Iron acquisition from host molecules by *Campylobacter jejuni.*

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Abstract

The ability of pathogenic organisms to obtain iron in the host is critical for pathogenesis. Limited research has been carried out into iron acquisition from host molecules by the important gastro-intestinal pathogen *Campylobacter jejuni*. The objective of this study was to investigate the ability of *C. jejuni* to acquire iron from host molecules and the nature of iron responsive regulation of gene expression in *C. jejuni*.

Four *C. jejuni* genes encoding putative haem uptake proteins are arranged in a predicted operon. Data presented in this study confirms that ChuA, a protein similar to haem receptors, functions as an outer membrane haem receptor in *C. jejuni* and is expressed from a promoter in the region upstream of *chuA*, under iron-responsive control by Fur. The *chuB*, *C* and *D* genes are non-essential for iron acquisition from haem, however involvement of *chuB*, *C* and *D* in haem uptake cannot be dismissed due to the possible complementation of *chu* mutation by another system. Identification of alternative haem uptake systems in *C. jejuni* was unsuccessful.

For the first time it was demonstrated that *C. jejuni* can acquire iron from both transferrin (Tf) and lactoferrin (Lf) in a contact dependent, substrate specific manner which does not require noradrenaline. Iron acquired from Tf and Lf accumulates in the soluble cellular fractions and is utilised for cellular growth. The molecular basis of iron acquisition from Tf or Lf has not been elucidated.

C. jejuni fur is co-transcribed with the *lysS* and *glyA* housekeeping genes from two distal promoters (adjacent to the *gatC* and Cj0399 genes). Expression from either promoter is not controlled by Fur in response to iron and therefore is not autoregulated.

This study demonstrates the presence of several sytems involved in iron acquisition from host molecules. Future research is essential to investigate the importance of these systems during host colonisation.

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Abbreviations

The following abbreviations are used in this thesis.

A	Adenine
A (mA)	Ampère (milliAmpère)
aa	Amino acid residue
Amp	Ampicillin
Amp^R	Resistant to ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
Cm	Chloramphenicol
Cm^R	Resistant to chloramphenicol
CO_2	Carbon dioxide
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Disodium ethylenediaminetetraacetate
EtBr	Ethidium Bromide
F	Faradays
Fe	Iron

g	Angular velocity as multiples of the acceleration due to gravity (*g*=9.8 $m.s^{-2}$)
g	Grams
G	Guanine
hrs	Hours
H_2O	Water
IPCR	Inverse polymerase chain reaction
IPCRM	Inverse polymerase chain reaction mutagenesis
J	Joules
K	Kilo (10^3)
kb	Kilobase pairs
kDa	KiloDaltons
Km	Kanamycin
Km^R	Resistant to kanamycin
L	Litre(s)
LA	Luria-Bertani agar
LB	Luria-Bertani broth
Lf	Lactoferrin
μ	Micro (10^{-6})
m	Milli (10^{-3})
M	Mole (s)
Mbp	Megabase pairs (10^6 bp)
MH	Mueller-Hinton media

MHA	Mueller-Hinton agar
min	Minute(s)
M_r	Molecular Weight
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
OD_{600}	Optical Density at a wave length of 600 nm
OM	Outer Membrane
ORF	Open reading frame
p	Pico (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RR	Response regulator
sec(s)	Second(s)
T	Thymine
TCR	Two-component regulatory system
Tf	Transferrin
tRNA	Transfer RNA
UV	Ultraviolet
V	Volts
VAIN	Variable atmosphere incubator
v/v	Volume per volume
w/v	Weight per volume.

1. General introduction.

1.1. Overview.

Campylobacter jejuni is currently the most frequently identified cause of bacterial diarrhoea and is the source of great financial burden to society, due to the cost of treatment and hours lost due to sickness (PHLS., 1999). Treatment of subsequent neurological sequelae such as Guillian-Barré syndrome may also be financially costly (Busby and Roberts., 1997). In numerous pathogenic organisms the ability to obtain nutrition and grow in the host has been demonstrated to be critical to pathogenesis. The ability to obtain iron is vital for the growth and survival of a micro-organism within the host (Ratledge and Dover., 2000). Limited research has been carried out on *Campylobacter* iron uptake systems and the mechanisms by which *C. jejuni* obtains the iron it requires whilst in the host remain to be characterised in detail. The focus of this study was to investigate both the ability of *C. jejuni* to acquire iron from a number of host molecules and the nature of iron responsive regulation of gene expression in *C. jejuni*.

1.2. Necessity of iron.

In order to grow all organisms, with the possible exception of certain Lactobacilli, have an absolute requirement for iron (Archibald, 1986). Iron is useful in biological systems due to its ability to exist in a range of oxidation states, the ability to switch principally between the 2+ and 3+ oxidation states makes iron a vital component of many functional proteins. This ability allows

iron-containing proteins to be involved in electron transfer reactions and interaction with oxygen/reactive oxygen species (Beard, 2000). In addition to the general functions of iron, oxidation of ferrous iron is used to provide energy for lithotrophic bacteria and ferric iron is used as an electron acceptor by anaerobic bacterial species (Brock and Madigan, 1991). In biological systems iron is found primarily in three types of protein: 1) Iron-sulphur enzymes, 2) haem containing proteins/enzymes and 3) non-iron-sulphur, non-haem containing proteins/enzymes. Bacteria must acquire sufficient iron for the production of enzymes such as ribonucleotide reductase and those involved in the carboxylic acid cycle and the respiratory chain (Abdul-Tehrani *et al.*, 1999). Even the few organisms that do not require iron must find an alternative solution to the necessity of iron. Such solutions include the substitution of manganese for iron in certain essential enzymes (Archibald, 1986) and the replacement of iron-containing ribonucleotide reductase with an enzyme containing adenosylcobalamin (Neilands, 1995).

1.2.1. Availability of iron.

Despite being one of the most common elements in the earth's crust, the availability of iron for an organism in aerobic environments is limited by the formation of an insoluble iron (III) hydroxide at neutral pH (Brock and Madigan, 1991). Under conditions of low pH ferrous iron is stable and soluble in water, therefore organisms that live in acid conditions are not subject to iron limitation to the same extent as organisms that inhabit higher pH environments (Brock and

Madigan, 1991). Consequently micro-organisms that live in neutral and alkaline pH environments may be subject to conditions of severe iron limitation. Iron may therefore be the growth-limiting nutrient in many environments. Bacteria can use a number of strategies to obtain iron, which include: -

a) Production and uptake of high affinity iron chelating molecules.

b) Expression of iron acquisition systems for iron containing molecules produced by other organisms.

c) Production of toxins/lysins to remove competing organisms and liberate their iron stores.

d) Lowering pH to convert insoluble ferric iron to soluble ferrous iron.

e) Production of proteins that reduce insoluble ferric to soluble ferrous iron.

1.2.2. Iron toxicity.

The presence of intracellular iron particularly in the aerobic environment causes problems for an organism. During respiration electron donors may reduce oxygen to a number of toxic reactive oxygen intermediates as shown below.

Chapter1. General introduction.

$$O_2 + e^- \rightarrow O_2^- + e^- (+ 2H^+) \rightarrow H_2O_2 \rightarrow HO^{\cdot} + e^- (+ H^+) \rightarrow H_2O$$

A B C D

By stepwise addition of electrons molecular oxygen firstly becomes superoxide O_2^- (A), then hydrogen peroxide, H_2O_2 (B) which may be converted (by transition metals or ionising radiation) to the hydroxyl radical, $HO^{\cdot}$ (C) before it is finally converted to water, H_2O (D) (Brock and Madigan, 1991). The presence of Fe(II) exacerbates this problem by catalysing the reduction of molecular oxygen to the superoxide ion (Haber-Weiss reaction) and on to hydrogen peroxide (see figure 1.1a and 1.1b). The accumulation of reactive iron(II) in the cell leads to production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction, (see figure 1.1c) (Pierre and Fontecave, 1999; Touati, 2000). Presence of the superoxide radical in a cell may cause liberation of further iron(II) by reduction of ferric iron, causing its release from iron(III) containing proteins. Inside the cell, unchecked accumulation of Hydroxyl radicals ($OH^{\cdot}$) can be immensely destructive. Hydroxyl radicals can damage biological macromolecules and may lead to the death of the cell/organism if vital proteins (Tamarit *et al.*, 1998), DNA, or membranes are severely damaged (Henle *et al.*, 1996).

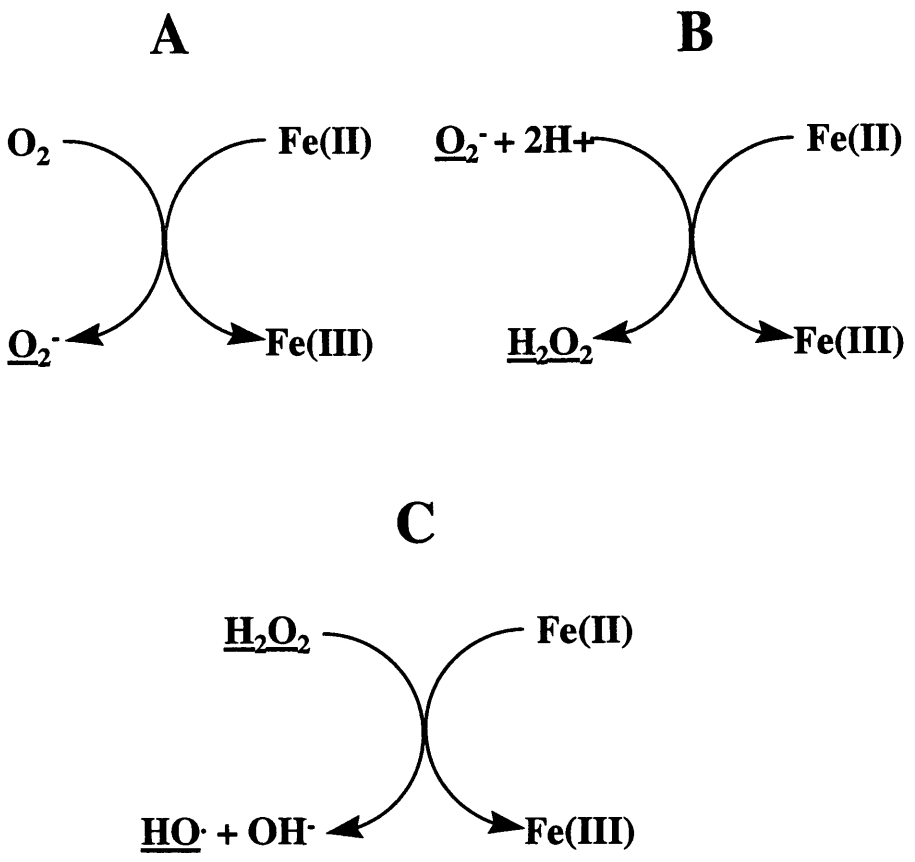

Figure 1.1. Catalytic reduction of molecular oxygen by ferrous iron, causing the production of reactive oxygen species (underlined). A, the production of superoxide from molecular oxygen (Haber-Weiss reaction). B the catalysis of hydrogen peroxide production from superoxide (and hydrogen ions). C, production of the hydroxyl radical from hydrogen peroxide (Fenton reaction) (Pierre and Fontecave, 1999).

1.2.3. Iron storage and metabolism.

The necessity of iron means that oxidative stress due to the presence of iron must either be prevented or dealt with. All organisms that live in aerobic environments must have oxidative stress systems to deal with the toxic products and radicals of reactive oxygen chemistry. Oxidative stress defence is particularly important for invasive pathogenic organisms that may be subject to an oxidative burst during host immune response by macrophages (Zwilling *et al.*, 1999). Pathogenic

microorganisms must possess systems that act by breaking down toxic oxygen species. Oxidative stress defence systems may include superoxide dismutases (SODs) to remove Superoxide radicals, and catalases and peroxidases to remove hydrogen peroxide, preventing destructive hydroxyl radical formation (Pierre and Fontecave, 1999). In order to deal efficiently with the oxidative stress 'load' produced by the presence of iron organisms may regulate expression of their oxidative stress systems in response to the presence of oxidative stress or iron. In *Escherichia coli* the protein OxyR controls expression of genes in response to hydrogen peroxide (Storz and Imlay., 1999). OxyR is inactive in its native form but upon oxidation by hydrogen peroxide an intra-molecular disulphide bond is formed, activating OxyR (Aslund *et al.*, 1999). Oxidised OxyR protein activates transcription from a number of promoters of oxidative stress response genes, including alkylhydroperoxide reductase (*ahpCF*) and hydroperoxidase I (*katG*). OxyR also activates transcription of glutaredoxin (*grxA*) and GrxA can in turn reduce OxyR, deactivating it to provide negative feedback on OxyR activity (Aslund *et al.*, 1999). Regulation of genes in response to superoxide is carried out by the SoxRS system in *E. coli* (Touati, 2000). The SoxR protein contains an iron-sulphur cluster that is oxidised by superoxide, causing activation of this protein. Oxidised SoxR activates transcription of SoxS, which in turn activates transcription of a number of proteins that deal with oxidative stress. These include the Manganese superoxide dismutase *sodA* (MnSOD), ferredoxin *fpr*, fumarase *fumC* and aconitase *acnA* genes (Zheng *et al.*, 1999). Due to the link between Oxidative

stress and iron, the regulators OxyR and SoxRS interact with iron regulatory proteins (Zheng *et al.*, 1999); this will be discussed the Section 1.2.14.

1.2.4. Bacterioferritins.

Another way of avoiding the potentially destructive effects of free intracellular iron is to store it by attaching it to a protein, which will render the iron unavailable to reactive oxygen chemistry until it is needed. Ferritins are a widely distributed family of proteins that form spherical molecular cages for the storage of iron in a soluble, easily released form. Ferritin 'cages' can accommodate 4500 iron atoms (Abdul-Tehrani *et al.*, 1999). There are two types of ferritins found in nature, bacterioferritins found almost exclusively in prokaryotes and ferritins which can be found in animals, plants, fungi and prokaryotes. *E. coli* possesses both a ferritin and bacterioferritin, mutation of which leads to differing phenotypes. Mutation of the ferritin gene leads to a strain that accumulates a lowered total cellular iron concentration, compared to that of the wild type parent strain, demonstrating that ferritin acts by storing the iron taken up in stationary phase. Ferritin therefore allows the bacteria to store large amounts of otherwise toxic iron for future growth under iron-limited conditions (Abdul-Tehrani *et al.*, 1999). Ferritin mutants do not show increased sensitivity to hydrogen peroxide but this is thought to be due the increased free intracellular iron levels affecting iron dependent negative-regulation of gene expression. Although the role of bacterioferritin remains unclear in *E. coli*

(Abdul-Tehrani *et al.*, 1999), bacterioferritin mutants of *N. gonorrhoeae* grow slowly in iron deficient media and are sensitive to hydrogen peroxide (Chen and Morse, 1999). This demonstrates a role for bacterioferritin in iron storage and protection from oxidative stress in some bacteria (Chen and Morse, 1999).

1.2.5. Iron in the Host.

As previously mentioned, in many environments iron is not available due to its presence as an insoluble iron (III) hydroxide. Iron limitation is compounded inside the vertebrate host by the expression in host tissues of a whole array of non-specific host defence mechanisms that sequester iron, which would otherwise be available to pathogenic micro-organisms. The first point of contact with the host for most pathogens including enteric pathogens will be the mucosa. Within the mucus lining of the mucosa secretion of lactoferrin by mucosal cells will limit available iron. Lactoferrin is a member of the transferrin family of proteins and acts in an almost identical manner to other transferrins. This family of proteins are all monomeric glycoproteins that are found ubiquitously in vertebrates (Ratledge and Dover., 2000). They have an approximate molecular mass of 80 kDa and, when folded, form two globular domains capable of reversible iron binding. Lactoferrin differs from other transferrins in its ability to form stable complexes with iron at a wide range of pHs. Iron remains complexed to lactoferrin down to pH of 2-4, whereas iron is released by serum transferrin between pH 4-6 (Ward *et al.*, 1996b). Highly

stable iron binding coupled with microbiocidal activity and proposed immune modulatory activity by lactoferrin, act to prevent colonisation of the mucosa by potentially harmful micro-organisms. The addition of excess lactoferrin has been shown to limit the severity of gastrointestinal infection (Levy, 2000. The addition of excess lactoferrin has been shown to limit the severity of gastrointestinal infection [Edde, 2001 #22) and both lactoferrin and lactoferrin-based molecules have been proposed as chemotherapeutic agents (Levy, 2000).

Should an enteric pathogen manage to invade the epithelial cells of the gastro-intestinal tract, iron will also be limited by the storage of iron by intracellular host ferritin. Once in the blood, an invading microorganism encounters an enormous potential reservoir of iron. Most of the iron in the mammalian host will be incorporated in haemoglobin in the erythrocytes. Haemoglobin is found widely in animals and has been found in some algaes, higher plants and prokaryotes. In vertebrate organisms, haemoglobin is primarily used for the transport of respiratory gasses. (Weber and Vinogradov, 2001). Haemoglobin is not readily available to an organism invading the blood stream, due to its compartmentalisation and because any free haemoglobin will be rapidly bound by haptoglobin in preparation for recycling by hepatocytes (Wong *et al.*, 1994). Liberated haem groups in the blood, that could present another potential iron source, are bound by haemopexin and serum albumin. Any free iron in the blood is bound by transferrin, the archetypal member of the transferrin protein family. Transferrin has a high affinity for iron having an association constant of $10^{22}M^{-1}$ in the presence of bicarbonate ions. In

order to maintain iron limited conditions serum transferrin is never 100% iron saturated so that there is sufficient iron free (apo) transferrin to bind any transient increases in iron level (e.g. after a meal) (Ward *et al.*, 1996a). The effectiveness of iron limitation as a form of innate immunity is apparent from a number of opportunistic pathogens that only become pathogenic in patients with an abnormal amount of iron loading of tissues and blood (reviewed in (Weinberg, 2000)).

1.2.6. Iron uptake by bacteria.

If soluble iron(II) is present (usually in anaerobic conditions or conditions of low pH) many organisms will take up iron directly. Ferrous iron uptake in *E. coli* and *Salmonella* Typhimurium is achieved by expression of the proteins FeoA and B (Ratledge and Dover., 2000)). Ferrous iron passes freely through outer membrane pores and the ferrous iron uptake system transports ferrous iron across the cytoplasmic membrane utilising energy from the hydrolysis of ATP (Kammler *et al.*, 1993). The Feo system is termed an ATP Binding Cassette system or ABC transport system. The ABC superfamily of uptake systems are involved in a diverse range of import and export processes and typically comprise of a hydrophobic membrane spanning subunit and a hydrophilic ATP binding component (Jones and George., 1999). These sub-units come together at the cytoplasmic membrane and import/export their specific substrate using the hydrolysis of ATP.

Plants and micro-organisms release citric acid into the soil in order to solubilise iron from ferric hydroxide. Gram negative bacteria that possess ferric citrate uptake systems may therefore be able to utilise not only the iron in soil but also ferric citrate that may be present in the tissues and blood of a mammalian host (Pierre and Gaultier-Luneau, 2000).

1.2.7. Siderophores.

In environments where iron may not be directly available, bacteria can obtain iron by the production of siderophores. Siderophores are low molecular weight molecules produced by bacteria or fungi that have a high affinity for iron (III). The high affinity for iron is due to three binding ligands present in the right three-dimensional configuration in each siderophore molecule (figure 1.2) (Brock and Madigan, 1991). A micro-organism will secrete siderophores into the immediate environment, free siderophores can then chelate any free iron or scavenge iron from other molecules. The siderophore and its chelated iron are then taken up by the bacteria using a specific uptake system. Siderophores fall into two groups defined by the chemical groups in their makeup, the catechols and hydroxamates. A typical catechol siderophore is enterobactin (figure 1.2.a), which is produced ubiquitously amongst enteric bacteria (Ratledge and Dover., 2000). Even at neutral pH enterobactin has an association constant of $10^{37}M^{-1}$, which is more than sufficient to remove iron from transferrin. Siderophore uptake systems comprise of a specific

outer membrane binding protein and associated energy system, a periplasmic binding protein and a cytoplasmic ABC transporter (Clarke *et al.*, 2001; Neilands, 1995).

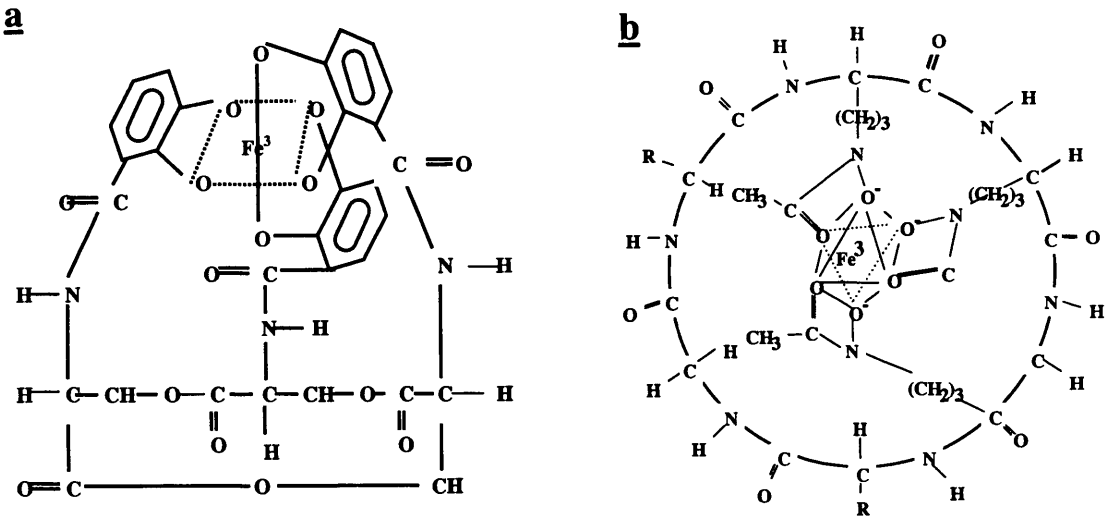

Figure 1.2. The general structures of a) a catecholate siderophore (enterobactin) and b) a hydroxamate siderophore (ferrichrome) demonstrating the 3D configuration of multiple chelating ligands with respect to iron.

The Outer membrane- (OM) receptor protein is localised in the outer membrane and specifically binds its designated ferri-siderophore (figure 1.3, step1). Siderophore receptors have a gated pore structure and utilise the energy provided by a TonB system (Clarke *et al.*, 2001) (figure 1.3, step2) to open the pore allowing the ferri-siderophore into the periplasm. The function of TonB systems is the transduction of energy across the periplasm in Gram-negative bacteria. Once in the periplasm the ferri-siderophore will be bound and shuttled across the periplasm by a specific periplasmic binding protein (figure 1.3, step3). The ferri-siderophore can then be transported across the inner membrane by an ABC transporter system using

energy provided by the hydrolysis of ATP (figure 1.3, step 4). Inside the cytoplasm, iron is liberated for use by the cleavage of the siderophore or reduction of the iron (figure 1.3, step5).

As previously mentioned a functional TonB system is an integral part of a ferri siderophore uptake system. TonB and its accessory proteins ExbB and ExbD are thought to harness the proton motive force across the cytoplasmic membrane, TonB then supplies this energy to the outer membrane binding protein (Larsen *et al.*, 1999). TonB interacts with outer membrane receptors via a specific 5 amino acid sequence at their N-terminus called the TonB box (Kadner, 1990). In this way one single TonB system may provide energy to any of a number of outer membrane proteins, that all have an identical motif at their N-terminus.

1.2.8. Host iron-protein utilisation.

Another way to obtain iron in the host is the direct uptake or removal of iron from host iron-containing proteins. Various systems to obtain iron from host molecules have been described in a number of bacterial species. The actual mechanism of uptake of host iron containing proteins is remarkably similar to that of siderophore uptake systems (compare figures 1.3 and 1.4). Bacteria may utilise host iron containing molecules in a number of ways. Firstly, by removal of iron from host molecules at the outer membrane via an enzymic type reaction e.g the Tbp system

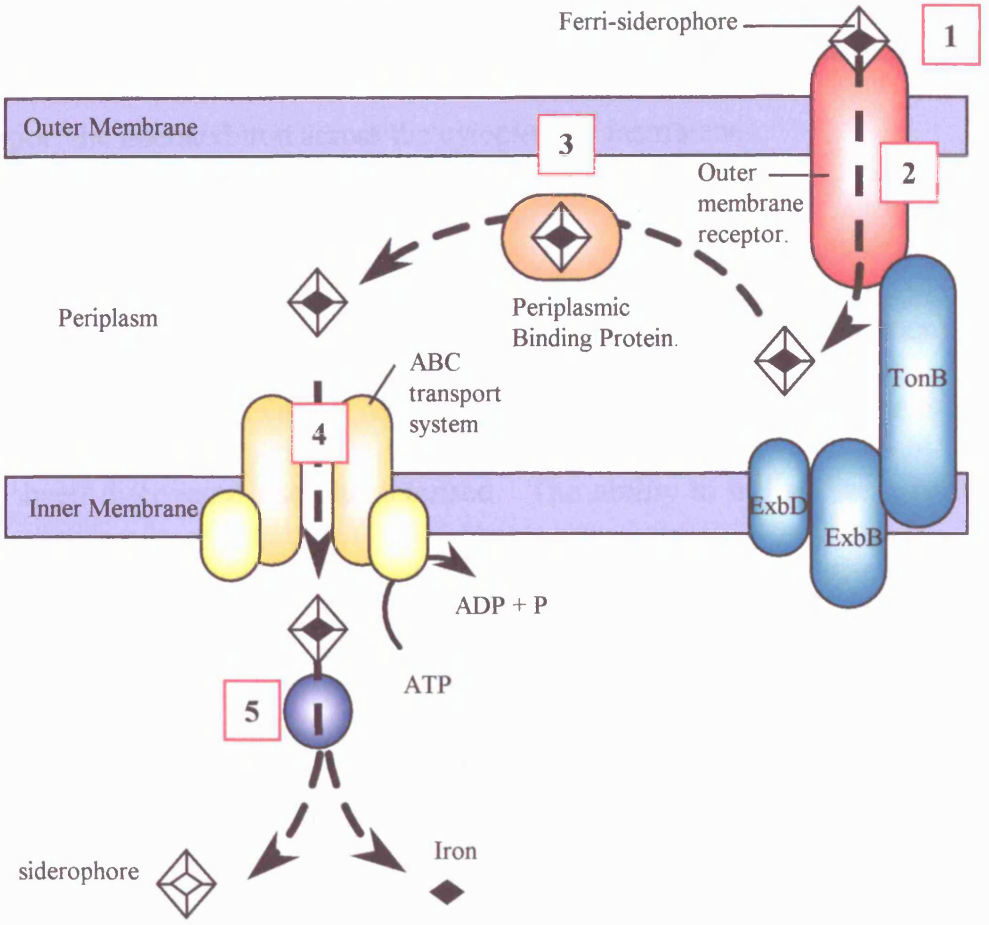

Figure 1.3. A generalised Gram-negative bacterial Siderophore uptake system. Mechanistic steps are numbered in red boxes. Step 1) ferric siderophore complex is bound by the outer membrane binding protein. Step 2) The ferri-siderophore is transported across the outer membrane using energy provided by the TonB energy transduction system and deposited in the periplasm. Step 3) the ferri siderophore is bound by the periplasmic binding protein and shuttled across the periplasmic space. Step 4) ferri-siderophore is transported across the cytoplasmic membrane by an ABC transport system comprising of a membrane permease and an ATPase. The energy for this step is provided by the hydrolysis of ATP. Step 5) iron is released from the ferri-siderophore complex by reduction of the iron by a ferric reductase (blue sphere) (Ratledge and Dover., 2000). The siderophore can then be recycled.

Such systems are dependent upon a functioning ferrous iron transport system to transport the liberated iron across the cytoplasmic membrane.

Secondly, a system may take up the whole molecule e.g. haem uptake in *Shigella dysenteriae* (see figure 1.4) (Mills and Payne, 1995). Haemin/haemoglobin uptake systems from a variety of Gram-negative and positive pathogenic bacteria have been discovered and characterised. The ability to utilise haem-containing compounds have been shown to be important in establishing human infection by pathogenic *Haemophilus* species (Al Tawfi *et al.*, 2000). In Gram-negative organisms most haemin uptake systems comprise of a TonB dependent outer membrane receptor, which acts in a similar manner to receptors of siderophore uptake systems. OM haem receptors have been described in many Gram-negative bacteria including *E. coli* (Torres and Payne, 1997), *Shigella dysenteriae* (Mills and Payne., 1997), *Neisseria meningitidis* (Stojiljkovic *et al.*, 1995) *Vibrio cholerae* (Henderson and Payne., 1994), *Ps. aeruginosa* (Ochsner *et al.*, 2000), *Yersinia pestis* (Thompson *et al.*, 1999), *Yesinia enterocolitica* (Stojiljkkovic and Hantke., 1994) and *Haemophilus influenzae* (Cope *et al.*, 1995). Haem binding by these proteins requires close contact of haem to the bacterium. These systems act in a manner similar to siderophore uptake systems, whereby iron is taken up by the binding of haem by the OM receptor. The OM receptor functions as a gated pore, that allows haem to pass through into the periplasm, using energy provided by a TonB system (Clarke *et al.*, 2001). A notable exception to this mechanism of haem uptake is the Has system in *Ps. aeruginosa*. *Ps. aeruginosa*

not only possesses a conventional contact dependent haem receptor (PhuR (Ochsner *et al.*, 2000)) but also an additional extracellular haemophore (HasA) which is released into the surrounding environment. HasA can remove haem from haemoglobin (Letoffe *et al.*, 1999) and delivers the haem to an OM bound receptor, HasR, the haem removed from HasA by HasR and imported into the periplasm in the conventional manner using energy from a TonB system (Letoffe *et al.*, 1999). As with siderophore uptake systems, haem uptake systems require a functional periplasmic binding protein based ABC transport system and TonB system to take up haem. The whole haem molecule (in a manner similar to a siderophore) is internalised, unlike a siderophore however, after removal of iron the rest of the molecule may be used in alternative biochemical pathways. The porphyin provided by the degradation of haem has been demonstrated to satisfy the internal requirement for porphyrin, in a number of auxotrophic mutant strains of different bacterial species (Hornung *et al.*, 1996; Mills and Payne., 1997; Stojiljkovic *et al.*, 1995).

Organisms that take up iron containing molecules may require accessory proteins to remove the iron once the molecule has entered the cell (e.g. HemO haem oxygenase in *Neisseria* (Zhu *et al.*, 2000)). Haem ABC transport systems and their mechanisms of action have been described in a number of bacterial species, a comparative overview of such systems would be difficult to contrive due to the differing properties of such systems in different organisms. ABC transport systems for haem/haem-containing molecules are present in *Ps. aeruginosa* (PhuT, U, and V (Ochsner *et al.*, 2000)), *Y .pestis* (HmuT, U and V (Thompson *et*

al., 1999)) *Y. enterocolitica* (HemT, U and V (Stojiljkkovic and Hantke., 1994)) and *V. cholerae* (HutB, C and D). Mutation of the ABC transporter genes in *Ps. aeruginosa* leads to impaired growth when haemin is present as the sole iron source (Ochsner *et al.*, 2000). Mutation of a homologous system in *Y.pestis* leads to the loss of ability to utilise certain haem containing compounds but not haemoglobin based proteins (Thompson *et al.*, 1999). Similarly *E. coli* K12 gained the ability to utilise haem when the *Y.enterocolitica* outer membrane haem receptor gene (*hemR*) alone was present on a plasmid. This contrasts with the observation that an *E. coli* siderophore mutant required not only the *V. cholerae* haem receptor but also the entire ABC system (*hutB*, *C* and *D*) and associated TonB system to show similar growth stimulation on agar with hemin as a sole iron source (Occhino *et al.*, 1998). In *Bradyrhizobium japonicum* mutation of the haem ABC transport genes had no effect on the ability to utilise haemoglobin suggesting the presence of a system, which can functionally complement mutation in this system (Nienaber *et al.*, 2001). It is apparent that in order to functionally analyse the haem uptake genes of an organism, consideration must be taken of other homologous genes and accessory factors such as putative haem degradation proteins e.g. PhuW and S (Ochsner *et al.*, 2000), HmuS and P' (Thompson *et al.*, 1999). Functional complementation by analogous or homologous systems is obviously also significant in an organisms ability to utilise haem containing compounds.

1.2.9. Regulation of iron uptake.

The limited availability of environmental iron, coupled with its potential toxicity in oxidative environments, means that it is vital for an organism that possesses high affinity iron uptake systems to regulate their expression to occasions when iron is scarce. If an iron acquisition system functioned at all times, potentially toxic concentrations of iron would build up in the cell. Among Gram-negative bacteria, iron uptake systems are controlled in response to iron by the Ferric uptake regulator (Fur) protein. The Fur protein has been most widely studied in *E. coli* where it was found to be a 17kDa polypeptide that appears to be a dimer in solution (Coy and Neilands, 1991). Homologues of the Fur protein have been discovered in a wide range of pathogenic bacterial species including *Haemophilus influenzae*, *Neisseria* species (Berrish *et al.*, 1993; Thomas and Sparling., 1994), *Pseudomonas* species (Prince *et al.*, 1993), *Salmonella typhimurium*, *Shigella dysenteriae*, *Vibrio* species (Litwin *et al.*, 1992; Tolmasky *et al.*, 1994), *Yersinia* species (Staggs and Perry., 1992), *Bordetella* species (Ratledge and Dover., 2000), *Staphylococcus* species (Heidrich *et al.*, 1996) and *Bacillus subtilis* (Bsat *et al.*, 1998).

1.2.10. Classical regulation by the Fur protein.

The *E. coli* Fur protein has an Fe(II) binding site at the carboxyl terminal domain which when bound to intracellular ferrous iron allows the N-terminus of Fur to bind to a specific DNA operator sequence, close to the promoter of a gene (Coy

and Neilands, 1991; Hantke, 2001). In *E. coli* the Fur protein was also found to have another metal binding site at its carboxy terminal domain, which is occupied by zinc, this motif is essential for function (Hantke, 2001). Initially it was assumed that the Fur dimer is bound to an operator sequence (called the Fur box), and expression from that promoter is blocked in a similar manner to classical repressors e.g. the *lac* repressor. Analysis of frequently occurring base pairs in Fur box regions of several *E. coli* iron regulated promoters allowed a 19bp consensus sequence to be elucidated. The Fur-box consensus sequence 5'GATAATGATAATCATAATC3', was originally thought to be recognised by the Fur protein as a palindromic sequence, due to the dimeric nature of the Fur protein (figure 1.5). Further analysis of Fur boxes by hydroxyl radical footprinting and construction of synthetic Fur binding sites revealed that Fur interacts with three 6bp repeat sequences within this consensus sequence (figure 1.5) (Escolar *et al.*, 1998). Electron microscopy appears to show polymerised Fur wrapping around the operator sequence in a helical manner demonstrating how Fur may interact with the multiple 6bp binding sites (Le Cam *et al.*, 1994). Binding in this modular manner may explain the observation that some *E. coli* iron regulated promoters appear to contain several overlapping 19bp consensus sequences. The presence of multiple Fur binding sites may also allow Fur regulation to occur in a concentration dependent manner, allowing global gene regulation by the Fur protein (Escolar *et al.*, 1998, 1999).

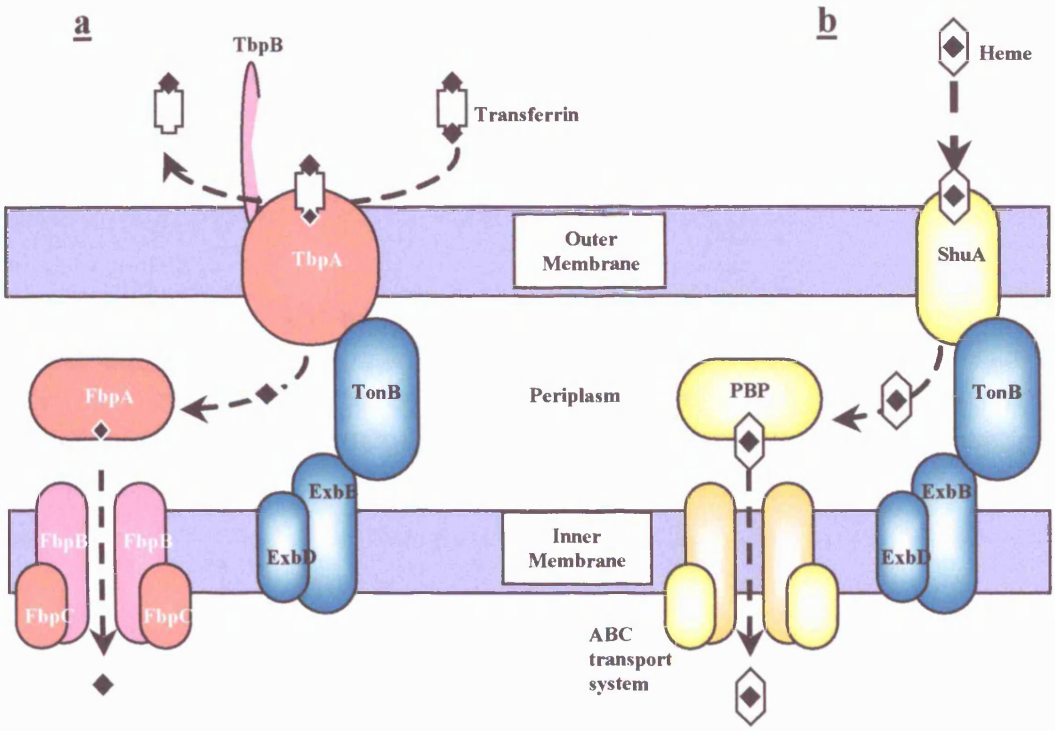

Figure 1.4. Examples of two systems involved in iron acquisition from host iron containing proteins, iron is represented by black diamonds. a) By removal of iron at the cell surface e.g. the Transferrin binding protein (Tbp) of *Neisseria* species. b) by uptake of the whole protein e.g. the haem uptake system of *Shigella dysenteriae*. Note that both systems require functional TonB and periplasmic binding protein dependent ABC transport systems. Also note the similarity between the components of the above two systems and with siderophore uptake systems (figure 1.3)

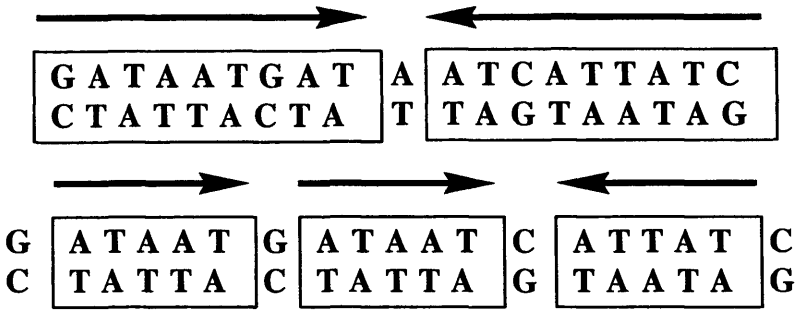

Figure 1.5. Two proposed models of Fur binding to the Fur box sequence. Either by a Fur dimer as a palindromic inverted repeat (top) a monomer binding each half of the palindromic sequence, or a sequence of three repeats (two direct and one inverted, bottom) by a polymer or aggregation of Fur protein monomers (Escolar *et al.*, 1998).

1.2.11. Other members of the Fur protein family.

A number of Fur-like proteins have been discovered which are responsible for several different regulatory functions in both Gram-negative and Gram-positive bacteria. In *E. coli*, Zur, a member of the Fur family, is adapted to regulate zinc uptake systems in response to Zn^{2+} (Patzer and Hantke., 2000). Several Fur homologues were discovered in *Bacillus subtilis*, a Fur homologue, a Zur homologue and a novel member of the Fur family of proteins. This novel Fur-like protein regulated expression of the oxidative shock defence proteins AhpC and KatA, and was designated PerR-peroxide stress regulator (Bsat *et al.*, 1998). To date the Fur family appears to be the most widespread group of proteins regulating gene expression in response to iron concentration (Touati, 2000).

In Gram-positive bacteria with a high G-C content, another type of iron responsive regulator has been described. This group of regulators was termed DtxR regulators after the first described member of the group, the diphtheria toxin regulator DtxR of β and ω temperate phages of *C .diphtheriae* (Hantke, 2001; Salyers and Whitt, 1994). At first glance Fur and DtxR seem dissimilar, DtxR at 25 kDa being slightly larger than Fur (17 kDa). Although Fur and DtxR share little sequence similarity they are structurally similar, both having a DNA binding N-terminus and two metal binding sites (Hantke, 2001). In *Treponema pallidum* TroR, a member of the DtxR family has evolved to regulate expression of manganese uptake systems in response to manganese (Hardham *et al.*, 1997).

1.2.12. Global regulation by the Fur protein.

The ability of Fur to act as both a positive and negative regulator of protein expression indicates that Fur acts in a more global manner than was initially thought. Analysis of protein expression in Fur mutant strains of some bacterial species revealed a number of genes to be de-repressed, showing that such genes were regulated in a conventional manner by Fur (Barton *et al.*, 1996; Hassett *et al.*, 1996). Some proteins however were expressed in lower quantities, meaning that Fur was regulating expression of these proteins in a positive manner. Expression of *sodB* in *E. coli* is activated by Fur in an iron dependent manner (Dubrac and Touati., 2000). Analysis of the *sodB* promoter revealed that Fur binding was

dependent upon a large sequence downstream of the -10 sequence (Dubrac and Touati., 2000). Interestingly, comparisons of *sodB* RNA stability in wild type and *fur* mutant strains demonstrated that the *sodB* RNA was stable far longer in the wild type parent strain than the *fur* mutant strain. This led to the conclusion that Fur may regulate SodB expression in a post-transcriptional manner. Fur mutants of *S.* Typhimurium were found to show increased sensitivity to acidic conditions and had altered expression of acid shock proteins (Hall and Foster., 1996). Acid tolerance was still present in an *S.* Typhimurium *fur* mutant that had one amino acid substitution causing abolished iron repression. This demonstrated that the positive regulation of gene expression by Fur was distinct from its iron dependent negative regulation (Hall and Foster., 1996). Other proteins that were expressed in lower amounts in the *E. coli fur* mutant strain than the wild type parent strain include iron containing enzymes such as aconitases and fumarases. Aconitases are enzymes that contain an iron sulphur cluster and in their native form are involved in the conversion of citrate to iso-citrate in the citric acid and glyoxylate cycles. If subjected to oxidative stress however, the enzymes can lose iron from the iron sulphur cluster causing aconitases to lose their enzymatic activity and to gain an mRNA binding activity. *E. coli* possesses two aconitases, AcnA which is expressed in response to iron and oxidative stress, and AcnB which is expressed during exponential growth (Tang and Guest., 1999). If these aconitases are exposed to oxidative stress the apo-iron forms of the enzymes can bind to aconitase mRNA, stabilising them and giving rise to a form of positive post-translational auto-regulation. Fumarases are tricarboxylic acid cycle enzymes, which catalyse

the conversion of fumarate to L-malate. FumC, a non-iron containing fumarate, is expressed in response to iron limitation and superoxide stress, compensating for loss of iron containing FumA (Park and Gunsalus, 1995). More recently a posttranscriptional regulation mechanism was discovered by which Fur indirectly regulates expression of SodB FumA and AcnA via a small regulatory RNA (Masse and Gottesman., 2002). Expression of the small RNA RyhB is regulated by Fur in response to iron, RhyB acts as an antisense RNA to the RNAs of SodB, FumA, AcnA, Bfr (bacterioferritin) and FtnA (ferritin) and succinate hydrogenase. By acting as an antisense RNA RhyB prevents translation of SodB, FumA, AcnA, Bfr, FtnA and succinate hydrogenase, providing a mechanism for the bacterium to limit expression of non-essential iron-containing proteins in times of iron-limitation (Masse and Gottesman., 2002).

The Fur protein may also allow indirect regulation of iron acquisition gene expression by controlling the expression of extracytoplasmic function (ECF) sigma factors (Braun *et al.*, 2003). ECF sigma factors allow transcription of iron acquisition genes in response to the presence of their respective extracellular ligand. The negative regulation of ECF sigma factors by Fur allows an organism to express iron uptake systems under iron-limited conditions, in the presence of their iron containing ligand only. Exaples of this mechanism of gene regulation include regulation of the ferric citrate transport genes (*fecABCDE*) by the FecI sigma factor of *E. coli* (Braun *et al.*, 2003) or regulation of ferri-siderophore transport genes by the PvdS and FpvI sigma factors of *Ps. aeruginosa* (Ravel and Cornelis., 2003).

1.2.13. The Fur regulon.

In *E. coli* the expression of Fur is auto-regulated and is also regulated by the presence of the catabolite cAMP under control of the cAMP receptor protein Crp (De Lorenzo *et al.*, 1988). *E. coli* Fur is also expressed in response to oxidative stress by the OxyR and SoxS transcriptional regulators (Zheng *et al.*, 1999). The types of genes regulated by Fur fall into three categories; those involved in iron transport, oxidative stress response and pathogenicity (figure 1.6).

