding protein of secretions. *Protides of the Biological Fluids* **14**, 115-124.
- Masson, P. L., Heremans, J. F. and Dive, C. H. (1966).** An iron-binding protein common to many external secretions. *Clinica Chimica Acta* **14**, 735-739.

- McCord, J. M., Keele, B. B. and Fridovich, I. (1971).** An enzyme-based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 499-506.
- McCord, J. M. and Day, E. D. (1978).** Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Letters* **86**, 139-142.
- McFadyean, F. and Stockman, S. (1913).** *Report of the departmental committee appointed by the board of agriculture and fisheries to enquire into epizootic abortion, III.* London: HMSO.
- McHugh, J. P., Rodríguez-Quinones, F., Abdul-Tehrani, H., Svistunenko, D. A., Poole, R. K., Cooper, C. E. and Andrews, S. C. (2003).** Global iron-dependent gene regulation in *Escherichia coli*: A new mechanism for iron homeostasis. *The Journal of Biological Chemistry* **278**, 29478-29486.
- McIntyre, A. S. and Thompson, D. G. (1992).** Adrenergic control of motor and secretory function in the gastrointestinal tract. *Alimentary Pharmacology and Therapeutics* **6**, 125-142.
- McSweegan, E. and Walker, R. I. (1986).** Identification and characterization of two *Campylobacter jejuni* adhesins for cellular and mucous substrates. *Infection and Immunity* **53**, 141-148.
- Menzio, F. D., Gantiez, C. and Locht, C. (1991).** Identification and purification of transferrin- and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Infection and Immunity* **59**, 3982-3988.
- Methner, U., Rabsch, W., Reissbrodt, R. and Williams, P. H. (2008).** Effect of norepinephrine on colonisation and systemic spread of *Salmonella enterica* in infected animals: Role of catecholate siderophore precursors and degradation products. *International Journal of Medical Microbiology* **298**, 429-439.
- Meyer, F. and Payne, S. M. (2001).** Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. *Molecular Microbiology* **42**, 835-849.
- Miethke, M. and Marahiel, M. A. (2007).** Siderophore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Reviews* **71**, 413-451.
- Miller, C. E., Rock, J. D., Ridley, K. A., Williams, P. H. and Ketley, J. M. (2008).** Utilization of lactoferrin-bound and transferrin-bound iron by *Campylobacter jejuni*. *Journal of Bacteriology* **190**, 1900-1911.
- Miller, J. F., Dower, W. J. and Tompkins, L. S. (1988).** High-voltage electroporation of bacteria: Genetic transformation of *Campylobacter jejuni* with plasmid DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 856-860.
- Miller, J. H. (1972).** *Experiments in molecular genetics*: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

- Miller, W. G. and Mandrell, R. E. (2005).** Prevalence of *Campylobacter* in the food and water supply: Incidence, outbreaks, isolation and detection. In *Campylobacter: Molecular and Cellular Biology*, pp. 101-163. Edited by J. M. Ketley and M. E. Konkel. Norfolk: Horizon Bioscience.
- Modun, B. J., Cockayne, A., Finch, R. and Williams, P. (1998).** The *Staphylococcus aureus* and *Staphylococcus epidermidis* transferrin-binding proteins are expressed *in vivo* during infection. *Microbiology* **144**, 1005-1012.
- Modun, B., Kendall, D. and Williams, P. (1994).** Staphylococci express a receptor for human transferrin: Identification of a 42-kilodalton cell wall transferrin-binding protein. *Infection and Immunity* **62**, 3850-3858.
- Modun, B. and Williams, P. (1999).** The staphylococcal transferrin-binding protein is a cell wall glyceraldehyde-3-phosphate dehydrogenase. *Infection and Immunity* **67**, 1086-1092.
- Mongkolsuk, S. and Helmann, J. D. (2002).** Regulation of inducible peroxide stress responses. *Molecular Microbiology* **45**, 9-15.
- Morgan, R. W., Christman, M. F., Jacobson, F. S., Storz, G. and Ames, B. N. (1986).** Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 8059-8063.
- Morita, T., Mochizuki, Y. and Aiba, H. (2006).** Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4858-4863.
- Morris, G. K., el Sherbeeney, M. R., Patton, C. M., Kodaka, H., Lombard, G. L., Edmonds, P., Hollis, D. G. and Brenner, D. J. (1985).** Comparison of four hippurate hydrolysis methods for identification of thermophilic *Campylobacter* spp. *Journal of Clinical Microbiology* **22**, 714-718.
- Moss, C. W., Lambert-Fair, M. A., Nicholson, M. A. and Guerrant, G. O. (1990).** Isoprenoid quinones of *Campylobacter cryaerophila*, *C. cinaedi*, *C. fennelliae*, *C. hyointestinalis*, *C. pylori* and '*C. upsaliensis*'. *Journal of Clinical Microbiology* **28**, 395-397.
- Nachamkin, I., Yang, X. H. and Stern, N. J. (1993).** Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: Analysis with flagellar mutants. *Applied and Environmental Microbiology* **59**, 1269-1273.
- Nachamkin, I., Allos, B. M. and Ho, T. (1998).** *Campylobacter* species and Guillain-Barré syndrome. *Clinical Microbiology Reviews* **11**, 555-567.
- Naikare, H., Palyada, K., Panciera, R., Marlow, D. and Stintzi, A. (2006).** A major role for FeoB in *Campylobacter jejuni* ferrous ion acquisition, gut colonization, and intracellular survival. *Infection and Immunity* **74**, 5433-5444.