1.2.14. Iron transport.

Many of the genes under control of the Fur protein in *E. coli* were found to be involved in the production and uptake of siderophores and iron uptake systems. Such proteins in *E. coli* include genes involved in ferrichrome uptake (*fhuA* and *fhuF*), enterochelin production (*entA* and *entF*) and uptake (*fepA*) and degradation (*fiu* and *cir*) (Stojiljkovic *et al.*, 1994). Proteins involved in iron acquisition are also regulated by Fur in many other bacterial species. In *Pseudomonas aeruginosa* production of both pyochelin and pyoverdin siderophores are repressed by Fur in iron-replete conditions (Hassett *et al.*, 1996; Ochsner *et al.*, 1995). Fur regulation of siderophore iron acquisition systems has also been demonstrated in *Y .pestis* (Yersiniabactin synthesis and uptake YptP, Q, X and S (Fetherstone *et al.*, 1999)), *Vibrio anguillarum* (Anguibactin (Tolmasky *et al.*, 1994)), *Staphylococcus aureus* (ferrichrome uptake Fhu (Xiong *et al.*, 2000)) and *B. subtilis* (dihydroxy benzoate

biosynthesis (Bsat *et al.*, 1998)). Fur regulation has also been implicated in control of expression of systems involved in iron acquisition from host iron-containing molecules. Fur regulated haem acquisition systems include genes from *Y.pestis* (Hornung *et al.*, 1996), *Y. enterocolitica* (Stojiljkkovic and Hantke., 1994), *V. cholerae* (Henderson and Payne., 1994), *Ps. aeruginosa* (Ochsner *et al.*, 2000), *E. coli* O157:H7 (Torres and Payne, 1997) and genes involved in haem acquisition from haemoglobin in *N. meningitidis* (Stojiljkovic *et al.*, 1995).

1.2.15. Oxidative stress response.

Due to the inextricable linkage of iron with oxidative stress, Fur was also found to control expression of proteins produced to combat the action of reactive oxygen species. In fact much of the early characterisation of oxidative stress systems in *E. coli* was achieved by the study of proteins de-repressed in *fur* mutants (Touati, 2000). Superoxide Dismutases (SODs) are enzymes responsible for the oxidation of superoxide ions within the cell. *E. coli* contains three types of SOD, an iron-containing enzyme (FeSOD) encoded by the gene *sodB*, a manganese-containing enzyme (MnSOD) encoded by the gene *sodA* and a Cu/Zn containing enzyme encoded by the gene *sodC*. In the presence of intracellular iron expression of *sodB* (FeSOD) is induced and expression of *sodA* (MnSOD) is repressed by *fur*. It is thought that FeSOD is expressed in the presence of iron to deal with the superoxide radicals produced by iron. MnSOD however is expressed in the absence of iron in

order to counter the inability to make FeSOD (due to lack of Fe) and to counter any transient oxidative shock produced upon iron uptake before FeSOD is expressed (Touati *et al.*, 1995). Expression of the *fur* gene itself has been shown to be activated by the oxidative stress regulators OxyR and SoxRS (Zheng *et al.*, 1999). As Fur has been implicated in regulation of expression of oxidative shock defence genes, the regulation of Fur expression by oxidative shock regulators demonstrates the role of Fur as an important component of the regulation of cell homeostasis.

1.2.16. Pathogenesis.

A number of virulence determinants are controlled by Fur in response to iron limitation. Expression of virulence determinants in response to iron limitation, allows co-ordination of bacterial pathogenic mechanisms with entry into the iron-limited conditions that predominate in the host. Virulence determinants shown to regulated by Fur include exototoxin A production in *Ps. aeruginosa* (Barton *et al.*, 1996), a Shiga toxin in *Shigella* (Litwin and Calderwood., 1993). A review of such systems can be found in Litwin and Calderwood, 1993(Litwin and Calderwood., 1993).

Bacterial acquisition of iron is important, in particular uptake of iron is vital for pathogens in order to colonise host organisms and overcome host defences. Iron uptake in bacteria is closely regulated and many aspects of bacterial physiology are expressed in response to iron. Understanding iron uptake systems and the

regulation of their expression is essential in understanding the biology and pathogenic status of an organism. Iron uptake and its regulation is well studied in *E. coli* (De Lorenzo *et al.*, 1988; Torres and Payne, 1997; Touati *et al.*, 1995) and other pathogens including *Ps. aeruginosa* (Hassett *et al.*, 1996; Prince *et al.*, 1993), (Ochsner *et al.*, 1995) and the *Vibrio* (Colquhoun and Sorum, 2002; Ochsner *et al.*, 2000; Okujo *et al.*, 1998). The role of iron uptake systems and iron dependent regulation in the important enteric pathogen *Campylobacter jejuni* is only now being addressed.

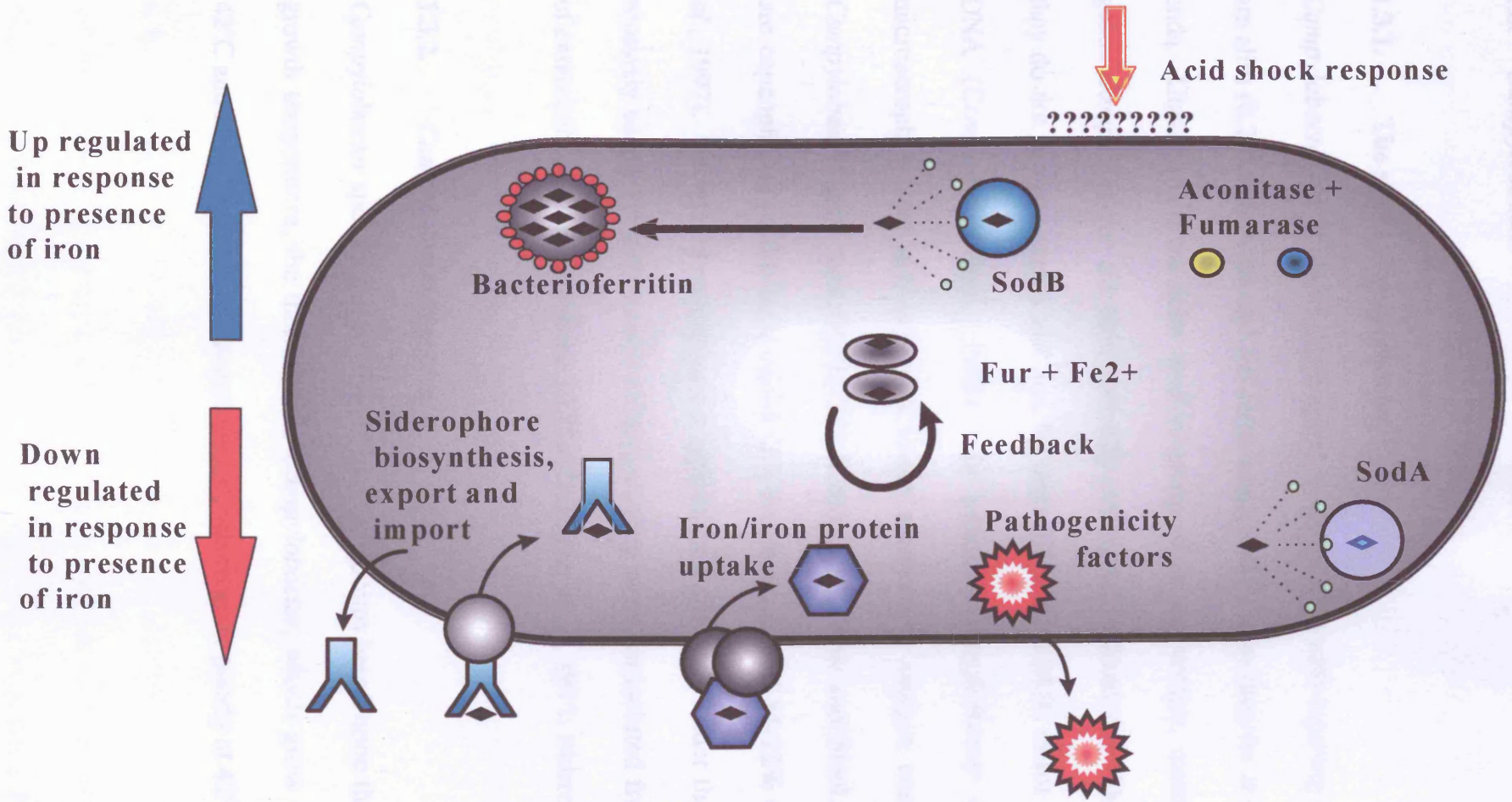

Figure 1.6. Generalised model of the types of systems regulated by the Fur protein in the presence of iron in Gram-negative bacteria. Iron is symbolised by black diamonds, reactive oxygen species are symbolised by small green circles.

1.3. *Campylobacter.*

1.3.1. The genus *Campylobacter.*

Campylobacter are Gram-negative spiral or curved rods with tapering ends. They are slim (0.2-0.5μm wide and 1.6-6μm long), and possess flagella at one or both ends. Classified in the delta epsilon group of proteobacteria, members of the genus *Campylobacter* are biochemically distinguished from the *Vibrio* because they do not ferment sugars and also by their differing AT/GC ratios of genomic DNA (Cowan and Steel., 1993). In general *Campylobacter* species are microaerophilic and will not grow under atmospheric oxygen concentrations, *Campylobacter* grow typically at 5-11% oxygen (Cowan and Steel., 1993) and are capnophillic, preferring a raised CO_2 concentration of 11-12% (Annable *et al.*, 1997). Because Campylobacters typically do not grow under the conditions routinely used to culture enteric pathogens, they were not isolated from the stool of enterocolitis patients until the 1970's (Dekeyser *et al.*, 1972; Skirrow, 1977).

1.3.2. *Campylobacter* Species.

Campylobacter species can be divided into two groups based upon their optimum growth temperature, the thermophilic *Campylobacter*, which grow optimally at 42°C and non-thermophilic *Campylobacter*, which grow poorly at 42°C.

1.3.3. Non-thermophilic Campylobacters.

Non-thermophilic Campylobacters were probably responsible for the first reports of pathogenic *Campylobacter* isolation from farm livestock following spontaneous epizootic abortion (McFadyean and Stockman, 1913). Based on the clinical features described, such infections can probably be attributed to *Campylobacter fetus* infection (Mishu *et al.*, 1992). In humans *C. fetus* infection is usually restricted to immunocompromised individuals. It is acquired through ingestion but tends to cause disseminated foci of infection (Lastovica and Skirrow., 2000), such as septic abortion (Sauerwein *et al.*, 1993), meningitis and septic arthritis. Non-thermophilic *Campylobacter* may be found as commensal organisms of the gastrointestinal and genital tract of domestic and farm livestock and the oral cavity (Everest and Ketley, 2001). Although the significance of non-thermophilic *Campylobacter* in animal pathogenesis may be well known their significance, as human pathogens may be difficult to assess. Differing reported isolation frequencies and variation in culture procedures in studies from various sources means the true significance and frequency as human pathogens remains unclear (Lastovica and Skirrow., 2000).

1.3.4. Thermophilic Campylobacters.

The species principally associated with disease in humans are differentiated from other *Campylobacter* by their high optimum growth temperature (42°C). A high optimum growth temperature appears to be an adaptation to colonisation of the avian gut, indeed birds, especially farm poultry show a high frequency of

colonisation (PHLS., 1999). Thermophilic Campylobacters include *C. jejuni*, *C. coli C. upsaliensis* (Bourke *et al.*, 1998) and *C. lari* (Cowan and Steel., 1993) of which *C. jejuni* and *C.coli* are most frequently associated with human disease.

C. lari is distinguished from *C. jejuni* and *C. coli* by its resistance to naladixic acid (Cowan and Steel., 1993) although occurrence of resistant strains of other *Campylobacter* species may result in miss-identification (Steinhauserova *et al.*, 2001). *C. lari* causes diarrhoeal disease in both immuno-competent and immuno-compromised individuals, which may be fatal in the latter (Lastovica and Skirrow., 2000). *C. jejuni* and *C. coli* may be distinguished by the presence of the hippuricase gene in *C. jejuni*, which allows *C. jejuni* to hydrolyse hippurate (Steinhauserova *et al.*, 2001), although this gene may be absent in some *C. jejuni* strains.

C.upsaliensis has infrequently been associated with human disease, however the true significance and frequency of human infection remains to be proven (Bourke *et al.*, 1998). Both *C. upsaliensis* and *C. lari* may be frequently isolated from cases of enteric disease in livestock and domestic pets (Steinhauserova *et al.*, 2001).

Of the *Campylobacter* species *C. jejuni* and *C. coli* are responsible for the majority (80-95%) of cases of human enteritis (Vandamme, 2000). *C. jejuni* and *C. coli* enteritis cause presentation of similar disease symptoms and both species share many genetic elements.

1.3.5. *Campylobacter jejuni.*

C. jejuni is the most comprehensively studied *Campylobacter* and due to the fact that it is a leading cause of bacterial diarrhoea in industrialised nations (PHLS., 1999). *C. jejuni* infection is acquired primarily through consumption of undercooked meat particularly poultry, contamination of the carcass with faeces during slaughter is thought to play a major role in the transmission of *C. jejuni* (PHLS., 1999). Subsequent undercooking or poor food preparation methods allow the organism to survive and remain viable until consumption. Comparison of Pulse Field Gel Electrophoresis (PFGE) profiles of *C. jejuni* strains isolated from chickens and humans, have linked strains found in retail chickens to those prevalent in humans by (Hanninen *et al.*, 2000). Other sources of infection may include dairy products, pets or sewage contaminated water. It is often difficult to establish the source of infection due to an incubation period of 2-5 days prior to the development of symptoms (Karmali and Fleming, 1979).

C. jejuni causes food borne enterocolitis with a spectrum of possible symptoms. The symptoms range from acute inflammatory diarrhoea (blood and pus in the stool) with severe abdominal cramping, to mild watery diarrhoea. Symptoms are caused by disruption of the functioning of the intestinal epithelium, in particular the absorption of fluids. Disruption is due to the organism colonising and invading the intestinal epithelium, and the host's subsequent inflammatory and immune responses to its presence. Several reports of toxin production or toxic activities have been described from a variety of strains (Pickett *et al.*, 1996) (Pickett *et al.*, 1992). It is thought that severity of symptoms is lessened with

prior exposure to the organism, possibly due to the development of a protective antibody response by the host. Milder disease symptoms are seen with greater frequency in developing countries where prior exposure may be more frequent (Ketley, 1997). *Campylobacter* infection is usually self-limiting with symptoms lasting up to a week, however bacteria may be isolated from the faeces several weeks after the symptoms have ceased (Skirrow and Blaser., 2000). In the immuno-compromised or malnourished bacteremia may develop which can be life threatening (Reed *et al.*, 1996). In the past *Campylobacter* enteritis was treatable with erythromycin and quinolone antibiotics however the incidence of resistance is on the increase (Gaunt and Piddock., 1996).

A more serious complication of *C. jejuni* infection is Guillain-Barré syndrome, an auto-immune condition causing muscle paralysis and weakness. This is thought to be due to the exposed polysaccharide of certain *C. jejuni* strains being immunologically cross-reactive with neural gangliosides (Prendergast *et al.*, 1998), causing the sensitised immune system to attack nerves after the infection has cleared. The link between *C. jejuni* and Guillain-Barré syndrome is reviewed comprehensively elsewhere by Nachamkin et al (Nachamkin *et al.*, 1998).

1.3.6. The genome of *Campylobacter jejuni.*

The genome of *C. jejuni* NCTC11168, a clinical isolate, has been sequenced. *C. jejuni* has a circular chromosome of 1,641,481 base pairs (Parkhill *et al.*, 2000). The genomic DNA is AT rich (G/C-30.6%) and plasmids and phages have been reported, although no transposable elements have been found (Parkhill *et al.*,

2000). Notable features of the *C. jejuni* genome are that it contains a general lack of repetitive sequences, a factor which contributes to the maintenance of a compact genome. Also notable are several homopolymeric runs of G-Cs in genes for some surface proteins. Slipped-strand mispairing (during DNA replication), leading to different numbers of G-Cs in the tracts, has been suggested as a possible mechanism of antigenic variation during infection by this organism (Parkhill *et al.*, 2000). *C. jejuni* is also capable of natural transformation, preferentially taking up *C. jejuni* DNA. (Wang and .Taylor., 1990). The lack of transposons, A-T rich nature and general dissimilarity to *E. coli*, means that specialised procedures have been developed to investigate *C. jejuni* molecular biology (van Vliet *et al.*, 1998a). Indeed *Campylobacter* promoters may not be functional in *E. coli, C. jejuni* promoters contain -16 sequences (as well as -10 and -35 sequences), a feature reminiscent of Gram-positive bacteria (Wosten *et al.*, 1998).

1.3.7. The molecular basis of *Campylobacter* pathogenesis.

Although few aspects of *Campylobacter* pathogenesis have been characterised at a molecular level, *C. jejuni* has several molecular pathogenicity factors that contribute to its ability to cause disease. These are expressed during the transmission, colonisation of the host and induction of disease symptoms.

1.3.7.1. Factors expressed during transmission.

C. jejuni has developed a number of mechanisms that allow it to respond to and thus survive a number of environmental stress factors it will encounter during transmission. *C. jejuni* may also have to withstand oxidative stress produced both in oxidative environments and upon internalisation by professional phagocytes. This subject will be covered later due to the inextricable nature of oxidative stress with iron metabolism.

1.3.7.2. Factors expressed enabling host colonisation.

The characteristic "corkscrew" shape of *C. jejuni* and the possession of a polar flagellum gives this organism a high degree of motility in viscous fluids. This enables the organism to successfully penetrate and colonise viscous intestinal mucus allowing it to subsequently attach to, and invade the intestinal epithelium. The organism also moves chemotactically, being attracted to mucin, L-serine and L-fucose, and being repelled by bile salts (Hugdahl *et al.*, 1988) an ability that is believed to allow *C. jejuni* to leave the intestinal lumen and reach the epithelium. The *C. jejuni* flagellum and associated motility are also linked with attachment to, and invasion of the intestinal epithelium. *C. jejuni* possess polar flagella composed of two similar flagellar sub-units, FlaA and FlaB. It was demonstrated that a strain variant that has a flagella solely composed of FlaA but poor motility, could still efficiently colonise chicken caeca (Wassenaar *et al.*, 1993). Conversely, mutation of the *flaA* (flagellar subunit) gene, leads to loss of motility and innabilty to attach to and invade human intestinal cells. The invasive

phenotype can be partially restored by centrifugation of bacteria onto the culture cells, demonstrating necessity of motility to *C. jejuni* pathogenesis (Wassenaar *et al.*, 1991).

The ability of a pathogen to respond to the heat shock encountered upon entering the host is critical in establishing host colonisation. Several proteins involved in the *C. jejuni* heat shock response have been shown to be vital to host colonisation including the DnaJ heat shock protein (Konkel *et al.*, 1998) and the RacR reponse regulator (Bras *et al.*, 1999). A *C. jejuni dnaJ* mutant was unable to colonise chickens (Konkel *et al.*, 1998) and a *C. jejuni racR* mutant showed decreased growth at 42°C and displayed reduced colonisation of chickens (Bras *et al.*, 1999).

Campylobacter cells and purified outer membrane proteins have been demonstrated to adhere to tissue culture cells. Also *C. jejuni* has been shown to produce fimbriae in response to bile salts. (Doig *et al.*, 1996). It remains doubtful that such fimbriae are involved in attachment to host cells, fimbrial formation appearing to be an artefact of the use of media containing bile salts (Gaynor *et al.*, 2001). Several *Campylobacter* proteins have been suggested as putative adhesins based on their adhesion to eukaryotic cells, subsequent analysis of their genetic and amino acid structural similarities shows them to be unlikely to be designated adhesins (van Vliet and Ketley, 2001). A fibronectin binding protein has been characterised and designated CadF, it displays similarity to a root binding protein from *Pseudomonas flourescens*. Mutants that lacked this protein can no longer bind fibronectin (Konkel *et al.*, 1997) and were unable to colonise

the cecae of newly hatched chicks (Ziprin *et al.*, 1999). Flagellin is involved in *C. jejuni* adhesion to eukaryotic cells, both by virtue of the flagellum itself (Yao *et al.*, 1994) and by motility resulting from a functional flagellum (Wassenaar *et al.*, 1991).

1.3.7.3. Factors causing the production of disease symptoms.

C. jejuni is capable of invading human intestinal cells (and a number of other cell lines) in vitro (Everest *et al.*, 1992), a factor that contributes to the invasiveness appears to be the motility of *C. jejuni* cells. Indeed mutation in the *flaA* flagellin gene, which results in non-motile mutants, which can no longer invade cultured human intestinal cells (Wassenaar *et al.*, 1991). It has been demonstrated that *C. jejuni* also produces a range of proteins when incubated with human cultured epithelial cells and that bacterial de-novo protein synthesis is essential for invasion (Konkel and Cieplak, 1992). Various host cell factors (actin filaments, micro tubules and clathrin pits) and associations with different host cell types (colonic enterocytes, M-cells, Poly-morphonucleocytes, monocytes and cell junctions) have been implicated as being important in *C. jejuni* infection. Such reports are often strain dependent and sometimes contradictory, an overview of *Campylobacter*-host cell interactions can be found in (Wooldridge and Ketley., 1997).

Although invasion of intestinal cells and subsequent host immune response will produce significant inflammation during the course of infection other tissue damaging pathogenicity factors have been suggested. Although various

enterotoxic, cytotoxic, hemolytic and hepatotoxic activities have been reported for various *C. jejuni* strains, these reports are at best unverified and sometimes contradictory (Wassenar, 1997). Cytolethal distending toxin (CDT) remains the only *C. jejuni* toxin to be fully characterised at the genetic level (Pickett *et al.*, 1996). CDT is so called due to the distended appearance of cultured cells (Vero, HeLa, Hep-2 and Chinese Hamster Ovary) after 2-4 days incubation. The CdtB component is prosed to halt the cell cycle at G2 phase by the production of DNA damage, which is suggeted by its DNAase activity (Cortes-Bratti *et al.*, 2001). Three functional genes are needed for production of this toxin (*cdtA*, *B* and *C*) of which *ctdB* at least, shows widespread distribution amongst *Campylobacter* species (Pickett *et al.*, 1996). From the genome sequence two genes containing putative hemolysin domains and a phospholipase gene have been found (Grant *et al.*, 1997; van Vliet and Ketley, 2001). Although little is known of the clinical significance of toxin production in *C. jejuni* strains, it is thought that toxins could be significant in liberating iron, and killing leucocytes and other bactericidal host cells.

1.4. Iron and Campylobacters.

Few iron acquisition systems have been characterised in *C. jejuni* compared with other enteric pathogens. Research into *C. jejuni* iron acquisition has provided evidence for the existence of several systems involved in acquisition of iron from a number of exogenous molecules. Evidence for siderophore uptake systems,

haem acquisition and a number of proteins with similarity to proteins involved in iron acquisition in other bacterial species has been presented.

1.4.1 Siderophore uptake systems.

Although siderophore production systems have not been characterised in *C. jejuni*, the ability to utilise the exogenous siderophores enterochelin and ferrichrome has been demonstrated (Field *et al.*, 1986). A putative operon was discovered in *C. jejuni* containing genes that showed homology to the components of an enterochelin periplasmic binding protein dependent ABC transport system (Park and Richardson., 1995). The genes encoded two cytoplasmic membrane permease proteins (CeuB and C) an ATP binding protein (CeuD) and a periplasmic binding protein CeuE. *C.coli* strains mutated in components of this system showed an impaired ability to utilise enterochelin as an iron source (Park and Richardson., 1995). At the point of commencing this study the *ceu* genes remained the only *C. jejuni* genes proven to function as an iron-uptake system.

Recently three ORFs have been discovered that are homologous to the *E. coli* ferrichrome locus (Fhu). The genes were homologous to an outer membrane ferrichrome receptor (FhuA) the cytoplasmic membrane permease (FhuB) the periplasmic binding protein (FhuD) (Galindo *et al.*, 2001). The function of these genes in the ability of *C. jejuni* to utilise ferrichrome remains to be proven, however this system does seem anomalous due to its G-C rich content (In contrast

to the A-T rich *C. jejuni* genome) and because it is absent from several *C. jejuni* strains including the genome strain (NCTC11168). One of the proteins found to be de-repressed in the outer membrane of the *fur* mutant was CfrA, a protein that displayed similarity to a ferric-siderophore receptor from *Bordetella bronchiseptica* (Guerry *et al.*, 1997). Unfortunately little else was discovered about this protein and its possible ligand. It was also found that *C. jejuni* possesses a gene (Cj1398 (Parkhill *et al.*, 2000)) with homology to the ferric uptake gene *feoB* of *E. coli*. FeoB is involved in ferrous iron uptake in *E. coli* (Kammler *et al.*, 1993), and *feoB* mutants of both *E. coli* and *H.pylori* were unable to colonise the gastro-intestinal tracts of mice (Stojiljkovic *et al.*, 1993; Velayudhan *et al.*, 2000).

1.4.2. *C. jejuni* genes with homology to iron acquisition genes.

The advent of publication of the *C. jejuni* genome sequence (Parkhill *et al.*, 2000) led to the discovery of a number of genes with similarity to the iron acquisition genes of other bacterial species. An Open reading frame (ORF) was discovered which shows sequence homology to the transferrin binding protein (TbpA) of *Neisseria* species (van Vliet *et al.*, 1998b). This discovery was of interest because previous *C. jejuni* studies have failed to demonstrate the ability to utilise transferrin as an iron source (Pickett *et al.*, 1992) (a mechanistic overview of the Tbp system can be seen in figure 1.4). This ORF is surrounded on the *C. jejuni* genome, by homologues of genes related to iron acquisition in other bacterial species. Proximally located upstream of *tbpA* is a homologue of the *phuW*, a

gene of unknown function that is co-expressed with a haem uptake ABC transporter system in *Ps. aeruginosa* (Ochsner *et al.*, 2000). In *Ps. aeruginosa* it was co-expressed in response to iron limitation with the haem uptake system *phuS*, *T*, *U* and *V*, under the control of Fur. The PhuW protein is located at the inner membrane and is required for optimal efficiency of haem uptake in *Ps. aeruginosa* (Ochsner *et al.*, 2000). Downstream of the *tbpA* homologue, *exbB*, *exbD* and *tonB* homologues are present, these are one set of three homologues of *tonB* genes (and two *exbB/exbD* genes) in *C. jejuni*. By searching the genome data (Parkhill *et al.*, 2000) three ORFs were discovered in an apparent operon, which encodes a putative periplasmic binding protein dependent (ABC) transport system. These genes encoded proteins with amino acid homology to HitA, B and C from *H. influenzae*. Mutation of *hitC* in *H. influenzae* produced a strain that could no longer use iron and protoporphyrin IX as a source of haem (*H. influenzae* cannot synthesise haem like some bacterial species) and so was proposed to be involved in the uptake of ferrous iron in this organism (Sanders *et al.*, 1994).

1.4.3. Regulation of iron uptake.

A key discovery concerning iron metabolism in *C. jejuni* was the discovery and cloning of the *fur* genes of two separate *C. jejuni* strains (Chan *et al.*, 1995; Wooldridge *et al.*, 1994). The *C. jejuni fur* gene encoded an 18kDa protein that was 35% identical to *E. coli* Fur protein at the amino acid level. *C. jejuni fur* could partially complement an *E. coli fur* mutation in a *lacZ* reporter gene assay

(Wooldridge *et al.*, 1994). Unusually amongst Gram-negative bacteria, the *C. jejuni fur* gene appeared to be in an operon upstream of the housekeeping genes lysyl-tRNA synthetase (*lysS*) and serine hydroxymethyltransferase (*glyA*). By attempting to mutate the *fur* gene of *C. jejuni* by insertion of an antibiotic resistance cassette, it was discovered that mutants could only be isolated if the antibiotic resistance cassette was inserted in the same orientation as the *fur* gene. This confirms previous observations that *fur* appears to be in an operon containing housekeeping genes, because the polar effects of mutant construction causes disruption of essential gene expression. Mutation of the *C. jejuni fur* gene results in a strain that displays slower growth in both high and low iron conditions than parental wild type strains (van Vliet *et al.*, 1998b). Unlike *fur* mutant strains of certain other bacterial species, *C. jejuni fur* mutants do not show significantly increased sensitivity to iron-replete conditions (van Vliet *et al.*, 1998b). Comparison of expression profiles of wild type and *fur* mutant strains highlighted a number of de-repressed proteins (observed under iron replete conditions) in the *fur* mutant. These included a protein homologous to CfrA, a putative unspecified siderophore receptor previously characterised in *C.coli* (Guerry *et al.*, 1997), a protein thought to be CeuD the periplasmic binding protein component of an enterochelin uptake system (Park and Richardson., 1995), p19, an uncharacterised 19kDa protein similar to a putative copper binding protein from magnetotactic bacteria (Dubbels *et al.*) and an number of other previously uncharacterised proteins. Interestingly two iron-regulated proteins were not de-repressed in the *fur* mutant, these were found to be the oxidative

stress proteins AhpC (Alkyl Hydroperoxide reductase (Baillon *et al.*, 1999)) and a catalase (KatA (Grant and Park., 1995)).

1.4.4. Oxidative stress and iron storage.

C. jejuni possesses several mechanisms to combat the oxidative stress it may suffer in the various environments it may inhabit. As previously mentioned *C. jejuni* has a catalase and an alkyl hydroperoxide reductase, both expressed in response to iron limitation but not under control of Fur. It was later demonstrated that *C. jejuni* possessed another gene with homology to *C. jejuni fur*, which due to its similarity (32% amino acid identity) to the *perR* gene from *Bacillus subtillis* (Bsat *et al.*, 1998), it was designated *perR* (van Vliet *et al.*, 1999). Mutation of the *perR* gene leads to over-expression of AhpC and KatA and the production of a strain resistant to oxidative stress (van Vliet *et al.*, 1999). The observation that expression levels of KatA and AhpC in the *perR* mutant exceeds those in iron replete conditions, has led to the hypothesis that iron is not the only stimulus affecting KatA and AhpC expression (van Vliet *et al.*, 1999). Catalase confers resistance to hydrogen peroxide and has been shown to contribute to the survival of *C. jejuni* in macrophages but not epithelial cells (Day *et al.*, 2000). AhpC degrades alkyl hydroperoxide intermediates thus preventing damage to the organism by these toxic metabolites and catalysis of toxic radicals by them. Mutation of the *ahpC* gene renders the organism susceptible to atmospheric oxygen and cumene hydroperoxide (Baillon *et al.*, 1999).

C. jejuni also possesses a number of oxidative stress defence genes which are not under transcriptional control of Fur or PerR. The presence of an Iron Superoxide Dismutase (SOD), designated SodB, has been demonstrated in *C. jejuni* (Pesci *et al.*, 1994). SOD catalyses the breakdown of superoxide radicals to hydrogen peroxide and oxygen. The removal of superoxide radicals prevents a rise in intracellular levels of Fe(II), which in turn would cause production of hydroxyl radicals by the Fenton reaction. *C. jejuni* SOD activity is induced by the presence of iron, the mechanism of this is unclear and appears to be independent of the activity of both Fur and PerR (van Vliet *et al.*, 1999). *C. jejuni* SodB mutants have a significantly reduced survival rate subsequent to invasion of human cultured cells (Pesci *et al.*, 1994). *C.coli* SodB mutants show reduced survival of stationary phase cells in atmospheric oxygen, both in *vitro* and in food, and decreased colonisation potential of day old chicks (Purdy *et al.*, 1999). Whilst investigating the genome sequence of *C. jejuni* a putative ferredoxin gene was discovered upstream of the *ahpC* gene (Baillon *et al.*, 1999). Ferredoxins are iron sulphur proteins thought to be involved as electron carriers in metabolic reactions. Mutation of the ferredoxin gene, designated *fdxA*, was found to produce a strain sensitive to atmospheric oxygen. The FdxA protein was found to be expressed in response to high intracellular iron levels (van Vliet *et al.*, 2001). It is not known at present whether regulation of FdxA expression is dependent on known iron regulatory proteins.

1.4.5. Haem protein utilisation.

Early studies into iron uptake demonstrated that *C. jejuni* could utilise haemin, haemoglobin, haemin-haemopexin and haemoglobin-haptoglobin (Pickett *et al.*, 1992). An undefined chemically mutagenised *C. jejuni* strain that lost the ability to utilise haem containing iron sources, was found to no longer express a 70kDa iron-regulated OM protein found in wild type strains. The 70kDa protein was also found to be de-repressed in a *fur* mutant, showing that expression of the 70kDa protein was repressed by both iron and Fur (van Vliet *et al.*, 1998b). Amino acid sequence analysis of the 70kDa protein found similarity to siderophore receptor proteins, both from other bacterial species and from *C. jejuni* itself. Due to the size similarity to the protein initially associated with haemin uptake, the 70kDa protein was hypothesised to be an outer membrane haemin/haemoglobin receptor and was designated Campylobacter Haemin Uptake protein A (or ChuA) (van Vliet *et al.*, 1998b). In strain NCTC 11168, it was found that three other genes followed the *chuA* gene, the 4 genes were arranged in an apparent operon. Analysis of predicted amino acid sequence homology showed that the three genes appear to encode an ABC transporter containing a permease, an ATP binding protein and a periplasmic binding protein. These genes were designated *chuB* (inner membrane permease), *chuC* (an ATP binding protein) and *chuD* (periplasmic binding protein).

1.5. Aims.

To date the iron acquisition systems of *C. jejuni* have been poorly characterised with only the enterochelin uptake system characterised fully, to a molecular level. Other noted iron acquisition genes have not been linked with the ability to utilise their proposed substrate. In other cases the ability of *C. jejuni* to utilise an iron-containing compound has been observed but without further identification of the genes/proteins involved.

Research into the *C. jejuni* Fur regulon has been carried out (van Vliet *et al.*, 1998b), however the regulation of expression of the *C. jejuni fur* gene itself remains uncharacterised. Predictions have been made about the location of the *fur* promoter and the nature of control of Fur expression (Chan *et al.*, 1995) but these remain to be confirmed. The research carried out within this project characterises a number of iron acquisition systems in *Campylobacter jejuni*, focusing the ability to utilise host derived haem-containing compounds, and the ability to utilise lactoferrin and transferrin as iron sources. This study will involve not only investigation of the functions putative iron uptake genes and their modes of their expression but also investigation of the expression and regulatory activity of the Fur protein

2. Materials and Methods.

2.1. Strains and plasmids.

The bacterial strains used in this study can be found in table 2.1. The plasmid vectors used and plasmids constructed during the course of this study can be found in table 2.2a. Oligonucleotide primers constructed for PCR can be found in table 2.2b, restriction enzyme recognition sites are underlined.

2.1.1. Bacterial strains.

The bacterial strains used in this study may be found in table 2.1.

Table 2.1

Bacterial species.	**Strain.**	**Genotype/comments.**	**Source/ References.**
E.coli.	DH5α	*ϕ80lacZΔM15, recA1, endA1 gyrA96, thi-1, hsdR17($^{r}K^{-}$, $^{m}K^{+}$), supE44, relA1, deoI, Δ(lacZYA-argF)U169.*	(Hanahan, 1983)
E.coli.	MC4100	(F^{-} *araD139 Δ(argF-lac)U169, rpsL* 150 (Str^{r}) *relA1 flb5301, deoC1, ptsF25, rbsR.*	(Silhavy, 1984)
C. jejuni	NCTC11168		National Collections of Type Cultures and Pathogenic Fungi, Colindale, London, UK.
C. jejuni	81116	(NCTC11828)	(Palmer *et al.*, 1983)

C. jejuni	480	(NCTC 12744)	(King *et al.*, 1991)
C. jejuni	AV17	NCTC11168 *Δfur* (Kan^r)	(van Vliet *et al.*, 1998b)
C. jejuni	JDR5	NCTC11168 *ΔchuA*::cat..	This study.
C. jejuni	JDR6	NCTC11168 *ΔchuB*::cat.	This study.
C. jejuni	JDR7	NCTC11168 *ΔchuC*::cat.	This study.
C. jejuni	JDR8	NCTC11168 *ΔchuD*::cat.	This study.
C. jejuni	JDR11	NCTC11168 *ΔchuBCD*::cat. Cassette ligated in same orientation as deleted genes.	This study.
C. jejuni	JDR12	NCTC11168 *ΔchuBCD*::cat. Cassette ligated in opposite orientation to deleted genes.	This study.
C. jejuni	JDR18	NCTC11168 *ΔhitABC*::kan.	This study.
C. jejuni	JDR19	NCTC11168 *ΔhitABC*::kan, *ΔchuBCD*::cat.	This study.
C. jejuni	JDR20	NCTC11168 *ΔtonB*::cat.	This study.
C. jejuni	JDR21	NCTC11168 *ΔCj0178*::cat.	This study.

2.1.2. Vectors, plasmids and primers.

The vectors used and plasmids constructed in this study may be found in table 2.2a. Oligonucleotide primers used and produced during this study can be found in table 2.2b.

Table 2.2a

Name.	**Details.**	**Reference.**
pUC19	Cloning vector, Amp^R.	(Yanisch-Peron *et al.*, 1985)
pJMK30	1.5 kb *C. coli* (*alpha-3*) Km^R gene in pUC19 with copy of polylinker on both sides.	(van Vliet *et al.*, 1998b)
pAV35	pBluescript containing a Cm^R gene cassette.	(van Vliet *et al.*, 1998b)
pMW10	LacZ transcriptional assay vector	(Wosten *et al.*, 1998)
pAV201	pMW10 containing a *lacZ* transcriptional fusion of the *C. jejuni* NCTC81116 *katA*.	(van Vliet, 2003)

p23E5	promoter of *C. jejuni metK* inserted into *Bam*HI of pMW10.	(Wosten *et al.*, 1998)
pJDR1	pUC19 containing cloned *C. jejuni* NCTC11168 *chuA* gene.	This study.
pJDR2	pUC19 containing cloned *C. jejuni* NCTC11168 *chuB* gene.	This study.
pJDR3	pUC19 containing cloned *C. jejuni* NCTC11168 *chuC* gene.	This study.
pJDR4	pUC19 containing cloned *C. jejuni* NCTC11168 *chuD* gene.	This study.
pJDR5	pJDR1 with deletion in *chuA* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.	This study.
pJDR6	pJDR2 with deletion in *chuB* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.	This study.
pJDR7	pJDR3 with deletion in *chuC* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.	This study.
pJDR8	pJDR4 with deletion in *chuD* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.	This study.
pJDR9	pUC19 containing cloned *C. jejuni* NCTC11168 *chuB, C* and *D* genes.	This study.
pJDR11	pJDR9 with deletion in *chuB, C* and *D* open reading frames. Cm^r resistance cassette ligated (in the same orientation as the deleted genes) in a *Bam*H1 site created at the point of deletion.	This study.
pJDR12	pJDR9 with deletion in *chuB, C* and *D* reading frames. Cm^r resistance cassette ligated (in the opposite orientation to the deleted genes) in a *Bam*H1 site created at the point of deletion.	This study.
pJDR13	pMW10 containing the *chuA* promoter region.	This study.
pJDR14	pMW10 containing the Cj1613 promoter region.	This study.
pJDR15	pMW10 containing the *hitA* promoter region.	This study.
pJDR16	pMW10 containing the *phuW* promoter region.	This study.

pJDR17	pUC19 containing *C. jejuni* NCTC11168 *hitA, B* and *C* genes.	This study.
pJDR18	pJDR17 with deletion in *hitA, B* and *C* open reading frame. Kan^r resistance cassette ligated (in the same orientation as the deleted genes) in a *Bam*H1 site created at the point of deletion.	This study.
pJDR19	pUC19 containing cloned *C. jejuni tonB1* gene.	This study.
pJDR20	pJDR19 with deletion in *tonB1* reading frame. Cm^r resistance cassette ligated (in the same orientation as the deleted gene) in a *Bam*H1 site created at the point of deletion.	This study.
pJDR21	pUC19 containing cloned *C. jejuni* Cj0178 gene.	This study.
pJDR22	pJDR21 with deletion in Cj0178 reading frame. Cm^r resistance cassette ligated (in the same orientation as the deleted gene) in a *Bam*H1 site created at the point of deletion.	This study.

Table 2.2b.

Primer name.	**Sequence.**	**Restriction site.**	**Annealing site.**
M13F	gtaaaacgacggccagt	-	pUC19
M13R	ggaaacagctatgaccatg	-	pUC19
Mwf1	cttcgtcttggtagc	-	pMW10
LacR1	gatcgcactccagccagc	-	pMW10
CatR1	cccttatcgattcaagtgcatcatg	-	pAV35 (cat cassette)
CatF1	tagtggtcgaaatactcttttcgtg	-	pAV35 (cat cassette)
Hpo318R	cggggtaccgtgcatacgagcaa acaac	*Kpn*I	*C. jejuni* genome 1540262-1540280bp
ChuBR	aaactgcagctagcttcatcatctc cgc	*Pst*I	*C. jejuni* genome 1543572-1543554bp
ChuAF	cggggtaccgacctactatcatag actc	*Kpn*I	*C. jejuni* genome 1542113-1542131bp

ChuCR	aaactgcaggcaagatagcaacaacggc	*Pst*I	*C. jejuni* genome 1544485-1544467bp
ChuBF	cggggtacccatagatggacgcttaccac	*Kpn*I	*C. jejuni* genome 1543075-1543094bp
ChuDR	aaactgcagctgctgatataacaggta	*Pst*I	*C. jejuni* genome 1545261-1545245bp
ChuCF	cggggtaccgccaaatggctcaggcaaaagc	*Kpn*I	*C. jejuni* genome 1543998-1544019bp
Hpo586F	aaactgcaggaaatcactacaagtggc	*Pst*I	*C. jejuni* genome 1546096-1546079bp
ChuAF2	cggggatccgatactacacctatcaagac	*Bam*HI	*C. jejuni* genome 1542848-1522867bp
ChuAR2	cgcggatccgccttctatgctgattac	*Bam*HI	*C. jejuni* genome 1540980-1540963bp
ChuBF2	cgcggatccatgtacagcatttggagga	*Bam*HI	*C. jejuni* genome 1543744-1543762bp
ChuBR2	cgcggatccgtggtaagcgtccatctatg	*Bam*HI	*C. jejuni* genome 1543094-1543075bp
ChuCF2	cgcggatcctagcttctatattctgcgat	*Bam*HI	*C. jejuni* genome 1544498-1544517bp
ChuCR2	cgcggatccgcttttgcctgagccatttggc	*Bam*HI	*C. jejuni* genome 1544019-1543998
ChuDF2	cgcggatcccgtcttagtcctaagataatag	*Bam*HI	*C. jejuni* genome 1545408-1545429bp
ChuDR2	cgcggatccgcaggatcaagcactacaaggc	*Bam*HI	*C. jejuni* genome 1544749-1544728bp
Cj1613R2	cgcggatccgctcgctctttgcactcatgc	*Bam*HI	*C. jejuni* genome 1540370-1540390bp
ChuAR4	cgcggatccagatctttgccttctatgc	*Bam*HI	*C. jejuni* genome 1540988-1540970bp
Cj1618F2	gcaagatcgcccactgtatc	-	*C. jejuni* genome 1546830-1546811bp
PhuWR1	cggggatccccaagcagtatcacatctgc	*Bam*HI	*C. jejuni* genome 173103-173084bp
HitAR1	aaaggatccattaagctcactagcacct	*Bam*HI	*C. jejuni* genome 172504-172522bp

PhuwR4	cggggtaccgccaagcagtatca catctg	*Kpn*I	*C. jejuni* genome 173104-173085bp
Cj0172R4	aaactgcagagcatagccatgatc acctgc	*Pst*I	*C. jejuni* genome 168479-168499bp
HitCF2	cgcggatccggtaactcatgatatt gaagatgc	*Bam*HI	*C. jejuni* genome 169441-169418bp
HitAR2	cgcggatccccgtcattagctata atagatgct	*Bam*HI	*C. jejuni* genome 172052-172057bp
Cj0172R5	cacaggtttaatccctagc	-	*C. jejuni* genome 168118-169206bp
PhuwR5	ccctttaagggctgagcccc	-	*C. jejuni* genome 173436-173417bp
Cj0752F1	cggggtaccgattgcaaagaaaa caaagcagg	*Kpn*I	*C. jejuni* genome 703814-703836bp
CfrAR1	aaactgcagccgccaataccctg acgacgccc	*Pst*I	*C. jejuni* genome 705772-705750bp
TonBR2	cgcggatccgggttgcacattcga aggc	*Bam*HI	*C. jejuni* genome 704892-704910bp
TonBF1	ggcggatccatgcgtatgagtgg ggagg	*Bam*HI	*C. jejuni* genome 704612-704625bp
CfrAR2	ccattagggcccacctctc	-	*C. jejuni* genome 705790-705772bp
Cj0752F2	tgcgttatcacagagatttaccaa	-	*C. jejuni* genome 703567-703590bp
PhuWF	cggggtaccgcagatgtgatactg cttggcg	*Kpn*I	*C. jejuni* genome 173084-173105bp
ExbBR	aaactgcagccaggcgcaacga ctgcc	*Pst*I	*C. jejuni* genome 176632-176615bp
Tbp1F	ggaagattctggagcaggtgatcc tatctatc	*Bgl*II	*C. jejuni* genome 175950-175972bp
Tbp1R	ggaagatctctctcttgtgcttgttg c	*Bgl*II	*C. jejuni* genome 173919-173902bp
ExbDR	aaactgcagccaggcgcaacga ctcgg	*Pst*I	*C. jejuni* genome 176632-176618bp

Chapter 2. Materials and methods

2.2. Growth media and conditions.

E.coli strains were routinely cultured at 37 °C using Luria-Bertani (L) (Roth, 1970) medium: 1% Bacto-tryptone (Oxoid), 0.5% Bacto-yeast extract (Oxoid), 0.5% Sodium chloride, adjusted to pH7.2.

C. jejuni strains were regularly cultured using Mueller-Hinton (MH) media (supplied by Oxoid): 0.6% Meat infusion, 1.75% Casein hydrolysate and 0.15% Starch.

Dialysis partition experiments were performed using SAPI medium, made using 6.25mM NH_4NO_3, 1.84mM KH_2PO_4, 3.35mM KCl and 1.01mM MgSO4, pH adjusted to 7.5. To provide a carbon source for Campylobacters 0.1 % filter-sterilised L-serine or L-aspartate was added.

Liquid media was incubated with shaking at 240 rpm on a G10 Gyrotory Shaker (New Brunswick Scientific Co, Inc., Edison, NJ, USA). Solid media was made by addition of 2% Bacto-agar to liquid media. All media was sterilised by autoclaving at 10 psi for 15 mins. Bacterial growth in liquid culture was monitored by the measurement of optical density using a spectrophotometer. An OD_{600} of 1 corresponds to a cell density of $8x10^8$ cells.ml^{-1}.

C. jejuni strains were routinely cultured at 37 °C, in a variable atmosphere incubator (VAIN, Don Whitley, Shipley, UK) in an atmosphere containing 85% N_2, 10% CO_2 and 5% O_2. *C. jejuni* strains were maintained on media containing vancomycin and trimethoprim Sigma (Poole, UK) in table 2.3. Liquid media was

incubated with shaking at 240 rpm. Antibiotics were included in media where necessary (table 2.3).

Table 2.3. Concentrations of stock antibiotic solutions and antibiotics routinely added to media.

Antibiotic	Stock concentration	Media concentration
vancomycin	10mg/ml	10μg/ml
trimethoprim	5mg/ml	5μg/ml
ampicillin	100mg/ml	100μg/ml
kanamycin	50mg/ml	50μg/ml
chloramphenicol	20mg/ml	20μg/ml

Iron limited growth conditions were achieved by the supplementation of media with 20μM deferoxamine mesylate (Desferal; Sigma chemical co). Alternatively, minimal essential media alpha modification (MEMα: Invitrogen Ltd) was used as a defined low iron growth medium. Iron replete growth conditions were achieved by the supplementation of media with 40μM $Fe(III)SO_4$. The iron containing compounds used in this study (hemin, haemoglobin, haemoglobin-haptoglobin) were obtained from Sigma (Poole, UK) prepared as described previously (Dyer *et al.*, 1987). Ferri and apo-lactoferrin (Sigma) and ferri and apo-transferrin (Sigma) were dissolved in PBS at a concentration of 0.1 mg/ml. Storage of bacterial strains was achieved by the harvesting of cells from a plate containing confluent growth containing selective

antibiotics, and suspended in 0.5 ml of liquid broth (*E.coli*-L broth, *C. jejuni*-MH broth). The cell suspension was then mixed with 0.5ml of 100 % glycerol in a labelled cryotube, snap frozen in a dry ice/isopropanol bath and stored at –80°C.

2.2.1. Miscellaneous buffers and solutions.

All reagents used in solutions were of analytical grade, supplied by Fisons Scientific Equipment, Loughborough, UK unless otherwise stated.

10 x Phosphate buffered saline (PBS): 1.37M NaCl, 0.27M KCl, 0.015M KH_2PO_4 and 0.08M Na_2HPO_4. After adjusting the pH of the buffer to pH7.4 with 2N HCl, the final volume was adjusted with distilled water. The buffer was autoclaved at 121°C for 15 mins.

0.5M EDTA was prepared by dissolving 0.5M of EDTA (disodium diaminoethane tetra acetate) in distilled water. 5M NaOH was added to pH8.0 to dissolve the EDTA. The final volume was adjusted with distilled water. The solution was autoclaved at 121°C for 15 mins.

Tris-HCl buffers: 1M Tris was dissolved in distilled water to give a volume of approximately 60% the final volume required. Concentrated HCl was added to give a pH just above the desired values of 6.8, 7.2, 7.5, 8.0 and 9.5. The buffer was allowed to equilibrate overnight with mixing before final adjustments were made to the pH. The final volume of buffer was adjusted with distilled water and autoclaved at 121°C for 15 mins.

TAE buffer: 0.04M Tris [hydroxymethyl] aminomethane (Tris), 0.001M EDTA. After adjusting the pH of the buffer to pH7.8 with glacial acetic acid, the final volume was adjusted with distilled water. The buffer was autoclaved at 121°C for 15 mins.

TE Buffer: 0.01M Tris-HCl, 0.001M EDTA, pH8.0. The buffer was autoclaved at 121°C for 15 mins.

CTAB/NaCl solution: This solution was prepared by dissolving 0.7M of NaCl in distilled water, followed by the slow addition of 10% ($^{w}/_{v}$) of CTAB (hexadecyltrimethyl ammonium bromide; Sigma), whilst heating and stirring. The final volume was adjusted by the addition of distilled water. The solution was not autoclaved.

IPTG (isopropylthio-β-D-galactoside) was prepared by dissolving 200mg/ml IPTG in distilled water. The solution was filter sterilised and dispensed in small aliquots which were then stored at -20°C.

Phenol/chloroform was prepared by mixing 200ml of liquefied phenol and 200ml of chloroform. 0.4g 8-hydroxyquinoline (Sigma) were added as an antioxidant. The pH was equilibrated to 7.5 by extracting twice with 150ml of 1M Tris pH7.5, followed by one extraction with 150ml of 0.1M Tris pH7.5 and finally 150ml of 0.01M Tris pH7.5. The pH was checked with pH-sensitive indicator papers (BDH). The phenol/chloroform mix was stored in a shatter-proof bottle at 4°C in the dark under 0.01M Tris-HCl pH7.5.

Chloroform/isoamyl alcohol 24:1 was prepared by mixing 240ml of chloroform with 10ml of isoamyl alcohol.

Proteinase K (Sigma) was prepared by dissolving 20mg/ml proteinase K in distilled water at a concentration of 20mg/ml and stored in small single-use aliquots at -20°C.

RNAse A (Sigma) was prepared by dissolving 10mg/ml RNAse A in distilled water to a final concentration of 10mg/ml and aliquoted into 1.5ml tubes. The samples were boiled for 15 mins to inactivate DNAses and stored at -20°C.

3M sodium acetate pH5.2 was prepared by adding 3M sodium acetate to distilled water. The pH was adjusted to pH 5.2 with glacial acetic acid and the final volume adjustments were made with distilled water. The solution was autoclaved at 121°C for 15 mins.

10% ($^{w}/_{v}$) SDS was prepared by adding sodium lauryl sulphate to distilled water and heating until dissolved. The solution was not autoclaved.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was prepared by dissolving 20mg/ml X-gal in dimethylformamide. The solution was stored at -20°C in the dark to prevent damage by light.

20 x SSC: 3M Nacl, 0.3M $C_6H_5Na_3O_7.2H_2O$ (*tri*-sodium citrate) (pH7.0)

STE: 0.1M NaCl, 10mM Tris (pH 8.0) and 1mM EDTA. The solution was autoclaved.

***Campylobacter* electroporation buffer (CEB)**: 0.272M sucrose and 15% ($^{v}/_{v}$) glycerol). The solution was autoclaved.

2.3. Recombinant DNA techniques.

In vitro manipulation of DNA was performed using standard protocols (Ausubel *et al.*, 1992; Sambrook *et al.*, 1989) for the following procedures. Removal of proteins from DNA solutions was carried out by the with phenol:chloroform DNA extraction (Sambrook *et al.*, 1989).

2.3.1. Ethanol precipitation.

Ethanol precipitation of DNA was carried out in order to increase DNA concentration in solution, remove excess salt or during purification procedures. DNA was precipitated by adding 1/10th volume of 3M sodium acetate pH5.2 and 2.5 volumes of 100% ethanol to the sample. If a small DNA fragment or small amounts of DNA was to be precipitated, 1µl of 10mg/ml tRNA (type A from *E. coli*, Sigma) was added to the tube as a co-precipitant. The sample was incubated for 20 mins on ice and subsequently centrifuged at 11600*g* in a microcentrifuge. The supernatant was discarded and the pellet was gently washed in 1ml of 70% (v/v) ethanol. The supernatant was removed after 5 mins centrifugation and the DNA was either air dried or dried in a 37°C heating block or water bath. The resulting dried sample was resuspended in an appropriate volume of dH_2O and stored at -20°C.

2.3.2. Agarose gel electrophoresis of DNA.

Horizontal agarose gel electrophoresis tanks, well combs and casting trays were manufactured to a standard design by the Leicester University School of Biological Sciences workshop. The gel was prepared by dissolving agarose (SeaKem, FMC bioproducts,UK) in 1 x TAE buffer to a final concentration of 0.6%-2.0% ($^{w}/_{v}$) depending on the DNA fragment size range to be separated. EtBr was added to the molten agarose (cooled to 60°C) to a final concentration of 0.5μg/ml. The gel was cast on the perspex tray with a comb inserted in one end to a depth of 0.5-0.75cm. Gels were allowed to set and then submerged in electrophoresis tanks containing 1 x TAE buffer. Prior to loading, 2μl of 5 x TAE sample buffer (5 x TAE buffer, 15% ($^{v}/_{v}$) glycerol, 0.3% ($^{w}/_{v}$) orange G (Sigma)) were added to 0.01ml of DNA sample. The samples were loaded into the wells created by removal of the comb. Molecular weight markers (250 ng) were used and consisted of commercially prepared λ DNA restricted with *Hin*dIII (Gibco-BRL) and ϕX174 DNA restricted with *Hae*III (Gibco-BRL). The gels were run at a constant voltage of 11V/cm. The gels were electrophoresed until the orange dye had travelled through approximately 80% of the gel. DNA was visualised by placing the gel on an ultraviolet (UV) transilluminator (UV Products Ltd) and exposing it to UV light (290nm). DNA bands were photographed using a Polaroid MP4 land camera.

2.3.3. Transformation procedures.

Standard protocols were used for the transformation of *C. jejuni* (van Vliet *et al.*, 1998a) and *E.coli* (Ausubel *et al.*, 1992; Sambrook *et al.*, 1989) by electroporation.

2.3.4. Cloning procedure.

Restriction enzymes and T4 DNA ligase were purchased from Invitrogen Ltd BRL, all enzymes were used according to the manufacturer's instructions.

Cloning of *C. jejuni* genes was carried out using the following standard protocol. Specific oligonucleotide primers were designed to anneal to *C. jejuni* chromosomal DNA, at least 500bp upstream (on the coding strand) and 500bp downstream (on the non-coding strand) of the gene to be cloned. The 500 bp of flanking sequence at either end of the gene was required for later recombination of clone-derived constructs back into the *C. jejuni* chromosome. The upstream primer incorporated a *Kpn*I site and the downstream primer a *Pst*I site at their 5' ends. The use of these rarely occurring restriction sites enabled the ligation and orientation of cloned genes in standard cloning vectors. The *C. jejuni* gene to be cloned was amplified by PCR using Expand High Fidelity polymerase (Boehringer Mannheim) in accordance with the manufacturer's instructions to produce a final reaction volume of 150µl. The reaction was carried out in an Omnigene Thermal Cycler (Hybaid, Ashford, U.K.). The high fidelity polymerase reduced the occurrence incorporation errors in cloned DNA. The

presence of an amplified fragment of the correct size was verified by running an aliquot of the reaction mixture on an agarose gel (Sambrook *et al.*, 1989) and visualisation of the fragment on a UV transilluminator. The amplified fragment was then purified by phenol/chloroform extraction (Sambrook *et al.*, 1989), followed by removal of dNTPs and unincorporated primer using the Nucleotrap PCR purification kit (Clontech). The amplified fragment was cut firstly with *Pst*I (Invitrogen Ltd BRL), phenol chloroform extracted and ethanol precipitated. Then the fragment was cut with *Kpn*I (Invitrogen Ltd BRL). At the same time pUC19 was also cut with the same enzymes in accordance with the manufacturers instructions, and after cutting with *Kpn*I was de-phosphorylated with shrimp alkaline phosphatase (Amersham). This treatment reduces re-circularisation of vector DNA when ligated. The restricted fragment and pUC19 were cleaned by running on an agarose gel and cutting out of the correct size bands. The DNA bands were removed from the gel and purified using either Nucleotrap gel extraction kit (Clontech) or Qiaex kit (Qiagen) in accordance with the manufacturers instructions. After visualisation on an agarose gel to assess approximate concentrations of insert and vector DNA, ligation of vector and insert was carried out (Sambrook *et al.*, 1989) using approximately twice the number of insert molecules to vector DNA molecules. Once complete 1µl of tRNA was added to the ligation reaction and ethanol precipitation was carried out. The precipitated DNA was thoroughly dried and resuspended in 20µl of distilled water. 10µl of the re-suspended ligation mixture was transformed into *E.coli* DH5α by electroporation (Sambrook *et al.*, 1989). The transformed cells

were plated on L-agar plates containing ampicillin and 40g/ml X-Gal. The presence of ampicillin and X-Gal allowed the selection of pUC19 transformants containing an insert. Several white colonies were selected with a sterile toothpick, patched onto a reference plate and incubated in 5ml of L-broth containing ampicillin. Plasmid was prepared from the L-broth cultures (Qiaquick, miniprep), the presence of correct size insert was verified by PCR and restriction mapping. A plasmid preparation (Qiagen midi) was carried out from a transformant colony (reference plate, step j). Sequencing reactions (Perkin-Elmer Applied Biosystems *Taq* Dye Deoxy Terminator cycle sequencing kit) were carried out from both ends of the insert (using M13 forward and reverse primers) to verify that no incorporation errors were present in the flanking regions. Sequencing reactions were processed by PNACL (University of Leicester).

Cloning of promoter regions was performed using a similar methodology with a few modifications. Firstly no 500bp flanking region was needed on the cloned fragment because re-integration into the bacterial chromosome was not necessary. Secondly *Bam*HI sites were designed onto the 5' ends of each oligonucleotide primer to allow ligation of the cloned promoter into pMW10 in both orientations. A diagrammatic representation of this method may be seen in figure 3.6.

2.3.5. Construction of Mutants.

Creation of *C. jejuni* mutants was achieved by Inverse PCR mutagenesis, using the following method. The gene to be mutated was cloned using the method outlined in the previous section. Oligonucleotide primers were designed to anneal within the ORF to be mutated, and so that extension proceeds out from the ORF and into the flanking region as described in figure 4.1. *Bam*HI restriction sites were designed at the 5' ends of each oligonucleotide primer. PCR was carried out on the cloned gene using Expand High Fidelity polymerase (Boehringer Mannheim), with the primers designed in step b. PCR was carried out in accordance with the manufacturers instructions to produce a final reaction volume of 150µl. The reaction was carried out in an Omnigene Thermal Cycler (Hybaid, Ashford, U.K.). The high fidelity polymerase reduced the occurrence incorporation errors in cloned DNA. The production of the correct PCR product was verified by running an aliquot of the reaction mixture on an agarose gel (Sambrook *et al.*, 1989) and visualisation of the fragment on a UV transilluminator. The amplified fragment was then cleaned by phenol chloroform extraction (Sambrook *et al.*, 1989), followed by removal of dNTPs and unincorporated primer using the Nucleotrap PCR purification kit (Clontech). The amplified fragment was cut with *Bam*HI (Invitrogen Ltd BRL), phenol chloroform extracted and ethanol precipitated. At the same time a plasmid containing an antibiotic resistance cassette (pAV35 for Cm^r or pJMK30 for Kan^r) was also cut with *Bam*HI. The antibiotic resistance cassette and cut PCR deleted gene construct was cleaned by running on an agarose gel and cutting out of the

correct size bands. The DNA bands were removed from the gel and purified using either Nucleotrap gel extraction kit (Clontech) or Qiaex kit (Qiagen). Agarose gel electrophoresis of antibiotic resistance cassette and PCR deleted gene construct was carried out to assess their approximate concentrations (Sambrook *et al.*, 1989). A ligation reaction was set up using approximately twice the number of antibiotic resistance cassette molecules to PCR deleted gene constructs. Once complete, 1µl of tRNA was added to the ligation reaction and ethanol precipitation was carried out. The precipitated DNA was thoroughly dried and resuspended in 20µl of distilled water. 10µl of the re-suspended ligation mixture was transformed into *E.coli* DH5α by electroporation (Sambrook *et al.*, 1989). The transformed cells were plated on L-agar plates containing the appropriate antibiotic (dependent upon which antibiotic resistance cassette was used). Several resistant colonies were selected with sterile toothpicks, patched onto a reference plate and inoculated into 5ml of L-broth containing the relevant antibiotic. PCR and restriction mapping were used to verify the orientation of the resistance cassette and correct construct arrangement. A plasmid preparation (Qiagen midi) was carried out from a suitable transformant colony. Sequencing reactions (Perkin-Elmer Applied Biosystems *Taq* Dye Deoxy Terminator cycle sequencing kit) were carried out from both ends of the insert (using M13 forward and reverse primers) to verify that no incorporation errors were present in the flanking regions. Sequencing reactions were processed by PNACL (University of Leicester). 1-10µl of plasmid was electroporated into *C. jejuni* NCTC11168 (van Vliet *et al.*, 1998a). The

transformed bacteria were plated onto MH plates containing relevant antibiotic selection (dependent upon resistance cassette used). *C. jejuni* NCTC11168 cannot maintain plasmids and the presence of antibiotic selects for integration of the deleted gene into the bacterial chromosome. *C. jejuni* NCTC11168 will not maintain single crossover events and so only double crossovers are observed. Several resistant colonies were selected, and frozen stocks prepared form a swab plate of each. Chromosomal DNA was prepared from 4 swab plates of each resistant colony. Chromosomal DNA from each resistant colony was tested by PCR to verify that the deleted gene construct has integrated into the *C. jejuni* chromosome at the correct location. This was carried out by the PCR strategy outlined in figure 2.2, one verified mutant colony was selected and a frozen stock prepared.

2.3.6. DNA preparation.

Chromosomal DNA preparation of *C. jejuni* was performed as described by Ausubel et al (Ausubel *et al.*, 1992). Medium and small-scale plasmid preparation was performed using affinity columns in accordance with the manufacturers instructions (Qiagen). The concentration of DNA in solution was determined by diluting 0.01ml of the sample to be tested in 0.99ml of water and transferring it into a 1ml quartz cuvette. The absorbance was measured at 260 and 280nm, using a Pharmacia Ultraspec III spectrophotometer. The concentration was calculated given 1.0 OD_{260} unit equals 50µg/ml of double stranded DNA or 40µg/ml of single stranded DNA.

2.3.7. Preparation of *Campylobacter* genomic DNA.

Chromosomal DNA preparation of *C. jejuni* was performed as described by previously (Ausubel *et al.*, 1992). *Campylobacter* cells were harvested from four swabbed plates in 12ml of MHB, pelleted by spinning at 3020*g* for 20 mins at 4°C and resuspended in 9ml of PBS. 2ml of 10% SDS was added and mixed with the resuspended cells and a further 100µl of 20 mg/ml proteinase K was then added to the cells prior to incubation for 1 h at 37°C. After this incubation step, 1.8ml of 5M NaCl was added and gently mixed with the cell suspension. A volume of 1.5ml 10% CTAB/0.7M NaCl was added, mixed and incubated for 20 mins at 65°C. The solution was then extracted with an equal volume of chloroform/isoamyl-alcohol. Genomic DNA was then precipitated by the addition of 0.7 volumes isopropanol, recovered using a glass rod, washed in 1ml of 70% ethanol and dried in air for 5 mins. The DNA was gently resuspended in the minimum volume of 1 x TE (typically 4ml) and incubated at 37°C overnight to ensure that the genomic DNA had completely redissolved in the TE.

2.4. Protein preparation.

Fractionation of bacterial cells produces a number of protein extracts corresponding to proteins from the cytoplasm, periplasm, outer membrane and inner membrane of bacterial cells. Cell fractionation was achieved by the following protocol (all centrifugation steps use a micro-centrifuge unless stated).

Bacteria were harvested from four agar plates covered with confluent bacterial growth in PBS. The OD_{600} of harvested bacterial cells were measured, the cells were pelleted, washed with PBS and suspended in 1ml of TES (10mM Tris-Cl pH 7.5, 10mM EDTA, 25% (w/v) sucrose, filter sterilised). The cells were then spun (11600*g*) for longer than 3 mins (due to the presence sucrose). The cells were again suspended in 1ml of TES and spun down (11600*g* for longer than 3 mins). The cells were then re-suspended in ice-cold dH_2O. The volume used was calculated using the following formula:-

Vol dH_2O in µl = [Volume of cells in µl (used in step a) x OD_{600}] /30

Cells were incubated on ice for 10 mins on ice then spun (11600*g*) for 2 mins at room temperature. The supernatant was gently removed, this being the periplasmic protein fraction. The spheroplasts pellet was re-suspend in a volume (the same volume calculated of water) of 10mM Tris-Cl pH 7.5. The spheroplasts were disrupted by sonication (Soniprep sonicator (MSE) small diameter tip, amplitude 1, low output) by 4-5, 5-second sonic bursts and cooled on ice for 30secs between bursts. Lysed spheroplasts were spun (11600*g*) for 1 min to remove unbroken cells and the supernatant ultra-centrifuged at 50,000 rpm (Beckman TL-100 ultracentrifuge), for 10min at 20°C, to pellet the membranes.

The supernatant is the cytoplasmic fraction and is retained, the pellet of crude membranes is re-suspended in $1/6^{th}$ of a volume (the volume calculated earlier) of solubilisation buffer (10mM Tris-Cl pH 7.5, 7mM EDTA, 0.6% (w/v) sarcosyl). Membranes were incubated for 30 min at 37°C, and mixed once

during incubation. Outer membranes were pelleted by ultra-centrifugation 50,000rpm, 10min, 20°C. The supernatant is the inner membrane fraction and was retained, the pellet is the outer membranes. The pellet was washed once with solubilisation buffer, ultra-centrifuged at 50,000 rpm, 10min, 20°C, then the pellet was washed once with PBS, ultra-centrifuged at 50,000rpm, 10min, 20°C. Finally the outer membrane pellet was re-suspended in $1/8^{th}$ of a volume (the volume calculated earlier) of PBS.

2.5. Protein manipulation.

Proteins were analysed by electrophoresis on SDS-polyacrylamide gels. Polyacrylamide gels were prepared according to standard protocols (Ausubel *et al.*, 1992), cytoplasmic and periplasmic fractions were run on 10-12% gels, membrane fractions on 6% gels. Protein samples were prepared by adding 40% (v/v of sample volume) loading buffer and boiling for 3-5mins. Whilst protein samples were being prepared, 2 aliquots of Low molecular weight size standard markers (Pharmacia Biotech) were prepared by dissolving markers in a volume of sample buffer (in accordance with the manufacturers instructions) and boiling for 3-5 mins. SDS-PAGE was performed either using Mini-Protean II (BioRad) apparatus producing "mini-gels" with dimensions of 7.2 x 10.2cm or a large Protean II apparatus producing "maxi-gels" with dimensions of 16 x 16cm. "Mini-gels" were electrophoresed at a constant current of 15mA. "Maxi-gels" were electrophoresed overnight at a voltage of 40V (constant). Gels were stained using Coomassie brilliant blue (Ausubel *et al.*, 1992) and dried using a BioRad

gel air dryer. Proteins to be N-terminal sequenced were not stained with Coomassie brilliant blue but electro-blotted on to PVDF. The blotters Scotch pads and 2 pieces of 3MM paper (cut to the size of the Scotch pads) were pre-soaked in transfer buffer A (48mM Tris, 39mM Glycine, 10% Methanol, 0.3% SDS). A piece of PVDF (Fluorotrans, Pall Biosupports) was cut to the size of the gel, wet completely in methanol, and then soaked in transfer buffer A until needed. When gel has finished running take a blotting cassette assemble the blotting 'sandwich' according to the order shown in figure 2.1. A pre-soaked Scotch pad was placed on one side with a piece of pre-soaked 3MM paper on top. The gel was then placed on the centre of the 3MM paper and remove any air bubbles from between the two. A second piece of 3MM paper was placed on top of the gel and any remaining air bubbles removed by gently rolling a test-tube over the sandwich. A second Scotch pad was placed on the top and the cassette closed. The cassette was placed in the blotter tank full of transfer buffer with the gel toward the anode and the PVDF toward the anode taking care ensure the level of buffer completely covers the gel. The lid was placed on the tank and the blot run for 2-3hours at 250mA constant current. When blotting was complete the blotter was disconnected and the cassette removed. The blot was stained (1% Acetic acid, 0.2% solution of Ponceau S) for 2 minutes and destained (5% acetic acid). The blot was rinsed in distilled water several times, left to dry, and stored between two sheets of 3MM paper in a plastic bag at room temperature.

N-terminal sequencing of protein bands was carried out at PNACL, the University of Leicester.

Figure 2.1.

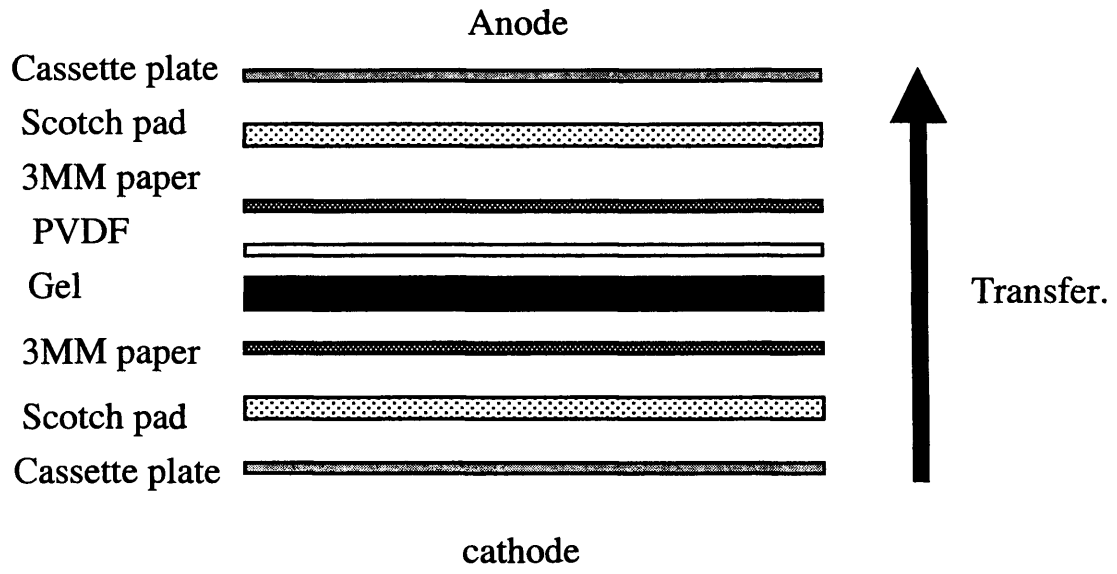

Figure 2.1. Construction of the gel cassette used in electro-blotting of polyacrylamide gels.

2.5.1. Isolation of proteins by lactoferrin binding affinity.

Lactoferrin-binding proteins were isolated using Elugent detergent (Calbiochem) and human Lf-sepharose chromatography, using a methodology similar to that used previously (Gorringe *et al.*, 1995). Extracts of membrane proteins of *C. jejuni* were prepared as described above from 50 ml of whole cells (500 µl final volume containing approximately 250 µg of protein) and were solubilised by addition of 2 % Elugent in PBS, and mixed by gentle inversion for 4 hours with 50 µl of Lf-sepharose equilibrated in PBS containing 2 % Elugent (the Lf-sepharose was a kind gift of Dr Rob Evans, United Medical and Dental School of Guy's and St Thomas's Hospitals, Guy's Hospital, London). The Lf-affinity resin was pelleted by centrifugation at 5,000*g* for 5 minutes, and the supernatant fraction (containing non-bound proteins) retained for protein analysis. To remove

non-specifically bound proteins, the resin was washed with 3 x 1ml of PBS containing 2 % Elugent, followed by 5,000g for 5 minutes. Lf-binding proteins were eluted by mixing the resin for 45 minutes with 2 separate additions of 100 µl of 0.2 M glycine, pH 2 containing 2 % Elugent. Supernatants were neutralised using 20 µl of 1 M Tris-HCl, pH 8.8, and analysed by SDS-PAGE on 12 % gels as described above. To confirm the specificity of Lf-binding, an identical *C. jejuni* membrane protein extract was also incubated for 1 hour with 100 µg/ml of diferric lactoferrin prior to Lf-sepharose affinity chromatography.

For analysis of protein profiles, gels were fixed and stained after electrophoresis in 0.05% (w/v) Coomassie blue in 40% (v/v) methanol and 10% (v/v) acetic acid, and destained in 10% (v/v) methanol and 10% (v/v) acetic acid. For identification of proteins by N-terminal sequencing, gels were electroblotted onto PVDF membranes at 0-4°C in 25 mM Tris, 192 mM glycine, 0.037% (w/v) SDS and 10% (v/v) methanol. Protein bands of interest were excised and sequenced using an Applied Biosystems 470A gas-phase sequencer.

2.5.2. Demonstration of Tf/Lf binding by *C. jejuni*.

Demonstration of Tf/Lf binding by *C. jejuni* was carried out by the following blotting method. *C. jejuni* cells were harvested from eight Mueller-Hinton swab plates with confluent bacterial growth. The bacterial cells were washed twice in SAPI minimal medium buffered with 50 mM Tris-HCl, pH 7.5, and re-suspended in the same medium to a cell density of approximately 2-3 $x10^8$ CFU/ml. Bacteria were incubated in this medium without shaking at 37°C for 30 minutes,

before addition of Fe-Tf (or Fe-LF). Bacteria were incubated with Tf/Lf for 60 minutes, after which the cell suspensions were harvested by centrifugation at 10000rpm for 10 mins, washed 3 times in PBS, and mixed with 50 µl of 100 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol and 2% (w/v) sodium dodecyl sulfate (SDS). This suspension was heated to 100°C for 15 minutes, centrifuged (11600g) for 10 mins, electrophoresed on 10% SDS-polyacrylamide gels, and electroblotted onto PVDF membranes, as described previously (methods section 2.6.). Blots were probed with anti-transferrin polyclonal antisera and cross-recognition determined using enhanced chemi-luminescence (Freestone *et al.*, 2000).

2.6. Growth assays.

Both solid and liquid media growth assays were carried out during the course of this study.

2.6.1. Solid media growth assays.

The plate iron utilisation assay is based upon the procedure described previously (Pickett *et al.*, 1992). *C. jejuni* cells were cultured for 24 hrs in Mueller-Hinton broth, harvested and suspended in PBS. *C. jejuni* cells were mixed with molten soft agar (50°C, MH broth, 1% agar) containing 20µM desferal to a calculated OD of 0.05. This mixture was then poured into a Petri dish allowed to set and dry, aseptically at room temperature. The agar surface was then spotted with

10µl of $FeSO_4$ dilutions (1M, 0.1M, 0.01M and 0.001M), Hemin dilutions (1µg/ml, 0.1µg/ml, 0.01µg/ml and 0.001µg/ml) or dH_2O (negative control).

2.6.2 Growth curves in liquid media.

The quantitative measurement of iron utilisation by *C. jejuni* was achieved by either MH-des (Mueller-Hinton broth, 20µM desferal) to produce iron limitation) growth curve assays, or MEMα based growth curve assays.

MH broth based assay were performed by harvesting the *C. jejuni* strain to be tested from a confluently grown agar plate containing antibiotics. The bacteria were washed in PBS and inoculated into a flask containing 250 ml of MH broth containing relevant antibiotic selection. The flask was then incubated at 37°C overnight in a VAIN cabinet. After incubation, the OD_{600} of the culture was measured and the cells harvested. The cells were re-suspended in 5ml of PBS, and the volume of this cell suspension needed to make an OD_{600} of 0.05 in the volume of broth used to for the growth curve (5ml if in conical based tubes, or more if larger volume vessels are used). For each strain tested three vessels were set up (conical based tubes if only small volumes were needed). One vessel containing MH broth supplemented with 40µM $FeSO_4$ (iron replete conditions), one containing 20µM desferal (iron limited conditions) and one with 20µM desferal and 1µg/ml hemin (hemin as sole iron source). A set of vessels containing *C. jejuni* NCTC11168 was also set up as a control. The cultures were then incubated at 37°C with shaking, samples were taken every 2hrs and the

OD_{600} measured. Note: Absorbance was blanked against the supplemented media type due to the strong colour of hemin supplement.

The MEMα based growth curves were carried out in a similar manner to the MH broth growth curves with the following differences. Firstly, Minimal Essential Media, modification α (MEMα Invitrogen Ltd BRL) was supplemented for MH broth in all steps. Secondly, desferal supplementation was not needed because MEMα is a defined iron limited media. Instead iron limited conditions were provided using un-supplemented media. 1μg/ml hemin may be substituted with 25μg/ml (~0.3μM) Fe-Lf (Sigma) or 100μg/ml (~1.25μM) Fe-Lf. Additionally further cultures were included in this experiment, containing MEMα supplemented with 25μg/ml and 100μg/ml apo-lactoferrin (Sigma).

2.7. Detection of promoter activity.

Detection of promoter activity was achieved by the use of the pMW10 LacZ assay system, using β-galactosidase activity as a measure of promoter activity. *C. jejuni* 480 containing the desired pMW10/promoter clones were grown for 18hrs on MH Agar swab plates at 37°C. The cells were harvested and resuspended in 1 ml of PBS. The cells were then added to a flask containing 5ml MH broth supplemented with 40μM FeSO4 and 5ml MH broth supplemented with 20μM desferal. These cultures were then incubated for 4hrs to allow induction of promoter expression in response to the iron limited and iron replete conditions. After incubation the bacteria were pelleted and re-suspended in PBS

at a "calculated" OD_{600} value of 0.4. Before starting the assay the "actual" OD_{600} value was determined, which typically varied between 0.37 and 0.45. The samples were maintained on ice.

To 50 µl of cell sample, 450µl of Z-Buffer (0.06M $Na_2HPO_4.7H_2O$, 0.04M $NaH_2PO_4.H_20$. 0.01M KCl, 0.001M $MgSO_4.7H_2O$ and 0.05m β-Mercaptoethanol), 8µl of 0.1% SDS and 15µl of chloroform were added and vortexed together for 30 secs. Each sample mixture was placed in a 28°C waterbath and allowed to equilibrate for 5 mins. At this point, 100µl of *O*-nitrophenyl-β-galactopyranoside (ONPG, 4 $mgml^{-1}$) was added, mixed thoroughly and the solution was incubated at 28°C for a set time for the yellow colour to develop, typically between 7 and 12 mins. The activity of β-galactosidase in *C. jejuni* was measured by the conversion of *O*-nitrophenyl-β-galactopyranoside to nitrophenol as described by Miller, (Miller, 1972). The reaction was terminated by addition of 250µl of 1M Na_2CO_3 and the colour development was measured at two different wavelengths, OD_{420} and OD_{550} using a spectrophotometer (Pharmacia LKB, Ultraspec III). Miller units were calculated using the following formula.

$$\frac{1000 \text{ X } (OD_{420}\text{-}(1.75 \text{ X } OD_{550}))}{OD_{600} \text{ X } 0.1 \text{ X t (s)}}$$

2.8. Radioactive iron uptake assays.

Observation of the uptake of lactoferrin/ transferrin bound iron was achieved by the use of dialysis partition experiments. These experiments were based on assays designed previously (Freestone *et al.*, 1999), to investigate the involvement of adrenaline and noradrenaline in bacterial iron uptake from lactoferrin/transferrin (Freestone *et al.*, 2000).

2.8.1. ^{55}Fe labelling of Tf.

This method of Tf ^{55}Fe-labelling results in a mixture of both di-ferric, and mono-ferric forms of ^{55}Fe Tf. 25 μCi of $^{55}FeCl_3$ (Amersham Pharmacia Biotech. Little Chalfont, Buckinghamshire U.K.) per mg apoTf was used. Incorporation of Fe into Tf is Fe-concentration-dependent. Because there is only a tiny amount of free Fe in the $^{55}FeCl_3$ (9 μg/l), cold $FeCl_3$ was added, to give a final ratio of a minimum of 1.4 μg total Fe per mg/apoTf. To achieve this a ^{55}Fe-citrate mix containing 60 μg/ml Fe was made to give 1.5 μg Fe/mg Tf:-

0.5 ml of $^{55}FeCl_3$ (500 μCi)

3 μl of 10 mg/ml $FeCl_3$ (filter-sterilised)

50 μl of 0.8 M Citrate, pH 7.5 (filter-sterilised)

The ^{55}Fe-citrate mix was added to the following components to ^{55}Fe label Tf, 1.1 μl of this mixture corresponds to 1 μCi ^{55}Fe:-

apoTf (50 mg/ml)	40 µl	(2 mg)
1 M Tris-HCl, pH 8.0	30 µl	(120 mM)
$^{55}FeCl_3$-Citrate mix	55 µl	(25 µCi ^{55}Fe/mg Tf, 1.5 ugFe/mg Tf)
dH_20 to 250 µl	125 µl	

The mixture was stored at -20°C.

This reaction was incubated at 37°C for a minimum of 5hrs. Unincorporated ^{55}Fe was removed by passing the completed reaction through a Biorad Bio-Spin 6 chromatography column [cat no 732-6221] (washed four times in PBS to remove azide storage buffer).

2.8.2. Dialysis partition experiments.

The dialysis partition experiment was carried out by placing 5 ml of fortified SAPI medium (SAPI with 0.1 % filter-sterilised L-serine, 0.1 % filter-sterilised 0.1 % filter-sterilised L-aspartate and 50 mM Tris-HCl, pH 7.5 added after the SAPI is autoclaved) in a 25 ml flask. If desired Noradrenaline may then be added to the medium at least 100µM final concentration. The sterile SAPI medium (in the flasks) was supplemented with 2×10^5 cpm/ml of filter-sterilized ^{55}Fe-Tf, either directly into the medium or enclosed within 1-cm diameter dialysis membrane (4 kDa cut-off, Scientific Industries International inc., Loughborough, UK). Three 20 µl samples of the ^{55}Fe-labelled protein mix were taken and mixed with 2ml Emulsifier-safe scintillant (Canberra-packard, Pangbourne, U.K.). The radioactivity of the sample was measured by

scintillation counting (Minaxi Tri-Carb 400 series scintillation counter, Canberra-packard). Approximately 5 ml (2-3 x10^8 cfu/ml) of an overnight MH Broth culture of *C. jejuni* was washed (once in PBS, the 3 times in SAPI to reduce carry-over of iron-scavenging metabolites) bacteria are then added to the sterilin. In this way bacteria iron incorporation is measured from two flasks, one containing bacteria in contact with ^{55}FeTf/Lf, and another where bacteria are partitioned away from the ^{55}FeTf/Lf (figure 2.3.). This allows investigation whether the uptake mechanism is contact-based. Each sterilin is then incubated for 4 hours at 37 °C in a VAIN cabinet. After incubation 3x 1 ml aliquots of the bacteria containing media are removed, pelleted (3x100µl of culture supernatant were retained to measure radioactive count), washed once in PBS, re-suspended in 50 µl of PBS. The bacterial cells were then measured for ^{55}Fe-incorporation as described below. The residual ^{55}Fe-labelled culture may be retained for later immuno-analysis of Tf-binding. Radioactivity was measured on the tritium counting channel, using 2 ml of Emulsifier Safe scintillation fluid per [original volume] ml aliquot of culture, or 100 µl of supernatant.

Poisoning experiments to discover whether Tf/Lf iron utilisation is energy dependent was carried out using the same method except with incubation of *C. jejuni* in SAPI medium containing 6 mM sodium azide for 30 min prior to addition of the ^{55}FeTf.

2.9. Gel retardation experiments.

The methods devised in this section were based upon previous mobility shift assays (Goldberg., 2001). Detection of Fur protein binding to *C. jejuni* promoter sequences was achieved by agarose gel retardation assays. Promoter DNA was prepared by PCR as previously described. Promoter DNA (50-100 ng) was mixed with a series of tenfold dilutions of cytoplasmic protein extract (prepared as described above) from both *C. jejuni* NCTC11168 and *C. jejuni* AV17 (*C. jejuni* NCTC11168 *Δfur*) in the following amounts:-

1µl promoter DNA (50-100ng).

1µl of protein dilution

1µl of 10x TBE (Sambrook *et al.*, 1989).

1µl NaCl solution*.

5µl dH_2O.

*Several 10x NaCl solutions were prepared to give final NaCl concentrations of 0, 10, 100 and 1000 mM, this was carried out to minimise the effects of salt concentration on protein binding.

This mixture was incubated for 15mins at 37°C to allow protein binding, all the mixtures were then run on a 0.6% TBE buffered agarose gel containing no ethidium bromide. When the gel was run it was stained with ethidium bromide for 10 mins and visualised on a UV-transilluminator.

Detection of promoter sequences in the agarose gel retardation experiment, was carried out by Southern hybridisation. A detailed methodology for Southern

hybridisation can be found in Sambrook et al (Sambrook *et al.*, 1989). The agarose gel was washed twice in denaturing solution for 10 mins, twice in neutralising solution for 10 mins and finally in 2 x SSC for 10 mins, all at room temperature. DNA was transferred from the agarose gel to a nitrocellulose membrane by capillary transfer overnight. The DNA was fixed to the membrane by exposure to UV radiation using a calibrated UV transilluminator. Detection of target DNA bound to the nylon membrane was accomplished using the Gene images random-prime labelling module, (Amersham life sciences) according to the manufacturers instructions. The fluorescent blot was exposed to autoradiography film in an X-ray cassette for exposure times of 5min to 24 hours to achieve the clearest image possible.

2.10. Bio-informatics.

Multiple protein sequence alignments were performed at the following site.

http://www2.ebi.ac.uk/clustalw

Sequence analysis and was performed using the following computer programmes and web sites.

Gene Runner *version 3.05.* (1994) copyright Hastings software.

Stemloop, Wisconsin Package Version 10.3, Accelrys Inc, San Diego CA

Sci Ed Central, Clone Manager 7, version 7.01, copyright© 1997-2002 Scientific and Educational Software

http://www.ncbi.nlm.nih.gov/BLAST

http://www.sanger.ac.uk/cgi-bin/Pfam/nph-search.cgi

http://expasy.hcuge.ch/sprot/prosite.html

Campylobacter jejuni genome sequence data was retrieved from the following web site.

http://www.sanger.ac.uk/projects/C. jejuni

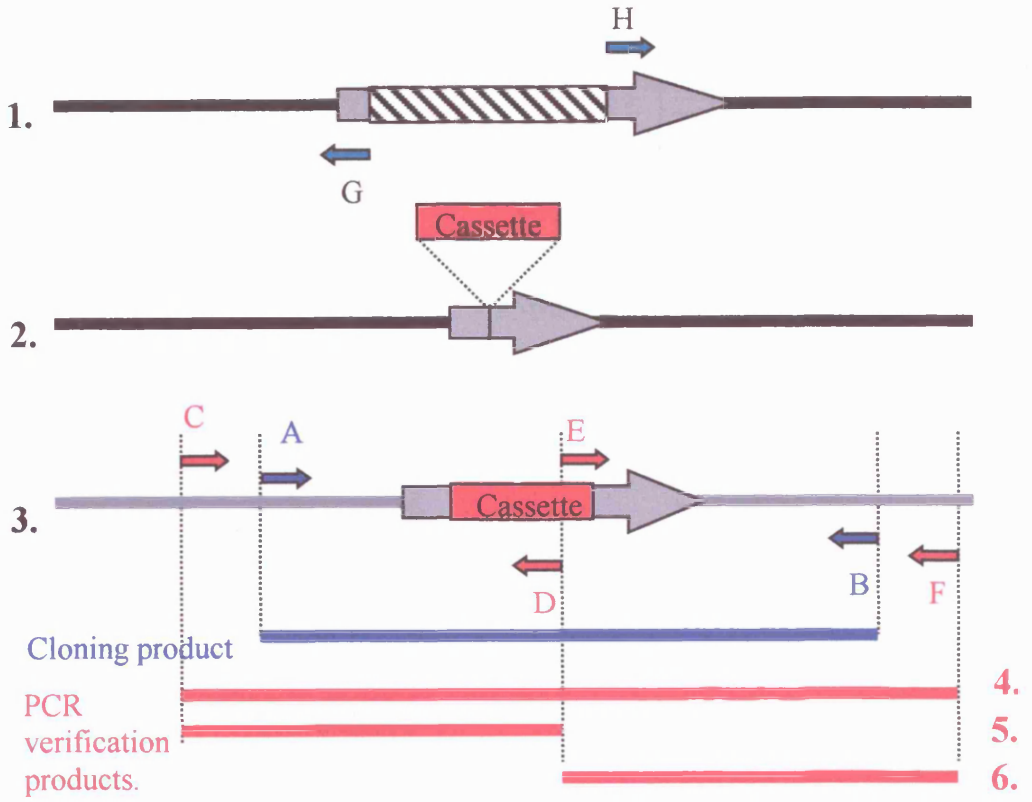

Figure 2.2. The PCR based method used to delete cloned gene sequences and verify correct recombination of deleted gene products into the *C. jejuni* chromosome to produce directed mutation. **1.** Primers G and H (green arrows) are designed to anneal to the cloned gene (grey arrow) and allow deletion of the diagonal striped area by PCR. PCR extension proceeds in the direction of the green arrows into the plasmid vector (thick black line). The PCR product is cut at a restriction site encoded on primers G and H and re-ligated, producing a truncated gene sequence (**2.**). This deleted gene is re-cut and an antibiotic resistance cassette (red box) ligated in place of the deleted sequence in the same orientation as the cloned gene (**3.**). The deleted gene construct is transformed into *C. jejuni* NCTC11168 where it integrates into the chromosome. Chromosomal DNA (thick grey line) can then be verified. The smaller blue arrows represent oligonucleotide primers used to clone the gene initially (A-B), the size of a PCR product produced by the original cloning primers is represented by the blue line. Red arrows represent primers used to verify the recombination event, PCR verification products are represented by red lines, the uppermost of these lines (4) is a PCR product across the cloned region (primers C-F). If more 0.7kbp is deleted there will be an observable size difference between PCR product obtained from mutant strain DNA and that from wild type parent strain. The lower two red lines (5 and 6) represent products (primers C-D and E-F) allowing the antibiotic resistance cassette to be located with respect to flanking DNA (outside the cloned region) in the mutant strain.