- Naikare, H. and Stintzi, A. (2006).** TonB dependent ferric iron transport in *Campylobacter jejuni*. In *ASM 106th General Meeting*. Florida.
- Nakayama, K. (1992).** Nucleotide sequence of *Streptococcus mutans* superoxide dismutase gene and isolation of insertion mutants. *Journal of Bacteriology* **174**, 4928-4934.
- Neal, C. P., Freestone, P. P., Maggs, A. F., Haigh, R. D., Williams, P. H. and Lyte, M. (2001).** Catecholamine inotropes are growth factors for *Staphylococcus epidermidis* and other coagulase-negative staphylococci. *FEMS Microbiology Letters* **194**, 163-169.
- Newell, D. G. and Wagenaar, J. A. (2000).** Poultry infections and their control at the farm level. In *Campylobacter*, pp. 497-509. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.
- Niederhoffer, E. C., Naranjo, C. M., Bradley, K. L. and Fee, J. A. (1990).** Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. *Journal of Bacteriology* **172**, 1930-1938.
- Nieuwenhuijzen, G. A. P., Deitch, E. A. and Goris, R. J. A. (1996).** Infection, the gut and the development of the multiple organ dysfunction syndrome. *European Journal of Surgery* **162**, 259.
- Nuijten, P. J. M., van Asten, F. J. A. M., Gaastra, W. and van der Zeijst, B. A. M. (1990).** Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. *Journal of Biological Chemistry* **265**, 17798-17804.
- Nunoshiba, T., Hidalgo, E., Amabile-Cuevas, C. F. and Demple, B. (1992).** Two-stage control of an oxidative stress regulon: The *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. *Journal of Bacteriology* **174**, 6054-6060.
- Oberhelman, R. A. and Taylor, D. N. (2000).** *Campylobacter* infections in developing countries. In *Campylobacter*, pp. 139-153. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.
- Ochsner, U. A., Johnson, Z. and Vasil, M. L. (2000).** Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*. *Microbiology* **146**, 185-198.
- Oelschlaeger, T. A., Guerry, P. and Kopecko, D. J. (1993).** Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 6884-6888.
- Ogunnariwo, J. A. and Schryvers, A. B. (1990).** Iron acquisition in *Pasteurella haemolytica*: expression and identification of a bovine-specific receptor. *Infection and Immunity* **58**, 2091-2097.

- Oke, M., Sarra, R., Ghirlando, R., Farnaud, S., Gorringe, A. R., Evans, R. W., Buchanan, S. K. (2004).** The plug domain of a neisserial TonB-dependent transporter retains structural integrity in the absence of its transmembrane beta-barrel. *FEBS Letters* **564**, 294-300.
- Okujo, N., Akiyama, T., Miyoshi, S.-I., Shinoda, S. and Yamamoto, S. (1998).** Involvement of vulnibactin and exocellular protease in utilisation of transferrin- and lactoferrin-bound iron by *Vibrio vulnificus*. *Microbiology and Immunology* **40**, 595-598.
- On, S. L. W. (1996).** Identification methods for campylobacters, helicobacters, and related organisms. *Clinical Microbiology Reviews* **9**, 405-422.
- On, S. L. W. (2005).** Taxonomy, phylogeny, and methods for the identification of *Campylobacter* species. In *Campylobacter: Molecular and Cellular Biology*, pp. 13-42. Edited by J. M. Ketley and M. E. Konkel. Norfolk: Horizon Bioscience.
- Palyada, K., Threadgill, D. and Stintzi, A. (2004).** Iron acquisition and regulation in *Campylobacter jejuni*. *Journal of Bacteriology* **186**, 4714-4729.
- Park, R. Y., Sun, H. Y., Choi, M. H., Bai, Y. H. and Shin, S. H. (2005).** *Staphylococcus aureus* siderophore-mediated iron-acquisition system plays a dominant and essential role in the utilization of transferrin-bound iron. *The Journal of Microbiology* **43**, 183-190.
- Park, S.-J. and Gunsalus, R. P. (1995).** Oxygen, iron, carbon, and superoxide control of the fumarase *fumA* and *fumC* genes of *Escherichia coli*: Role of the *arcA*, *fnr*, and *soxR* gene products. *Journal of Bacteriology* **177**, 6255-6262.
- Park, S. F. and Richardson, P. T. (1995).** Molecular characterization of a *Campylobacter jejuni* lipoprotein with homology to periplasmic siderophore-binding proteins. *Journal of Bacteriology* **177**, 2259-2264.
- Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M.-A., Rutherford, K. M., van Vliet, A. H. M., Whitehead, S. and Barrell, B. G. (2000).** The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665-668.
- Patzer, S. I. and Hantke, K. (1998).** The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Molecular Microbiology* **28**, 1199-1210.
- Payne, S. M. and Mey, A. R. (2004).** Pathogenic *Escherichia coli*, *Shigella*, and *Salmonella*. In *Iron transport in bacteria*, pp. 199-218. Edited by J. H. Crosa, A. R. Mey and S. M. Payne. Washington, DC: ASM Press.
- Pearson, B. M., Gaskin, D. J. H., Segers, R. P. A. M., Wells, J. M., Nuijten, P. J. M. and van Vliet, A. H. M. (2007).** The complete genome sequence of *Campylobacter jejuni* strain 81116 (NCTC11828). *Journal of Bacteriology* **189**, 8402-8403.

- Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X. Z., Kopecko, D. J., Bourgeois, A. L., Fauchère, J. L. and Blaser, M. J. (1998).** Mutation in *peb1A* of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infection and Immunity* **66**, 938-943.
- Pellaud, J., Schote, U., Arvinte, T. and Seelig, J. (1999).** Conformation and self-association of human recombinant transforming growth factor-B3 in aqueous solutions. *The Journal of Biological Chemistry* **274**, 7699-7704.
- Perkins-Balding, D., Ratliff-Griffin, M. and Stojiljkovic, I. (2004).** Iron transport systems in *Neisseria meningitidis*. *Microbiology and Molecular Biology Reviews* **68**, 154-171.
- Perna, N. T., Plunkett III, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., Mayhew, G. F., Evans, P. S., Gregor, J., Kirkpatrick, H. A., Posfai, G., Hackett, J., Klink, S., Boutin, A., Shao, Y., Miller, L., Grotbeck, E. J., Davis, N. W., Lim, A., Dimalanta, E. T., Potamousis, K. D., Apodaca, J., Anantharaman, T. S., Lin, J., Yen, G., Schwartz, D. C., Welch, R. A. and Blattner, F. R. (2001).** Genome sequence of enterohaemorrhagic *Escherichia coli* 0157:H7. *Nature* **409**, 529-533.
- Pesci, E. C., Cottle, D. L. and Pickett, C. L. (1994).** Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infection and Immunity* **62**, 2687-2694.
- Peterson, P. K., Chao, C. C., Molitor, T., Murtaugh, M., Strgar, F. and Sharp, B. M. (1991).** Stress and pathogenesis of infectious disease. *Reviews of Infectious Diseases* **13**, 710-720.
- Pettersson, A., van der Ley, P., Poolman, J. T. and Tommassen, J. (1993).** Molecular characterization of the 98-kilodalton iron-regulated outer membrane protein of *Neisseria meningitidis*. *Infection and Immunity* **61**, 4724-4733.
- Pettersson, A., Maas, A. and Tommassen, J. (1994).** Identification of the *iroA* gene product of *Neisseria meningitidis* as a lactoferrin receptor. *Journal of Bacteriology* **176**, 1764-1766.
- Pettersson, A., Prinz, T., Umar, A., van der Biezen, J. and Tommassen, J. (1998).** Molecular characterization of LbpB, the second lactoferrin-binding protein of *Neisseria meningitidis*. *Molecular Microbiology* **27**, 599-610.
- Pickett, C. L., Auffenberg, T., Pesci, E. C., Sheen, V. L. and Jusuf, S. S. (1992).** Iron acquisition and hemolysin production by *Campylobacter jejuni*. *Infection and Immunity* **60**, 3872-3877.
- Pickett, C. L., Cottle, D. C., Pesci, E. C. and Gikah, G. (1994).** Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. *Infection and Immunity* **62**, 1046-1051.

Pickett, C. L., Pesci, E. C., Cottle, D. C., Russell, G., Erdem, A. N. and Zeytin, H. (1996). Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. *Infection and Immunity* **64**, 2070-2078.

Pickett, C. L. (2000). *Campylobacter* toxins and their role in pathogenesis. In *Campylobacter*, pp. 179-190. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.

Pidcock, K. A., Wooten, J. A., Daley, B. A. and Stull, T. L. (1988). Iron acquisition by *Haemophilus influenzae*. *Infection and Immunity* **56**, 721-725.

Pierre, J. L. and Fontecave, M. (1999). Iron and activated oxygen species in biology: The basic chemistry. *Biometals* **12**, 195-199.

Posey, J. E. and Gherardini, F. C. (2000). Lack of a role for iron in the Lyme disease pathogen. *Science* **288**, 1651-1653.

Postle, K. (1993). TonB protein and energy transduction between membranes. *Journal of Bioenergetics and Biomembranes* **25**, 591-601.

Prince, R. W., Cox, C. D. and Vasil, M. L. (1993). Coordinate regulation of siderophore and exotoxin A production: Molecular cloning and sequencing of the *Pseudomonas aeruginosa* *fur* gene. *Journal of Bacteriology* **175**, 2589-2598.

Purdy, D. and Park, S. F. (1994). Cloning, nucleotide sequence and characterization of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. *Microbiology* **140**, 1203-1208.

Purdy, D., Cawthraw, S., Dickinson, J. H., Newell, D. G. and Park, S. F. (1999). Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization. *Applied and Environmental Microbiology* **65**, 2540-2546.

Qadri, F., Raqib, R., Ahmed, F., Rahman, T., Wenneras, C., Das, S. K., Alam, N. H., Mathan, M. M. and Svennerholm, A. M. (2002). Increased levels of inflammatory mediators in children and adults infected with *Vibrio cholerae* O1 and O139. *Clinical and Diagnostic Laboratory Immunology* **9**, 221-229.

Querijnjean, P., Masson, P. L. and Heremans, J. F. (1971). Molecular weight, single-chain structure and amino acid composition of human lactoferrin. *European Journal of Biochemistry* **20**, 420-425.

Rabsch, W., Methner, U., Voigt, W., Tschäpe, H., Reissbrodt, R. and Williams, P. H. (2003). Role of receptor proteins for enterobactin and 2,3-dihydroxybenzoylserine in the virulence of *Salmonella enterica*. *Infection and Immunity* **71**, 6953-6961.

Raphael, B. H. and Joens, L. A. (2003). FeoB is not required for ferrous iron uptake in *Campylobacter jejuni*. *Canadian Journal of Microbiology* **49**, 727-731.

Ratledge, C. and Dover, L. G. (2000). Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology* **54**, 881-941.

Raymond, K. N. and Dertz, E. A. (2004). Biochemical and physical properties of siderophores. In *Iron Transport in Bacteria*, pp. 3-17. Edited by J. H. Crosa, A. R. Mey and S. M. Payne. Washington DC: ASM Press.

Redhead, K., Hill, T. and Chart, H. (1987). Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. *Journal of General Microbiology* **133**, 891-898.

Reissbrodt, R., Erler, W. and Winkelmann, G. (1994). Iron supply of *Pasteurella multocida* and *Pasteurella haemolytica*. *Journal of Basic Microbiology* **34**, 61-63.