Figure 2.3.

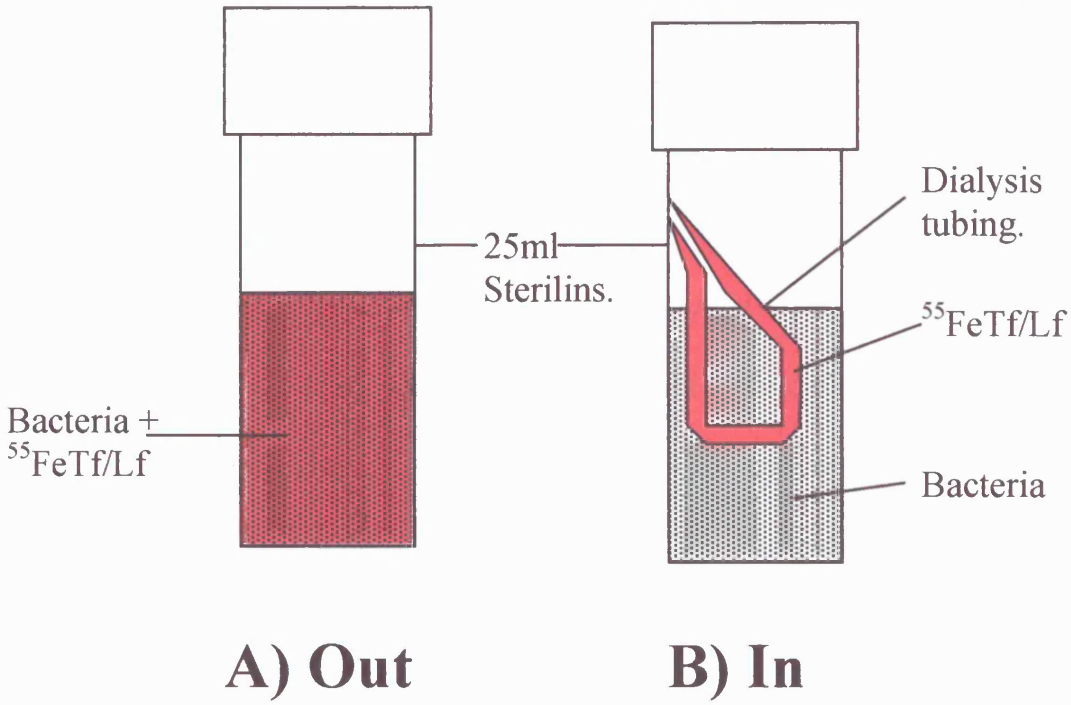

Figure 2.3. The dialysis partition experiment to assess the ability of *C. jejuni* to obtain iron from ^{55}Fe labelled Tf/Lf. The sterilin A (out) contains bacteria (black spotted area) in contact with ^{55}Fe labelled Tf/Lf (red shaded area) and demonstrates the ability of an organism to obtain iron from Tf/Lf. Sterilin B (In) contains bacteria partitioned away from the ^{55}Fe labelled Tf/Lf using dialysis tubing. This vessel allows investigation of the contact dependent nature of any acquisition mechanism. Sterilin B can also be used to investigate the ability to use small diffusible molecules (siderophores or catecholamines) to obtain iron by the addition of such molecules with the bacteria.

3. Results: Analysis and cloning of *chu* genes.

3.1. Introduction.

Initial studies into the ability of *C. jejuni* to utilise iron compounds had proven that a number of *C. jejuni* strains could utilise hemin and a number of haem-containing proteins as iron sources (Pickett *et al.*, 1992). Analysis of OM proteins produced by chemically mutated *C. jejuni* strains that could not utilise hemin, presented one class of mutant that lacked an iron-regulated 71kDa OM protein (Pickett *et al.*, 1992). The construction of a *C. jejuni fur* mutant strain allowed comparison of protein expression between wild type and *fur* mutant strains (van Vliet *et al.*, 1998). This comparison highlighted a number of de-repressed proteins (observed under iron replete conditions) in the *fur* mutant including a number of OM proteins. Among the Fur regulated OM proteins was a 70kDa protein that was N-terminal sequenced and identified as a siderophore receptor homologue (van Vliet *et al.*, 1998). This protein was predicted by its size to be the same OM protein found absent in hemin utilisation mutants (Pickett *et al.*, 1992). It was predicted that the 70kDa OM protein was the receptor for hemin and other haem-containing proteins and it was designated ChuA (Campylobacter Hemin Uptake protein A). The preliminary genome sequence (formerly at http://www.sanger.ac.uk.) was available at this point and the region downstream of the *chuA* gene could be analysed. Downstream of this ORF are three other ORF's arranged in an apparent operon, which appear to encode a typical ABC transport system (van Vliet *et al.*, 1998). These three ORF's encoded a putative cytoplasmic membrane permease (*chuB*), a putative ATP

binding protein (*chuC*) and a putative periplasmic binding protein (*chuD*). Visualisation of a de-repressed protein of 29kDa in the Fur mutant was predicted to be ChuD by its size by one-dimensional SDS-PAGE (van Vliet *et al.*, 1998). The previous work on the 70 kDa OM protein and Fur regulated 70 kDa protein designated as ChuA raises a number of questions about the *chu* system and the ability of *C. jejuni* to utilise hemin. Functional analysis of the *chu* system must establish whether ChuA is involved in the uptake of hemin and haem-containing proteins. The involvement of the ABC transport system (ChuB, ChuC and ChuD) in the uptake of hemin must also be investigated. Finally how expression of the Chu proteins are regulated may be investigated, it is possible that Chu protein expression is Fur regulated in response to iron. Finally whether the four genes are arranged in an operon as they appear may be addressed.

3.2. Results.

3.2.1. Sequence analysis of the *chu* genes and proteins.

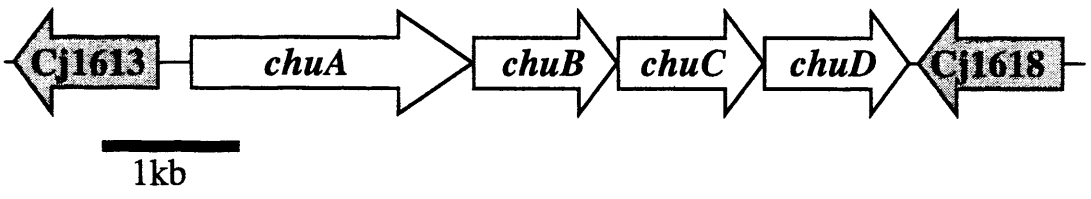

Figure 3.1. The organisation of genes around *chuA* on the chromosome of *C. jejuni* NCTC11168.

Chapter 3. Analysis and cloning of the *chu* genes.

Computational analysis of the *chu* gene sequences was carried out initially before publication of the *C. jejuni* genome (Parkhill *et al.*, 2000). The four *chu* genes appear to be arranged in an apparent operon (figure3.1). All four genes are expressed on the same DNA strand, either overlapping the adjacent ORFs by a few bp or with no gaps between ORFs. The four genes are 30.1% GC compared with a genome average of 30.6% (Parkhill *et al.*, 2000). There was no sign of any T-loop structural motif between *chuA* and *chuB* (Stemloop, Wisconsin Package Version 10.3, Accelrys Inc, San Diego CA) so the genes would appear to be co-expressed. A region of about 200bp exists between *chuA* and the divergently orientated Cj1613, which is a possible location of the *chu* promoter. Within this non-coding region, approximately 10bp upstream of the *chuA* start codon (TTG) is a putative ribosomal binding site (AGGAG) and 47bp upstream is a sequence that conforms to the *C. jejuni* consensus Fur-box sequence (nATnATnATnATnATnATn) (van Vliet *et al.*, 2002).

5'-GTTAATAAAGGATAAATTTATTTCTAAATTATTATGATATAAATTATCATTATTTATTTT
ATTTAAGGAGTAAAATTTG

Figure 3.2. Analysis of the region upstream of *chuA*. A putative fur box is shown (shaded grey) and a putative ribosomal binding site (shaded black, white script), the start codon (**TTG**) is shown in bold script.

The *chuA* ORF is 2130bp in length (www.sanger.ac.uk/projects/C. jejuni/CDS/Cj1614.shtml) and has a 30.6 % GC content (Gene Runner, version 3.05, Hastings Software inc). The *chuA* ORF sequence displays little similarity

with other bacterial genes and appeared to show greatest similarity to human gene sequences (http://www.ncbi.nlm.nih.gov). The ChuA protein is 709 amino acids in length and has a predicted molecular weight of 80.9kDa. Analysis of ChuA by Blast protein alignment (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), demonstrates regions of similarity to the hemin receptors of *Fusobacterium nucleatum* (28-24% identity), *Vibrio vulnificus* (22.9% identity) and *Vibrio cholerae* (23% identity). Pfam analysis highlighted the similarity of ChuA to the TonB dependent family of receptors (http://www.sanger.ac.uk/cgi-bin/Pfam/nph-search.cgi). ClustalW alignment of ChuA with a number of OM receptors (TonB dependent) from other bacterial species can be seen in figure 3.3. Some limited areas of conserved sequence may be observed at the N-terminal regions of the proteins represented some of which may be due to protein regions which interact with the TonB protein. Much of the conserved sequence however appears to be within structural and functional regions away from the N-terminal TonB interaction sequences (Grey shaded area, VB12R).

```
Y.pIutA         ----------------------------MKHKHL-WVLNPCLLVMLT--PAAWAED---Q 26
E.cIutA         MAQRQPEKTAAGGCCFNSLYNKYSGITMMRKKYMPRALGPLLLVVLS--PAVAQQNDDNE 58
VB12R           ----------------------------MQKSALAIALASLLTPISY--LHANEAQPQET 30
chuA            --------------------------LHPNKKAFKLSLFALLLITNLNAQESNKAINLQK 34
Hem             ---------------------------------MANGYGEIINLGEK------------- 14
CfrA            ------------------------------MKKICLSVCAIGLLASN---AISQNVELDS 27
                                              . :          .:          .

Y.pIutA         LVVSANRSHRSVAEMAQTTWVIEGQELEQQVQGGLEIKDILAQLIPGIDVS-SQGRTNYG 85
E.cIutA         IIVSASRSNRTVAEMAQTTWVIENAELEQQIQGGKELKDALAQLIPGLDVS-SQSRTNYG 117
VB12R           VVVTANRFEQKASSTLADVEIITRQDIEQQAKTLPELLRRLTGVQITQNGGRGQLASLFV 90
chuA            VVVSTTGFEQDADSNLRNVISIEGKDLQNKGYVSLEQALERISSISFVNFGLGRNIDMRG 94
Hem             NIYSETGFEKNLRNSTTSPYIITSKDIETKGYTSVSEILDSVPGVN-IQEGLHPAVDVRG 73
CfrA            SIISASGFAQDIKEAPATINVISKKELQSKPYRDVEEAIADIPGVDLYASKGKTGSYNIT 87
                 : : .   :  .       *   ::: :     .

Y.pIutA         MN-MRGRS-IMVMIDG--VRLNSSRSDSRQLDSID------PFNIAHIEVISGATS-LYG 134
E.cIutA         MN-MRGRP-LVVLIDG--VRLNSSRSDSRQLDSVD------PFNIDHIEVISGATA-LYG 166
VB12R           RG-TSSDQ-VLVLVDG--IRFARAAKGAVDFNQIP------LTYVDRIEYVRGARASLYG 140
chuA            QGNKSNIA-VKVMIDGHAINVLDNSHGVTPLDSIN------LDNVERIEIIPGGGSVLYG 147
Hem             QGFQKAKATVQLLVDGVPANMLDTSHMNVPIDVVN------INEIERIEVIPGGGAVLYG 127
CfrA            MRGITGYT--LVLIDGR-RQGIGGEVGPNGFNEISNSFLPPISSIERIEVIKGPMSTLYG 144
                        :::** . .          :: :          : :** : *  : ***
```

```
Y.pIutA         GGSTGGLINIVTKKGQEGKQVELQIGGKTGFNSHNDHDENISAAMSGGTERAFGRFSVSY 194
E.cIutA         GGSTGGLINIVTKKGQPETMMEFEAGTKSGFNSSKDHDERIAGAVSGGNDHISGRLSVAY 226
VB12R           SEAIGGVINIITKARSQQQGTTVSAG----LGSLDYQELSIASGVAIGE----------- 185
chuA            SGTRGGVINIITKKQKSDAFAINLKSSAYDHGGLG-GNLGINGAKQINENLAFSFDIQSF 206
Hem             SGTSGGVINIITKKYKGNNNVRGGVG--YQVASFRNNKFDVSAGTSVGDFDFDINYSKNR 185
CfrA            SEALGGVVNIITKKVSDKWETSVSLDALLNENKDWGNTYGTSIYSSGPLMNDKLGLTLRF 204
                . : **::**:**  .         .

Y.pIutA         QRYGGWYDGKGNEVLIDNTQTGLQYSNRLDVMGTGTLNIDENQQLQLTTQYFNSESDGKH 254
E.cIutA         QKFGGWFDGNGDATLLDNTQTGLQHSNRLDIMGTGTLNIDESRQLQLITQYYKSQGDDNY 286
VB12R           --------------------------------KGQMNVALG----------TESD--- 198
chuA            NLDGYQEGYNEKGYFIN---------------TKTYIDINDNSDLTLGYNYFKSKNTS-- 249
Hem             KYGYRDYDFTNSDYFSG----------------RINYNINKTSNIAFKYSGYRDKYTYP- 228
CfrA            REFYRQ---------------------------QSNVEFTNGSGQ--------------- 222
                      . . .    .                    :.         .    .

Y.pIutA         GLYLGQNFSAVTGTGQASNSAALNSDRIPGTERHLINLQYSNTDFWGQDLVAQVYYRDES 314
E.cIutA         GLNLGKGFSAISGSSTPYVSKGLNSDRIPGTERHLISLQYSDSDFLRQELVGQVYYRDES 346
VB12R           --------------------KGYNVRPVPGVNDGDRHGFRSDN-----ALLGYVHQFDES 233
chuA            ------------SGYLTKAQAQSDPTQKGKSDNITQINRPEIS------LDYHYYFDDI 290
Hem             --------------NFLDQKELDENRRQSGIEKEAKENNRIKKD---EFTLTYNTKIGDK 271
CfrA            --------------RVQGDQAQSPTKANNFNIGTRISYLANDYNTFIFDIDFSRNHYDN 267
                          :.       .    . .        .       :       :

Y.pIutA         LTFYPFPTLKDGK---VSTIGASQQKTDFYGSKLTLNSEPIDSLTLTYGIDLEHESFNAN 371
E.cIutA         LRFYPFPTVNANKQ--ATAFSSSQQDTDQYGMKLTLNSQLMDGWQITWGLDAEHERFTSN 404
VB12R           WSLFAN----------ARAYENIYQYDNSYGTRDYKEAE-KDDLSFTIGTQYQSERWVSE 282
chuA            WEFNLEAFWQNQKI--NYLKDVSTMSYMNMSLPVYQNGSGFEDTLTGISLKNKLNYANNS 348
Hem             NDLNILGFYQKTDIPSESIEDYTSEYKGMLAGQAARLRKALRNPRLPARARTAMQNRLNA 331
CfrA            KQGQLGTITSPGRTPGSLTGGYADIMEVDKFVTYLSHEGVYENFSITSGLQYNRVSNDGR 327
                          .                          .

Y.pIutA         QQFFNLAKAQQSGGM---------TLENAYNVGRYPSYTTTNLAPFLQTRYDINPIFTLS 422
E.cIutA         QMFFDLAQASASGGL---------NNHKIYTTGRYPSYDITNLAAFLQSSYDINDIFTVS 455
VB12R           LQLTTQKQKSWDYTQ---------SKGKYSDT----SDNLEQRNIQWINRYLVNDVWTFA 329
chuA            YFIFGYEFANHDAKRKSLVYYSVPSVINYHRMTTLIDMTKQSHSIFALDSHNFNEFFTLS 408
Hem             LLAELGSTNSVDFKKFS----QFKDTKKAIKIKDKFTYDNAGSNVIVGLGYTDNDMLRVS 387
CfrA            EVVGQSTQPFLGENR--------DIVAEDIILDTKSVIPLGQSHILSVRGEYRLEKMQDK 379
                          .    :    .    :

Y.pIutA         ----------GGVRYQYTENKVDD--FVGYAQQ-----------QAIASGSATSADPVPG 459
E.cIutA         ----------GGVRYQYTENRVDD--FIDYTQQ-----------QKIAAGKAISADAIPG 492
VB12R           ----------GGVDWRDES-------YIDKTAD-----------KEFDR---SNTAAFAV 358
chuA            ----------GGARYEFSSYNTDRSYRNEMSMN-----------GRPPSPTIIDSTTLFD 447
Hem             KMELVGKRVMADTKIDLSKKTFEVFALNTFKVNRFELIQGLRFENSKYDGTRKNNDDTLD 447
CfrA            ----------IASPTNFDQYLLAIFAEDEYSIKD---------DLRLTFGARYNHHEIFG 420
                               .              .                .

Y.pIutA         GKTDYNNFLFNAGLLAHLTESQQTWFNFSQGFEIPDLAKYYGSGSY-TLVNG-HYQLQNS 517
E.cIutA         GSVDYDNFLFNAGLLMHITERQQAWFNFSQGVALPDPGKYYGRGIYGAAVNG-HLPLTKS 551
VB12R           VAAEWQQWLLEASLRFDDNQEYGSQTTHNIALGYQFIPEFGVKASYGSAFKAPNLYQQYD 418
chuA            TNKNANNFAFEITPNFKYSDTGKLYIKYERGFVSPSPAQFVNKDKNSQKYYSANLNPEIF 507
Hem             IKKSKDNWAGSLAVNYLYSDTGNAYLKYERAFTSPAPGQLVDKVQTAPRVYTYKVNNLKS 507
CfrA            NNVSPRAYVVYN-PTNELTLKGGVSTGFRTPYANRLINGTYSYSGQGRFPTYGNPDLKEE 479
                   .   :          .          .                .      :

Y.pIutA         VNVNDSKLEGIKVDSYELGWRYTGDNLRTQMAGYYSLSDQTIS----INKTDMTINVLPD 573
E.cIutA         VNVSDSKLEGVKVDSYELGWRFTGDNLRTQIAAYYSLSNKSVE----RNK-DLTISVKDD 606
VB12R           PSYGNVNLQPEDADSAELSFYGLFSGIKWSITGYDYKINNLID----YNSTTKNYQNVIG 474
chuA            DTFELG-IDDFWWDFYGFNLTLFYTLSKDEIS---YLGNPHST----SGSWWKYYNIDQT 559
Hem             ESTNLFEIGWNDYLLGSLLSADVFYAETKDEIATIFDGGAANA----HGTAFRSTNLGKT 563
CfrA            TSLNYEIAAIYNNDLFYVSATGFLTNFKDKISSQSYNNSEPIPGIGTCDADRCSRAINHG 539
                 .              .          .         .          .

Y.pIutA         KRRIYGVEGAVDYF-FDNSEWSAGATFNLIKSETKVSGKWQKLTIDAASPSKATAYIG-- 630
E.cIutA         RRRIYGVEGAVDYL-IPDTDWSTGVNFNVLKTESKVNGQWQKYDVKESSPSKATAYIN-- 663
VB12R           ESNIKGVEFTAEFA-TGIVQHQLSVDLKDADDSKGKTLQRRAEHMYKWNALVAFEQVD-- 531
chuA            RRLGVELSLSQNFL-DDDLIFRESLTYLDAKISKGVNDGMRIPYVSKIKATAGLEYAWNK 618
Hem             KR--YGFDLSAEQK-FEKFTFKEAYSFIETKILKDNSSSFEGKHIADVPKHKLVFSVDYD 620
CfrA            KVEYKGVELGAGISPLDNLNVNFAYTYLDTEVKEAQDRSVIGKPEQDSLKHNIMLKTEYS 599
                .     ..              .      .                          .
```

```
Y.pIutA         ----WAPGDWNLRVQSQQTFDVSD----SKGDKIDGYNTIDFLSSYALPVG-KLSFSIEN 681
E.cIutA         ----WAPEPWSLRVQSTTSFDVSD----AEGNDINGYTTVDFISSWQLPVG-TLSFSVEN 714
VB12R           ----WSIGYQYVGKRPDLDYNTYP----TQNITLDAYSLVDTSVSYYVTDSTTISARIDN 583
chuA            NFSNFIDLTYFSRAKDGGTIDENTGKM-SKNSWIRDYFLTDIGMKYNYKKLQILAG-IRN 676
Hem             -----ITSKFTVGADYEYRAAAFIDNA-NKYGKDKAKSVFNLRADYKITNSLNVYAGINN 674
CfrA            -----FYNKFTPWIKGEWQIDRYMGDTNINREYYKDIFLASMGVRYDINKQWSISAAIYN 654
                . :.                    ..   :         .   :       :   : *

Y.pIutA         LLDKEYTTVWGQRAPILYSPTYGSPNLYSYKGRGRTFGVNYSVLF 726
E.cIutA         LFDRDYTTVWGQRAPLYYSPGYGPASLYDYKGRGRTFGLNYSVLF 759
VB12R           LLDKEYETANG-----------------YPAAERAYYLNIGYQF 610
chuA            LFDKRYYTYQDSIN------------DQYLVGNGRNYYVEFKYAF 709
Hem             IFGAKYYNSVRGNS---------SGERFYDPAPKINYYVGFKYKF 710
CfrA            LFDNSFTNGWESYAS---GSGSTWVNTYNRIEEGRRMYISINGNF 696
                :::  : .                          :     *
```

Figure 3.3 Clustal alignment of the ChuA protein sequence with similar proteins from other bacterial species. Underneath the protein sequence, conserved areas of identical (represented by asterisks) and similar (represented by single or double points) are indicated. *C. jejuni* ChuA is abbreviated as chuA, similar genes that are aligned are the IutA (aerobactin) receptors from both *E. coli* (E.cIutA) and *Y.pestis* Kim strain (Y.pIutA), the hemin receptor from *Fusobacterium nucleatum* subsp. vincentii ATCC49256 (Hem), the Vitamin B12 receptor of *V. cholerae* strain N16961 biotype: el tor(VB12R) and the putative siderophore receptor CfrA of *C.coli* strain VC176. Within the *V. cholerae* Vitamin B12 receptor the region that interacts with the TonB protein is shaded grey and the TonB-Box (TXXVT) is underlined.

The *chuB*, *C* and *D* genes also display limited amino acid similarity to other bacterial genes. The *chuB* gene is 987bp and encodes a protein 328 amino acids long with a predicted molecular weight of 35.6 kDa. Protein sequence alignment of ChuB demonstrates its similarity to inner membrane permeases of ABC transport systems in a number of bacterial species. Greatest similarity (48% identity) is demonstrated to HmuU of *Fusobacterium nucleatum* (Kapatral *et al.*, 2002), a permease involved in hemin uptake. Pfam analysis of ChuB again found a match with the TonB dependent receptor protein family but also with the FecCD protein family. FecCD proteins are a binding protein dependent sub-family of transport proteins, which include membrane permeases.

Chapter 3. Analysis and cloning of the *chu* genes.

The *chuC* gene is 777bp encoding a protein of 258 amino acids in length with a predicted molecular weight of 29.5 kDa. Alignment of ChuC protein by Blast, demonstrated similarity to ATP binding proteins from ABC transport systems involved in iron acquisition in a large number of bacterial species. Greatest similarity (39% identity) was demonstrated to ATP binding proteins of the hemin uptake system of *F.nucleatum.* Pfam analysis matched ChuC to the ABC transport family of proteins, and Prosite pattern analysis detected an ATP/GTP-binding site motif within ChuC.

The *chuD* gene is 807 base pairs and encodes a protein of 268 amino acids with a predicted molecular weight of 30.1 kDa. Analysis of ChuD by protein alignment (Blast) demonstrated greatest similarity (33% identity) to hemin-binding periplasmic proteins from *F.nucleatum.* A summary of similarities between the Chu proteins of *C. jejuni* and to proteins from other bacterial species may be found in table 3.1.

Table 3.1.

C. jejuni protein.	Similar protein from other organism.	Function/putative function.	Percentage Identity
ChuA	Protein 710, (*Fusobacterium nucleatum*).	Hemin receptor	28% over 742 amino acids.
ChuA	IutA, (*Escherichia fergusonii*).	Aerobactin receptor.	22% over 515 amino acids.
ChuA	1..6116, (*Vibrio cholerae*).	Vitamin B12 receptor.	27% over 332 amino acids.
ChuA	CfrA, (*C. coli*)	Putative ferri-siderphore receptor.	35% over 144 amino acids.

ChuB	HmuU, (*Fusobacterium nucleatum*).	Hemin transport system permease.	48% over 321 amino acids.
ChuB	HutC, (*V. cholerae*).	Hemin transport system permease.	31% over 322 amino acids.
ChuB	FhuB, (*Bacillus cereus*).	Ferrichrome transport system permease	29% over 313 amino acids.
ChuC	HmuV, (*Fusobacterium nucleatum*).	Hemin transport system ATP binding protein.	39% over 253 amino acids.
ChuC	FecE, (*Clostridium tetani*).	Putative hemin system ATP binding protein.	38% over 250 amino acids.
ChuC	FhuC (*Rhizobium leguminosarum*).	Ferrichrome transport system ATP binding protein.	32% over 248 amino acids.
ChuD	HmuT, (*Fusobacterium nucleatum*).	Hemin binding periplasmic protein.	33% over 257 amino acids.
ChuD	CeuE, (*C. coli*)	Enterochelin binding periplasmic protein.	24% over 234 amino acids.

3.2.2. Assessing *chu* gene distribution among *C. jejuni* strains by PCR.

Initially it was desirable to gain a rapid assessment of distribution of *chu* genes in a number of *C. jejuni* and other *Campylobacter* strains. This was carried out by PCR using three primer combinations. For each strain three PCR reactions were carried out on chromosomal DNA preparations, using the primer hpo568 plus another primer in each reaction. Preliminary PCR reactions were carried out using chromosomal DNA from *C. jejuni* lab strains to assess the best primer combinations to use. The primer hpo568 was designed to anneal to DNA outside the *chu* operon in the ORF Cj1618 and was selected as the universal primer in these reactions because it was found to produce PCR products when used in combination with the other test primers for all strains tested. This demonstrated

not only the presence of the *chu* operon in all strains initially tested but also conservation of the genomic organisation. Interestingly a primer designed to anneal to the 3'region of *chuA* (ChuAF) failed to give PCR products using DNA from strains other than NCTC11168, demonstrating sequence variation between *C. jejuni* strains within this region (data not shown).

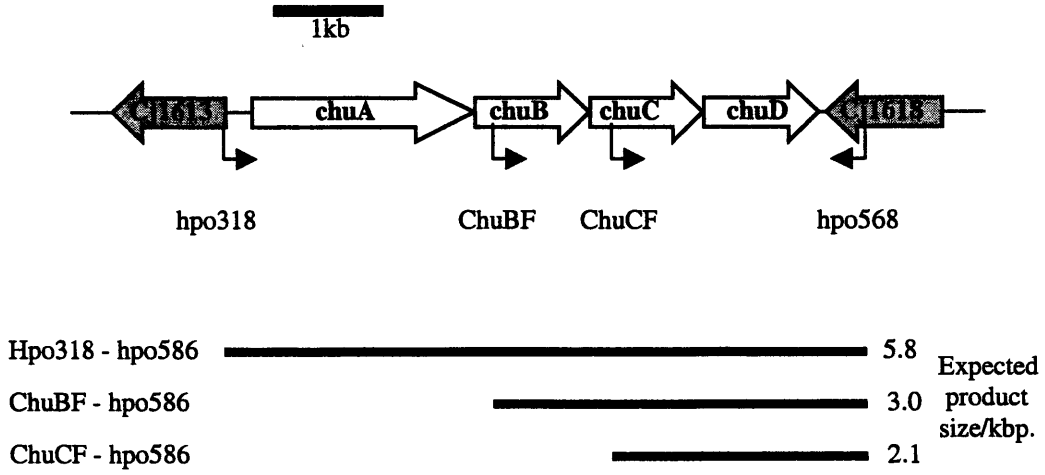

Figure 3.4. The sites of primer annealing and PCR products produced to investigate the presence of *chu* genes in a number of *C. jejuni* strains. Block arrows represent the arrangement of genes on the *C. jejuni* NCTC11168 genome; white arrows represent *chu* genes, grey arrows represent flanking ORFs. Primer annealing position and orientation (5' to 3') are represented by bent line arrows. The relative sizes of PCR extension products can be seen in the lower part of the diagram denoted by black lines.

The presence of several *chu* gene sequences was demonstrated by primer annealing and production of several PCR products from different regions of the *chu* operon. In order to achieve this hpo568 was used in combination with hpo318, ChuBF and ChuCF, to show that gene sequence was present from the regions flanking the *chu* operon, *chuB* and *chuC*. In *C. jejuni* NCTC 11168

these primer combinations produced products of 5.8 kbp (encompassing the entire operon), 3.0kbp (inside *chuB* to *Cj01618*) and 2.1kbp (inside *chuC* to *Cj01618*) respectively. The results produced from a variety of *Campylobacter* strains are shown in table 3.2.

Table 3.2

Strain.	**Presence of PCR products of expected size.**			**References.**
	hpo568-ChuCf	**hpo568-ChuBf**	**hpo568-hpo318**	
11168	+	+	+	R.Owen, National Collections of Type Cultures and Pathogenic Fungi, Colindale, London, UK.
11351	+	+	+	
81-176	+	+	+	(Russel *et al.*, 1989)
11828 (81116)	+	+	+	T.Wassenaar, University of Mainz, Germany.
27/F155	+	+	+	A.Swann, Public Health Laboratory, Leicester Royal Infirmary, Leicester, UK.
4F/182	+	+	+	
2F/68	+	+	+	
8F/169	+	+	+	
N82	+	+	+	(Everest *et al.*, 1992)
K85	+	+	+	

C. coli O73	+	+	+	
E206	+	+	+	
J75	+	+	+	
B404`	-	-	-	
HS10	+	+	+	N. Oldfield, university of Leicester, Leicester, UK.
H53	+	+	+	
G1	+	+	+	N.Gregson, Guy's Medical School, London, UK
G 2	+	+	+	
2561/90	+	+	+	D.Wareing, Public Health Laboratory, Royal Preston Hospital, Preston, UK.
2608/90	+	+	+	
2523/90	+	+	-	
306/90	-	-	-	
1915/91	-	-	-	
2258/90	-	+	-	
C. fetus 10842	-	-	-	National Collections of Type Cultures and Pathogenic Fungi, Colindale, London, UK.

As can be seen from table 3.2, the majority of *Campylobacter* strains tested produced products of the right size when analysed using the primer combination described. Many of the template DNA preparations used originate from strains obtained during routine clinical screening of patients. In order ensure the maximum genetic variation possible between strains; a number of strains were

selected that each expressed a different heat stable serotype. It seems therefore that this strain is widely distributed amongst *C. jejuni* strains.

The only evidence suggesting that possession of the *chu* system is not essential to pathogenesis, is the lack of some or all of the bands in a small number of strains. In the strains that have produced some bands but not others (2258/90 and 2523/90) it is likely that the absent bands were not produced due to slight sequence variation at the sites of primer annealing. Variation in gene sequence at the 5' region of the *chuA* ORF has already been observed during preliminary PCR experiments (data not shown), so sequence variation among the *chu* genes of *C. jejuni* strains is possible.

3.2.3. Cloning of the *chuA* promoter region.

Previously ChuA protein expression had been observed to be under the control of Fur in response to iron limitation (van Vliet *et al.*, 1998). It was hypothesised that the four *chu* genes were co-expressed from a single promoter present in the small intergenic region between *Cj1613* and *chuA*, under the control of the Fur protein. In order to demonstrate promoter activity the Cj1613/*chuA* intergenic region was analysed by an established *Campylobacter* promoter assay described previously (Wosten *et al.*, 1998). By this method the Cj1613/*chuA* intergenic region was amplified by PCR (using the Cj1613R2 and ChuAR4 oligonucleotide primers) and cloned into the vector pMW10 at a *Bam*HI site. This would produce two plasmid species each comprising of the pMW10 vector containing the intergenic region ligated in either orientation. Upon transformation of the

ligation mixture into *E. coli* DH5α, transformants were then selected for by plating the bacteria on media containing kanamycin. The presence of either desired plasmid species was checked by PCR (using Cj1613R2-LacR1 and Cj1613R2-MwF1 oligonucleotide primer combinations). The inserts of promoter/plasmid constructs were then sequenced to check for possible PCR incorporation errors (using LacR1 and MwF1 oligonucleotide primers). Once both the plasmid species were obtained they were named pJDR13 and pJDR14, pJDR13 had the promoter insert ligated into the plasmid so that p*chuA* was a transcriptional fusion with *lacZ* gene (see figure 3.4). The plasmid pJDR14 had the insert ligated in the opposite orientation so that pCj1613 was a transcriptional fusion with the *lacZ* gene (see figure 3.4). In this manner, LacZ assays carried out on strains containing pJDR13 would measure the *chuA* (and hypothetically all the other *chu* genes) promoter activity and pJDR14 would measure the Cj1613 promoter activity. Diagrams of pJDR13 and pJDR14 may be found in the appendix.

3.2.4. Assaying promoter activity of the *chuA* promoter region.

Once transformed into *C. jejuni* 480, strains containing pJDR13 and pJDR14 were cultured to OD 0.4 in Mueller-Hinton broth made iron restricted using desferal (20μM) and iron replete using iron sulphate (40μM). In this way control of promoter activity in response to iron could also be measured. The experiment was repeated at least three times (to ensure consistency of results and obtain standard deviation values). The results can be seen in figure 3.5

Chapter 3. Analysis and cloning of the *chu* genes.

Three strains of *C. jejuni* 480 containing plasmids were used as controls. *C. jejuni* 480 containing the vector plasmid, pMW10 was included as a negative control. *C. jejuni* 480 containing p23E5 (pMW10 containing the constitutively expressed *metK* promoter (Wosten *et al.*, 1998)) was included as a non-iron responsive positive control. *C. jejuni* 480 containing pAV201 (pMW10 containing the *C. jejuni* 81116 *katA* promoter region, (van Vliet, 2003)) was included as an iron responsive positive control. From the β-galactosidase activity derived from 480(pJDR13) demonstrates greater promoter activity in iron-limited conditions, than any of the control plasmids. In iron-replete conditions however LacZ activity is less than 3% of the level in iron-limited conditions. This demonstrates that the *chuA* promoter is clearly controlled in response to iron limitation, this confirms previous observations of ChuA protein expression (van Vliet *et al.*, 1998) by direct analysis of the *chu* promoter region. Transcription of *chuA* is controlled by Fur in an iron responsive manner. The 480(pJDR14) *lacZ* activity under iron limited condition is high, at least twice the *lacZ* activity of pJDR13. Under iron-replete conditions *lacZ* activity is 14% of that demonstrated under iron-limited conditions. The *lacZ* activity in iron replete conditions 14% of that under iron-limited conditions, this compares with 3% for *chuA*. Comparison of iron regulation demonstrates that *Cj1613* expression is also regulated in response to iron level albeit not as tightly as *chuA*.

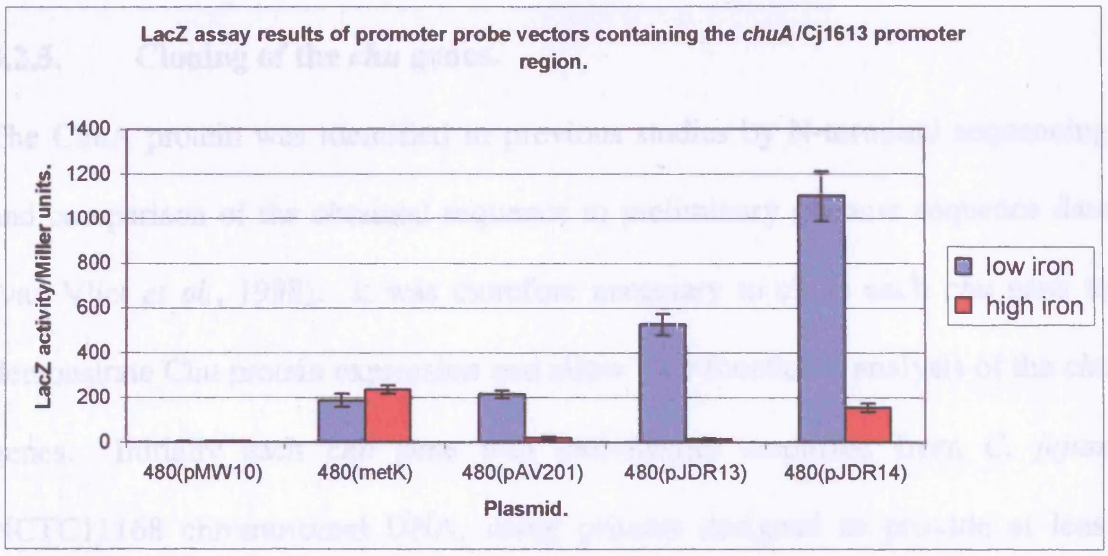

Figure 3.5 LacZ assays on pJDR13 and pJDR14, with negative control pMW10 only, positive control pMW10 containing the constitutively expressed *metK* promoter and an iron responsive control pAV201 (pMW10 containing the *C. jejuni* NCTC81116 *katA* promoter region) promoter. These results measure LacZ activity into *chuA* (pJDR13) in both iron limited and iron-replete conditions, and into Cj1613 (pJDR14) in both iron limited and iron-replete conditions.

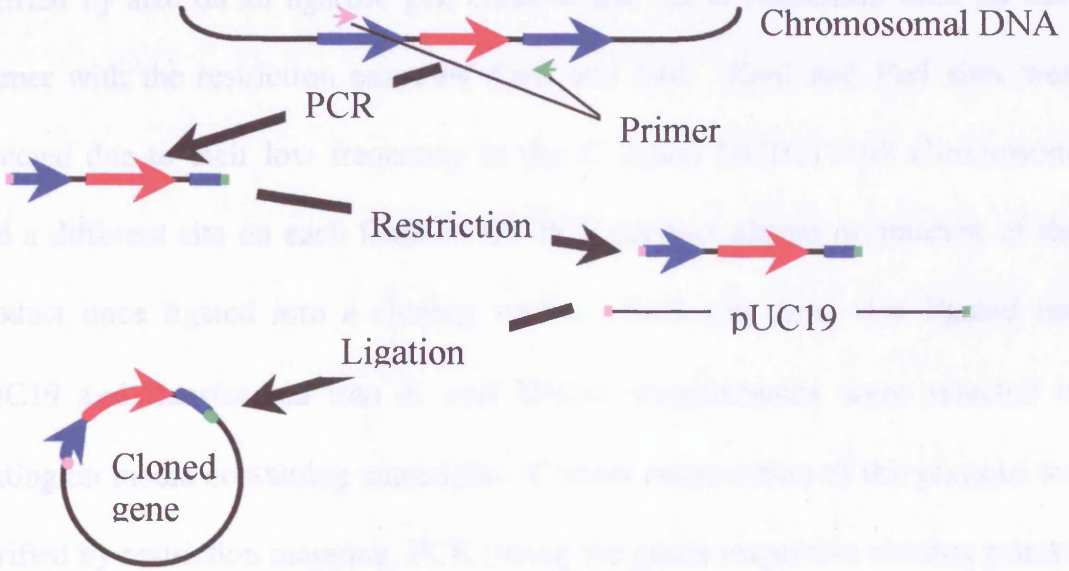

Figure 3.6. A graphic representation of the cloning strategy used in this chapter. Firstly the target gene (red arrow) is amplified by PCR from chromosomal DNA, using primers designed to give 500bp of flanking sequence (blue arrows). The primers had a *Kpn*I (pink arrow) and *Pst*I (green arrow) restriction site incorporated at the 5' end. This allowed restriction of the PCR product for ligation into *Kpn*I and *Pst*I cut pUC19 in a particular orientation. The ligation products were electro-transformed into E.coli DH5α and plated on L-agar containing X-gal. White colonies (plasmid containing an insert) were selected and the plasmid they contained was extracted and verified by restriction mapping, PCR and sequencing.

3.2.5. Cloning of the *chu* genes.

The ChuA protein was identified in previous studies by N-terminal sequencing and comparison of the obtained sequence to preliminary genome sequence data (van Vliet *et al.*, 1998). It was therefore necessary to clone each *chu* gene to demonstrate Chu protein expression and allow later functional analysis of the *chu* genes. Initially each *chu* gene was individually amplified from *C. jejuni* NCTC11168 chromosomal DNA, using primers designed to provide at least 500bp either side of the ORF (see figure 3.6). The flanking DNA is required for homologous recombination of the mutated gene back into the *C. jejuni* chromosome at a later date. Once amplified, each *chu* gene PCR product was verified by size on an agarose gel, cleaned and cut at restriction sites on each primer with the restriction enzymes *Kpn*I and *Pst*I. *Kpn*I and *Pst*I sites were selected due to their low frequency in the *C. jejuni* NCTC11168 chromosome and a different site on each flank of the PCR product allows orientation of that product once ligated into a cloning vector. Each *chu* gene was ligated into pUC19 and transformed into *E. coli* DH5α, transformants were selected by plating on media containing ampicillin. Correct construction of the plasmid was verified by restriction mapping, PCR (using the genes respective cloning primers with M13F or M13R primer combinations) of the insert and sequencing. Each plasmid containing a cloned gene was named (see table 3.3).

Table 3.3 Construct names of each cloned *chu* gene in pUC19.

Plasmid designated name.	Contents	Cloning primer used
pJDR1	pUC19 containing cloned *chuA*	Hpo318-ChuBR
pJDR2	pUC19 containing cloned *chuB*	ChuAF-ChuCR
pJDR3	pUC19 containing cloned *chuC*	ChuBF-ChuDR
pJDR4	pUC19 containing cloned *chuD*	ChuCF-Hpo586

3.3. Discussion.

Sequence analysis of the region containing the *chu* genes demonstrated the presence of four *chu* genes arranged in an apparent operon with an upstream putative promoter region. The organisation of the *chu* genes and the lack of T-loop structures between *chuA* and *chuB* indicate the chu genes are probably co-expressed from a single promoter. It is possible that embedded promoters exist within the *chu* genes meaning that *chuB*, *C* or *D* may be expressed from a different promoter to *chuA*. This was in the process of being investigated at the end of the project, by cloning of the *chu* intergenic regions into pMW10 and *lacZ*. Unfortunately the project ended before this work was completed. The presence of a putative Fur box upstream of *chuA* indicates that expression of the *chu* proteins are regulated by Fur. The presence of a Fur box supports the

observation that ChuA expression is de-repressed in the *C. jejuni* fur mutant (van Vliet *et al.*, 1998).

Analysis of putative protein sequence expressed from the *chu* genes, reinforced the results of previous sequence analysis of *chu* genes and proteins (van Vliet *et al.*, 1998). Based on similarity to proteins from other organisms it may be predicted that *chu* genes encode a hemin uptake system, comprising of a TonB dependent outer membrane receptor (ChuA) and a periplasmic binding protein dependent ABC transport system. The ABC transport system comprised of genes with amino acid similarity to putative membrane permeases (ChuB), ATP binding proteins (ChuC) and periplasmic binding proteins (ChuD). The Chu proteins appear to show greatest amino acid sequence similarity to proteins involved in the uptake of haem in other organisms, principally *Fusobacterium nucleatum*. *Fusobacterium nucleatum* is a Gram-negative bacterium principally found in the oral micro-flora, which also has a low GC% (27%) of its genome sequence (Kapatral *et al.*, 2002). The amino acid sequence similarity of *chu* proteins to *F. nucleatum* haem uptake proteins may be a result of codon preference in AT rich organisms or may imply DNA exchange between these organisms.

The results of the PCR which tested the distribution of *chu* gene sequences among *C. jejuni* strains demonstrated that possession of *chu* gene sequences appears to be widespread amongst *C. jejuni* strains, and that *chu* gene sequences are present in at least 1 *C.coli* strain (O73). *C. jejuni* infection produces causes inflammatory diarrhoea, involving tissue damage, excretion of blood and pus and

the possible invasion of tissues and blood. The course of infection would bring *C. jejuni* into contact with significant amounts of host and food derived haem-containing molecules. This being the case, then *C. jejuni* within the host will experience significant advantage from the possession of a haem uptake system. The ability to utilise haem-containing molecules has shown to be important for virulence in a human model by pathogenic *Haemophilus* species (Al Tawfi *et al.*, 2000). The ability to use haem as an iron source allows the pathogen access to an enormous potential reservoir of host iron.

Some chromosomal DNA preparations did not produce the expected PCR products, possibly due to failure of primer annealing. This is probably caused by sequence variability between strains at the site of primer annealing because sequence variation between strains was observed during preliminary PCR reactions in the 5' region of the *chuA* ORF (section 3.2.2). This experiment was designed to give rapid assessment of the distribution of *chu* genes amongst *C. jejuni* strains and so the results were not followed up. Once molecular characterisation of other *C. jejuni* iron acquisition systems has been achieved it will be possible to correlate the possession of an iron uptake system (or combination of iron uptake systems) with frequency of infection and severity of symptoms produced by a *C. jejuni* strain. This could be carried out by the PCR system described in this chapter with the use of DNA hybridisation on strains failing to produce PCR products. Southern blotting would be more time consuming but would minimise false negatives produced by sequence variation in primer annealing regions.

From the analysis of the *chu* 'promoter' region it is possible to observe the presence of two iron regulated promoters allowing transcription into the *chu* genes and into Cj1613. Fur regulated expression of the ChuA protein and the predicted ChuD protein had been previously demonstrated (van Vliet *et al.*, 1998). The promoter of the *chu* genes is tightly iron regulated, an observation consistent with the promoter of an iron uptake system. The extremely low level of expression under iron-replete conditions assists in maintaining the iron homeostasis by preventing any further import of iron into the cell. High levels of expression under iron -restricted condition produce an effective response to this form of environmental stress. The possible effect of multiple plasmid copies, supercoiling and context-based effects (the presence of promoter sequences on a plasmid rather in their normal position on the chromosome) meant that further data was not extrapolated from this experiment.

Subsequent studies have achieved expression of the *C. jejuni* Fur protein using pASK-IBA vectors and have demonstrated *C. jejuni* Fur binding to the promoters of *cfrA*, *p19*, *ceuE* and *chuA* (Ketley, 2003). This supports the evidence presented in this study that Chu expression is regulated by Fur.

Expression of Cj1613 is repressed under iron replete conditions, although not as tightly as *chu* expression. The function of the Cj1613 gene product is not currently known, although the Cj1613 protein does display similarities to HugZ, a protein required for haem utilisation in *Plesiomonas shigelloides* (Henderson *et al.*, 2001). The Cj1613 gene is a target for future research into haem-iron acquisition in *C. jejuni*.

Chapter 3. Analysis and cloning of the *chu* genes.

In summary four genes are present on the *C. jejuni* genome arranged in an apparent operon. The first gene *chuA* encodes a protein with amino acid similarity to TonB dependent outer membrane haem receptors. The *chuB*, *C* and *D* genes display similarity to ABC-transport systems involved in haem uptake. Possession of these genes appears to be widely distributed among *C. jejuni* strains. ChuA is expressed from a promoter in the intergenic region upstream of *chuA* under control of the Fur protein in response to iron. A gene arranged divergently from *chuA*, Cj1613, is expressed from a second Fur-regulated iron-responsive promoter within the intergenic region between *chuA* and Cj1613. This gene demonstrates similarity to an accessory protein involved in haem-iron acquisition from *Plesiomonas shigelloides* (Henderson *et al.*, 2001).

4. Results: Functional analysis of the *chu* genes.

4.1. Introduction.

Cloning of the individual *chu* genes makes it possible to assess the function of each gene and its respective protein. Prior to this study the function of ChuA was linked to haem uptake due to its amino acid sequence similarity to the haem receptors of other bacterial species (van Vliet *et al.*, 1998) and abolition of expression of a 70kDa protein in a haem-uptake mutant (Pickett *et al.*, 1992). ChuB, C and D had been linked to haem uptake due to their genetic organisation and presumed co-expression with ChuA. In order to demonstrate the function of the *chu* genes it was necessary to individually inactivate each *chu* gene by mutation. This can be achieved by directed mutagenesis of each individual *chu* gene by insertional inactivation. Phenotypic analysis may then be carried out to assess the ability of each mutant to grow using hemin as an iron source. This will allow analysis of the function of each Chu protein in the hemin uptake pathway to be assessed based on the phenotype of each mutant and prior characterisation of the Chu proteins (Chapter 3, section 3.2.1)

4.2. Results.

4.2.1. Construction of *C. jejuni chu* mutants.

The cloned *chu* genes on plasmids pJDR1, pJDR2, pJDR3 and pJDR4 (constructed in chapter 3, section 3.2.5) were used to create *C. jejuni chu* gene mutants. The cloned genes were deleted using Inverse PCR Mutagenesis (IPCRM), and an antibiotic resistance cassette ligated in place of the deleted region (materials and methods, section 2.3.1) (Wren *et al.*, 1994). In this case a Chloramphenicol Actetyl-Transferase (CAT) cassette was ligated in place of the *chu* gene. When the ligation mixture is transformed into *E. coli* DH5α the presence of a CAT cassette allows selection of transformants with chloramphenicol. Plasmids pJDR1, pJDR2 pJDR3 and pJDR4 containing insertionally inactivated *chu* genes were verified by PCR and restriction mapping, and sequenced to ensure no PCR errors were present in the flanking DNA. Four verified plasmid preparations were selected which each contained a deletion of one *chu* gene and each was named (see table 4.1)

Table 4.1

Designated plasmid name	Contents	Deletion primers used	Mutant strain produced.	Verification primers used
pJDR5	pUC19 containing deleted *chuA* gene, ligated with a CAT cassette. *chuA* mutation construct.	ChuAR2-ChuAF2	JDR5	CatR1, CatF1, PrfAF1, ChuCR.
pJDR6	pUC19 containing deleted *chuB* gene, ligated with a CAT cassette. *chuB* mutation construct.	ChuBR2-ChuBF2	JDR6	CatR1, CatF1, Hpo318R, ChuDR
pJDR7	pUC19 containing deleted *chuC* gene, ligated with a CAT cassette. *chuC* mutation construct.	ChuCR2-ChuCF2	JDR7	CatR1, CatF1, ChuAF, Hpo568F.
pJDR8	pUC19 containing deleted *chuD* gene, ligated with a CAT cassette. *chuD* mutation construct.	ChuDF2-ChuDR2	JDR8	CatR1, CatF1, ChuBF, Cj1618F2.

An overview of the cloning and deletion strategy up to this point can be found in figure 4.2. Deleted gene constructs of each *chu* gene were selected and transformed back into *C. jejuni* NCTC11168. Transformants were plated on media containing chloramphenicol to select for recombinant strains containing the CAT marker cassette. Mutant strains were verified by PCR characterisation of chromosomal DNA, the mutant strains produced using each deletion construct were named (table 4.1). An example of the PCR verification strategy used to verify *chuA* allelic exchange had occurred can be seen in figure 4.1, the primers used to verify each *chu* gene allelic exchange can be seen in table 4.1.

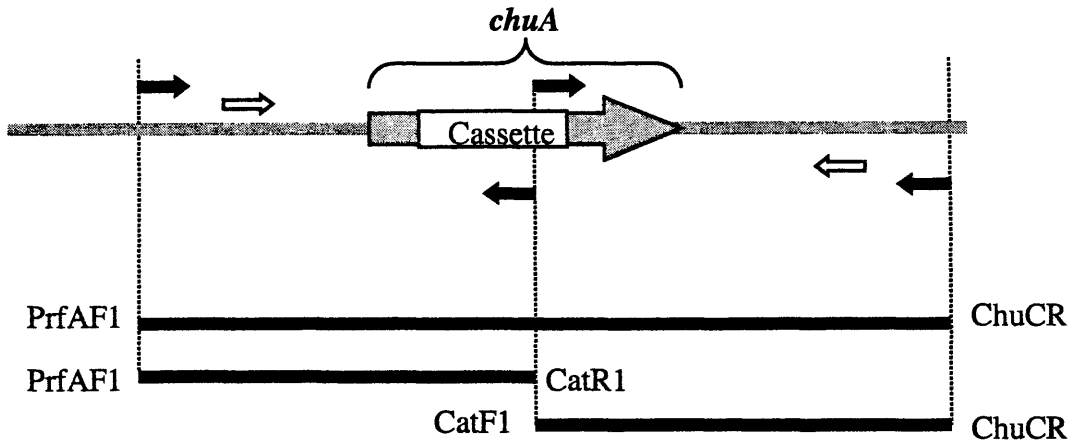

Figure 4.1. An example of the PCR strategy used to verify that a *chu* gene had been mutated correctly. PCR reactions were set up containing three primer combinations, one primer combination produced a PCR product that spanned the entire cloned region (PrfAF1-ChuCF), note that these primers anneal outside the region originally cloned (white arrows are the annealing positions of the original cloning primers). The two other primer combinations produce PCR products that begin within the Cat cassette and extend to outside the originally cloned region (CatR1-PrfAF1 and CatF1-ChuCR).

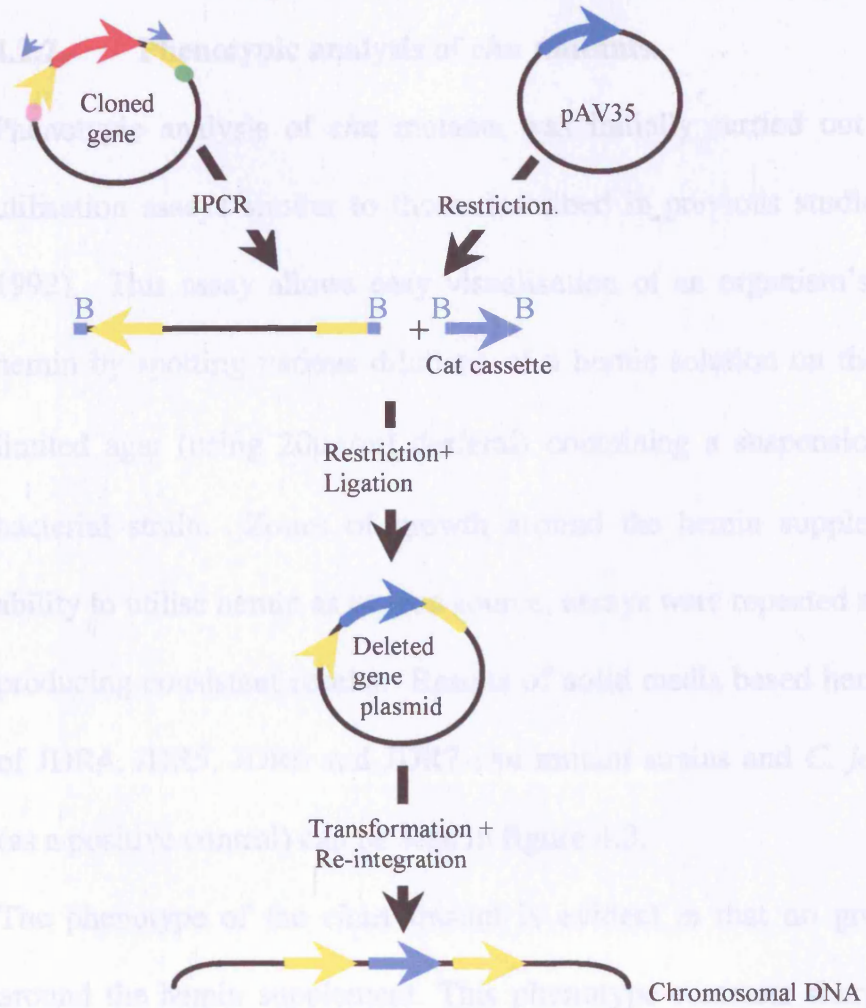

Figure 4.2, an overview of the mutation procedure starting from the cloned gene (chapter 3). Primers are designed which anneal to either end of the cloned gene (small blue arrows), allowing PCR extension out into the flanking regions (yellow lines). IPCR produces a linear product that comprises of vector DNA, cloned flanking regions and novel *Bam*HI sites at each end (blue Bs). The *Bam*HI sites allow restriction of the IPCR product producing "sticky ends" which facilitates ligation of an antibiotic resistance marker gene (blue arrow) in place of the original cloned gene. The re-circularised deleted gene product is then transformed back into *C. jejuni* NCTC11168, which cannot maintain plasmids. The plasmid may re-integrate back into the chromosome by a double crossover recombination event between flanking regions on the construct and flanking regions on chromosomal DNA. Antibiotic resistance due to the presence of an antibiotic resistance marker may identify bacteria containing *chu* gene insertional deletions. Allelic exchange replaces the gene originally cloned with a deleted copy, producing a mutant strain.

4.2.2. Phenotypic analysis of *chu* mutants.

Phenotypic analysis of *chu* mutants was initially carried out using plate iron utilisation assays similar to those described in previous studies (Pickett *et al.*, 1992). This assay allows easy visualisation of an organism's ability to utilise hemin by spotting various dilutions of a hemin solution on the surface of iron-limited agar (using 20μg/ml desferal) containing a suspension of the relevant bacterial strain. Zones of growth around the hemin supplement indicate an ability to utilise hemin as an iron source, assays were repeated at least three times producing consistent results. Results of solid media based hemin growth assays of JDR4, JDR5, JDR6 and JDR7 *chu* mutant strains and *C. jejuni* NCTC11168 (as a positive control) can be seen in figure 4.3.

The phenotype of the *chuA* mutant is evident in that no growth can be seen around the hemin supplement. This phenotype contrasts sharply with *C. jejuni* NCTC11168 results, where it is possible to see a zone of growth stimulation around the hemin supplements. This indicates that the ChuA outer membrane receptor is absolutely required for the uptake of hemin and that mutation of *chuA* abolishes the ability of *C. jejuni* to use hemin as an iron source. Significant variability of growth zone size was observed between experimental repetitions demonstrating that comparison of zone size between repetitions may be a poor indicator of uptake ability, therefore presence /absence of a growth zone only was noted.

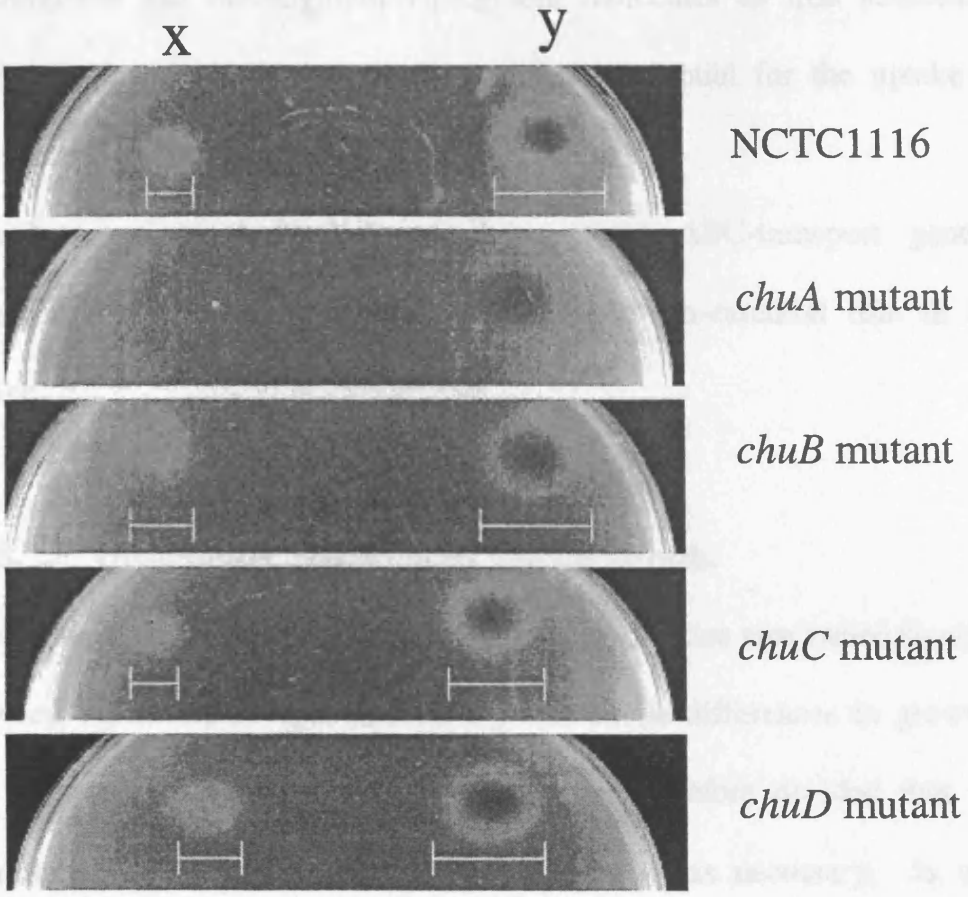

Figure 4.3 *C. jejuni* growth stimulation provided by hemin supplementation on the surface of iron limited (20μg/ml deferral) MH agar. The results are shown for *C. jejuni* NCTC11168 and the four *chu* mutant strains, JDR5 (*chuA* mutant), JDR6 (*chuB* mutant), JDR7 (*chuC* mutant) and JDR8 (*chuD* mutant). The left spot (x) is a supplement of 10μl of 0.1μg/ml hemin solution and the right spot (y) is 10μl supplement of 1μg/ml hemin solution. Pale zones of growth stimulation can be seen around the dark hemin supplement, where such zones are present the width of the zone of growth stimulation is delineated by a white line.

The *chuB* mutant, *chuC* mutant and *chuD* mutant are indistinguishable from the wild type parent strain in their ability to utilise hemin as an iron source, meaning none of these genes are essential for hemin uptake. Repetition of this experiment using haemoglobin or haemoglobin-haptoglobin solution as a supplement gave identical patterns of growth as seen with hemin for all mutant strains (data not shown). This indicates that ChuA is essential for the utilisation of hemin,

haemoglobin and haemoglobin-haptoglobin molecules as iron sources. The *chuB*, *chuC* and *chuD* gene products are non-essential for the uptake of the haem-based molecules used in this study.

Complementation of function of the mutated ABC-transport genes was considered as a possible explanation for their non-essential role in haemin uptake.

4.2.3. Quantitative assays of *chu* mutant growth.

In solid media growth assays (section 4.2.2) growth zone size varied significantly between experimental repeats, meaning that subtle differences in growth zone size between strains may be missed. It was therefore decided that a more quantitative method of measuring hemin uptake was necessary. In order to measure quantitatively hemin growth stimulation of *C. jejuni* strains in iron-limited media it was decided to measure growth over a time period. This was achieved by culturing the wild type strain and the four *chu* mutant strains over a 24hr period, using iron replete Mueller-Hinton broth (40μM FeSO4), iron limited conditions (20μM desferal) and iron-limited Mueller-Hinton broth (20μM desferal, 1μg/ml hemin) supplemented with hemin. Little variation in growth rate and cell density was observed between *C. jejuni* NCTC11168 and the mutant strains (JDR5, JDR6, JDR7and JDR8) during growth in iron replete media and iron restricted media over a 24hr period (an example is shown in figure 4.4a and figure 4.4b).

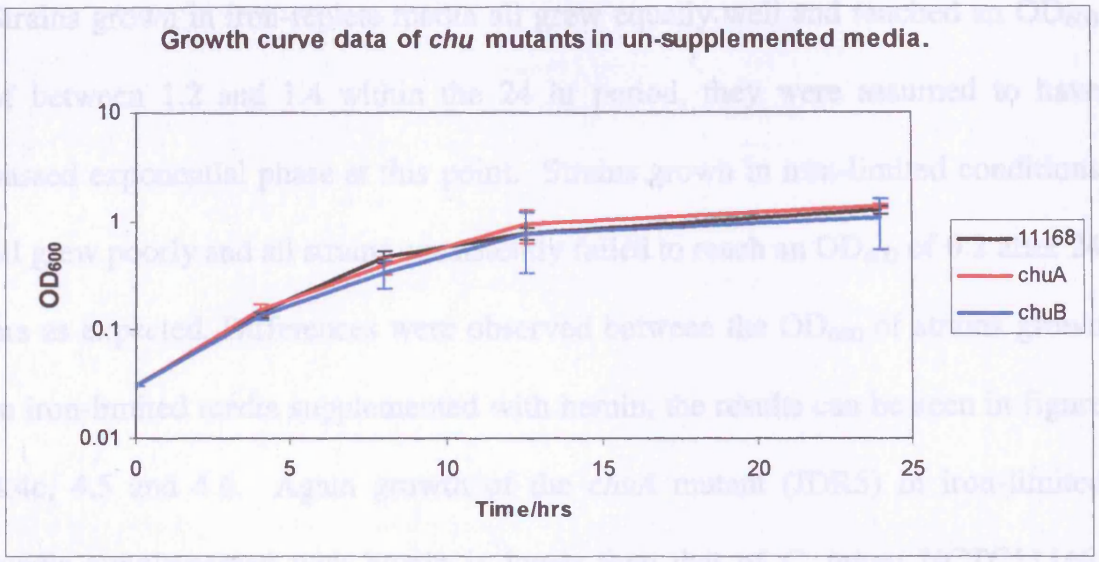

Figure 4.4.a Growth of *C. jejuni* NCTC11168, *chuA* (JDR5) and *chuB* (JDR6) mutants in un-supplemented Mueller-Hinton broth, over a 24hour time period.

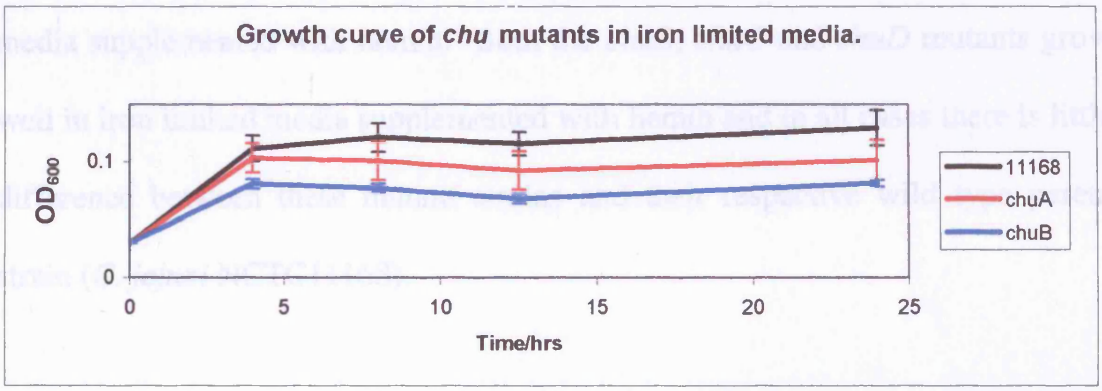

Figure 4.4.b Growth of *C. jejuni* NCTC11168 (black line), *chuA* (JDR5; red line) and *chuB* (JDR6; blue line) mutants in iron-limited media Mueller-Hinton broth (20μM desferal) over a 24hour time period.

Strains grown in iron-replete media all grew equally well and reached an OD_{600} of between 1.2 and 1.4 within the 24 hr period, they were assumed to have passed exponential phase at this point. Strains grown in iron-limited conditions all grew poorly and all strains consistently failed to reach an OD_{600} of 0.2 after 24 hrs as expected. Differences were observed between the OD_{600} of strains grown in iron-limited media supplemented with hemin, the results can be seen in figure 4.4c, 4.5 and 4.6. Again growth of the *chuA* mutant (JDR5) in iron-limited media supplemented with hemin is lower than that of *C. jejuni* NCTC11168. Little difference is observed between the ability of the *chuA* mutant to grow in iron limited media alone (by comparison of figure 4.4b and 4.4c) or iron limited media supplemented with hemin. Both the *chuB*, *chuC* and *chuD* mutants grow well in iron limited media supplemented with hemin and in all cases there is little difference between these mutant strains and their respective wild type parent strain (*C. jejuni* NCTC11168).

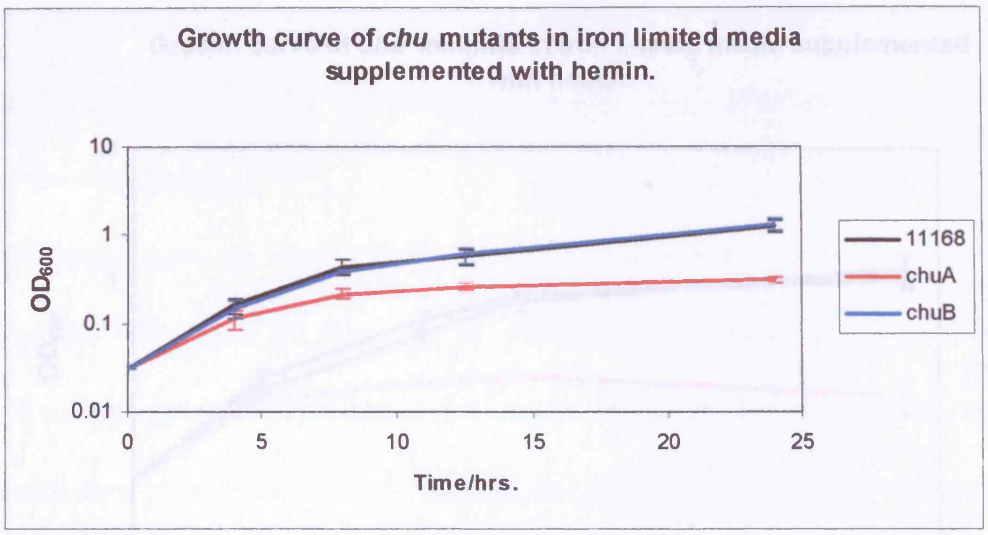

Figure 4.4.c Comparison of the optical densities of cultured strains over a 24hr period, the optical density is plotted on a logarithmic scale. The growth of *C. jejuni* NCTC11168, the *chuA* mutant strain (JDR5) and the *chuB* mutant strain (JDR6) was measured (OD_{600}) in iron-limited media supplemented with 1μg/ml hemin over a 24hr time course. Mean growth rates and standard error values were calculated from data produced from three experimental runs.

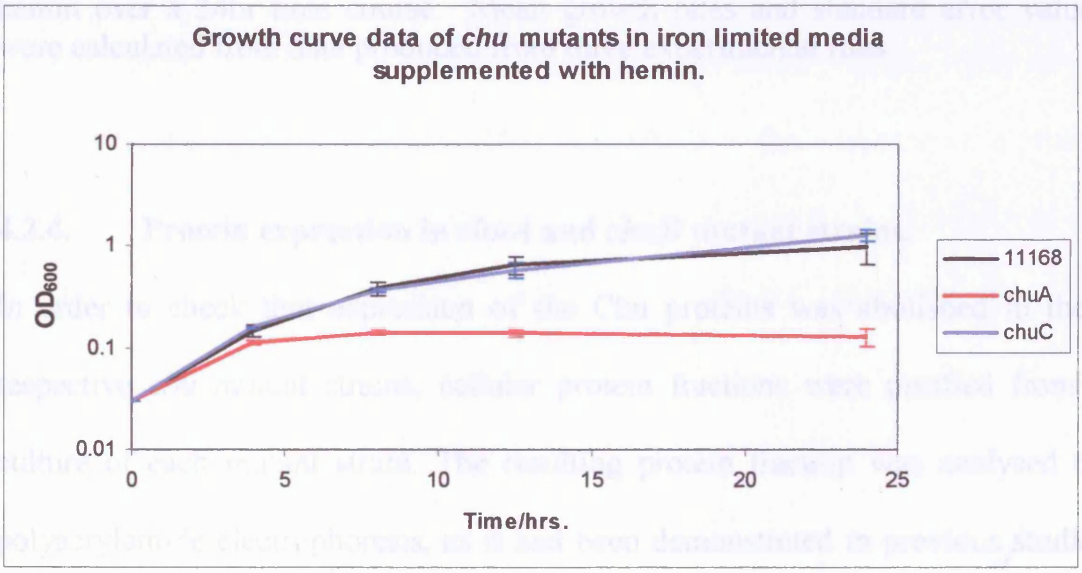

Figure 4.5. Comparison of the optical densities of cultured strains over a 24hr period, the optical density (y-axis) is on a logarithmic scale. The growth of *C. jejuni* NCTC11168, the *chuA* mutant strain (JDR5) and the *chuC* mutant strain (JDR7) was measured (OD_{600}) in iron-limited media supplemented with 1μg/ml hemin over a 24hr time course. Mean growth rates and standard error values were calculated from data produced from three experimental runs.

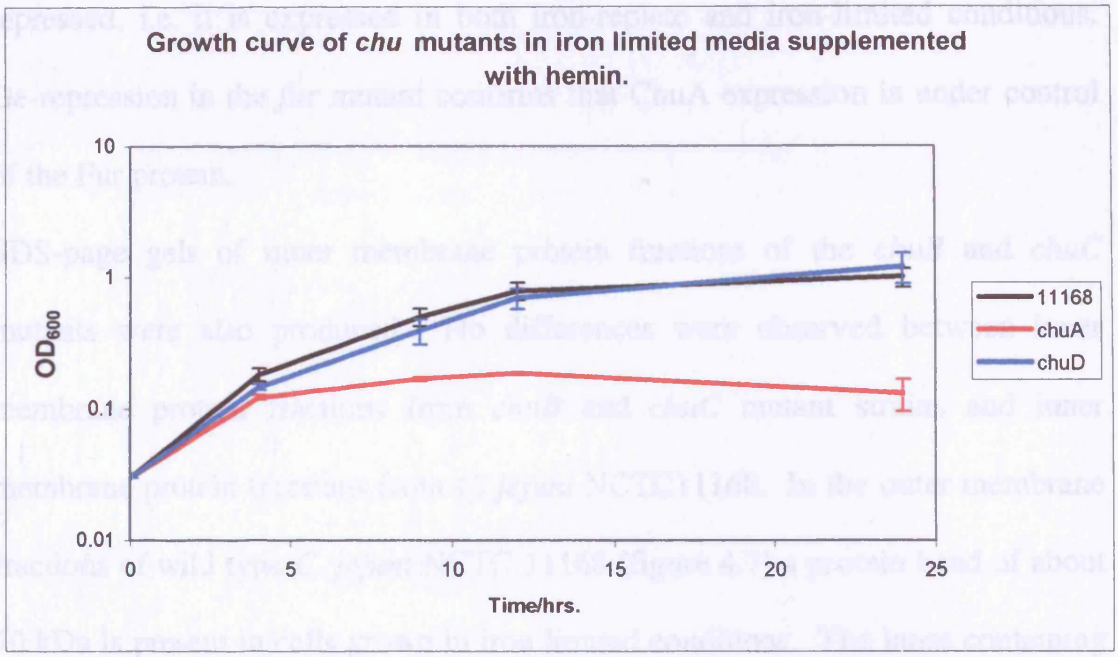

Figure 4.6. Comparison of the optical densities of cultured strains over a 24hr period, the optical density (y-axis) is on a logarithmic scale. The growth of *C. jejuni* NCTC11168, the *chuA* mutant strain (JDR5) and the *chuD* mutant strain (JDR8) was measured (OD_{600}) in iron-limited media supplemented with 1μg/ml hemin over a 24hr time course. Mean growth rates and standard error values were calculated from data produced from three experimental runs.

4.2.4. Protein expression in *chuA* and *chuD* mutant strains.

In order to check that expression of the Chu proteins was abolished in their respective *chu* mutant strains, cellular protein fractions were purified from a culture of each mutant strain. The resulting protein fraction was analysed by polyacrylamide electrophoresis, as it had been demonstrated in previous studies that ChuA could be visualised in the outer membrane fraction, and a protein proposed to be ChuD visualised in the periplasmic fraction of *C. jejuni* NCTC11168. In AV17, a previously constructed *fur* mutant strain of *C. jejuni* NCTC 11168 (van Vliet *et al.*, 1998) expression of ChuA is seen to be de-

repressed, i.e. it is expressed in both iron-replete and iron-limited conditions. De-repression in the *fur* mutant confirms that ChuA expression is under control of the Fur protein.

SDS-page gels of inner membrane protein fractions of the *chuB* and *chuC* mutants were also produced. No differences were observed between inner membrane protein fractions from *chuB* and *chuC* mutant strains and inner membrane protein fractions from *C. jejuni* NCTC11168. In the outer membrane fractions of wild type *C. jejuni* NCTC 11168 (figure 4.7) a protein band of about 70 kDa is present in cells grown in iron limited conditions. The lanes containing cells grown in iron-replete conditions were loaded with greater amounts of protein extract to demonstrate the negligible amounts of expression. Expression of the 70 kDa protein band is clearly absent in the *chuA* mutant confirming that *chuA* mutation abolishes the ability to express the 70kDa protein. Gels of the periplasmic fractions of *C. jejuni* NCTC 11168 show the presence of a protein band expressed only under iron limited conditions (figure 4.8). This protein can still be clearly seen in the *chuD* mutant strain cultured under iron-restricted conditions.

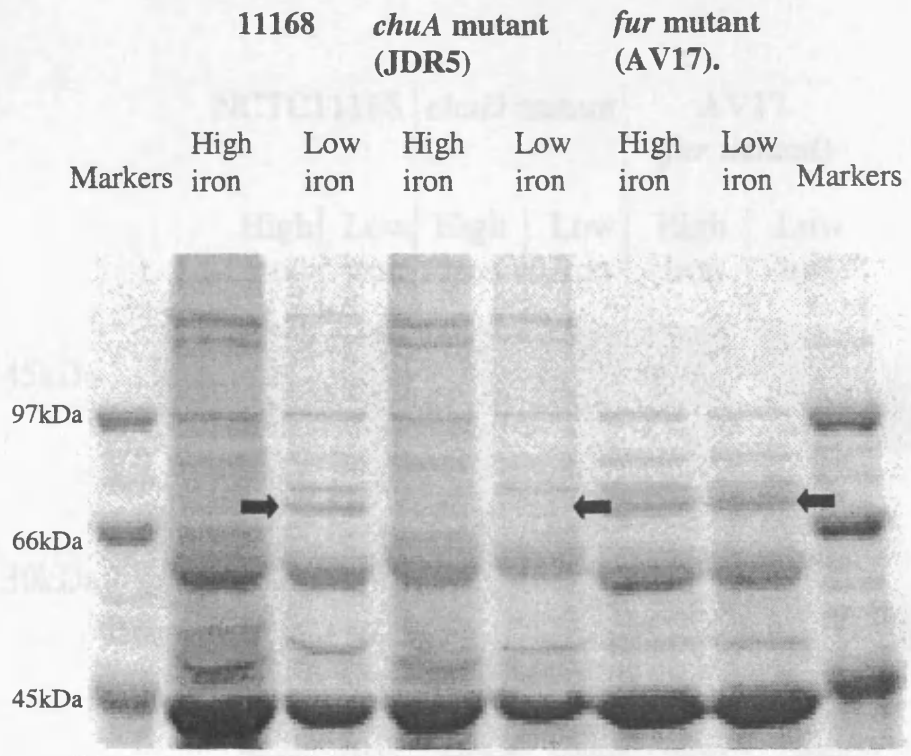

Figure 4.7. Electrophoresis of outer membrane proteins from *C. jejuni* NCTC11168, JDR5 a *chuA* mutant strain and AV17 a *fur* mutant strain on an 8% SDS-polyacrylamide gel stained with Coomassie brilliant blue stain. Proteins were harvested from each strain grown in iron-replete (high iron) and iron-limited (low iron) conditions. Black arrows demonstrate the position (or predicted position if protein is absent) of the ChuA protein.

This occurred consistently over several repetitions demonstrating this protein was not a contaminant. As the chromosomal deletion was verified at the DNA level showing that the mutation event had occurred, it was decided to N-terminal sequence the putative ChuD protein (Materials and Methods, section 2.6). Once sequenced it was discovered that this protein was not ChuD as previously assumed but the product of the ORF Cj1613 (figure 4.9). Cj1613 encodes a protein with a molecular weight of 29kDa and promoter analysis of this ORF (chapter 3) has shown Cj1613 expression is iron regulated.

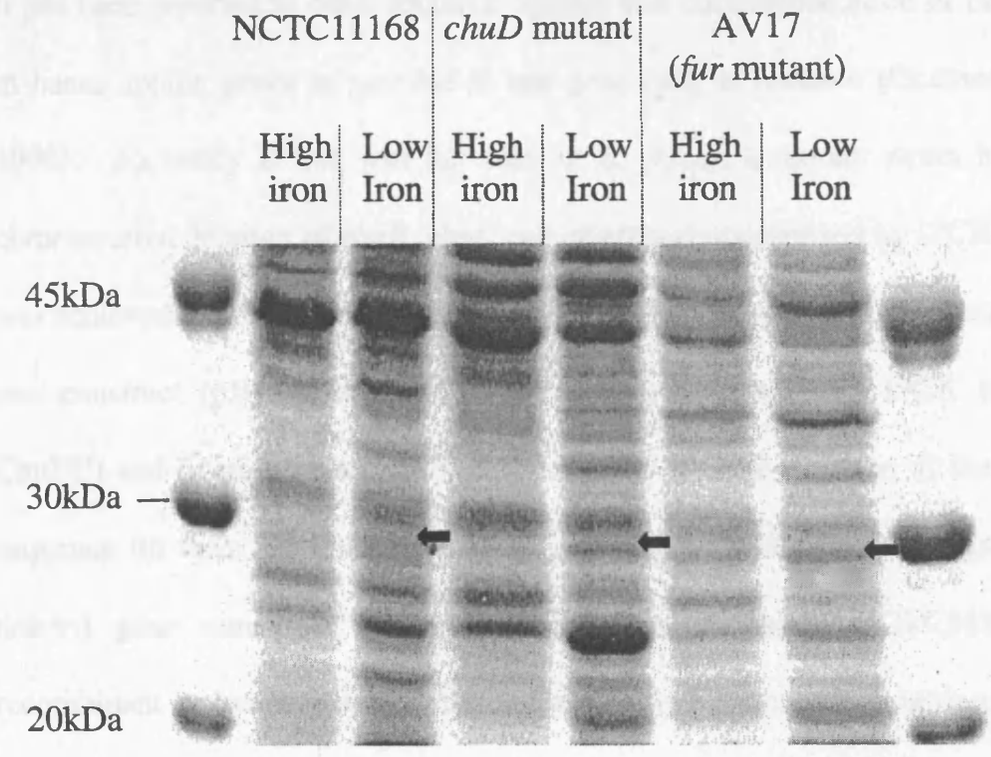

Figure 4.8. Electrophoresis of periplasmic proteins from *C. jejuni* NCTC11168, JDR8 a *chuD* mutant strain and AV17 a *fur* mutant strain on a 12% polyacrylamide gel stained with Coomassie brilliant blue stain. Proteins were harvested from each strain grown in iron-replete and iron-limited conditions. Black arrows demonstrate the position of the protein predicted to be ChuD.

N-terminal sequence.	M N F E S I I S
Cj1613 sequence.	M N F E S I I S
ChuD sequence.	M K K I L I I M

Figure 4.9. The results of N-terminal sequencing of the protein band predicted to be ChuD and alignment of the resulting sequence with the Cj1613 gene product

4.2.5. Construction of a *chuBCD* deletion mutant.

It has been reported in other bacterial species that complementation of mutations in haem uptake genes is possible if one gene only is mutated (Occhino *et al.*, 1998). To verify if this was the case in *C. jejuni*, a mutant strain having a chromosomal deletion of *chuB*, *chuC* and *chuD* was constructed by IPCRM. This was achieved by cloning *chuB*, *C* and *D* (PCR using ChuAF-HPO586 primers) in one construct (pJDR9) deletion of the coding sequences by IPCR (ChuBR-ChuDF) and ligation of an antibiotic resistance cassette in place of the deleted sequence (to form pJDR11 and pJDR12, see appendix for plasmid maps). The deleted gene construct was transformed into *C. jejuni* NCTC11168 and recombinant colonies selected by antibiotic resistance. An overview of the cloning and mutation procedure is in the materials and methods section 2.3.1. Two mutant strains were produced, one (JDR11) had the cat cassette ligated in the same transcriptional orientation as *chuA* and a second strain (JDR12) had the cassette ligated in the opposite transcriptional orientation. Once constructed and checked (by PCR using primers HPO316, CatR1, CatF1 and Cj1618F2), the growth phenotype of this strain was investigated using the solid media growth assays assays. The results for the plate assays can be seen in figure 4.10.

Comparison of the growth of the wild type strain with the *chuBCD* deletion mutant strain again fails to demonstrate a significant difference between the *ΔchuBCD* strains and *C. jejuni* NCTC11168.

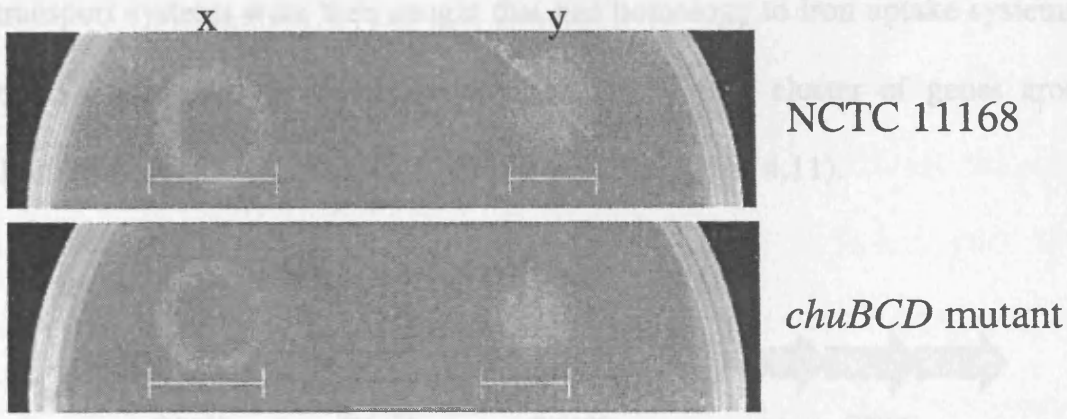

Figure 4.10. *C. jejuni* growth stimulation provided by hemin supplementation on the surface of iron limited (20μg/ml deferral) MH agar. The results are shown for *C. jejuni* NCTC11168 and JDR11 the *chuBCD* mutant strains. The left spot (X) is a supplement of 10μl of 1μg/ml hemin solution and the right spot (Y) is 10μl supplement of 0.1μg/ml hemin solution.

No difference was observed between JDR11 and JDR12 in the ability to grow on iron-limited MH-agar supplemented with hemin, demonstrating a lack of polar effects of the mutation of *chuB*, *C* and *D*. Subtle differences were observed such as the indistinct edge of growth stimulation zone in the wild type when compared with the *chuBCD* mutant strain. The significance of this observation is unknown at present.

4.2.6. Investigation of other genes possibly involved in hemin uptake.

The lack of a demonstrable phenotype for the *chuBCD* mutants (JDR11 and JDR12) lead to the search for *C. jejuni* genes that may be complementing this mutation. The *C. jejuni* NCTC11168 genome contains just fewer than 60 genes that encode proteins of putative ABC transport systems (Parkhill *et al.*, 2000), many of these are uncharacterised and their ligands unknown. It was decided that a targeted approach was needed when creating further mutants, ABC

transport systems were then sought that had homology to iron uptake systems in other organisms. In particular attention turned to a cluster of genes around 170000bp on the *C. jejuni* NCTC11168 genome (figure 4.11).

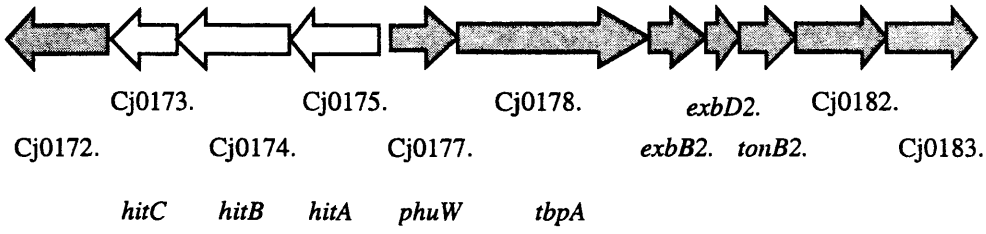

Figure 4.11, the organisation of Cj0173, Cj0174 and Cj0175, and surrounding genes on the chromosome of *C. jejuni* NCTC11168. Cj0173, Cj0174 and Cj0175 are represented by unshaded arrows, surrounding genes are represented by grey arrows. The names of the genes/ORFs can be seen in black script, bacterial proteins showing sequence similarity to the predicted protein product of a *C. jejuni* gene/ORF can be seen underneath in italic script.