Reissbrodt, R., Kingsley, R., Rabsch, W., Beer, W., Roberts, M. and Williams, P. H. (1997). Iron-regulated excretion of α -keto acids by *Salmonella typhimurium*. *Journal of Bacteriology* **179**, 4538-4544.

Richardson, P. T. and Park, S. F. (1995). Enterochelin acquisition in *Campylobacter coli*: Characterization of components of a binding-protein-dependent transport system. *Microbiology* **141**, 3181-3191.

Richardson, W. P. and Sadoff, J. C. (1977). Production of a capsule by *Neisseria gonorrhoeae*. *Infection and Immunity* **15**, 663-664.

Ridley, K. A., Li, Y., Rock, J. D. and Ketley, J. M. (2006). Heme utilization in *Campylobacter jejuni*. *Journal of Bacteriology* **188**, 7862-7875.

Robinson, D. A. (1981). Infective dose of *Campylobacter jejuni* in milk. *British Medical Journal (Clinical Research Ed)* **282**, 1584.

Rock, J. D. (2003). Iron acquisition from host molecules by *Campylobacter jejuni*. *Department of Genetics*. Leicester: University of Leicester.

Rogolsky, M. (1979). Nonenteric toxins of *Staphylococcus aureus*. *Microbiological Reviews* **43**, 320-360.

Rohde, K. H., Gillasp, A. F., Hatfield, N. D., Lewis, L. A. and Dyer, D. W. (2002). Interactions of haemoglobin with the *Neisseria meningitidis* receptor H: The role of TonB and an intact proton motive force. *Molecular Microbiology* **42**, 335-354.

Rollins, D. M. and Colwell, R. R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Applied and Environmental Microbiology* **52**, 531-538.

Rossi, M. S., Paquelin, A., Ghigo, J. M. and Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: The inducer and the transported substrate are different molecules. *Molecular Microbiology* **48**, 1467-1480.

- Roth, J. R. (1970).** Genetic techniques in studies of bacterial metabolism. *Methods in Enzymology* **17A**, 3-35.
- Sahney, N. N., Lambe, B. C., Summersgill, J. T. and D., M. R. (1990).** Inhibition of polymorphonuclear leukocyte function by *Legionella pneumophila* exoproducts. *Microbial Pathogenesis* **9**, 117-125.
- Sallmann, F. R., Baveye-Descamps, S., Pattus, F., Salmon, V., Branza, N., Spik, G. and Legrand, D. (1999).** Porins OmpC and PhoE of *Escherichia coli* as specific cell-surface targets of human lactoferrin. *The Journal of Biological Chemistry* **274**, 16107-16114.
- Sambrook, J. and Russell, D. (2001).** *Molecular cloning: a laboratory manual.*, Third edn. New York: Cold Spring Harbor Laboratory Press.
- Sampathkumar, B., Napper, S., Carrillo, C. D., Willson, P., Taboada, E., Nash, J. H. E., Potter, A. A., Babiuk, L. A. and Allan, B. J. (2006).** Transcriptional and translational expression patterns associated with immobilized growth of *Campylobacter jejuni*. *Microbiology* **152**, 567-577.
- Sanders, J. D., Cope, L. D. and Hansen, E. J. (1994).** Identification of a locus involved in the utilisation of iron by *Haemophilus influenzae*. *Infection and Immunity* **62**, 4515-4525.
- Sandstedt, K., Ursing, J. and Walder, M. (1983).** Thermotolerant *Campylobacter* with no or weak catalase activity isolated from dogs. *Current Microbiology* **8**, 209-213.
- Sandstedt, K. and Ursing, J. (1991).** Description of *Campylobacter upsaliensis* sp. nov. previously known as the CNW group. *Systematic and Applied Microbiology* **14**, 39-45.
- Sansonetti, P. J., Ryter, A., Clerc, P., Maurelli, A. T. and Mounier, J. (1986).** Multiplication of *Shigella flexneri* within HeLa cells: Lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. *Infection and Immunity* **51**, 461-469.
- Schade, A. L. and Caroline, L. (1944).** Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. *Science* **100**, 14-15.
- Schaffer, S., Hantke, K. and Braun, V. (1985).** Nucleotide sequence of the iron regulatory gene *fur*. *Molecular and General Genetics* **200**, 110-113.
- Schaible, U. E. and Kaufmann, S. H. E. (2004).** Iron and microbial infection. *Nature Reviews Microbiology* **2**, 946-953.
- Schalk, I. J., Kyslik, P., Prome, D., van Dorselaer, A., Poole, K., Abdallah, M. A. and Pattus, F. (1999).** Copurification of the FpvA ferric pyoverdinin receptor of *Pseudomonas aeruginosa* with its iron-free ligand: Implications for siderophore-mediated iron transport. *Biochemistry* **38**, 9357-9365.
- Schalk, I. J., Yue, W. W. and Buchanan, S. K. (2004).** Recognition of iron-free siderophores by TonB-dependent iron transporters. *Molecular Microbiology* **54**, 14-22.