Three ORFs discovered in an apparent operon (Parkhill *et al.*, 2000), encoded proteins with amino acid homology (37% identity over 243 amino acids) to HitA, B and C from *H. influenzae* (Sanders *et al.*, 1994). Mutation of *hitC* in *H. influenzae* produced a strain that could no longer use iron and protoporphyrin IX as a source of haem (*H. influenzae* cannot synthesise haem like some bacterial species). HitC has been proposed to be involved in the uptake of ferrous iron in *H. influenzae* (Sanders *et al.*, 1994). Consequently the Cj0175, Cj0174 and Cj0173 ORFs were designated *hitA*, *hitB* and *hitC* respectively, due to their predicted protein sequence similarities. Arranged in a divergent manner to *hitA*, *B* and *C*, are several other genes with homology to genes of iron-associated function in other organisms (figure 4.11). Cj0177 is an ORF with homology to

phuW, a gene of unknown function that in *Ps. aeruginosa* is co-expressed with a haem uptake ABC transporter system in response to iron limitation (Ochsner *et al.*, 2000). In *Ps. aeruginosa* the PhuW protein is located at the inner membrane and is required for optimal efficiency of haem uptake. Cj0178 is an ORF, which shows sequence homology to the transferrin binding protein (TbpA) of *Neisseria* species. Downstream of the *tbpA* homologue, *exbB*, *exbD* and *tonB* genes are present.

4.2.7. Location of the *hit* gene promoter.

Prior to functional analysis of the *hit* genes it was decided to analyse the *hit* gene promoter to discover whether expression was iron-regulated, which may provide an indication of whether the *hit* gene function is linked to iron uptake. The promoter region between Cj0177 and *hitA* was cloned into pMW10 (using primers PhuWR1-HitAF1) as described in materials and methods, section 2.3.1). This produced two verified constructs, pJDR15 a construct containing a transcriptional fusion of *hitA* with *lacZ* and pJDR16, which contained a transcriptional fusion of Cj0177 with *lacZ*. These constructs were transformed into *C. jejuni* 480 (King *et al.*, 1991) and the resulting transformants assayed for LacZ activity as described in the Materials and Methods chapter, section 2.10. The assay results are shown in figure 4.12.

As with previous LacZ assays three control plasmids in *C. jejuni* 480 were used, pMW10 as a negative control, p23E5 (pMW10 containing the constitutively expressed *metK* promoter (Wosten *et al.*, 1998)) as a positive control and

pAV201 (pMW10 containing the *C. jejuni* NCTC81116 *katA* promoter region, (van Vliet, 2003)) as an iron responsive positive control. Both *C. jejuni* 480 containing pJDR15 and *C. jejuni* 480 containing pJDR16 demonstrated promoter activity, both with greater LacZ activity in iron limited conditions. This means that *hitA* and Cj0177 are expressed from two promoters between these two genes and that both *hitA* and Cj0177 are expressed in response to iron limitation.

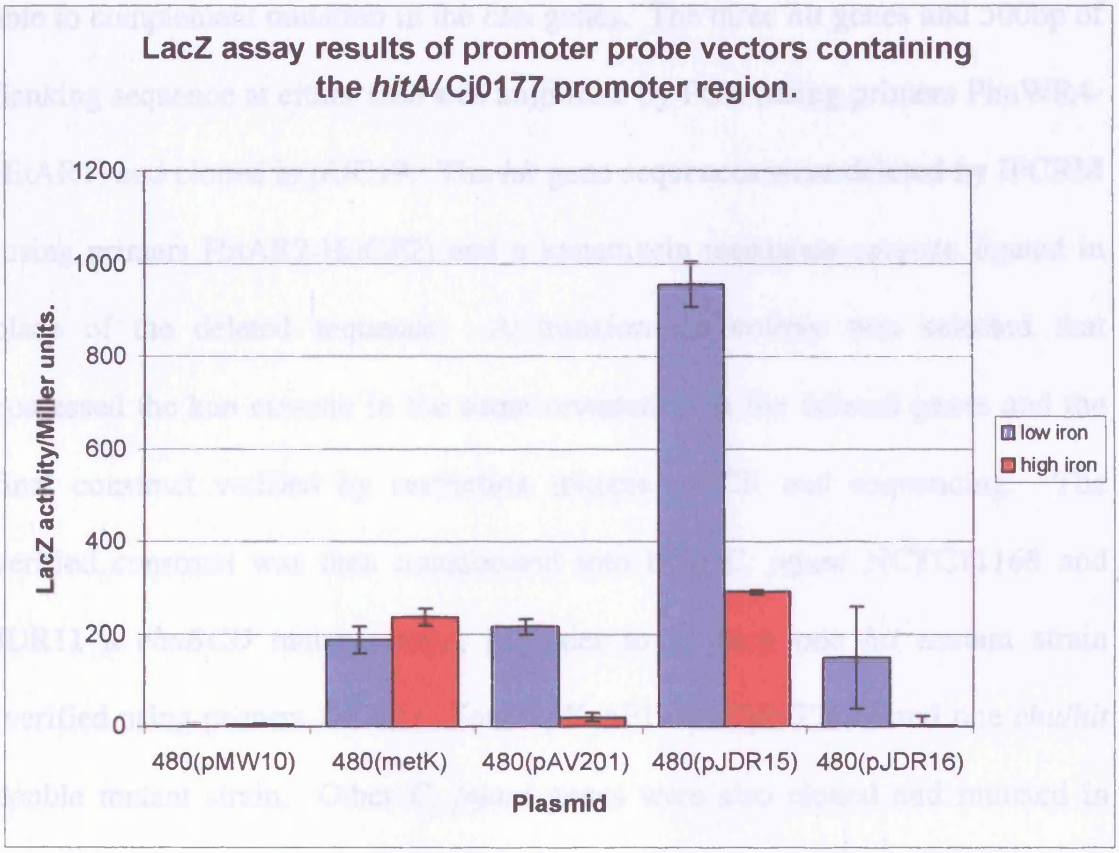

Figure 4.12. LacZ assays of 480(pJDR15) and 480(pJDR16), with negative control pMW10 only, positive control pMW10 containing the constitutively expressed *metK* promoter and an iron responsive control pAV201 (pMW10 containing the *C. jejuni* NCTC81116 *katA* promoter region) promoter. These results measure LacZ activity into *hitA* (pJDR15) in both iron limited and iron-replete conditions, and into Cj0177 (pJDR16) in both iron limited and iron-replete conditions.

4.2.8. Construction and haem uptake phenotype of other mutant strains.

Due to the fact that *hitA, B and C* encode an ABC transport system with protein sequence similarity to iron transport systems of other organisms, it was decided to analyse the function of the *hit* genes by mutation. The growth phenotype of any mutants on media containing hemin as a sole iron source would indicate if these genes were essential for haem uptake. The growth phenotype of a *chuB, C, D/ hitA, B, C* double mutant strain would demonstrate whether the *hit* genes are able to complement mutation in the *chu* genes. The three *hit* genes and 500bp of flanking sequence at either side was amplified by PCR (using primers PhuWR4-HitAR1) and cloned in pUC19. The *hit* gene sequences were deleted by IPCRM (using primers HitAR2-HitCF2) and a kanamycin resistance cassette ligated in place of the deleted sequence. A transformant colony was selected that possessed the kan cassette in the same orientation as the deleted genes and the final construct verified by restriction mapping, PCR and sequencing. The verified construct was then transformed into both *C. jejuni* NCTC11168 and JDR11 a *chuBCD* mutant strain, in order to produce one *hit* mutant strain (verified using primers HitAR1, KanR1, KanF1 and Cj0172R5) and one *chu/hit* double mutant strain. Other *C. jejuni* genes were also cloned and mutated in order investigate their possible role in haem uptake and iron acquisition. Genes mutated included the Cj0178 (cloned with primers PhuWF-ExbBR, deleted with primers Tbp1F and Tbp1R) which encodes a protein that displayed amino acid sequence similarity to the TbpA outer membrane transferrin receptor (Cornelissen *et al.*, 1992). Also assayed was a *tonB* mutant strain, which was

produced using the strategy outlined in Materials and Methods, sections 3.2.4 and 3.2.5 (*tonB* cloned using primers Cj0752F1- CfrAR1, *tonB* deleted using primers TonBR2- TonBF1). *C. jejuni* contains 3 *tonB* genes, one of which is adjacent to a putative siderophore receptor *cfrA*. A *tonB* gene also exists next to *cfrA* in *C.coli* and mutation of this *tonB* gene in *C.coli* results in a strain that is unable to utilise hemin, ferrichrome and enterochelin as iron sources (Guerry *et al.*, 1997). The mutant strains produced were assayed for the ability to grow using hemin as a sole iron source (figure 4.13).

Little difference is observed between the growth phenotype of the wild type strain (*C. jejuni* NCTC11168), the *hit* mutant, the *chu* mutant or the *chu/hit* mutant strains. The *chuA* mutant was included as a negative control for hemin uptake. This means that neither *chuBCD*, nor *hitABC* are essential for haem uptake.

4.3. Discussion.

From the results of plate assays and growth curve assays it can be seen that ChuA is essential for the uptake of haem in *C. jejuni* NCTC11168. This means that ChuA is the exclusive haem-outer membrane receptor in *C. jejuni* NCTC11168 and is able to act on a wide range of haem-based molecules including hemin, haemoglobin and haemoglobin-haptoglobin. The absolute requirement for ChuA in hemin uptake confirms previous observations that mutant strains that have lost the ability to utilise hemin, no longer express a 70kDa protein (Pickett *et al.*, 1992).

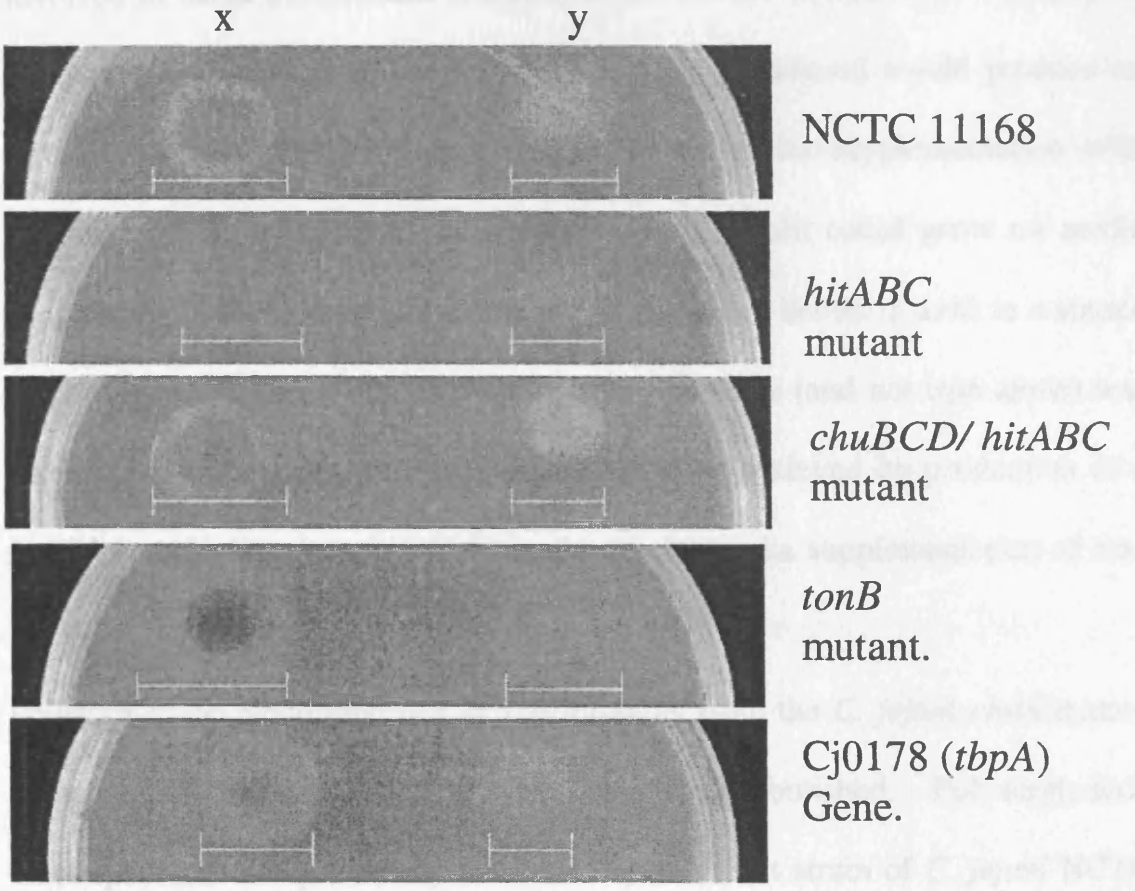

Figure 4.13. *C. jejuni* growth stimulation provided by hemin supplementation on the surface of iron limited (20μg/ml deferral) MH agar. The results are shown for *C. jejuni* NCTC11168 and JDR18 the *hitABC* mutant strain and JDR19 the *hitABC/chuBCD* double mutant strain. The left spot (x) is a supplement of 10μl of 1μg/ml hemin solution and the right spot (y) is 10μl supplement of 0.1μg/ml hemin solution.

ChuA is likely to function by removal of the haem-group haem containing molecules, followed by internalisation of the haem moiety by a conformational change in ChuA. This model of haem uptake by ChuA is based on models developed in other Gram-negative bacteria (Mills and Payne., 1997; Torres and Payne, 1997). Confirmation that haem is taken up whole by *C. jejuni* could be achieved by mutation of the *C. jejuni* porphyrin biosynthetic enzymes. *C. jejuni* contains seven genes which have been identified as proteins or putative proteins

involved in haem biosynthesis (Parkhill *et al.*, 2000). Mutation of a porphyrin biosynthesis gene such as *hemA* (glutamyl-tRNA reductase) would produce an auxotrophic mutant strain which would require media supplementation with aminolaevulinic acid (ALA) for growth. If this strain could grow on media supplemented with hemin alone, this would show that hemin is used as a source of porphyrin, indicating that the whole haem molecule (and not iron alone) was taken up. Confirmation of haem uptake could be achieved by production of a *hemA/chuA* double mutant, which would require media supplementation of iron and ALA for growth.

Polyacrylamide electrophoresis of OM proteins from the *C. jejuni chuA* mutant demonstrates that expression of ChuA is clearly abolished. Polyacrylamide electrophoresis of a previously constructed *fur* mutant strain of *C. jejuni* NCTC 11168 (van Vliet *et al.*, 1998) demonstrates that expression of ChuA is de-repressed, i.e. it is expressed in both iron replete and iron limited conditions. This confirms that ChuA expression is under control of the Fur protein.

ChuA shows amino acid sequence similarity to TonB dependent outer membrane receptors (Chapter 3 section 3.2.1). It is therefore likely that haem uptake by ChuA requires energy from a functional TonB system, *C. jejuni* has three *tonB* genes and two pairs of associated *exbB/exbD* accessory genes. A *tonB* gene exists at 704500bp on the *C. jejuni* NCTC11168 chromosome next to a siderophore receptor homologue *cfrA*. These two genes are also adjacent on the chromosome of *C.coli* (Guerry *et al.*, 1997) and mutation of this *tonB* gene in *C.coli* resulted in a strain that could no longer utilise hemin, enterochelin and

ferrichrome (Guerry *et al.*, 1997). Mutation of the same *tonB* gene was carried out in *C. jejuni* NCTC11168 but does not lead to the abolition of hemin uptake (see Chapter 3, section 5.2.8). This may mean that the alternative *tonB* gene is involved in hemin uptake in *C. jejuni* or that both *tonB* gene products may be utilised for hemin uptake in *C. jejuni.*

The observation that ChuB, C, or D are not necessary for the utilisation of haem as an iron source indicates that either they are not involved in haem uptake, or mutation of their genes can be complemented by a similar system. Similar observations have been made in the organism *Bradyrhizobium japonicum*, where the haem receptor uptake was shown to be essential for haem utilisation but mutation of the haem uptake ABC-transport genes had no effect on the ability to utilise haemoglobin as an iron source (Nienaber *et al.*, 2001). The failure to demonstrate a reduction of growth stimulation by hemin in the multiple *chu* gene mutant provides no evidence that ChuB, C or D are involved in haem uptake but does not preclude the possibility that *chu* gene mutation is complemented by the function of another transport system. The possession of a number of alternate systems for the acquisition of haem has been demonstrated in *H. influenzae* (Morton *et al.*, 1999), *V. cholerae* (Occhino *et al.*, 1998) *Ps. aeruginosa* (Ochsner *et al.*, 2000) and the pathogenic *Neisseria* (Schryvers and Stojiljkovic, 1999). The expression of *chuA*, *B*, *C* and *D* genes in a *C. jejuni* strain or other bacterial species that cannot utilise haem may be necessary to absolutely confirm whether these genes are involved in haem uptake. This approach was successfully carried out in *V. cholerae* when mutation in the haem ABC transport

genes failed to show a growth phenotype on media containing haemoglobin as a sole iron source (Occhino *et al.*, 1998). The expression of *V. cholerae* ABC haem transport genes and an OM haem-receptor in *E. coli* K12 (which cannot utilise haemoglobin) conferred the ability to utilise hemin as an iron source.

Little difference is observed between the growth phenotype of the wild type strain (*C. jejuni* NCTC11168), the *hit* mutant, the *chu* mutant or the *chu/hit* mutant strains on media containing hemin as an iron source. This means that neither *chuBCD*, nor *hitABC* are essential for haem uptake. Unfortunately this data leaves us with little evidence whether either ABC transport system is involved in the uptake of haem. The most simple explanation for these findings is that neither *chuBCD* nor *hitABC* are involved in hemin uptake, which is carried out by an existing system not yet linked to hemin uptake. Alternatively it may also be possible that *chuBCD* (but not *hitABC*) may be involved in hemin uptake but its mutation is complemented by such an alternative ABC transport system. Finally the results described in this chapter do not preclude the possibility that both systems may be involved in hemin uptake but mutation of both systems may be complemented by a system not yet linked to hemin uptake. Functional redundancy has been observed in *H. influenzae* among haemoglobin binding proteins, as mutation in *hgpA*, *hgpB* and *hgpC* does not completely eradicate the ability to utilise haemoglobin as an iron source (Morton *et al.*, 1999). Similar observations have been made in the organism *Bradyrhizobium japonicum*, the outer membrane haem receptor was essential for the utilisation of haemoglobin (Nienaber *et al.*, 2001). Mutation of the haem uptake ABC-

transport genes however, had no effect on the ability to utilise haemoglobin as an iron source (Nienaber *et al.*, 2001). It has also been observed that *V. cholerae* is able to complement mutation of any one of the ABC haem transport genes but not mutation of multiple genes (Occhino *et al.*, 1998).

Functional redundancy among characterised haem uptake systems has also been described in *Bradyrhizobium japonicum* (Nienaber *et al.*, 2001) *Ps. aeruginosa* (Letoffe *et al.*, 1999; Ochsner *et al.*, 2000) and *Y.pestis* (Thompson *et al.*, 1999). Whether complementation of mutation in two systems involved in haem-uptake in *C. jejuni* occurs, remains to be proven. Targeted mutagenesis of likely systems may not be the most effective way of isolating systems involved in haem uptake at this point. If a non-hemin utilising strain of *C. jejuni* can be isolated from a mutant library (which is currently under construction (Ketley, 2003)) or by transposon mutagenesis (Hendrixson *et al.*, 2001) may be a better way to demonstrate involvement of a system in haem-uptake.

SDS-page gels of the periplasmic fractions of *C. jejuni* NCTC 11168 show the presence of a protein band of 29kDa expressed only under iron limited conditions. This protein can still be clearly seen in the *chuD* mutant strain cultured under iron-restricted conditions. Previously this protein was identified by one-dimensional SDS-PAGE as ChuD on the basis of Mr (van Vliet *et al.*, 1998). N-terminal sequencing of this protein band showed this protein was not ChuD (figure 4.9) as previously assumed but the product of the ORF Cj1613. Characterisation of promoter activity in the region upstream of Cj1613 demonstrates that the Cj1613 gene product is expressed under iron-restricted

conditions (Chapter3 figure 3.5). Due to the similarity in Mr of the two proteins it is possible that the Cj1613 gene product will mask ChuD expression in one-dimensional electrophoresis. ChuD and the Cj1613 gene product may be differentiated by two-dimensional SDS-PAGE due to differences in pI (the pI ChuD of is 9.57, the pI of Cj1613 is 6.16). The function of the Cj1613 gene product is unknown at present, however the amino acid sequence of this protein does display similarity to proteins involved in haem-uptake in other organism. Cj1613 is a target for future investigation and may be involved in haem or iron acquisition. Initial attempts were made to mutate Cj1613 but were unsuccessful. This may mean that Cj1613 is essential to cellular function in *C. jejuni* but more investigation would be required to establish this. The function of the Cj1613 gene product is not currently known, although the Cj1613 protein does display similarities to HugZ, a protein required for haem utilisation in *Plesiomonas shigelloides* (Henderson *et al.*, 2001). This sequence similarity means that Cj1613 will be of interest to the future research of haem uptake and utilisation in *C. jejuni.*

In summary data produced in this study confirms that ChuA functions as the sole OM haem receptor in *C. jejuni* NCTC11168. The *chuB*, *C* and *D* and the *hitA, B* and *C* genes are not essential for the acquisition of iron from haem. The involvement of *chuB*, *C* and *D* or the *hit* genes in haem uptake cannot be ruled out due to the possibility of complementation of mutation by another system. How *C. jejuni* utilises haem as an iron source subsequent to its import into the periplasm remains uncharacterised.

5 Results: Lactoferrin and transferrin utilisation in *C. jejuni*.

5.1. Introduction

The vertebrate host produces a range of proteins to inhibit the colonisation of its tissues by potentially pathogenic microorganisms. One mechanism used to prevent colonisation is the production of proteins to limit the amount of extracellular iron available to microorganisms. In the vertebrate host, iron sequestration is principally carried out by the transferrin protein family (Ward *et al.*, 1996a). The transferrin family of proteins all share a number of common molecular traits. They all have approximate molecular masses of 80 kDa, and when folded form two globular domains capable of reversible iron binding. Transferrins comprise of N-terminal (amino acids 1-333) and C-terminal (amino acids 342-679) domains, both domains possessing the ability to bind iron, the C-lobe with slightly higher affinity (10^{-22}M (Boulton *et al.*, 1999)). In this manner a single transferrin molecule is able to bind two molecules of ferric iron.

In the blood transferrin (Tf) chelates free ferric iron resulting in less than 10^{-18}M of free iron in blood plasma (Ratledge and Dover., 2000), this results in the bacteriostatic nature of blood plasma on many bacterial species. Transferrin in the blood maintains iron-limited conditions, preventing an organism from multiplying in the blood stream by withholding iron, and restricting microbial colonisation of the tissues. Tf acts as an iron carrier to all growing host cells, which all possess surface Tf receptors. Ferric-Tf is taken up by host cells by receptor mediated endocytosis and the iron liberated from Tf by acidification of the endocytic vesicle to pH5, causing the iron to dissociate (Lodish *et al.*, 1995).

Chapter 5. Lactoferrin and transferrin utilisation in *C. jejuni*.

Transferrin is the most thoroughly studied member of the transferrin family, in both its action and its utilisation by pathogens (Ward *et al.*, 1996a).

Lactoferrin is secreted at mucous membranes and is present in tears, breast milk, and the mucous secretions of the respiratory, gastrointestinal and reproductive tracts (Gachon and Lacazette., 1998). In contrast to transferrin, which dissociates from iron at pH 6-4, lactoferrin is able to form stable complexes with iron over a wider pH range. Lactoferrin forms complexes with iron as low as pH 4-2 (Ward *et al.*, 1996b) and is proposed to possess microbiocidal and immune modulatory activities (Levy, 2000). Lactoferrin is vital in the prevention of mucosal colonisation by harmful microorganisms.

In the intestine lactoferrin chelates any ferric iron released from food or in intestinal secretions (Ward *et al.*, 1996a). Lactoferrin inhibits intestinal colonisation by microorganisms creating an iron-limited environment that gastrointestinal pathogens must overcome in order to colonise the gut mucosa. The transferrin family of proteins are comprehensively reviewed in Ward et al, (Ward *et al.*, 1996a).

5.1.1. Bacterial utilisation of transferrins.

The ability to utilise the transferrin family of proteins as a source of iron allows a significant competitive advantage to any colonising microorganism. Utilisation of lactoferrin and transferrin as iron sources has been described in a wide range of Gram-positive and negative bacterial species including the pathogenic *Neisseria* (Schryvers and Stojiljkovic, 1999), *Staphylococcus aureus* (Modun and

Williams, 1999) and *E. coli* (Freestone *et al.*, 2000). Bacteria have devised a number of strategies to utilise iron from lactoferrin and transferrin, which can be grouped into four main mechanisms: -

a) By proteolytic cleavage of the transferrin/lactoferrin molecule. Cleavage of lactoferrin or transferrin at sites critical to iron binding or protein structure may result in the liberation of iron. Surface associated and extracellular proteases that liberate iron from Tf/Lf have been reported in several bacteria including *Porphyromonas gingivalis* (Brochu *et al.*, 2001), *Vibrio vulnificus* (Okujo *et al.*, 1998) and *Pseudomonas aeruginosa* (Wolz *et al.*, 1994).

b) By reduction of the chelated iron.

The affinity of Lf and Tf for Fe^{2+} is much lower than for Fe^{3+}. Cell surface associated reductases cause reduction of lactoferrin (Lf) or transferrin (Tf) bound ferric iron, causing the ferrous iron to dissociate from Lf or Tf. The use of ferric reductases to obtain iron has been reported in Gram-negative organisms such as *E. coli*, *Ps. aeruginosa* and S. Typhimurium (Vartivarian and Cowart., 1999).

c) By the production of a higher affinity iron chelator. In order to remove iron from host iron chelating proteins, an organism may produce low molecular weight soluble molecules with a higher affinity for iron than Tf or Lf such as siderophores. Siderophores have been described in a number of pathogenic microorganisms (Ratledge and Dover., 2000). A description of siderophores and their mode of action is covered in the General introduction section.

d) By possession of a specific energy dependent uptake system. Specific uptake systems for Lf and Tf have been extensively described in the pathogenic

Neisseria (Schryvers and Stojiljkovic, 1999). In *N.meningitidis* utilisation of transferrin or lactoferrin is achieved by an OM receptor complex comprising of several proteins. The receptor complex was shown to comprise of a homo-dimer of two TbpA sub-units and one associated TbpB lipoprotein sub-unit (Boulton *et al.*, 1999). TbpA function as OM receptors and are capable of iron removal from Tf/LF without TbpB (Schryvers and Stojiljkovic, 1999). TbpB is an accessory protein that is not essential for iron acquisition but does increase the efficiency of Tf/Lf binding (Anderson *et al.*, 1994). The genes for these two sub-units are co-expressed in response to iron limitation, mutational inactivation of both genes leads to the loss of ability to utilise iron from transferrin (Schryvers and Stojiljkovic, 1999).

The presence of a similar multi-component receptor for lactoferrin was also confirmed in *N.meningitidis*. This receptor also consisted of two A subunits and a B subunit (Petterson *et al.*, 1998). Mutation of the LbpB subunit demonstrated a reduced ability to utilise lactoferrin (Petterson *et al.*, 1998). A similar system has been described in *N.gonnorhoeae*, and it was demonstrated using a human urethritis model, that transferrin utilisation is vital to virulence (Cornelissen *et al.*, 1998).

5.1.2. Evidence for Tf or LF uptake systems in *C. jejuni.*

In 1992, a study by Pickett et al (Pickett *et al.*, 1992), investigated the ability of a number of *C. jejuni* strains to utilise a number of iron sources present in host tissues. Stimulation of *C. jejuni* growth was observed by hemin, haemoglobin,

hemin-hemopexin and haemoglobin-haptoglobin. Molecules found to be unable to stimulate *C. jejuni* growth in this study included ferritin, lactoferrin and transferrin.

Subsequently a molecular basis was found for systems involved in the uptake of enterochelin (Park and Richardson., 1995), ferrichrome (Field *et al.*, 1986) and the hemin/haemoglobin (chapter 3 and 4, this study). The advent of publication of the *C. jejuni* genome sequence (Parkhill *et al.*, 2000) confirmed the existence of enterochelin and hemin/haemoglobin uptake systems but also highlighted a number of novel genes related to iron acquisition. These included: -

a) Cj0175, Cj0174 and Cj0173, three ORFs with similarities to a ferrous iron uptake system (*hitA, B and C*) in *H. influenzae* (Sanders *et al.*, 1994) (chapter4).
b) p19, a gene encoding a 19kDa protein with similarities to a protein found in marine magnetotactic bacteria proposed to be involved in copper handling (Dubbels *et al.*, 2003; van Vliet *et al.*, 2002).
c) Cj0177, an ORF showing similarity to *phuW* of *Ps. aeruginosa*, a gene of unknown function which is co-expressed with a haem uptake system (Ochsner *et al.*, 2000).
d) Cj0178, an ORF that shows similarities to the Tf binding-protein (TbpA) of *Neisseria* species (Boulton *et al.*, 1999).

The discovery of Cj0178 was of interest as previous studies failed to demonstrate the ability of *C. jejuni* to utilise Tf or Lf as iron sources (Pickett *et al.*, 1992).

It had been demonstrated that *Listeria monocytogenes* could use a number of

exogeous siderophores, natural iron binding compounds and catechols to promote growth (Simon *et al.*, 1995). In an attempt to elucidate the mechanism by which human catechol stress hormones enhance bacterial growth in serum-based media, a method was developed to demonstrate the ability of the organism acquire iron from Lf and Tf using noradrenaline (Freestone *et al.*, 2000). Bacteria assayed in this manner utilised noradrenaline in much the same way as a bacterium might utilise an exogenous catecholate siderophore. In this study, assays were performed using ^{55}Fe-labelled Tf or Lf as iron sources, allowing the monitoring of bacterial iron uptake by measuring cellular incorporation of radioactivity (^{55}Fe). This was a simple assay that produced quantitative results on the ability of a bacterium to acquire iron from Lf/Tf.

The presence of a sensitive, quantitative assay system of the ability of a bacterium to take iron from ^{55}Fe-labelled Lf/Tf, allows a re-evaluation of the ability of *C. jejuni* to utilise Lf and Tf as iron sources. It also allows the involvement of noradrenaline and the nature of the utilisation system (contact/non-contact dependent) to be assessed, by partitioning ^{55}Fe-labelled Lf/Tf away from the bacteria using dialysis membrane. The ability to utilise Tf/Lf bound iron for growth can also be investigated by the use of growth curve assays in a similar manner to those performed for hemin utilisation (Chapter 4). Such assays may reveal a possible source of growth promoting iron available in the intestine, the site where *C. jejuni* infection is established. The ability to obtain iron during intestinal colonisation will be critical to the outcome of infection. The work presented in this chapter was carried out in collaboration

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with Dr Primrose Freestone (University of Leicester).

5.2. Results.

5.2.1. *C. jejuni* iron acquisition from transferrin.

C. jejuni as a pathogen shares many of the same niches that are colonised by the gastro-intestinal pathogens that have been demonstrated to utilise nor-adrenaline to acquire transferrin and lactoferrin bound iron (Freestone *et al.*, 2000). It was decided to investigate the ability of *C. jejuni* to utilise nor-adrenaline to acquire transferrin and lactoferrin bound iron using the newly described assay (Freestone *et al.*, 2000). In order to investigate whether *C. jejuni* can take up transferrin-bound iron, an assay was used that measured the ability to take up ^{55}Fe from ^{55}Fe labelled-transferrin (^{55}Fe-Tf). Comprehensive descriptions of the methods used and diagrams of the experimental set up can be found in the materials and methods section (chapter 2, section 2.11). Radiolabelled iron-uptake assays and demonstration of Tf binding (by blotting) and Lf binding (using Lf-sepharose affinity columns) was carried out by Dr Primrose Freestone, *Campylobacter* propagation, mutant construction, production of cellular protein fractions and growth curve assays were carried out by myself.

Measurement of ^{55}Fe uptake, both when the bacteria are in contact with the ^{55}Fe-Tf or partitioned away from the iron source using dialysis membrane, allows the mechanism of uptake (cell associated or soluble factor dependent) to be investigated. ^{55}Fe-uptake assays were also set up that were supplemented with

noradrenaline in order to assess whether gut associated stress hormones may be used by *C. jejuni* to obtain iron from Tf. After completion of the assay, association of the ^{55}Fe either within the bacterial cells or in the assay medium was assessed by measuring the radioactivity of the cell pellet and of the assay medium.

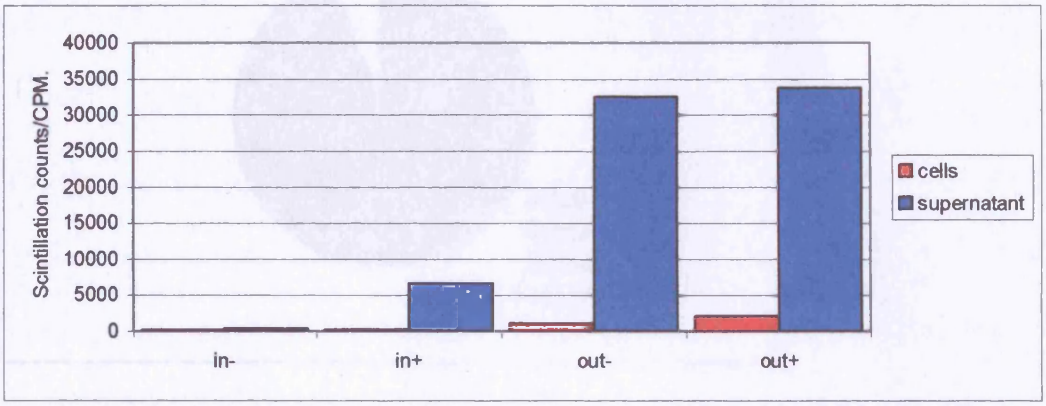


Figure 5.1a. .Radioactivity (CPM) associated with the *C. jejuni* cell pellet (red bars) and assay medium supernatant (blue bars) from a single dialysis partition experiment using ^{55}Fe-Tf (2×10^5 cpm/ml final concentration) as an iron source. The results from four experimental assays are represented; firstly an assay in which ^{55}Fe-Tf was partitioned away from the bacteria using dialysis membrane (in-). The second bar group (in+) represent radiation measurements of cells and supernatants from an assay in which the ^{55}Fe-Tf was partitioned as before but with the addition of Noradrenaline (NA, 100 μM final concentration). The third bar group represent radiation measurements of cells and supernatants from an assay in which bacteria are in contact with ^{55}Fe-Tf (out-). The fourth bar group also represent radiation measurements of cells and supernatants from an assay in which bacteria are in contact with ^{55}Fe-Tf (out+), with the addition of Noradrenaline (NA, 100 μM final concentration).

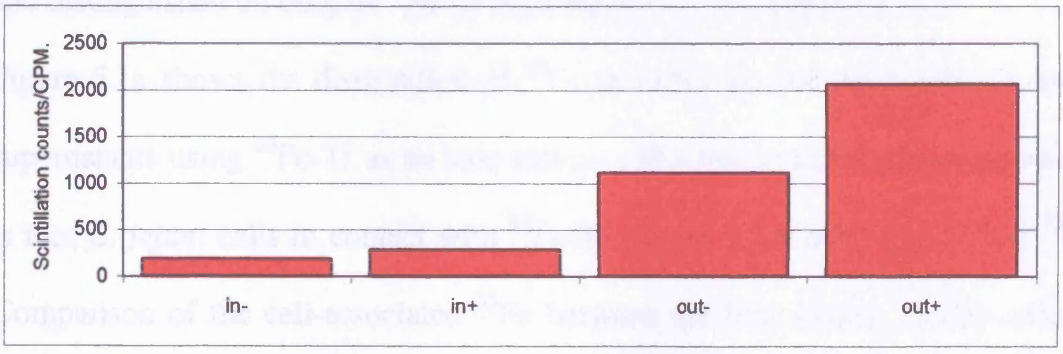


Figure 5.1b. Comparison of cell associated radioactivity from the ^{55}Fe-Tf dialysis partition experiment represented in figure 5.1a.

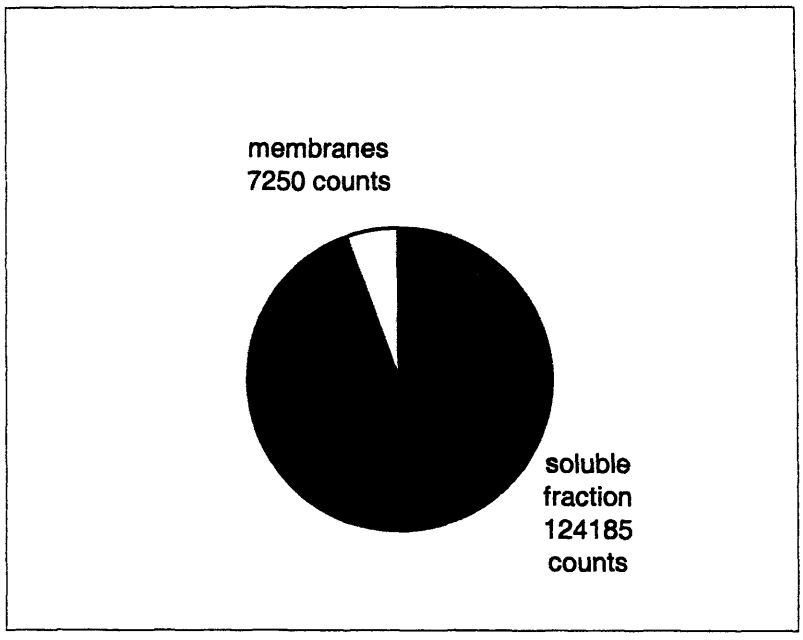


Figure 5.1.c. Localisation of the radioactivity associated with the membranes (white segment) and cytoplasm/periplasm (grey segment) of fractionated *C. jejuni* cells from the iron uptake assay using ^{55}Fe-Tf as an iron source. After the assay cells (1ml~10^8 cfu/ml) were harvested and fractionated by sonication and ultracentrifugation. The radioactivity measured from both fractions is represented as a percentage of the total counts obtained from whole bacteria.

The radioactivity observed in bacterial cells and assay medium from each experimental vessel can be seen in figure 5.1.a and b. Extraction and fractionation of the ^{55}Fe-labelled cells by ultracentrifugation, also allowed the location of ^{55}Fe obtained from ^{55}Fe-Tf and within the different cellular fraction and distinguished binding of ^{55}Fe-Tf from uptake of ^{55}Fe (figure 5.1c).

Figure 5.1a shows the distribution of ^{55}Fe in either the bacterial cells or assay supernatants using ^{55}Fe-Tf as an iron source. The main noteworthy observation is that *C. jejuni* cells in contact with ^{55}Fe-Tf (figure 5.1.a out-) can obtain ^{55}Fe. Comparison of the cell-associated ^{55}Fe between the four assays can be seen in

figure 5.1.b. It can be seen that cells in contact with the ^{55}Fe-Tf accumulate far greater amounts of ^{55}Fe, than those partitioned away from the ^{55}Fe-Tf. This indicates that acquisition of ^{55}Fe from ^{55}Fe-Tf requires direct contact with Tf at the bacterial cell surface. The presence of noradrenaline in the growth medium, both in contact and partitioned away from the iron source does increase the amount of ^{55}Fe obtained. This may be due to noradrenaline causing the release of small amounts of ^{55}Fe from ^{55}Fe-Tf but noradrenaline does not appear essential for *C. jejuni* uptake of ^{55}Fe from ^{55}Fe-Tf. Figure 5.1c shows the location of cell-associated ^{55}Fe in either the membrane fraction or cytoplasm/periplasm protein fraction of *C. jejuni* cells after incubation with ^{55}Fe-Tf. Radioactivity measured in the various cellular fractions is displayed as a percentage of the total cell associated radiation. Figure 5.1c shows that 94% of the total cell associated radioactivity is present in the periplasm and cytoplasm of the bacterial cells, thus indicating that it has passed through the outer membrane. This means the majority of cell-associated radiation observed is due to ^{55}Fe inside the cell and may indicate the presence of an uptake system for Tf-bound iron, rather than simple adherence of transferrin to the outer membranes or exposed surface structures.

It was also possible to observe binding or adherence of transferrin to *C. jejuni* NCTC11168, by incubating cells in media containing Tf for 60 mins and the subsequent electrophoresis of lysates of the washed cells (methods section 2.5). Blotting of the polyacrylamide gels and probing of the blots with polyclonal anti-transferrin allowed the presence of bound transferrin to be visualised (figure 5.2).

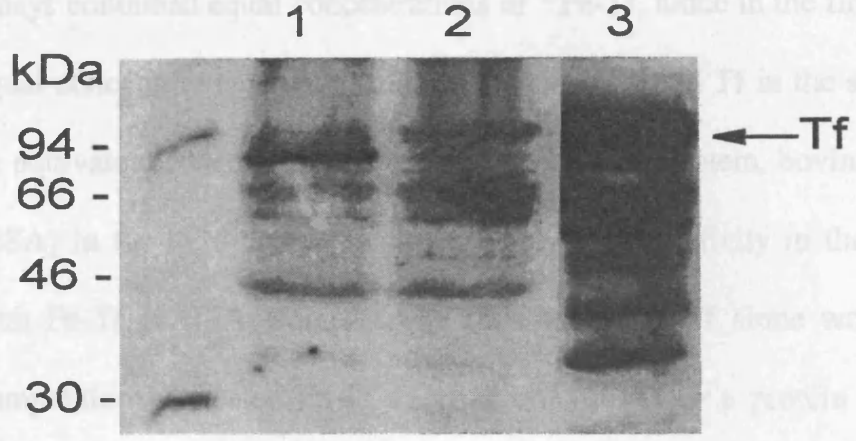


Figure 5.2. Transferrin binding to *Camplylobacter jejuni*. Western blot analysis of solubilised *C. jejuni* proteins (approximately 25 μg of protein separated by SDS-PAGE) prepared from cells incubated in the presence (lane 1) and the absence (lane 2) of transferrin, and probed with anti-transferrin antibodies as described in section 2.5.2. Lane 3 (Tf) contains 1 μg of purified human transferrin; the arrow indicates the position of the mature Tf protein.

This confirms the interaction of Tf with *C. jejuni* cell surface structures, which may be due to simple adherence of Tf to the *C. jejuni* cell surface or if taken with the ^{55}Fe location data (figure 5.1.c) may hint at the presence of a specific Tf-bound iron acquisition system.

5.2.2 Investigating specificity of Tf-bound iron acquisition in *C. jejuni*.

Results from the previous section suggest the existence of a *C. jejuni* iron acquisition system, allowing *C. jejuni* to acquire iron bound to Tf. It was therefore important to confirm that the ability of *C. jejuni* to obtain iron from Tf was achieved by a specific receptor rather than general adherence to bacterial outer membrane structures. In order to achieve this, ^{55}Fe uptake assays were set up in three vessels with *C. jejuni* in direct contact with ^{55}Fe-Tf. All ^{55}Fe uptake

assays contained equal concentrations of ^{55}Fe-Tf, alone in the first assay, with an equal concentration of non radioactive iron saturated Tf in the second assay and an equivalent concentration of a non-iron binding protein, bovine serum albumin (BSA) in the third assay. A lower level of radioactivity in the cells incubated with Fe-Tf or BSA, compared to cells with ^{55}Fe-Tf alone would demonstrate competition between ^{55}Fe-Tf and that cold Fe-Tf or a protein present in BSA. The results of the competition assay can be seen in figure 5.3.

C. jejuni cells incubated with ^{55}Fe-Tf alone (figure 5.3, first column) are again able to obtain substantial amounts of iron from Tf when incubated in direct contact with the Tf. The *C. jejuni* cells incubated with both ^{55}Fe-Tf and cold Fe-Tf obtained less ^{55}Fe (48% lower radioactivity) than cells incubated with ^{55}Fe-Tf alone. This demonstrates that the cold Fe-Tf is competing with radioactive ^{55}Fe-Tf for binding sites on the surface of the *C. jejuni* cells. The *C. jejuni* cells incubated with both ^{55}Fe-Tf and BSA show little difference in the radioactivity accumulated than cells incubated ^{55}Fe-Tf alone. This shows that BSA does not compete with transferrin for binding sites on the *C. jejuni* outer membrane, indicating that the binding of transferrin is a specific process.

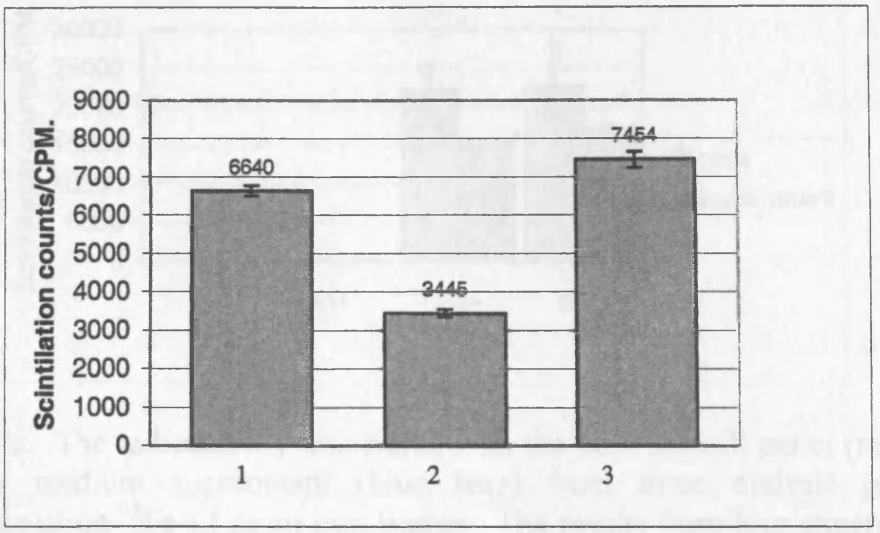


Figure 5.3. *C. jejuni* incorporation of ^{55}Fe when incubated in direct contact with ^{55}Fe-Tf either alone (1), with an equivalent concentration of non-radioactive iron saturated Tf (2), or an equivalent concentration of BSA (3). Each column displays the mean values of three scintillation counts sampled from one experimental run. The three assays carried out to demonstrate competition of various proteins with ^{55}Fe-Tf for a system involved in Fe acquisition from Tf.