- Schryvers, A. B. and Morris, L. J. (1988a).** Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. *Infection and Immunity* **56**, 1144-1149.
- Schryvers, A. B. and Morris, L. J. (1988b).** Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. *Molecular Microbiology* **2**, 281-288.
- Schryvers, A. B. (1989).** Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. *Journal of Medical Microbiology* **29**, 121-130.
- Schryvers, A. B., Bonnah, R., Yu, R. H., Wong, H. and Retzer, M. (1998).** Bacterial lactoferrin receptors. *Advances in Experimental Medicine and Biology* **443**, 123-133.
- Schryvers, A. B. and Stojiljkovic, I. (1999).** Iron acquisition systems in the pathogenic *Neisseria*. *Molecular Microbiology* **32**, 1117-1123.
- Sébald, M. and Véron, M. (1963).** Teneur en bases de l'ADN et classification des vibrions. *Ann Inst Pasteur* **105**, 897-910.
- Shreeve, J. E., Toszeghy, M., Pattison, M. and Newell, D. G. (2000).** Sequential spread of *Campylobacter* infection in a multipen broiler house. *Avian Diseases* **44**, 983-988.
- Simonson, C., Brener, D. and DeVoe, I. (1982).** Expression of a high-affinity mechanism for acquisition of transferrin iron by *Neisseria meningitidis*. *Infection and Immunity* **36**, 107-113.
- Simpson, J. A., Smith, S. E. and Dean, R. T. (1989).** Scavenging by alginate of free radicals released by macrophages. *Free Radical Biology and Medicine* **6**, 347-353.
- Singh, P. K., Parsek, M. R., Greenberg, E. P. and Welsh, M. J. (2002).** A component of innate immunity prevents bacterial biofilm development. *Nature* **417**, 552-555.
- Skamene, E., Schurr, E. and Gros, P. (1998).** Infection genomics: *Nramp1* as a major determinant of natural resistance to intracellular infections. *Annual Review of Medicine* **49**, 275-287.
- Skirrow, M. B. (1977).** *Campylobacter* enteritis: a 'new' disease. *British Medical Journal (Clinical Research Ed)* **2**, 9-11.
- Skirrow, M. B. (1987).** A demographic survey of *Campylobacter*, *Salmonella*, and *Shigella* infections in England. *Epidemiology and Infection* **99**, 647-657.
- Skirrow, M. B. (1991).** Epidemiology of *Campylobacter* enteritis. *International Journal of Food Microbiology* **12**, 9-16.
- Skirrow, M. B. (1994).** Diseases due to *Campylobacter*, *Helicobacter* and related bacteria. *Journal of Comparative Pathology* **111**, 113-149.

Skirrow, M. B. and Blaser, M. J. (2000). Clinical aspects of *Campylobacter* infection. In *Campylobacter*, pp. 69-88. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.

Skirrow, M. B. (2006). John McFadyean and the century of the first isolation of *Campylobacter* species. *Clinical Infectious Diseases* **43**, 1213-1217.

Slader, J., Domingue, G., Jørgensen, F., McAlpine, K., Owen, R. J., Bolton, F. J. and Humphrey, T. J. (2002). Impact of transport crate reuse and of catching and processing on *Campylobacter* and *Salmonella* contamination of broiler chickens. *Applied and Environmental Microbiology* **68**, 713-719.

Šmajš, D. and Weinstock, G. M. (2001). The iron-and temperature-regulated *cjrBC* genes of *Shigella* and enteroinvasive *Escherichia coli* strains code for colicin Js uptake. *Journal of Bacteriology* **183**, 3958-3966.

Šmarda, J. and Šmajš, D. (1998). Colicins-exocellular lethal proteins of *Escherichia coli*. *Folia Microbiologica* **43**, 563-582.

Smith, M. A. and Bidochka, M. J. (1998). Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. *Canadian Journal of Microbiology* **44**, 351-355.

Smith, T. (1918). Spirilla associated with disease of the fetal membranes in cattle (infectious abortion). *Journal of Experimental Medicine* **28**, 701-721.

Smith, T. and Taylor, M. S. (1919). Some morphological and biochemical characters of the spirilla (*Vibrio fetus* n. sp.) associated with disease of the fetal membranes in cattle. *Journal of Experimental Medicine* **30**, 299-312.

Smythe, M. A., Melendy, S., Jahns, B. and Dmuchowski, C. (1993). An exploratory analysis of medication utilization in a medical intensive care unit. *Critical Care Medicine* **37**, 1319-1326.

Sriyosachati, S. and Cox, C. D. (1986). Siderophore-mediated iron acquisition from transferrin by *Pseudomonas aeruginosa*. *Infection and Immunity* **52**, 885-891.

Staggs, T. M. and Perry, R. D. (1991). Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. *Journal of Bacteriology* **173**, 417-425.

Staggs, T. M., Fetherston, J. D. and Perry, R. D. (1994). Pleiotropic effects of a *Yersinia pestis fur* mutation. *Journal of Bacteriology* **176**, 7614-7624.

Stanley, J., Burnens, A. P., Linton, D., On, S. L. W., Costas, M. and Owen, R. J. (1992). '*Campylobacter helveticus*' sp. nov., a new thermophilic group from domestic animals: characterization of the species and cloning of a species-specific DNA probe. *Journal of General Microbiology* **138**, 2293-2303.

- Stanley, K., Wallace, J., Currie, J., Diggle, P. and Jones, K. (1998a).** Seasonal variation of thermophilic campylobacters in lambs at slaughter. *Journal of Applied Microbiology* **84**, 1111-1116.
- Stanley, K. N., Wallace, J., Currie, J., Diggle, P. and Jones, K. (1998b).** The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. *Journal of Applied Microbiology* **85**, 472-480.
- Steele, T. W. and Owen, R. J. (1988).** *Campylobacter jejuni* subsp. *doylei* subsp. nov., a subspecies of nitrate-negative campylobacters isolated from human clinical specimens. *International Journal of Systematic Bacteriology* **38**, 316-318.
- Stintzi, A., Marlow, D., Palyada, K., Naikare, H., Panciera, R., Whitworth, L. and Clarke, C. (2005).** Use of genome-wide expression profiling and mutagenesis to study the intestinal lifestyle of *Campylobacter jejuni*. *Infection and Immunity* **73**, 1797-1810.
- Stintzi, A., van Vliet, A. H. M. and Ketley, J. M. (2008).** Iron metabolism, transport and regulation. In *Campylobacter*. Edited by I. Nachamkin, C. M. Szymanski and M. J. Blaser. Washington DC: ASM Press.
- Stojiljkovic, I., Cobeljic, M. and Hantke, K. (1993).** *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. *FEMS Microbiology Letters* **108**, 111-115.
- Stojiljkovic, I., Bäumlér, A. J. and Hantke, K. (1994).** Fur regulon in Gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. *Journal of Molecular Biology* **236**, 531-545.
- Stojiljkovic, I. and Hantke, K. (1995).** Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). *Molecular and General Genetics* **247**, 199-205.
- Storz, G., Christman, M. F., Sies, H. and Ames, B. N. (1987).** Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 8917-8921.
- Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveira, L. A. and Ames, B. N. (1989).** An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: Genetic characterization and cloning of *ahp*. *Journal of Bacteriology* **171**, 2049-2055.
- Suits, M. D. L., Pal, G. P., Nakatsu, K., Matte, A., Cygler, M. and Jia, Z. (2005).** Identification of an *Escherichia coli* 0157:H7 heme oxygenase with tandem functional repeats. *Proceedings of the national Academy of Sciences of the United states of America Early Edition*, 1-6.
- Szymanski, C. M., King, M., Haardt, M. and Armstrong, G. D. (1995).** *Campylobacter jejuni* motility and invasion of Caco-2 cells. *Infection and Immunity* **63**, 4295-4300.

Szymanski, C. M., Yao, R., Ewing, C. P., Trust, T. J. and Guerry, P. (1999). Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Molecular Microbiology* **32**, 1022-1030.

Takata, T., Fujimoto, S. and Amako, K. (1992). Isolation of nonchemotactic mutants of *Campylobacter jejuni* and their colonization of the mouse intestinal tract. *Infection and Immunity* **60**, 3596-3600.

Tardat, B. and Touati, D. (1993). Iron and oxygen regulation of *Escherichia coli* MnSOD expression: Competition between the global regulators Fur and ArcA for binding to DNA. *Molecular Microbiology* **9**, 53-63.

Tartaglia, L. A., Storz, G. and Ames, B. N. (1989). Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. *Journal of Molecular Biology* **210**, 709-719.

Taylor, D. N., Echeverria, P., Pitarangsi, C., Seriwatana, J., Bodhidatta, L. and Blaser, M. J. (1988). Influence of strain characteristics and immunity on the epidemiology of *Campylobacter* infections in Thailand. *Journal of Clinical Microbiology* **26**, 863-868.

Tee, W. and Mitch, A. (1998). *Campylobacter jejuni* bacteremia in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients: Comparison of clinical features and review. *Clinical Infectious Diseases* **26**, 90-96.

Tholozan, J. L., Cappellet, J. M., Tissier, J. P., Delattre, G. and Federighi, M. (1999). Physiological characterization of viable-but-nonculturable *Campylobacter jejuni* cells. *Applied and Environmental Microbiology* **65**, 1110-1116.

Thomas, C. E. and Sparling, P. F. (1994). Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. *Molecular Microbiology* **11**, 725-737.

Thomas, C. E. and Sparling, P. F. (1996). Isolation and analysis of a *fur* mutant of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **178**, 4224-4232.

Tom-Yew, S. A. L., Cui, D. T., Bekker, E. G. and Murphy, M. E. P. (2005). Anion-independent iron coordination by the *Campylobacter jejuni* ferric binding protein. *Journal of Biological Chemistry* **280**, 9283-9290.

Tomb, J.-F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzgerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M. and Venter, J. C. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539-547.

- Touati, D. (1988).** Transcriptional and posttranscriptional regulation of manganese superoxide dismutase biosynthesis in *Escherichia coli*, studied with operon and protein fusions. *Journal of Bacteriology* **170**, 2511-2520.
- Touati, D., Jacques, M., Tardat, B., Bouchard, L. and Despied, S. (1995).** Lethal oxidative damage and mutagenesis are generated by iron in *fur* mutants of *Escherichia coli*: Protective role of superoxide dismutase. *Journal of Bacteriology* **177**, 2305-2314.
- Towbin, H., Staehelin, T. and Gordon, J. (1979).** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354.
- Tsaneva, I. R. and Weiss, B. (1990).** *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *Journal of Bacteriology* **172**, 4197-4205.
- Tseng, C. P. (1997).** Regulation of fumarase (*fumB*) gene expression in *Escherichia coli* in response to oxygen, iron and heme availability: Role of the *arcA*, *fur*, and *hema* gene products. *FEMS Microbiology Letters* **157**, 67-72.
- Tsolis, R. M., Bäumler, A. J., Heffron, F. and Stojiljkovic, I. (1996).** Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. *Infection and Immunity* **64**, 4549-4556.
- Umbarger, H. E. (1978).** Amino acid biosynthesis and its regulation. *Annual Review of Biochemistry* **47**, 533-606.
- van Snick, J. L., Masson, P. L. and Heremans, J. F. (1974).** The involvement of lactoferrin in the hyposideremia of acute inflammation. *The Journal of Experimental Medicine* **140**, 1068-1084.
- van Vliet, A. H. M., Wood, A. C., Henderson, J., Wooldridge, K. and Ketley, J. M. (1998a).** Genetic manipulation of enteric *Campylobacter* species. In *Bacterial pathogenesis: Methods in Microbiology*. Edited by P. H. Williams, J. M. Ketley and G. Salmond: Academic press.
- van Vliet, A. H. M., Wooldridge, K. G. and Ketley, J. M. (1998b).** Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. *Journal of Bacteriology* **180**, 5291-5298.
- van Vliet, A. H. M., Baillon, M. L., Penn, C. W. and Ketley, J. M. (1999).** *Campylobacter jejuni* contains two Fur homologs: characterization of iron-responsive regulation of peroxide stress defense genes by the PerR repressor. *Journal of Bacteriology* **181**, 6371-6376.
- van Vliet, A. H. M., Rock, J. D., Madeleine, L. N. and Ketley, J. M. (2000).** The iron-responsive regulator Fur of *Campylobacter jejuni* is expressed from two separate promoters. *FEMS Microbiology Letters* **188**, 115-118.