5.2.3 Investigation of *C. jejuni* acquisition of lactoferrin-bound Fe.

It was decided to investigate whether *C. jejuni* possessed the ability to utilise iron from lactoferrin using the same assay used to measure Tf-bound iron uptake. The ability to utilise Lf may be more relevant to an organism colonising the intestinal mucosa due to the greater abundance of Lf compared with Tf in this environment. Investigation of the uptake of Lf-bound Fe was possible because Lf can also be ^{55}Fe labelled in the same way as Tf. The results from the first assay demonstrating the ability of *C. jejuni* to take up lactoferrin-associated iron can be seen in figure 5.4.

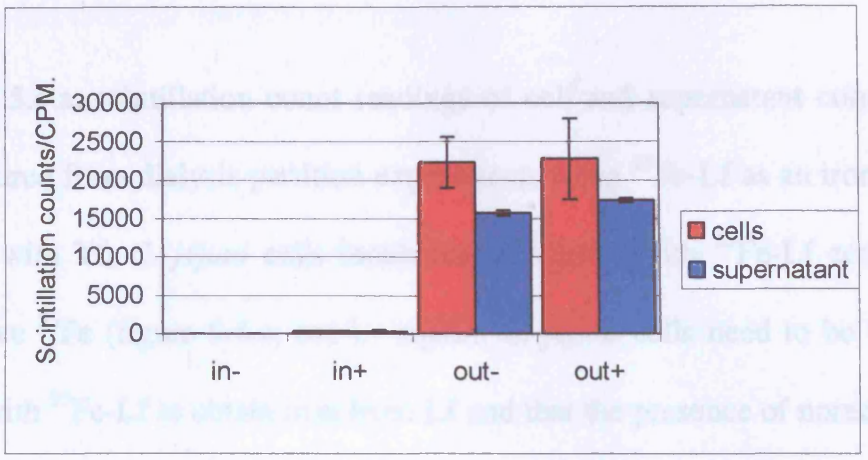


Figure 5.4.a. The radioactivity associated with the both the cell pellet (red bars) and assay medium supernatant (blue bars) from three dialysis partition experiments using ^{55}Fe-Lf as an iron source. The results from four experimental assays are represented, firstly an assay in which ^{55}Fe-Lf was partitioned away from the bacteria using dialysis membrane (in-). The second bar group (in+) represent radiation measurements of cells and supernatants from an assay in which the ^{55}Fe-Lf was partitioned as before but with the addition of Noradrenaline (NA, 100 μM final concentration) with the bacteria. The third bar group (out-) represent radiation measurements of cells and supernatants from an assay in which bacteria are in contact with ^{55}Fe-Lf. The fourth bar group (out+) also represent from an assay in which bacteria are in contact with ^{55}Fe-Lf, with the addition of Noradrenaline (NA, 100 μM final concentration).

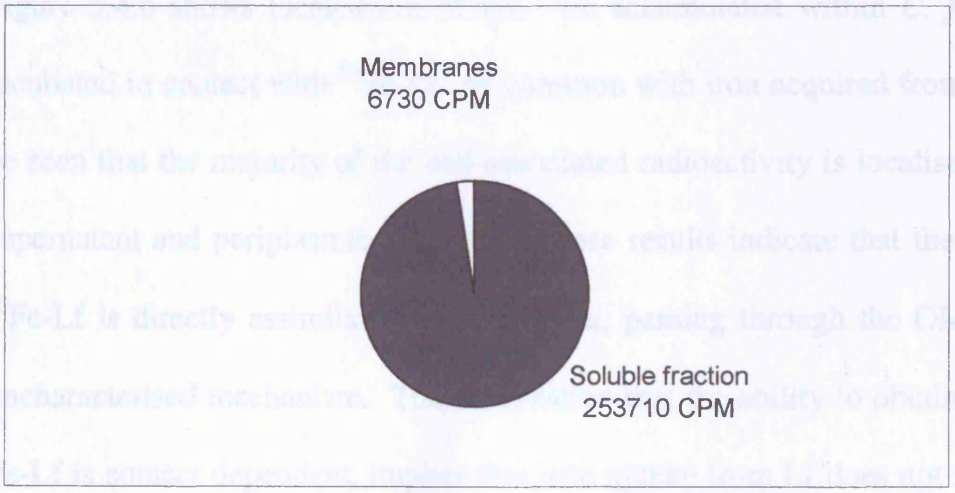


Figure 5.4.b. Measurement of radioactivity associated with the membranes (yellow segment) and soluble fraction (periplasm and cytoplasm-red segment) of fractionated *C. jejuni* cells from the dialysis partition experiments using ^{55}Fe-Lf as an iron source. Cells (1ml~10^8 cfu/ml) were harvested and fractionated by sonication and ultracentrifugation. The radioactivity measured from both fractions is represented as a percentage of the total radioactivity of whole bacteria.

In figure 5.4.a. scintillation count readings of cell and supernatant components are measured from dialysis partition experiments using ^{55}Fe-Lf as an iron source. As seen with Tf, *C. jejuni* cells incubated in contact with ^{55}Fe-Lf accumulate radioactive ^{55}Fe (figure 5.4.a, out-). Again, *C. jejuni* cells need to be in direct contact with ^{55}Fe-Lf to obtain iron from Lf and that the presence of noradrenaline is not essential in obtaining iron from Lf. What is striking from comparison of the data from figure 5.4.a to the same experiment carried out with Tf (figure 5.1.a), is the greater uptake of radioactive iron from ^{55}Fe-Lf. It can be seen from the assay containing *C. jejuni* cells partitioned away from ^{55}Fe-Lf, that the cells obtain little ^{55}Fe (figure 5.4a, in- and in+). Lactoferrin-associated iron uptake is therefore likely to be a contact dependent process that is minimally influenced by the presence of noradrenaline (figure 5.4.a, out+).

Figure 5.4.b shows localisation of the ^{55}Fe accumulated within *C. jejuni* cells incubated in contact with ^{55}Fe-Lf. In common with iron acquired from Tf, it can be seen that the majority of the cell-associated radioactivity is localised with the supernatant and periplasmic fractions. These results indicate that the ^{55}Fe from ^{55}Fe-Lf is directly assimilated into *C. jejuni*, passing through the OM by some uncharacterised mechanism. The observation that the ability to obtain iron from Fe-Lf is contact dependent, implies that iron uptake from Lf does not require the ability to utilise siderophores.

5.2.4. Lactoferrin competition assays.

As performed previously with Tf, specificity of Lf-binding by *C. jejuni* was investigated. This was achieved via competition for ^{55}Fe acquisition from ^{55}Fe-Lf as an iron source with equivalent concentrations of non-radioactive iron saturated lactoferrin or BSA. The results of this experiment can be seen in figure 5.5.

From figure 5.5., the cell associated radioactivity from each of three assays used to demonstrate competition for binding sites between ^{55}Fe-Lf and other proteins at the *C. jejuni* surface. From figure 5.5, it can be seen that *C. jejuni* cells incubated with ^{55}Fe-Lf and cold iron saturated lactoferrin showed significantly lower levels of ^{55}Fe incorporation than cells incubated with ^{55}Fe-Lf alone. No such reduction was seen when *C. jejuni* cells were incubated with ^{55}Fe-Lf and BSA, demonstrating that the competition between ^{55}Fe-Lf and Fe-Lf for binding sites present on *C. jejuni* cells was specific.

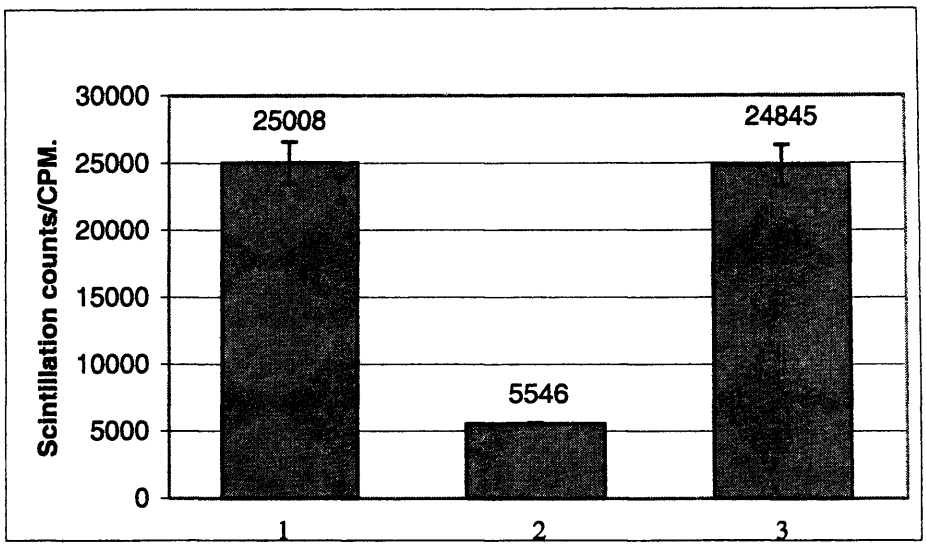


Figure 5.5. *C. jejuni* incorporation of ^{55}Fe when incubated in direct contact with ^{55}Fe-Lf either alone (1), with an equivalent concentration of non-radioactive iron saturated Lf (2), or an equivalent concentration of BSA (3). Each column displays the mean values of three scintillation counts sampled from one experiment. The three assays carried out to demonstrate competition of various proteins with ^{55}Fe-Lf for a system involved in Fe acquisition from Lf.

This supports the hypothesis that there is a specific binding site for Lf rather than non-specific adherence to the cell surface. By comparison of the amounts of iron accumulated, it may be seen that the amount of ^{55}Fe accumulated from ^{55}Fe-Lf (figure 5.5 column 1) is far greater than observed in the equivalent experiment using ^{55}Fe-Tf (figure 5.2). This may indicate that Lf is a preferred source of host-derived iron over Tf for *C. jejuni*.

The results described so far in this chapter provides evidence that *C. jejuni* does indeed possess a system that allows it to obtain Fe from Lf and Tf. Lf seems to be a preferred source of iron compared with Tf, which may be an indication of the preferred iron source utilised by *C. jejuni in vivo*. The iron obtained from Tf and Lf then becomes compartmentalised within *C. jejuni* cells.

5.2.5. Investigation of the utilisation of iron acquired from Lf.

The data shown in figures 5.1-5.5 showed that *C. jejuni* can acquire iron from Lf and Tf and import it into the cell. Determination of whether the iron obtained from Lf can be utilised for growth is essential, because previous studies concluded that Lf could not be utilised as a source of iron for growth (Pickett *et al.*, 1992). To investigate whether Lf can support growth of *C. jejuni*, an assay was designed to observe whether *C. jejuni* is able to use Lf-bound iron for growth. Lactoferrin was selected as an iron source because *C. jejuni* seemed to demonstrate a preference for Lf as an iron source, and would therefore be more likely to produce growth enhancement. It was desirable therefore to select a different strategy that would not to replicate previous experiments, which had not demonstrated utilisation of Lf derived iron for growth (Pickett *et al.*, 1992). It was decided to adopt a growth curve strategy using liquid media, which would allow a quantitative examination of growth stimulation by the presence of iron containing compounds. In order to negate any interference from the presence of iron chelators it was decided to use MEMα medium (Gibco). This liquid media is iron limited and had been previously demonstrated to support *C. jejuni* growth poorly in the absence of added iron and stimulate expression of iron regulated transport systems (van Vliet *et al.*, 1998). *C. jejuni* cells were cultured overnight in MEMα to deplete pre-existing iron stores, harvested and then re-suspended in fresh MEMα containing various concentrations of ferri-lactoferrin and apo-lactoferrin and growth observed over a 26hr period by monitoring changes in optical density. *C. jejuni* growth was also measured in MEMα alone as an iron-

limited control, and in MEMα supplemented with 40μM $FeSO_4$ as an iron-replete control. The mean results of three experiments can be seen in figure 5.5., standard error bars are not present due to variation in optical densities (OD_{600}) between experimental duplicates. It is noted however that relative patterns of growth between *C. jejuni* in media containing different iron sources was consistent.

From figure 5.6, it can be seen that *C. jejuni* is capable of limited growth in un-supplemented MEMα medium, reaching a final OD of approximately 0.17 after 26 hrs incubation. The growth of *C. jejuni* in $FeSO_4$ supplemented media can be seen to exceed the amount of growth in un-supplemented media. Supplementation of the media with 25μg/ml (~0.3μM) of ferri-lactoferrin stimulates the growth of *C. jejuni* to a final level greater than that of *C. jejuni* cells grown in un-supplemented media. This result indicates that *C. jejuni* can utilise iron bound to Lf for growth. Addition of a higher concentration of ferri-lactoferrin (100μg/ml,~1.25μM) results in a slightly higher amount of growth though still lower than the growth of *C. jejuni* in $FeSO_4$ supplemented media. It may be noted at this stage however that 40 μM $FeSO_4$ contains approximately 16 times the amount of iron as 100μg/ml Fe-Lf, depending upon iron saturation of Lf. The growth of *C. jejuni* in media supplemented with iron-free lactoferrin was consistently poor, resulting in a lower final cell density than *C. jejuni* grown in un-supplemented media. It is clear from the results of these experiments (Figure 5.6) that *C. jejuni* does indeed possess the ability to utilise the iron present in lactoferrin for growth.

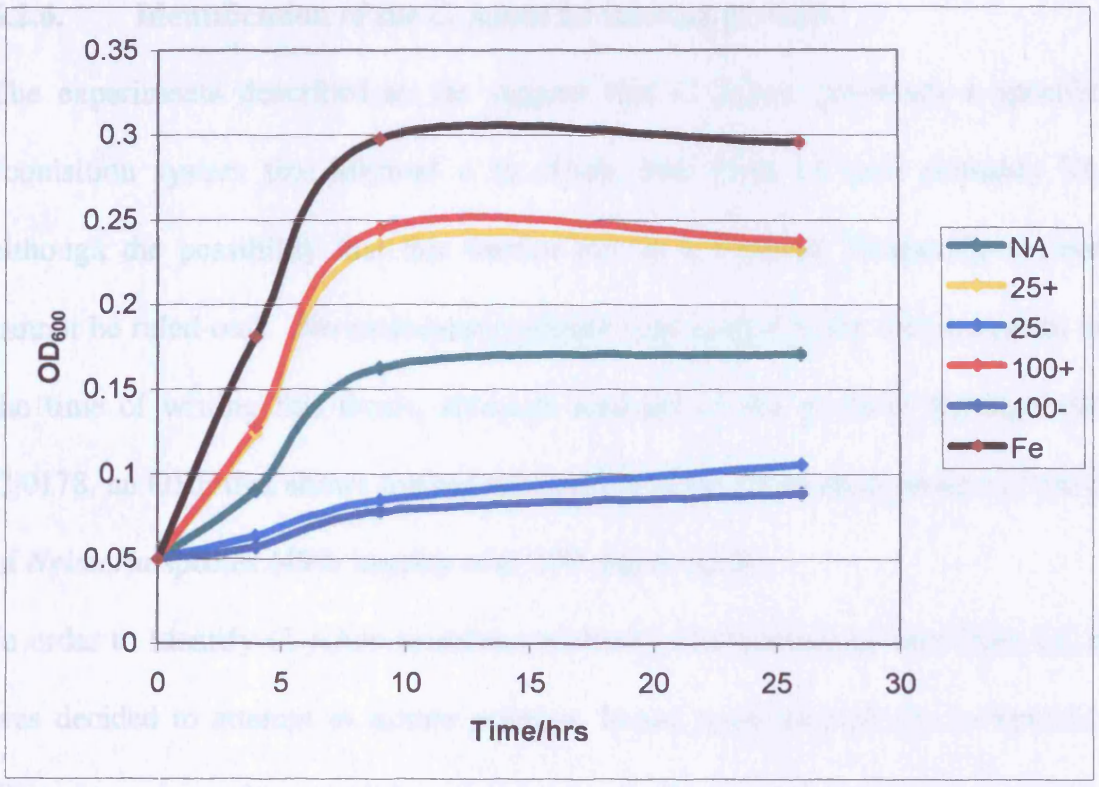


Figure 5.6. Growth of *C. jejuni* NCTC11168 in MEMα medium supplemented with a various iron sources measured by monitoring OD_{600}. The data set labelled NA in the legend (green line) represents growth of *C. jejuni* in un-supplemented media. The data set labelled 25+ in the legend (orange line) represents growth of *C. jejuni* in media supplemented with 25μg/ml ferri-lactoferrin. The time course labelled 25- in the legend (turquoise line) represents growth of *C. jejuni* in media supplemented with 25μg/ml apo-lactoferrin. The data set labelled 100+ in the legend (red line) represents growth of *C. jejuni* in media supplemented with 100μg/ml ferri-lactoferrin. The data set labelled 100- in the legend (blue line) represents growth of *C. jejuni* in media supplemented with 100μg/ml apo-lactoferrin. The data set labelled Fe in the legend (black line) represents growth of *C. jejuni* in media supplemented with 40μM $FeSO_4$.

5.2.6. Identification of the *C. jejuni* Lf-binding protein.

The experiments described so far suggest that *C. jejuni* possesses a specific acquisition system that allowed it to obtain iron from Lf (and probably Tf, although the possibility that this carried out by a separate Tf-specific system cannot be ruled out). No molecular evidence was available for such a system at the time of writing this thesis, although analysis of the genome did highlight Cj0178, an ORF that shows limited similarities to the Tf binding-protein (TbpA) of *Neisseria* species (49% identity over 199 amino acids).

In order to Identify *C. jejuni* proteins involved in acquisition of iron from Lf, it was decided to attempt to isolate proteins, based upon their ability to bind Lf. This was achieved by applying *C. jejuni* proteins to an Lf-sepharose column. Identification of bound proteins could then be determined by N-terminal sequencing

C. jejuni OM proteins were prepared (Materials and Methods, Section 2.4and passed down an Lf-sepharose column, and the run off (containing non-bound proteins) was collected. Any proteins that had bound to the column were eluted using a low pH buffer and collected. The run off, eluted proteins and a sample of the initial OM extract (that had not passed through the column), were run on a polyacrylamide gel and compared. The experiment was repeated with a sample of *C. jejuni* OM protein fraction that had been pre-incubated with lactoferrin in order to demonstrate specific lactoferrin binding in the non-lactoferrin incubated sample. The results can be seen in figure 5.7.

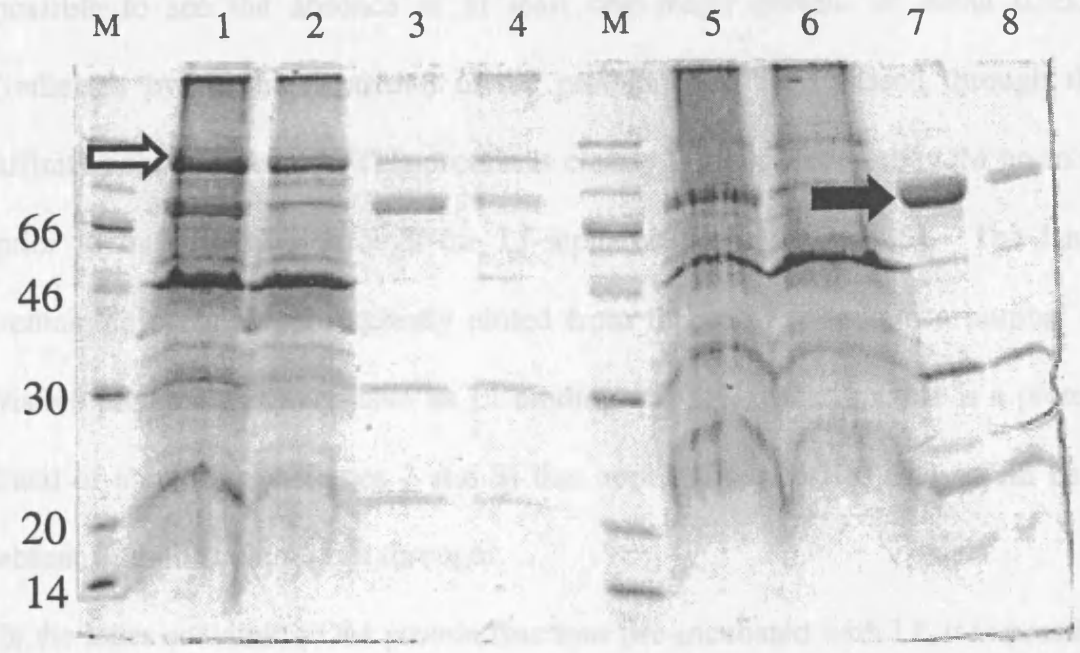


Figure 5.7. Polyacrylamide electrophoresis gel of OM proteins from *C. jejuni* NCTC11168, pre-incubated with lactoferrin (lanes 1-4) or without Lactoferrin pre-incubation (lanes 5-8). Molecular weight markers (M sizes in kDa are shown at the left side) are visible at the left end of the gel and between the two protein sets. Lane 1 contains OM proteins pre-incubated with Lf, lane 2 contains OM proteins pre-incubated with lactoferrin not bound to the Lf-sepharose column (column run off). Lane 3 and 4 contain OM proteins pre-incubated with Lf, bound to the Lf-sepharose column (first and second elution with low pH buffer). Lane 5 contains OM proteins not pre-incubated with Lf, lane 6 contains OM proteins not bound to the Lf-sepharose column (column run off). Lane 7 and 8 contains OM proteins bound to the Lf-sepharose column (first and second elution with low pH buffer). The black arrow marks the position of a protein that free Lf pre-incubation reduces binding to the Lf-sepharose column. The white arrow marks the position of the Lf band in the protein set incubated with Lf (lanes 1-4).

Figure 5.7 shows a polyacrylamide gel electrophoresis of *C. jejuni* NCTC11168 OM proteins passed through an Lf-sepharose column. Lanes 1-4 show the profiles of OM proteins pre-incubated with Lf and lanes 5-8 contain the same OM proteins applied straight to the Lf-sepharose column. Firstly by observing the proteins applied straight to the column without LF pre-treatment, it is

possible to see the absence of at least one major protein of about 65kDa (indicated by the black arrow) in the proteins that have passed through the affinity column (lane 6). This protein is clearly present among the OM proteins prior to their passing through the Lf-sepharose column (lane 5). The lanes containing proteins subsequently eluted from the column contain a number of visible proteins that may have an Lf binding affinity. Most notable is a protein band of about 65kDa (lanes 7 and 8) that appears identical to the protein band absent from the column run through.

In the lanes containing OM protein fractions pre-incubated with Lf, it is possible to see the Lf band clearly at about 80kDa (indicated by the white arrow). Pre incubation of the protein fraction with Lf does not appear to completely prevent binding of the 65kDa protein to the column, because the protein is again absent from the column run through (lane 2). Elution of any bound proteins from the column again yields a number of proteins visible by SDS page electrophoresis, the most conspicuous band being the previously noted 65kDa protein (lanes 3 and 4). It appears that the 65kDa protein does possess some specificity of Lf binding. However the failure of pre-incubation of Lf to completely abolish binding of this protein to the column may be characteristic of a non-specific binding affinity of this protein for Lf. Another explanation may be that this protein may form large insoluble structures that will not allow it to pass through the column substrate until eluted.

N-terminal sequencing of the 65kDa protein identified it as glycosylated flagellin A (figure 5.8), the primary flagellum protein subunit shown to be involved in adhesion to uncharacterised host motifs (Wassenaar *et al.*, 1993).

N-terminal	G F R I N T N V A A L N A K A N.
C. jejuni FlaA	M G F R I N T N V A A L N A K A N

Figure 5.8. Alignment of N-terminal sequence results of the 65kDa band with the first 17 amino acids of the *C. jejuni* FlaA protein sequence (www.sanger.ac.uk/projects/C. jejuni).

In order to investigate FlaA binding and minimise obscuring of other receptor protein bands on the electrophoresis gel by FlaA, it was decided to repeat this experiment using outer membrane protein fractions from a *C. jejuni* strain in which *flaA* and *flaB* were mutationally innactivated (Wassenaar *et al.*, 1991). Proteins from the *fla* mutant strain, passed through the lactoferrin affinity column can be seen in figure 5.9.

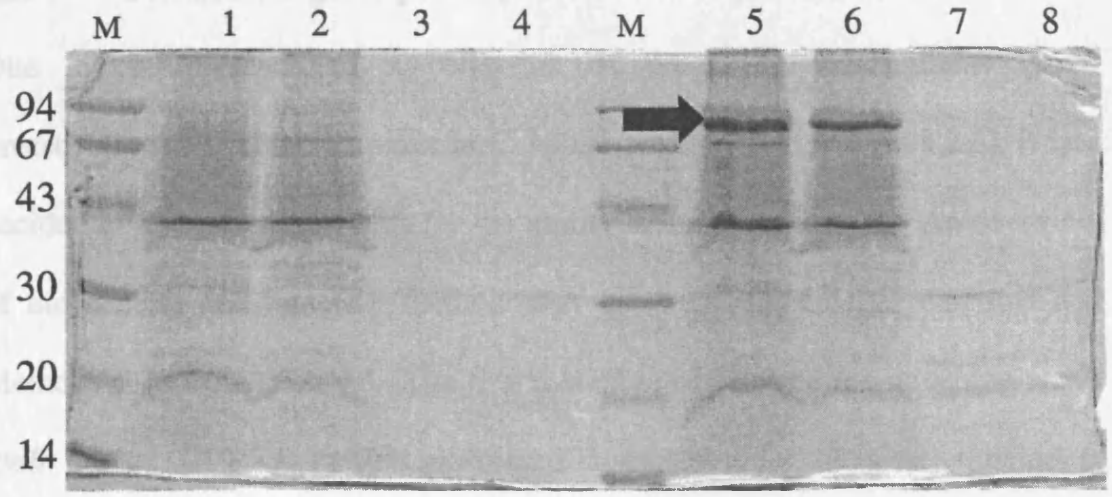


Figure 5.9. Polyacrylamide electrophoresis gel of OM proteins from *C. jejuni* flagellin mutant strain incubated with Lf (lanes 5-8) and OM proteins not pre-incubated with Lf (lanes 1-4). Molecular weight markers (M sizes in kDa are shown at the left side) are visible at the left end of the gel and between the two protein sets. Lane 1 contains OM protein fraction, lane 2 contains OM protein fraction not bound to the Lf-sepharose column (column run off). Lane 3 and 4 contain OM proteins bound to the Lf-sepharose column (first and second elution with low pH buffer). Lane5 contains OM proteins pre-incubated with Lf not bound to the Lf-sepharose column (column run off). Lane 6 and 7 contain OM proteins pre-incubated with Lf bound to the Lf-sepharose column (first and second elution). The black arrow marks the position of the Lf band in the protein set pre-incubated with Lf (lanes 5-8).

Figure 5.9 is a polyacrylamide gel showing *C. jejuni* flagellin mutant OM proteins passed through an Lf-sepharose column. Lanes 1-4 contain *fla* mutant OM proteins applied directly to the Lf-sepharose column, lanes 5-8 contain *fla* mutant OM proteins pre-incubated with Lf. In figure 5.9 lane 1, OM proteins can be seen and it is immediately noticeable that the 65kDa protein band observed in *C. jejuni* NCTC11168 (wild type) OM protein is no longer present. This confirms that the flagellin subunits are no longer expressed in this mutant strain. Unfortunately due to poor gel resolution it is not possible to visualise any absent protein bands in the column run through.

5.2.7. Mutation of genes possibly involved in acquisition of Fe from Lf.

Due to the presence of a collection of pre-constructed mutants (from investigation of the hemin uptake in *C. jejuni*, see chapter 4 section 4.2.8), it was decided to screen such mutants for the ability to utilise Lf and Tf. An overview of the cloning and mutation methodology can be found in the Materials and Methods chapter, section 2.3. The first mutant to be selected for screening was a *tonB* mutant (JDR20), which possessed a mutation in the gene situated at approximately 704500 base pairs on the *C. jejuni* NCTC11168 chromosome. This mutant was selected due to the probable involvement of a TonB system in energy transduction during the uptake process. Secondly this particular *tonB* gene was selected because mutation a homologous gene in *C.coli* had been reported to produce a strain that could no longer utilise many iron containing compounds including hemin, ferrichrome and enterochelin (Guerry *et al.*, 1997). Since Tf and Lf receptors from Gram-negative bacteria are generally TonB dependent proteins it was thought that mutation of this *tonB* gene could inactivate Tf and Lf-iron uptake. The *tonB* mutant strain was assayed for the ability to grow in MEMα using Lf and a number of other iron sources (figure 5.10).

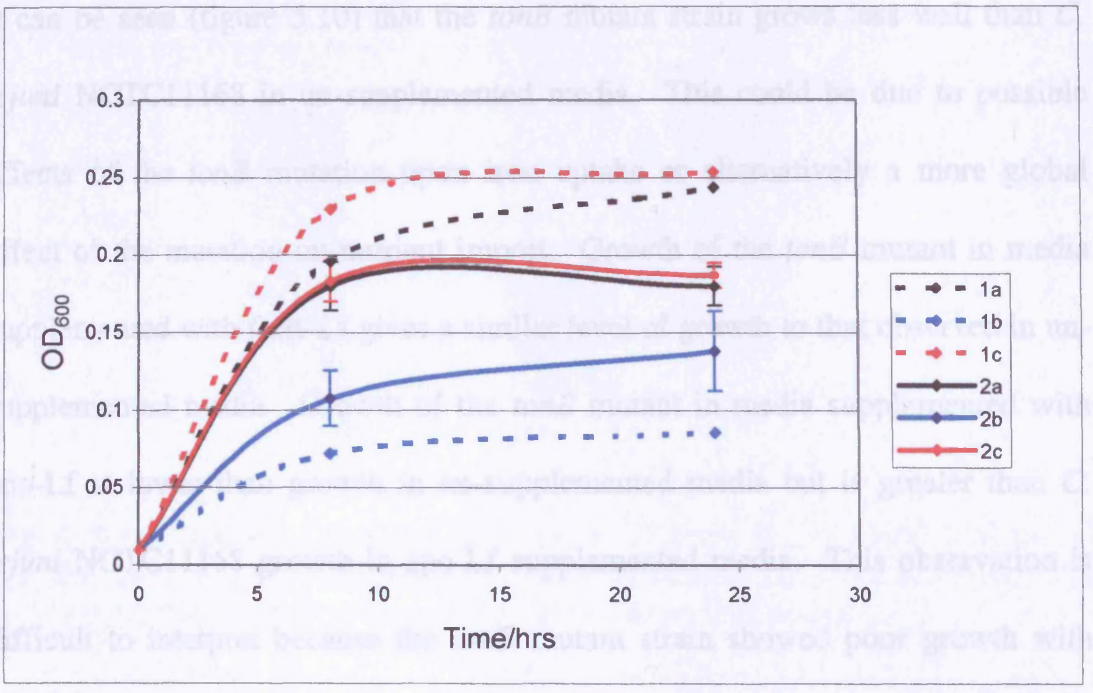


Figure 5.10. Growth of *C. jejuni* NCTC11168 and JDR20 (*C. jejuni tonB* mutant strain) in MEMα medium supplemented with a various iron containing compounds. *C. jejuni* NCTC11168 growth is represented in broken lines (1a, 1b and 1c). The data set labelled 2a in the legend (black line) represents growth of the *tonB* mutant strain in un-supplemented media, *C. jejuni* NCTC11168 grown in un-supplemented media, is represented by the black broken line (1a). The data set labelled 2b in the legend (blue line) represents growth of the JDR20 (*tonB* mutant) strain in media supplemented with 100μg/ml apo-Lf, *C. jejuni* NCTC11168 grown in 100μg/ml apo-Lf supplemented media is represented by the blue broken line (1b). The data set labelled 2c in the legend (red line) represents growth of the JDR20 (*tonB* mutant) strain in media supplemented with 100μg/ml ferri-Lf, *C. jejuni* NCTC11168 grown in 100μg/ml ferri-Lf supplemented media is represented by the red broken line (1c).

It can be seen (figure 5.10) that the *tonB* mutant strain grows less well than *C. jejuni* NCTC11168 in un-supplemented media. This could be due to possible effects of the *tonB* mutation upon iron uptake or alternatively a more global effect of the mutation on nutrient import. Growth of the *tonB* mutant in media supplemented with ferri-Lf gives a similar level of growth to that observed in un-supplemented media. Growth of the *tonB* mutant in media supplemented with apo-Lf is lower than growth in un-supplemented media but is greater than *C. jejuni* NCTC11168 growth in apo-Lf supplemented media. This observation is difficult to interpret because the *tonB* mutant strain showed poor growth with MEMα as a growth medium. Repetition of this experiment is required to ensure that this growth phenotype is consistently observed.

Another mutant selected for screening was a mutant possessing a deletion in the *tbpA* homologue Cj0178, at approximately 174000bp on the *C. jejuni* genome. This mutant was selected due to the similarity of Cj0178 to genes involved in transferrin uptake from *H.influenzae* (Loosmore *et al.*, 1996) and *N.meningitidis* (Pajon *et al.*, 1997). The *tbpA* gene was also mutated using the allelic replacement method outlined in the materials and methods chapter sections 2.3., producing a *tbpA* mutant strain (JDR22). The TbpA mutant was assayed for the ability to take up iron from ^{55}Fe-Lf using the experiment described in materials and methods section 2.11. Lf was used as an iron source due to the fact that ^{55}Fe-Lf appeared to be the host iron protein utilised most efficiently by *C. jejuni* NCTC11168 (this chapter, section 5.2.4.) The results from this assay can be seen in figure 5.11.

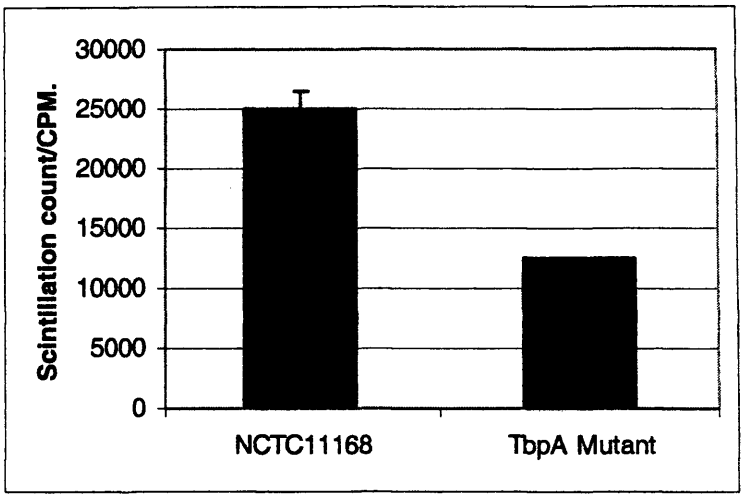


Figure 5.11. The cell associated radiation of *C. jejuni* NCTC11168 (column 1) and the Cj0178 (*tbpA*) mutant strain (JDR22, column 2) measured from cells incubated in SAPI medium containing ^{55}Fe-Lf as an iron source.

From figure 5.11 it can be seen that the ability of the *tbpA* mutant to take up radioactive iron was approximately half that of wild type *C. jejuni* NCTC11168, showing that the mutant retained a significant ability to obtain iron from lactoferrin. This may imply the involvement of TbpA in the ability to obtain iron from Lf, though the possibility that other systems for the acquisition of Lf-iron exist cannot be excluded.

5.3 Discussion.

The results presented in this chapter demonstrate for the first time that *C. jejuni* can acquire iron from the host iron binding proteins Tf and Lf. The uptake assays in which Tf/Lf was partitioned away from the bacterial cells, showed that *C. jejuni* must be in contact with Tf/Lf in order to obtain iron (figure 5.1.). This means that iron is obtained from Tf/Lf by an initial binding to *C. jejuni* membrane proteins, and soluble iron factors (siderophores) are not necessary for

iron acquisition. This is important because both synthesis and uptake of the siderophore enterochelin, was shown to be critical to the ability to acquire iron from Tf from *E. coli* (Burton *et al.*, 2002; Freestone, ; Freestone *et al.*, 2003).

The presence of noradrenaline increases iron acquisition from Tf and Lf, however noradrenaline is not essential for the acquisition process, in contrast with noradrenaline stimulation observed in *E.coli* (Freestone *et al.*, 2003). *C. jejuni* does not require noradrenaline to obtain iron from ^{55}Fe-Lf or ^{55}Fe-Tf, to the same extent as seen in other bacterial species (Freestone *et al.*, 2000). This does not mean the small increase in iron acquisition caused by noradrenaline is not important for pathogenesis but this may be difficult to investigate in vitro.

Analysis of fractionated *C. jejuni* cells incubated in the presence of ^{55}Fe-Tf or ^{55}Fe-Lf shows the iron obtained from these host proteins accumulates primarily in the periplasm/cytoplasm of cells (figure 5.1.c). It is probable that iron is liberated from Tf/Lf at the cell surface, transported across the outer membrane and taken up by a ferrous iron uptake system. Another possibility is that the ^{55}Fe-Tf/^{55}Fe-Lf is transported into the cell but this seems unlikely, based on models of iron acquisition from Tf/Lf in other Gram-negative bacteria (Schryvers and Stojiljkovic, 1999). Certainly among pathogenic *Neisseria* iron appears to be liberated from Tf and Lf at the OM and transported into the periplasm, the Lf/Tf molecule is then released (Boulton *et al.*, 1999).

Competition for an acquisition system between ^{55}Fe-Tf/^{55}Fe-Lf and non-radioactive Fe-Tf/Fe-Lf, demonstrates that iron acquisition from Tf/Lf is a specific process (figure 5.3.). This means that the majority of cell-associated

radioactivity observed is due to a transferrin specific process rather than general protein adherence to the *C. jejuni* cell envelope. Tf binding to *C. jejuni* NCTC11168 cells confirms the interaction of Tf with *C. jejuni* cell surface structures. This observation may be due to adherence of Tf to *C. jejuni* cell surface structures or may be due to the presence of a specific uptake mechanism. It must also be noted that Lf binding affinity of FlaA (figure 5.6) may be responsible for a proportion of the *C. jejuni* cell surface bound Tf observed.

The ability of *C. jejuni* to obtain iron from Lf shares a number of characteristics with the ability of *C. jejuni* to obtain iron from Tf. As with Tf, *C. jejuni* can obtain iron from Lf only when the cells are in contact with Fe-Lf (figure 5.4). Lf-bound iron acquisition has not been previously reported in *C. jejuni* although investigation of the ability to utilise iron Lf for growth was carried out in a prior study (Pickett *et al.*, 1992). This study failed to demonstrate the ability to utilise iron Lf for growth probably because it relied on visualisation of growth stimulation in solid media.

The ability to acquire iron from Tf and Lf could be due to the presence of Lf and Tf specific receptors/ receptor complexes as demonstrated in the pathogenic *Neisseria* (Schryvers and Stojiljkovic, 1999) or possibly the presence of a membrane bound ferric reductase, although there is no genetic evidence for the existence of such a protein in *C. jejuni*.

The main difference between the ability of *C. jejuni* to obtain iron from Lf and Tf is that *C. jejuni* appears to obtain iron more readily from Lf than Tf. This is apparent by comparison of the radioactivity of *C. jejuni* NCTC11168 incubated

in SAPI containing ^{55}FeTf (figure 5.1.) and the same bacterial strain incubated in SAPI containing ^{55}FeLf (figure 5.4a). The amount of ^{55}Fe acquired from ^{55}FeLf greater than the amount of ^{55}Fe acquired from ^{55}FeTf. The preference of Lf over Tf may reflect the adaptation by *C. jejuni* in order to effectively colonise the niche that this pathogen occupies in the host, i.e. the intestine.

The ability of *C. jejuni* to obtain iron from both Lf and Tf suggests two possibilities. Firstly *C. jejuni* may possess two separate systems: a high affinity system that acquires iron from Lf and a lower efficiency system that acquires iron from Tf. Alternatively *C. jejuni* may possess a single system that acquires iron from Lf, Tf being utilised as a less efficient substrate for the Lf-bound iron uptake system. There is little precedence among Gram-negative bacteria for the existence of a multifunctional Lf/Tf binding protein. It appears that the pathogenic *Neisseria* possess separate receptors for the acquisition of iron from Tf (Tbp) and Lf (Lbp) (Schryvers and Stojiljkovic, 1999). At present there is little to differentiate between a multifunctional receptor or two separate receptors and further investigation is required. Demonstrating competition between ^{55}Fe-Tf and different concentrations of cold lactoferrin for an acquisition system, could test these hypotheses.

This study demonstrates that the dialysis partition method of measuring iron acquisition from ^{55}Fe-Lf and ^{55}Fe-Tf by *C. jejuni* is reliable and reproducible. Establishment of a negative control is desirable but may not be possible until the system used to obtain iron from Lf/Tf is identified and mutated, or a non-Lf/non-Tf utilising strain is discovered.

Chapter 5. Lactoferrin and transferrin utilisation in *C. jejuni*.

Attempts to assess whether iron acquisition from Lf wer inconclusive at present The use of ATP in acquisition of iron from Lf may be necessary to transport liberated iron across the inner membrane, via the use of an ABC transport system (Jones and George., 1999). The use of ATP in acquisition of iron from Lf could be investigated by measuring *C. jejuni* iron uptake from ^{55}Fe-Lf in the presence of sodium azide (which poisons ATP based energy dependent systems e.g. ABC transport systems) and carbonylcyanide m-chlorophenylhydrazone (a protonophore which disrupts the proton-motive force at the inner membrane and should eliminate TonB dependent transport systems) (Rohde *et al.*, 2002).

The MEMα growth curve experiments in figure 5.6, show that *C. jejuni* NCTC11168 can utilise Lf bound iron for growth, whereas previous studies had failed to demonstrate that lactoferrin could be used for growth by a number of *C. jejuni* strains (Pickett *et al.*, 1992). The ability of *C. jejuni* to utilise iron acquired from Lf for cellular growth means that Fe acquired from Lf binding is entering the cytoplasm, to be incorporated into enzymes that require an iron co-factor (Abdul-Tehrani *et al.*, 1999). An interesting observation is that the growth of *C. jejuni* in MEMα containing apo-lactoferrin is consistently poor, resulting in a lower final optical density than *C. jejuni* grown in un-supplemented media. The lower *C. jejuni* growth in media containing apo-lactoferrin indicates that lactoferrin is competing with *C. jejuni* for the residual iron in MEMα medium. The lack of available iron in the growth medium, and the energy expenditure required to remove the small amounts of iron from lactoferrin could explain the reduction in observed growth. This hypothesis may be tested by the addition of

an iron-limited control (*C. jejuni* grown in media containing 20μM desferal) to the growth curve assay. If the growth of *C. jejuni* in media containing 20μM desferal, was lower than the growth in media containing apo-lactoferrin, then this would indicate that the iron bound by apo-Lf can be acquired but this requires energy expenditure.

It remains unclear why previous studies have failed to demonstrate the ability to utilise Lf-bound iron for growth, despite using higher concentrations of protein (30μM (Pickett *et al.*, 1992) compared with 1.25μM used in this study). The assay carried out by Pickett et al, was repeated by a project student under my supervision and who also failed to demonstrate iron acquisition from either Tf or Lf. This failure to demonstrate iron acquisition from either Tf or Lf may be due to the reliance on growth stimulation in solid media, which requires a clear zone of growth stimulation to be visualised i.e. slight growth stimulation may not be apparent. Growth may also be affected by the permeability of the solid media to Lf and the presence of iron chelators such as EDDA (Ethylenediamine di-*o*-hydrophenyl-acetic acid). The MEMα based growth curve assay is simple, reproducible and will allow the ability of *C. jejuni* to obtain iron from Tf and Lf to be investigated further in the future.

Attempts to isolate proteins involved in iron acquisition from Lf/Tf, were unsuccessful. It may be tempting to speculate that the FlaA binding of Lf is an integral part of iron acquisition from Lf because this protein has previously been implicated in the attachment and invasion of human cells (Wassenaar *et al.*, 1991). Pre-incubation of OM proteins (containing flagellin) with Lf reduces

retention of flagellin in the Lf-sepharose column, however it does not completely abolish binding. Although this does indicate some specificity of Lf binding by FlaA it may indicate that rather than binding to the Lf-sepharose, large pieces of flagellar material are simply too large to pass through the sepharose matrix. The subsequent elution of proteins with low pH buffer disrupts protein-protein interaction, causing large protein aggregates to disassociate, resulting in the prominent flagellin band in the column elutions (figure 5.7.). It is unlikely that flagellin is involved in iron acquisition from Lf, this possibility could be eliminated by testing the *fla* mutant's ability to acquire iron from Lf.