- van Vliet, A. H. M., Baillon, M. L., Penn, C. W. and Ketley, J. M. (2001).** The iron-induced ferredoxin FdxA of *Campylobacter jejuni* is involved in aerotolerance. *FEMS Microbiology Letters* **196**, 189-193.
- van Vliet, A. H. M. and Ketley, J. M. (2001).** Pathogenesis of enteric *Campylobacter* infection. *Journal of Applied Microbiology* **90**, 45S-56S.
- van Vliet, A. H. M., Ketley, J. M., Park, S. F. and Penn, C. W. (2002).** The role of iron in *Campylobacter* gene regulation, metabolism and oxidative stress defense. *FEMS Microbiology Reviews* **26**, 173-186.
- Vandamme, P. (2000).** Taxonomy of the family *Campylobacteraceae*. In *Campylobacter*, pp. 3-26. Edited by I. Nachamkin and M. J. Blaser. Washington, DC: ASM Press.
- Vartivarian, S. E. and Cowart, R. E. (1999).** Extracellular iron reductases: Identification of a new class of enzymes by siderophore-producing microorganisms. *Archives of Biochemistry and Biophysics* **364**, 75-82.
- Vassinova, N. and Kozyruv, D. (2000).** A method for direct cloning of Fur-regulated genes: Identification of seven new Fur-regulated loci in *Escherichia coli*. *Microbiology* **146**, 3171-3182.
- Velayudhan, J., Hughes, N. J., McColm, A. A., Bagshaw, J., Clayton, C. L., Andrews, S. C. and Kelly, D. J. (2000).** Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Molecular Microbiology* **37**, 274-286.
- Véron, M. and Chatelain, R. (1973).** Taxonomic study of the genus *Campylobacter* Sébald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sébald and Véron. *International Journal of Systematic Bacteriology* **23**, 122-134.
- Vinzent, R., Dumas, J. and Picard, N. (1947).** Septicémie grave eu cours de la grossesse, due à un vibron. Avortement consécutif. *C R Acad Med* **131**, 90.
- Vinzent, R. (1949).** Une affection méconnue de la grossesse l'infection placentaire à *Vibrio foetus*. *Presse Medicale* **81**, 1230.
- Vogel, H. J. (1960).** Two modes of lysine synthesis among lower fungi: Evolutionary significance. *Biochimica et Biophysica Acta* **41**, 172-174.
- Vogelstein, B. and Gillespie, D. (1979).** Preparative and analytical purification of DNA from agarose. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 615-619.
- Wai, S. N., Takata, T., Takade, A., Hamasaki, N. and Amako, K. (1995).** Purification and characterization of ferritin from *Campylobacter jejuni*. *Archives of Microbiology* **164**, 1-6.

- Wai, S. N., Nakayama, K., Umene, K., Moriya, T. and Amako, K. (1996).** Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: Contribution of ferritin to iron storage and protection against oxidative stress. *Molecular Microbiology* **20**, 1127-1134.
- Wandersman, C. and Stojiljkovic, I. (2000).** Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Current Opinion in Microbiology* **3**, 215-220.
- Wang, Y. and Taylor, D. E. (1990).** Natural transformation in *Campylobacter* species. *Journal of Bacteriology* **172**, 949-955.
- Ward, P. P., Zhou, X. and Conneely, O. M. (1996).** Cooperative interactions between the amino- and carboxyl -terminal lobes contribute to the unique iron-binding stability of lactoferrin. *Journal of Biological Chemistry* **271**, 12790-12794.
- Wardrop, S. L. and Richardson, D. (1999).** The effect of intracellular iron concentration and nitrogen monoxide on Nramp2 expression and non-transferrin-bound iron uptake. *European Journal of Biochemistry* **263**, 41-49.
- Wassenaar, T. M., Bleumink-Pluym, N. M. and van der Zeijst, B. A. M. (1991).** Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO Journal* **10**, 2055-2061.
- Wassenaar, T. M., Fry, B. N. and van de Zeijst, B. A. M. (1993a).** Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. *Gene* **132**, 131-135.
- Wassenaar, T. M., van der Zeijst, B. A. M., Ayling, R. and Newell, D. G. (1993b).** Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *Journal of General Microbiology* **139**, 1171-1175.
- Weinberg, E. D. (1978).** Iron and infection. *Microbiological Reviews* **42**, 45-66.
- Weinberg, E. D. (1997).** The Lactobacilli anomaly: Total iron abstinence. *Perspectives in Biology and Medicine* **40**, 1-6.
- Whitehouse, C. A., Balbo, P. B., Pesci, E. C., Cottle, D. L., Mirabito, P. M. and Pickett, C. L. (1998).** *Campylobacter jejuni* cytolethal distending toxin causes a G₂-phase cell cycle block. *Infection and Immunity* **66**, 1934-1940.
- Wilderman, P. J., Sowa, N. A., FitzGerald, D. J., FitzGerald, P. C., Gottesman, S., Ochsner, U. A. and Vasil, M. L. (2004).** Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 9792-9797.
- Williams, P. H., Rabsch, W., Methner, U., Voigt, W., Tschäpe, H. & Reissbrodt, R. (2006).** Catechol receptor proteins in *Salmonella enterica*: Role in virulence and implications for vaccine development. *Vaccine* **24**, 3840-3844.
- Wilson, K. (1987).** *Current protocols in molecular biology*. New York: Wiley.

Winkelmann, G. (2002). Microbial siderophore-mediated transport. *Biochemical Society Transactions* **30**, 691-696.

Wolz, C., Hohloch, K., Ocaktan, A., Poole, K., Evans, R. W. and Rochel, N. (1994). Iron release from transferrin by pyoverdine and elastase from *Pseudomonas aeruginosa*. *Infection and Immunity* **62**, 4021-4027.

Wooldridge, K. G. and Williams, P. H. (1993). Iron uptake mechanisms in pathogenic bacteria. *FEMS Microbiology Reviews* **12**, 325-348.

Wooldridge, K. G., Williams, P. H. and Ketley, J. M. (1994). Iron-responsive genetic regulation in *Campylobacter jejuni*: Cloning and characterization of a *fur* homolog. *Journal of Bacteriology* **176**, 5852-5856.

Wooldridge, K. G., Williams, P. H. and Ketley, J. M. (1996). Host signal transduction and endocytosis of *Campylobacter jejuni*. *Microbial Pathogenesis* **21**, 299-305.

Wooldridge, K. G. and van Vliet, A. H. M. (2005). Iron transport and regulation. In *Campylobacter: Molecular and cellular biology*, pp. 293-310. Edited by J. M. Ketley and M. E. Konkel. Norfolk: Horizon Bioscience.

Woolf, P. D., McDonald, J. V., Feliciano, D. V., Kelly, M. M., Nichols, D. and Cox, C. (1992). The catecholamine response to multi-system trauma. *Archives of Surgery* **127**, 899.

Worst, D. J., Gerrits, M. M., Vandenbroucke, G. C. and Kusters, G. (1988). *Helicobacter pylori* *ribBA*- mediated riboflavin production is involved in iron acquisition. *Journal of Bacteriology* **180**, 1473-1479.

Wösten, M. M. S. M., Boeve, M., Koot, M. G. A., van Nuenen, A. C. and van der Zeijst, B. A. M. (1998). Identification of *Campylobacter jejuni* promoter sequences. *Journal of Bacteriology* **180**, 594-599.

Wren, B. W., Henderson, J. and Ketley, J. M. (1994). A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *Biotechniques* **16**, 994-996.

Wu, J. and Weiss, B. (1991). Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. *Journal of Bacteriology* **173**, 2864-2871.

Wu, J. and Weiss, B. (1992). Two stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. *Journal of Bacteriology* **174**, 3915-3920.

Yanagawa, Y., Takahashi, M. and Itoh, T. (1994). The role of flagella of *Campylobacter jejuni* in colonization in the intestinal tract in mice and the cultured-cell infectivity. *Nippon Saikingaku Zasshi Japanese Journal of Bacteriology* **49**, 395-403.

Yao, R., Burr, D. H., Doig, P., Trust, T. J., Niu, H. and Guerry, P. (1994). Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: The role of motility in adherence and invasion of eukaryotic cells. *Molecular Microbiology* **14**, 883-893.

Young, N. M., Brisson, J. R., Kelly, J., Watson, D. C., Tessier, L., Lanthier, P. H., Jarrell, H. C., Cadotte, N., St Michael, F., Aberg, E. and Szymanski, C. M. (2002). Structure of the N-linked glycan present on multiple glycoproteins in the Gram-negative bacterium, *Campylobacter jejuni*. *Journal of Biological Chemistry* **277**, 42530-42539.

Yue, W. W., Grizot, S. and Buchanan, S. K. (2003). Structural evidence for iron-free citrate and ferric citrate binding to the TonB-dependent outer membrane transporter FecA. *Journal of Molecular Biology* **332**, 353-368.

Zhang, Z., Gosset, G., Barabote, R., Gonzalez, C. S., Cuevas, W. A. and Saier, J. M. H. (2005). Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. *Journal of Bacteriology* **187**, 980-990.

Zhao, G. H., Ceci, P., Ilari, A., Giangiacomo, L., Laue, T. M., Chiancone, E. and Chasteen, N. D. (2002). Iron and hydrogen peroxide detoxification properties of DNA-binding protein from starved cells - A ferritin-like DNA-binding protein of *Escherichia coli*. *Journal of Biological Chemistry* **277**, 27689-27696.

Zheng, M., Åslund, F. and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**, 1718-1721.

Zheng, M., Doan, B., Schneider, T. D. and Storz, G. (1999). OxyR and SoxRS regulation of *fur*. *Journal of Bacteriology* **181**, 4639-4643.

Zheng, M., Wang, X., Doan, B., Lewis, K. A., Schneider, T. D. and Storz, G. (2001a). Computation-directed identification of OxyR DNA binding sites in *Escherichia coli*. *Journal of Bacteriology* **183**, 4571-4579.

Zheng, M., Wang, X., Templeton, L. J., Smulski, D. R., A., L. R. and Storz, G. (2001b). DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *Journal of Bacteriology* **183**, 4562-4570.

Zhou, M., Hank Simms, H. and Wang, P. (2004). Increased gut-derived norepinephrine release in sepsis: Up-regulation of intestinal tyrosine hydroxylase. *Biochimica et Biophysica Acta Molecular Basis of Disease* **1689**, 212-218.

Zimmermann, L., Angerer, A. and Braun, V. (1989). Mechanistically novel iron (III) transport system in *Serratia marcescens*. *Journal of Bacteriology* **171**, 238-243.

Ziprin, R. L., Young, C. R., Stanker, L. H., Hume, M. E. and Konkel, M. E. (1999). The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expression bacterial fibronectin-binding protein. *Avian Diseases* **43**, 586-589.

Zwilling, B. S., Kuhn, D. E., Wikoff, L., Brown, D. and Lafuse, W. P. (1999). Role of iron in *Nramp1*-mediated inhibition of mycobacterial growth. *Infection and Immunity* **67**, 1386-1392.