No other proteins isolated from the Lf-sepharose column had specific Lf binding affinity. The results presented in section 5.2.7 were gathered from a limited number of experiments at the end of the project and time constraints prevented repetition. Repetition of SDS-PAGE of proteins derived from Lf-sepharose analysis of the *fla* mutant strain may lead to clearer images. Induction of protein expression by culturing *C. jejuni* in iron-limited conditions may be necessary to produce visible quantities of proteins involved in iron acquisition.

Analysis of the *tbpA* mutant's ability to acquire iron from Lf demonstrated that TbpA may be involved in iron acquisition from Lf. The ability of the *tbpA* mutant to take up radioactive Lf-bound iron were approximately half that of wild type *C. jejuni* NCTC11168. Although it seems unlikely that TbpA is the sole receptor for Lf (because a receptor mutant is unlikely to be able to obtain Fe from Lf), TbpA may play some role in lactoferrin-bound iron uptake. It may be that TbpA is a component of an Lf-receptor complex in a similar manner to LbpB of

N.meningitidis, which if mutated also displays a reduced iron uptake phenotype (Petterson *et al.*, 1998). Alternatively, a *tbpA* mutation may affect iron uptake in general, or may cause some impairment that decreases the ability to survive or grow, such as polar effects of the mutation causing on the downstream *exbB*, *exbD* and *tonB* genes. The ligation of resistance cassettes in the same orientation as the mutated gene was carried out to minimise polar effects but the effects of polar mutation cannot be ruled out without verifying correct levels of expression of the downstream genes. Another possibility is that TbpA is involved in obtaining iron from transferrin, the ability to obtain iron from lactoferrin being provided by a separate system. This may be tested by repeating the iron uptake assay with the *tbpA* mutant using ^{55}Fe-Tf as an iron source instead of ^{55}Fe-Lf.

Investigation of the ability of the *tonB* mutant strain to grow using Fe-Lf as an iron source (section 5.2.8) requires repetition to verify the results obtained in this chapter. The *tonB* mutant appears to grow poorly when compared to the growth of *C. jejuni* NCTC11168, possibly due to the effect of the *tonB* mutation upon iron uptake or alternatively a more global effect of the mutant on nutrient import. The only occasion when growth of the *tonB* mutant exceeded that of *C. jejuni* NCTC11168 was in media supplemented with apo-Lf. If this result occurs consistently upon repetition, then this observation is difficult to interpret. The results obtained by growing the *tonB* mutant in un-supplemented media and containing ferri-Lf suggest that the mutant does not display inactivation of all iron uptake systems, as observed in the respective *C.coli* mutant (Guerry *et al.*, 1997). This may be expected due to the presence of two other *tonB* genes in *C.*

jejuni NCTC11168 (Genome paper), which may be able to complement mutation of the *tonB* gene that was mutated in this study. It may also be noted that the JDR20 *tonB* mutant does not display inactivation of the ability to utilise hemin as an iron source as observed in the respective *C. coli* mutant (Guerry *et al.*, 1997). Phenotypic analysis of the *tonB* mutant may be better achieved by eliminating the pre-culture step of the MEMα growth curve experiment. It may be that the depletion of the bacterial iron stores may overstress what is already an iron stressed mutant strain. It may be noted that differences between TonB utilisation exist between the iron uptake systems of *C. jejuni* and *C. coli*, a factor, which may be, investigated in the course of future research. Molecular characterisation of the system involved in iron acquisition from Lf remains central to future research into acquisition iron from Lf by *C. jejuni*.

A gene similar to the *feoB* gene from *E. coli* was discovered in the *C. jejuni* genome sequence (Parkhill *et al.*, 2000; van Vliet and Ketley, 2001). The *feoB* gene in *E. coli* encodes a protein with a region of homology to ATPases and is involved in iron import across the inner membrane (Kammler *et al.*, 1993). If iron-acquisition from Tf/Lf in *C. jejuni* follows the model described in other Gram-negative bacteria (Schryvers and Stojiljkovic, 1999) then a functional ferric iron uptake system may be necessary to import the iron liberated from Lf/Tf across the inner membrane (Gomez *et al.*, 1998). This gene may be involved in iron acquisition from Tf/Lf in *C. jejuni* and is a possible target for future research

Chapter 5. Lactoferrin and transferrin utilisation in *C. jejuni*.

To summarise, *C. jejuni* can acquire iron from both Tf and Lf. Acquisition of iron from both Tf and Lf is contact dependent, does not require noradrenaline and is a specific for each substrate molecule. The iron acquired from Tf and Lf accumulates in the periplasm/cytoplasm and can be utilised for cellular growth. The molecular basis of acquisition has not been elucidated, however TbpA may be involved in the acquisition process.

6. Results: Characterisation of Fur expression.

6.1. Introduction.

Results presented in the previous chapters of this study demonstrate that the *chu* operon and Cj01613 are directly regulated by Fur (discussed in Chapter 3). A description of *C.jejuni* Fur and the *C.jejuni* Fur regulon can be found in the general introduction section (section 1.4.2). The discovery of a second iron responsive gene regulator, PerR, highlighted a second group of proteins with expression regulated in response to iron; these include AhpC (Alkyl Hydroperoxide reductase (Baillon *et al.*, 1999)) and KatA (Catalase (Grant and Park., 1995)).

To date detection of Fur-box sequences in *C.jejuni* has been complicated by the A-T rich nature of the *C.jejuni* genome (Parkhill *et al.*, 2000). Indeed the *E.coli* consensus of NATWAT-NATWAT-NATWAT-N (Escolar *et al.*, 1999) may conceivably be found many times in the *C.jejuni* genome. Although it had been demonstrated that *C.jejuni* Fur could complement mutation in *E.coli fur* (Wooldridge *et al.*, 1994), it is entirely possible that a *C.jejuni* Fur-box sequence may differ from the *E.coli* consensus. Analysis of the promoter regions of 9 Fur/iron regulated genes resulted in a *C.jejuni* Fur-box consensus of naTnAT-aAtwat-nAtnat-n to be predicted (van Vliet *et al.*, 2002).

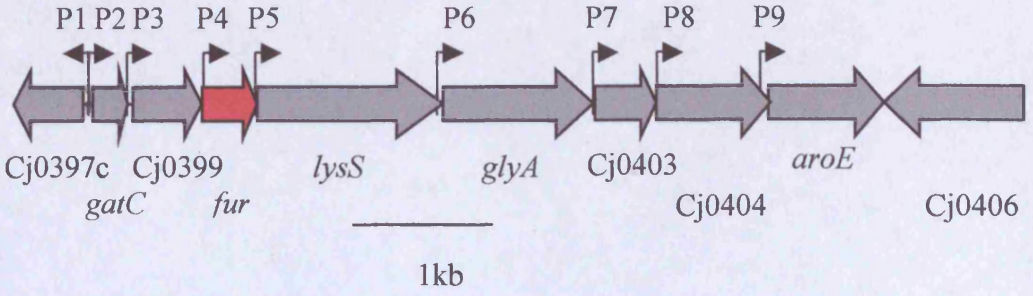


Figure 6.1. The organisation of genes surrounding the *fur* gene of *C.jejuni* NCTC11168. The *fur* gene is coloured red, surrounding ORF's are shown in grey. Note the apparent operon structure that runs from gatC and includes *gatC*, Cj0399, *fur*, *lysS*, *glyA*, Cj0403, Cj0404 and *aroE*. The position and orientation of putative promoters, are represented by black arrows.

When the region surrounding the *fur* gene on the *C.jejuni* genome was sequenced, the *fur* gene was found to be located in an apparent operon (Figure 6.1). The *fur* gene was situated upstream of the housekeeping genes lysyl-tRNA synthetase (*lysS*) and serine hydroxymethyltransferase (*glyA*) (Parkhill *et al.*, 2000). This genetic organisation appears conserved among *C.jejuni* strains (Chan *et al.*, 1995; Wooldridge *et al.*, 1994), and *Campylobacter* species (Bourke *et al.*, 1996). Previous primer extension experiments supported the hypothesis that Fur was expressed from a promoter directly upstream of the *fur* gene, in common with many other Gram-negative bacteria (Chan *et al.*, 1995). This upstream promoter hypothesis was supported by the detection of putative Fur binding sites and putative CAP binding sequences upstream of the *fur* gene (Chan *et al.*, 1995). The reproducibility of this experiment was questioned however, due to the apparent instability of the mRNA from which Fur is translated, leading to a truncated extension product (Ketley, 2003). The lack of reproducibility of the primer extension experiment, along with the unusual

genetic organisation observed suggested that the earlier theory of a single devoted upstream *fur* promoter (Chan *et al.*, 1995) may be an oversimplification.

In order to clarify whether the *C.jejuni fur* gene was transcribed from its own promoter directly upstream, the 370bp upstream region from the *fur* gene was cloned into pMW10 (van Vliet *et al.*, 2000). This plasmid had been used successfully to assay promoter activity (Baillon *et al.*, 1999), (Wosten *et al.*, 1998) by production of a transcriptional fusion of a promoter region to the *lacZ* gene. No promoter activity was detected in the region upstream of *fur* indicating that the *C.jejuni fur* gene is located within an operon and is unlikely to be the first gene expressed as was initially thought (van Vliet *et al.*, 2000). It would seem that the *fur* gene of *C.jejuni* was in an operon containing genes with differing functions, however the only way to test this was to locate the promoter of the *fur* gene.

The lack of promoter activity in the region upstream of the *fur* gene indicates that the location of the *fur* promoter remains uncharacterised. Location of the fur promoter would demonstrate the transcriptional organisation of *fur* and its surrounding genes and allow characterisation of the nature of its transcriptional regulation. Demonstration of Fur binding to the promoters of Fur regulated genes would lead to the possible discovery of further Fur regulated genes, would help reach a more accurate consensus Fur box sequence and discover what distinguishes Fur-boxes from PerR binding sites. Laetitia N. Madeleine, a visiting undergraduate project student on a Socrates Programme grant, carried out part of the work on characterisation of Fur expression.

6.2. Results.

The organisation of genes around *C.jejuni fur* and lack of a promoter directly upstream of the *C.jejuni fur* gene, suggest that the promoter is located further upstream of *fur*. Prior sequencing and analysis of the region upstream of *fur* in *C.jejuni* 81116 confirmed that the DNA sequence was 99% identical to the same region in *C.jejuni* NCTC11168 resulting in only two amino acid changes within *gatC* Cj0399 and *fur* (van Vliet, 2003), (van Vliet *et al.*, 2000). Location of the *C.jejuni* 81116 *fur* promoter was achieved by cloning various fragments of the proposed operon containing *fur* from *C.jejuni* 81116. Each fragment could then be tested for promoter activity using the established *LacZ* promoter assay method (see methods and chapter 3) (Wosten *et al.*, 1998). This would also allow investigation of whether Fur expression was auto-regulated and regulated in response to iron limitation.

Oligonucleotide primers were designed to produce a series of DNA fragments which, when ligated into pMW10, would allow measurements of promoter activity from each putative promoter region upstream of *fur* (P1-P5, figure 6.1). A series of overlapping fragments of different lengths were produced, allowing transcriptional read-through from a putative promoter into each downstream gene (figure 6.2). Each linear DNA fragment was individually amplified by PCR, purified using phenol chloroform extraction and restricted using *Bam*HI as described in chapter 3. Each cut fragment was then cleaned by phenol chloroform extraction, ethanol precipitated and re-suspended in a suitable

volume of distilled autoclaved water. Each fragment was individually ligated into *Bam*HI cut pMW10 and verified, to produce a series of overlapping constructs that would allow identification of any promoter activity in each region (the work described up to this point was carried out by Laetitia N. Madeleine). Attempts to clone any fragments that contained a complete *fur* gene (Figure 6.2, blue dashed lines) resulted in the production of fragments containing incorporation errors in the reading frame. This occurred repeatedly despite the use of a polymerase with a proof reading activity in the cloning PCR reaction. This indicates that multiple copies of *C.jejuni fur* are perturbing the vital cellular function of *E.coli*. Each construct was transformed into the *lacZ* assay strain *C.jejuni* 480 and an isolate containing each promoter construct was assayed for *lacZ* activity. Once transformed into *C.jejuni* 480, a strain containing each assay construct was cultured to OD 0.4 in both iron restricted Mueller-Hinton broth (20μM desferal), and iron replete Mueller-Hinton broth (40μM iron sulphate). The LacZ activities of *C.jejuni* 480 containing each promoter assay construct, grown in both iron-replete and iron-limited conditions were measured. In this way control of promoter activity in response to iron could also be measured. The experiment was repeated at least three times. The *lacZ* activities of each assay construct strain can be seen in figure 6.3. The same controls were used as in previous promoter assay experiments, i.e. *C.jejuni* 480 containing pMW10 as a negative control, p23E5 (*C.jejuni metK* promoter in pMW10) as a positive control or pAV201 (*C.jejuni katA* promoter in pMW10) as an iron responsive positive control.

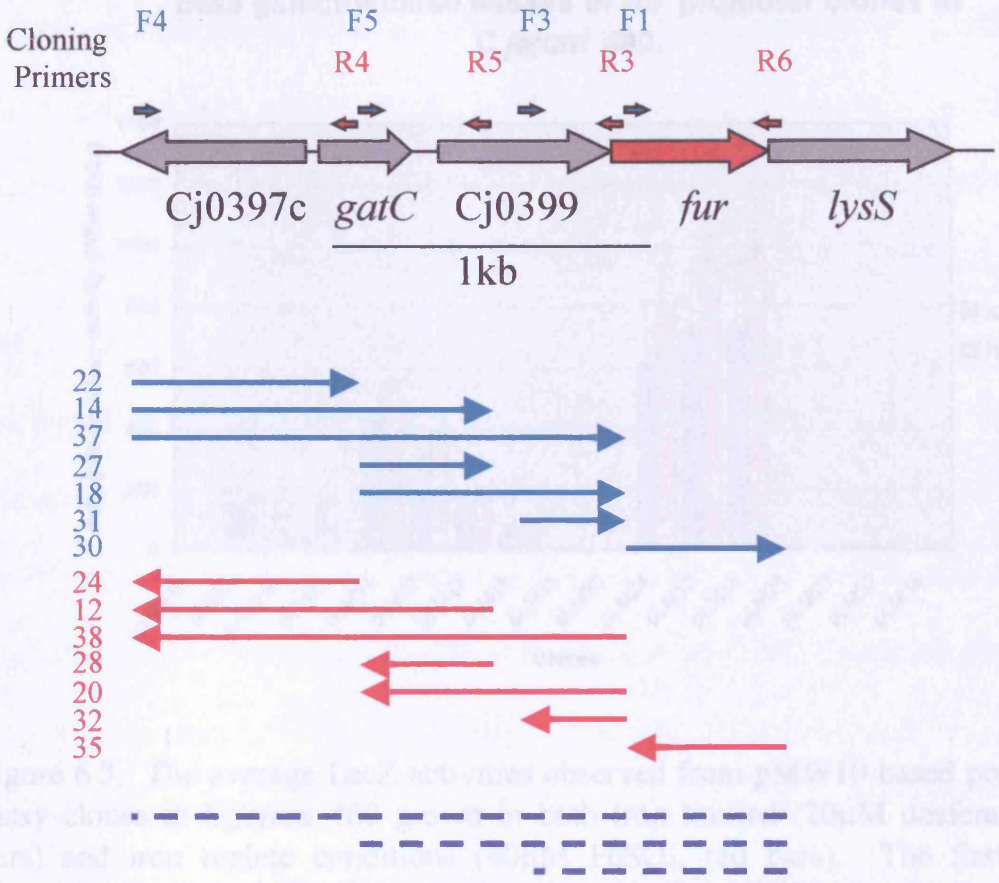


Figure 6.2. The cloning strategy used to investigate the promoter arrangement of the *fur* gene of *C.jejuni* NCTC11168. At the top can be seen the arrangement of genes around the *fur* gene, above this can be seen the positions of cloning primers used to PCR each overlapping fragment. Forward primers are represented by green arrows, reverse primers are represented as red arrows, each DNA segment was PCR amplified using one forward and one reverse primer. Below the gene representations can be seen the regions cloned and the corresponding plasmid number (left hand side) containing that region. Represented in green are so called forward clones, in red are the reverse clones the arrows point to the position that the *lacZ* gene will be once ligated into pMW10. This means promoter activity will be measured from this end of the cloned fragment. The blue dashed lines at the bottom represent sections of DNA that could not cloned successfully without mutation, due to the presence of the complete *fur* gene.

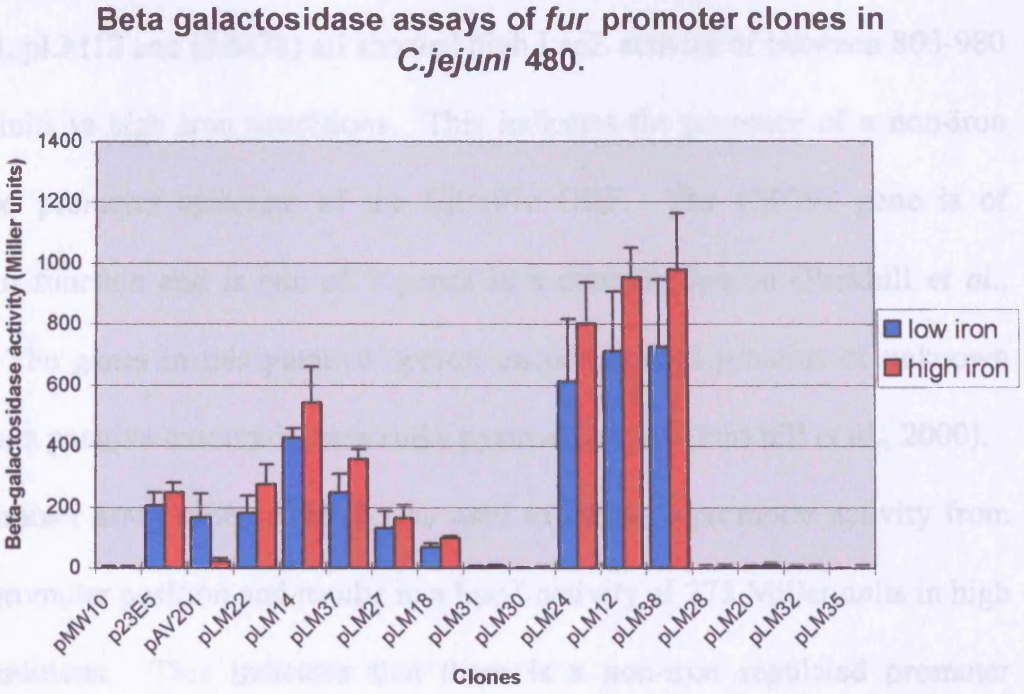


Figure 6.3. The average LacZ activities observed from pMW10 based promoter assay clones in *C.jejuni* 480 grown in both iron limited (20μM desferal, blue bars) and iron replete conditions (40μM $FeSO_4$, red bars). The first three plasmids act as controls, firstly a negative control (pMW10 alone), secondly a positive control (p25E5: pMW10 *metK* promoter) and lastly an iron responsive positive control (pAV201: pMW10 *katA* promoter). The plasmids pLM22, pLM14, pLM37, pLM27, pLM18, pLM31, pLM30 are forward clones (see figure 6.2) and pLM24, pLM12, pLM38, pLM28, pLM20, pLM32, and pLM35 are reverse clones (see figure 6.2).

From the results of the LacZ assay (figure 6.3.), it is noted that pMW10 gives little LacZ activity in high and low iron conditions and p23E5 has a consistent LacZ activity of between 200-250 Miller units in both high and low iron conditions. Cells containing pAV201 have an activity of around 23 Miller units in iron-replete condition and an activity of around 166 Miller units in iron limited conditions, displaying clear iron responsive promoter activity.

Promoter assay clones used to measure promoter activity at the P1 position (pLM24, pLM12 and pLM38) all showed high LacZ activity of between 803-980 Miller units in high iron conditions. This indicates the presence of a non-iron regulated promoter upstream of the Cj0397c ORF. The Cj0397 gene is of unknown function and is one of 7 genes in a putative operon (Parkhill *et al.*, 2000). The genes in this putative operon encode several proteins of unknown function, a putative oxidoreductase and a pyruvate kinase (Parkhill *et al.*, 2000). The promoter assay clone pLM22 was used to measure promoter activity from the P2 promoter position and results in a LacZ activity of 275 Miller units in high iron conditions. This indicates that there is a non-iron regulated promoter directly upstream of the *gatC* gene (putative Glutamine-tRNA Glycine amidotransferase subunit C).

The promoter assay clone pLM27 measures promoter activity from the P3 position, directly upstream of Cj0399. *C.jejuni* 480 containing pLM27 demonstrates a LacZ activity of around 164 Miller units in high iron conditions, indicating the presence of a promoter in this location. Interestingly promoter assay clones that contain both the P2 and P3 putative promoter regions demonstrate a higher LacZ activity than either of these two promoter regions alone. *C.jejuni* 480 containing pLM14 (P2 and P3) had a LacZ activity of 541 Miller units and pLM37 (P2, P3 and P4) had an activity of 358 Miller units (both in high iron conditions).

The promoter assay clone pLM31 measures promoter activity from the P4 position, i.e. a promoter directly upstream of the *fur* gene predicted to be the

position of the *fur* promoter in earlier studies (Chan *et al.*, 1995). As can be seen from figure 6.3 *C.jejuni* 480 containing pLM31 has a negligible LacZ activity in both high and low iron conditions and so is unlikely to be the site of a promoter responsible for Fur expression.

C.jejuni 480 containing pLM18 measures promoter activity from the P3 and P4 positions. A LacZ activity of 99 Miller units in high iron conditions was observed from pLM18, this observation, and the negligible promoter activity from the P4 position alone, indicates that *fur* is likely to be co-transcribed along with Cj0399, *lysS* and *glyA* from the P3 position.

The promoter assay clone pLM30 measures promoter activity from the P5 position i.e. any possible promoter directly upstream of the *lysS* gene. *C.jejuni* 480 containing pLM30 has negligible LacZ activity demonstrating that it is unlikely that *lysS* possesses its own promoter. This indicates that *lysS* and *glyA* are likely to be co-transcribed with the *fur* gene. What was striking about the results was that none of the promoters detected demonstrated any iron responsive activity, indicating that auto-regulation of Fur expression or PerR dependent regulation is unlikely.

Rather than using *C.jejuni* 480 this experiment was first attempted in an *E.coli* LacZ assay strain (MC4100) and produced entirely different results. Certain promoter assay clones that demonstrated promoter activities in *C.jejuni* 480 (notably pLM12, pLM14, pLM22, pLM24, pLM37 and pLM38) produced low LacZ activities in *E.coli* MC4100. Other promoter assay clones such as pLM28 produced greater LacZ activities in *E.coli* than *C.jejuni*. The differences

observed between LacZ assays carried out in *E.coli* and *C.jejuni* indicate that *C.jejuni* promoter sequences sequences may be different from those found in *E.coli* and that measurement of *C.jejuni* promoter activity must be carried out in *C.jejuni* strains (Wosten *et al.*, 1998). Also of interest was the fact that pAV201, the iron responsive positive control plasmid lost not only its promoter activity (typically under 50 Miller units in *E.coli* MC4100, compared with 166 Miller units in *C.jejuni* 480, low iron conditions) but also its iron-repressed nature.

6.3. Discussion.

As seen previously in this study (Chapter 3) and other studies (Wosten *et al.*, 1998) the use of the LacZ assay vector pMW10 provides a useful method of determining promoter activity. Despite the previous claims that a promoter exists directly upstream of the *fur* gene (Chan *et al.*, 1995), this study provides no evidence of this. There was no promoter activity detected in the region between the *fur* and Cj0399 genes using the LacZ assay system. The prior detection of a promoter directly upstream of the *fur* gene was based on two strands of evidence, firstly the primer extension results, secondly the detection of Fur-box and CAP sequences (Chan *et al.*, 1995). The primer extension results may be misleading as later primer extension experiments on *C.jejuni fur* (Wooldridge, 2003) produced a variety of truncated extension products, one of which may have been mistaken for the product of a promoter directly upstream of *fur*. The production of truncated products may have been due to instability or processing of the polycistronic mRNA from which Fur is translated. The detection of Fur-boxes

directly upstream of the *C.jejuni fur* gene (Chan *et al.*, 1995) may be due to the consensus sequence used to detect the Fur binding sites being based on Fur box sequences from *E.coli*. The *E.coli* genome is 50.79 %GC (Blattner *et al.*, 1997), Fur-boxes being A-T rich are prominent in the *E.coli* genome sequence. *C.jejuni* has an A-T rich genome and so the presence of a sequence that matches the *E.coli* Fur-box consensus may not signify the presence of a *C.jejuni* Fur-box. The results presented in this chapter indicate that there is no promoter directly upstream of the *fur* gene, at least in *C.jejuni* NCTC11168. The conflicting evidence presented in this study and previous studies (Chan *et al.*, 1995) may reflect some sequence variation between the *C.jejuni* strains used.

The negligible promoter activity detected in the region between the *fur* and *lysS* genes confirms assertions made in previous studies (Chan *et al.*, 1995; van Vliet *et al.*, 1998b) that *fur* is located in an operon containing *lysS* and *glyA* (and likely Cj0403, Cj0404 and *aroE*). The location of *C.jejuni fur* in an operon with essential house keeping genes is the reason why a *fur* mutant strain construction could only be constructed by insertion of an antibiotic resistance cassette in the same orientation as the *fur* gene (van Vliet *et al.*, 1998b). Ligation of an antibiotic resistance cassette in the opposite orientation to the *fur* gene causes a lethal polar mutation, disrupting expression of essential downstream genes. It is highly unusual among Gram-negative organisms that *fur* is co-transcribed with a number of housekeeping genes. In many Gram-negative organisms, Fur is expressed mono-cistronically (Prince *et al.*, 1993; Staggs and Perry., 1992) and

in an autoregulated manner (De Lorenzo *et al.*, 1988; Delany *et al.*, 2002; Sebastian *et al.*, 2002; Tsolis *et al.*, 1995).

The observation of *C.jejuni* Fur expression from a promoter upstream of Cj0399 indicates that Fur is expressed as part of a polycistronic message. The operon containing *fur* contains Cj0399, *lysS* and *glyA*, and this genetic organisation is conserved among *C.jejuni* strains (Chan *et al.*, 1995; Wooldridge *et al.*, 1994) and *Campylobacter* species (Bourke *et al.*, 1996).

Promoter activity discovered in the region upstream of the *gatC* gene results in Fur being expressed from a second promoter. Promoter assay clones that contained both promoters (upstream of Cj0399 and upstream of *gatC*) had a higher promoter activity than either single promoter, indicating production of two separate transcripts encoding Fur. Downstream of *glyA* are Cj0403 (hypothetical protein), Cj0404 (putative transmembrane protein) and *aroE* (shikimate 5-dehydrogenase), which are all directly adjacent to, or overlapping one another. It is possible to presume that these genes are also co-transcribed with *fur*, giving rise to the presence of two long transcript species. These transcripts contain Cj0399, *fur*, *lysS*, *glyA*, Cj0403, Cj0404, *aroE* and possibly *gatC* depending upon which promoter they were transcribed from. The long transcripts may correspond to the two long extension products observed in the primer extension experiments of previous studies (Chan *et al.*, 1995). The third shorter extension product that led earlier researchers to propose that *fur* possessed its own upstream promoter may be the result of RNA processing. It may be possible that

additional promoters exist downstream of *fur*, which may be verified in the future by cloning downstream regions into pMW10.

The presence of a promoter upstream of the partial ORF Cj0397c (hypothetical protein) was observed but this promoter was deemed unlikely to have any effect on Fur expression and so is outside the scope of this study.

The fact that none of the promoters observed in this study are transcriptionally regulated in response to iron means that Fur expression is unlikely to be auto-regulated in a conventional manner. This raises interesting questions about the regulation of Fur expression. This means that Fur could be constitutively expressed or could be expressed in response to an environmental signal other than iron. The presence of two upstream promoters may suggest that Fur expression may be under the control of 2 signals, allowing *C.jejuni* greater freedom of regulation in response to environmental signals. A similar organisation is present in *E.coli*, allowing Fur to be expressed from one promoter in response to peroxide and another in response to superoxide (Zheng *et al.*, 1999). This results in *E.coli* Fur being expressed in response to four environmental signals, autoregulation in response to iron restriction by Fur, presence of the catabolite cAMP by Crp (De Lorenzo *et al.*, 1988), peroxide by OxyR and superoxide by SoxS (Zheng *et al.*, 1999). The *fur* gene of *Ps aeruginosa* is also apparently transcribed from two promoters although it remains unclear whether *Ps.aeruginosa* Fur is expressed in response to environmental signals (Barton *et al.*, 1996). It is unknown whether oxidative stress may play a part in regulation of Fur expression in *C.jejuni* or but it may be unlikely that

Chapter 6. Characterisation of Fur expression

PerR is involved in regulation of Fur expression due to the lack of iron responsiveness of any of the promoters characterised.

The results observed when measuring *C.jejuni* promoter activity in *E.coli* highlight differences between the genomes and transcriptional regulation of these two organisms. It was observed that pAV201, the iron-responsive control plasmid, lost the iron-regulated nature of its promoter when assayed in *E.coli.* The loss of iron regulation may be explained by the fact that pAV201 contains the promoter from the *C.jejuni katA* gene, which is regulated in response to iron limitation (and oxidative stress) by PerR (van Vliet *et al.*, 1999). *E.coli* has no PerR, instead relying on OxyR and SoxRS to regulate genes in response to oxidative stress (Zheng *et al.*, 1999). The promoter activity detected upstream of *fur* in *E.coli* may have led to the observed ability of a 5Kb fragment cloned in pUC19 containing *C.jejuni fur* to complement an *E.coli fur* mutation (Wooldridge *et al.*, 1994) and the misidentification of a promoter directly upstream of *C.jejuni fur* (Chan *et al.*, 1995).

During the course of this study expression of the Fur protein was attempted in order to demonstrate Fur binding to promoters of iron responsive genes. The ability to demonstrate Fur binding would allow analysis of the composition of a *C.jejuni* Fur box, what differentiates it from PerR binding sequences and allow detection of a greater number of Fur regulated genes. Previous attempts to express His_6 tagged Fur ran into difficulty because Fur bound irreversibly to the nickel column used to purify His_6-tagged proteins (van Vliet, 2003). Initially attempts to express Fur were made using pGEX2T (Amersham Pharmacia

Biotech) because pGEX2T had been used to express a slightly truncated *C.jejuni* Fur in a previous study (Chan *et al.*, 1995), and doesn't involve purification using nickel (Makrides, 1996).

Upon transformation into *E.coli* there were very few transformants, all of which contained plasmid with incorrect or re-arranged inserts, or correctly verified inserts that did not produce expressed protein. It was thought that transcription must not be tightly regulated enough in this vector by the *lac* repressor, allowing small amounts of *C.jejuni* Fur protein to be expressed in the *E.coli* expression strain, interfering with metabolism.

As expressing pure Fur was proving difficult it was decided that a more simple way to visualise Fur-promoter binding. Fur/promoter binding was attempted by applying cytoplasmic protein extracts (van Vliet *et al.*, 1998a) from wild type *C.jejuni* NCTC11168 and a Fur- mutant strain (AV17) to PCR-amplified *chu* promoter DNA (primers ChuAR4 and Cj1613R2). The protein extracts and promoter DNA were incubated together and run on an agarose gel (Goldberg., 2001). The gel was then blotted onto Nylon membrane and non-radioactively hybridised (materials and methods, section 2.9.) with labelled random prime oligonucleotides (Gene images random prime labelling module, Amersham life sciences) produced from the PCR amplified *chu* promoter. The labelled oligonucleotides were then detected by exposure to the detection reagents and exposed to photographic film for varying lengths of time.

Upon examination a great deal of background staining of the photographic images was present, probably due to DNA contamination in the cytoplasmic

protein fractions. This made them difficult to analyse and consequently this data is not shown. A general reduction in DNA mobility was observed in all samples, which increased with larger amounts of cytoplasmic protein (both wild type and *fur* mutant). A reduction in gel mobility of the *chu* promoter was observed when incubated with neat *C.jejuni* NCTC11168 cytoplasmic protein extract. Unfortunately the results of *fur* mutant protein were obscured by background staining meaning that the contribution of the Fur protein to DNA binding was difficult to assess. The expression of *C.jejuni fur* in *E.coli* presents certain difficulties due to the toxic effects of *C.jejuni* Fur upon *E.coli* metabolism, which is apparent in the inability to clone fragments containing the complete *fur* gene in pMW10. Once cloned, expression of purified His_6 tagged Fur cannot be carried out due to the irreversible binding of Fur to the divalent nickel used to purify such proteins. The vector pGEX2T had already been used to express Fur that was slightly truncated at the N-terminal region but appeared to be fully functional (Chan *et al.*, 1995). The ability to clone, or clone and express *C.jejuni* Fur has been demonstrated in a number of previous studies (Chan *et al.*, 1995), (van Vliet *et al.*, 1998b; Wooldridge *et al.*, 1994). It is entirely possible that the *lac* promoter of pGEX2T is not tightly regulated enough, or that coding sequence of the *fur* gene is acting as a promoter. The failure to clone any fragments that contained an intact *fur* gene in pMW10 supports the theory that expression of multiple copies of *C.jejuni* Fur may be toxic to *E.coli*. In either case some expression of functional Fur may be occurring which coupled with the high plasmid copy number may lead to significant amounts of toxic *C.jejuni* Fur in

E.coli. The tighter regulation of *tet* promoter present in pASK-IBA vectors may may be more suitable for the expression of *C.jejuni* Fur. The use of these vectors may prove successful and allow the expression of *C.jejuni* Fur in the future. Since the completion of this study *C.jejuni* Fur has been successfully expressed using the pASK-IBA system (Ketley, 2003). The ability to express functional *C.jejuni* Fur has allowed demonstration of Fur binding to the promoters of the *chuA*, *cfrA*, *ceuB* and *p19* genes (Ketley, 2003).

Fur/promoter binding could be used in future studies to assess specificity of Fur/Fur-box interaction, discovery of Fur regulated genes of *C.jejuni* and to distinguish Fur-boxes from PerR recognition sequences. Greater characterisation of the *C.jejuni* Fur regulon could be achieved by gel shift assays and DNAase footprinting of a greater selection of promoters. Analysis of the transcriptome of *C.jejuni* may assist in characterisation of what constitutes a Fur-box sequence and allow the identification of further Fur-Boxes in the C.jejuni genome.

In summary the *C.jejuni* Fur protein is expressed with the *lysS* and *glyA* housekeeping genes from two distantly located promoters (adjacent to the gatC and Cj0399 genes). Expression from the two promoters is not controlled in response to iron and is therefore not autoregulated by the Fur protein.

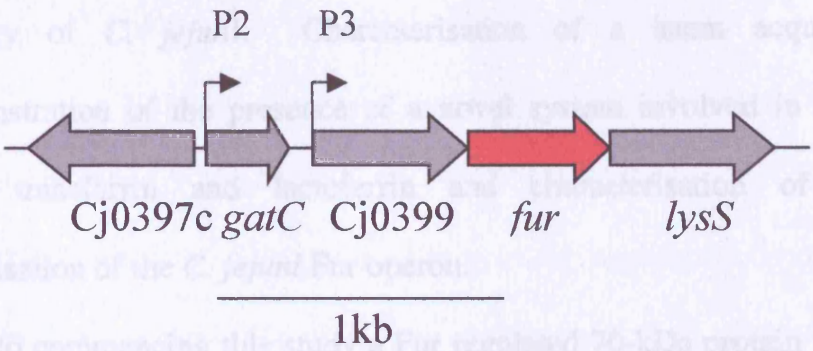


Figure 6.4. The actual positions of the 2 *fur* promoters within the *C.jejuni fur* operon, the promoters were found to be in positions 2 and 3 (figure 6.1) and are indicated by black arrows.

7. General Discussion

The work presented in this study allows an insight into three facets of the iron biology of *C. jejuni*. Characterisation of a haem acquisition system, demonstration of the presence of a novel system involved in iron acquisition from transferrin and lactoferrin and characterisation of transcriptional organisation of the *C. jejuni* Fur operon.

Prior to commencing this study a Fur regulated 70-kDa protein was predicted to be involved in haem uptake due to sequence similarity to haem receptors from other bacterial species (van Vliet *et al.*, 1998b). The *chuA* gene which encoded this protein was located on the *C. jejuni* chromosome situated upstream of three genes encoding genes with similarity to ABC transport systems (Parkhill *et al.*, 2000; van Vliet *et al.*, 1998b). Due to the organisation of the *chu* ABC transport genes in a predicted operon with *chuA*, the *chuB*, *C* and *D* genes were predicted to be involved in haem transport across the periplasm and inner membrane. The objective of this study was to test the hypothesis that the *chu* genes encode a *C. jejuni* haem uptake system. This hypothesis was investigated by functional analysis of the *chu* genes and characterisation of the *chu* promoter. The presence of *chu* gene sequences is widely distributed amongst *C. jejuni* strains. Functional analysis of the *chu* genes by mutational inactivation, confirmed that *chuA* encodes an OM haem receptor, able to acquire haem from hemin, haemoglobin and haemoglobin-haptoglobin as iron sources. Investigation of promoter activity in the region upstream of *chuA* showed that the ChuA protein was expressed from an iron-responsive promoter under transcriptional control of the Fur protein.

Chapter 7. General discussion.

The organisation of the *chu* genes and the lack of T-loop structures between *chuA* and *chuB* indicate the *chu* genes are probably co-expressed from a single promoter although this requires verification.

Functional analysis of the ABC transport genes by mutational inactivation showed that none of the *chuB*, *C* and *D* genes or *hitA*, *B* and *C* genes are essential for haem utilisation. The search for an inner membrane haem uptake system remains largely incomplete. *C. jejuni* contains a large number of uncharacterised and putative ABC transport system proteins (Parkhill *et al.*, 2000) that may be involved in the transport of haem across the inner membrane. Functional redundancy amongst proteins involved in haem acquisition is observed in a number of bacterial species including *H. influenzae* (Morton *et al.*, 1999), *V. cholerae* (Occhino *et al.*, 1998) *Ps. aeruginosa* (Ochsner *et al.*, 2000) and the pathogenic *Neisseria* (Schryvers and Stojiljkovic, 1999). This means that the most efficient way to discover which ABC transport system may be involved in haem uptake in *C. jejuni* may be expression of suspected ABC transport system proteins (plus ChuA) in a bacterial species that cannot utilise hemin. The fact that ChuB, C and D are not necessary for haem uptake may simply mean that import of haem into the cytoplasm is not necessary for iron acquisition from haem. It is possible that *C. jejuni* possesses a novel system that allows the liberation of iron from periplasmic haem, which although unlikely (there is no precedent for this in Gram-negative bacteria), remains to be disproven. The lack of a proven phenotype for the *chuBCD* and *hitABC* mutants may simply mean that ChuBCD and HitABC are not involved in the uptake of haem. The ChuBCD

and HitABC proteins may be involved in the uptake of ferrous iron (or iron citrate), other iron-containing compounds (ferritin or siderophores) or non iron-containing compounds. This provides characterisation of the haem iron acquisition system of *C. jejuni*, the only other fully characterised iron acquisition system being the *ceu* ferri-enterobactin uptake system (Park and Richardson., 1995). Other genes that may be involved in *C. jejuni* iron acquisition (due to sequence similarity to proteins involved in haem uptake in other bacterial species) include *cfrA*, *p19*, *tbpA* (Cj0178) Cj1613 and the *hit* genes (Cj0175, Cj0174 and Cj0173), although the function of these genes remains to be proven. Previous studies of iron-acquisition from host iron-containing molecules demonstrated that *C. jejuni* could utilise hemin, haemoglobin, hemin-hemopexin and haemoglobin-haptoglobin, and was unable to utilise ferritin, lactoferrin and transferrin (Pickett *et al.*, 1992). The presence of a gene with sequence similarity to *tbpA* of *H.influenzae* and *N.meningitidis,* and the development of a quantitative assay of bacterial iron-acquisition from Tf and Lf, allowed further investigation of the ability of *C. jejuni* to utilise Tf or Lf bound iron. The results presented Chapter 5 demonstrate for the first time that *C. jejuni* can acquire iron from the host iron binding proteins Tf and Lf. Although an acquisition system has not been identified or characterised, *C. jejuni* iron acquisition from Lf/Tf demonstrates several distinguishing characteristics. *C. jejuni* must be in contact with Tf/Lf in order to acquire iron, and the involvement of siderophores or catecholamines are not necessary for iron acquisition contrary to observations in *E.coli* (Burton *et al.*, 2002; Freestone *et al.*, 2000). Iron acquisition from Tf/Lf

appears to be energy dependent and is characterised by specific binding of Lf or Tf at the cell surface, followed by uptake of iron into the soluble fractions (periplasm/cytoplasm) of bacterial cells. Lf appears to be a preferred iron source to Tf because *C. jejuni* obtains iron more readily from Lf than Tf. The FlaA protein demonstrated a specific binding affinity for Lf, however it remains unlikely that FlaA is involved in iron acquisition from Lf.

Unless a completely novel mechanism of iron acquisition from Tf/Lf occurs in *C. jejuni* this leaves two possibilities: -

a) That *C. jejuni* acquires iron from Tf/Lf by the use of receptor based uptake systems as seen in the pathogenic *Neisseria* (Schryvers and Stojiljkovic, 1999).

b) That *C. jejuni* liberates iron from Tf/Lf by employment of OM bound enzymes, such as proteases or ferric reductases and *C. jejuni* then takes up the liberated iron.

At this time the presence of a receptor based uptake system in *C. jejuni* would seem more probable due to the lack of molecular evidence for ferric reductases and OM proteases at present in *C. jejuni* (Parkhill *et al.*, 2000). Also because mutation of Cj0178 lowers the ability of *C. jejuni* to obtain iron from Lf, this suggests the possible presence of a multi-component OM receptor as observed in the pathogenic *Neisseria* (Boulton *et al.*, 1999). Molecular identification and characterisation of the system/systems involved in iron acquisition from Lf/Tf seems the only way to test such hypotheses and so must remain central to future research on *C. jejuni* iron acquisition from Tf/Lf. A potential target for future

investigation is the *feoB* gene, which may be involved in the uptake of periplasmic iron liberated from Tf/Lf at the *C. jejuni* OM.

Previous sequence analysis of the *C. jejuni fur* gene demonstrated the presence of putative Fur-box and, by primer extension analysis, Fur expression was predicted to be initiated from a promoter directly upstream of the *fur* gene (Chan *et al.*, 1995). This study demonstrated that expression of *C. jejuni* Fur is regulated in an unusual manner compared with the Fur homologues of other Gram-negative bacteria. The results of *C. jejuni fur* promoter analysis showed that Fur is not expressed from a promoter directly upstream of the *fur* gene as previously supposed (Chan *et al.*, 1995), and observed in a number of bacteria including *E.coli* (De Lorenzo *et al.*, 1988), *Helicobacter pylori* (Delany *et al.*, 2002) and *Klebsiella pneumoniae* (Achenbach and Yang, 1997). Also *C. jejuni* Fur expression is not auto-regulated and not expressed in response to iron limitation in contrast to Fur expression observed *E.coli* (De Lorenzo *et al.*, 1988), *Helicobacter pylori* (Delany *et al.*, 2002) and the pathogenic *Vibrio* (Colquhoun and Sorum, 2002). The *C. jejuni fur* gene is situated within an operon of housekeeping genes and may be transcribed from either of two distal upstream promoters. When both promoters were present in a pMW10 construct containing a *fur:lacZ* fusion, expression was shown to be higher than when either promoter alone was present. This indicates that both promoters may be involved in expression of *C. jejuni* Fur. A similar observation has been noted with the promoter of *E.coli fur* (Zheng *et al.*, 1999) in a study that demonstrated that *fur* is encoded on two mRNA transcripts. One transcript is produced from a promoter

directly upstream of the *fur* gene in response to peroxide stress (OxyR) and a longer transcript, which also encodes a flavodoxin, is produced in response to superoxide (SoxS). This allows the *E.coli* Fur expression to be controlled by four different regulatory proteins (Fur, CAP, OxyR and SoxS (De Lorenzo *et al.*, 1988; Zheng *et al.*, 1999)) in response four different environmental stimuli (iron restriction, presence of catabolite, peroxide and superoxide). A similar purpose may be achieved *C. jejuni* by the possession of two different *fur* promoters allowing *C. jejuni* versatility in response to a number of different environmental stimuli. At present it is not possible to speculate which regulatory proteins or environmental stimuli may be involved in *C. jejuni* Fur expression. It is noted however that iron appears to have no effect on Fur expression meaning that neither Fur nor PerR (van Vliet *et al.*, 1999) seem to affect Fur expression in the conventional manner. It is likely that Fur functions as a global regulator of protein expression in *C. jejuni* as described in *E.coli* (Escolar *et al.*, 1998; Zheng *et al.*, 1999), rather than a specific iron responsive repressor. The benefit of *C. jejuni* Fur being co-expressed with a number of housekeeping genes and putative ORFs (Cj0399, *fur*, *lysS*, *glyA*, Cj0403, Cj0404, *aroE* and *gatC*) seems unclear at present. Many genes in the *C. jejuni* genome are arranged in operons and pathogenicity islands appear rare. It is possible that co-expression of genes is simply an adaptation to the maintenance of a compact genome and further investigation is required to establish the significance of the genetic organisation in the *fur* region.

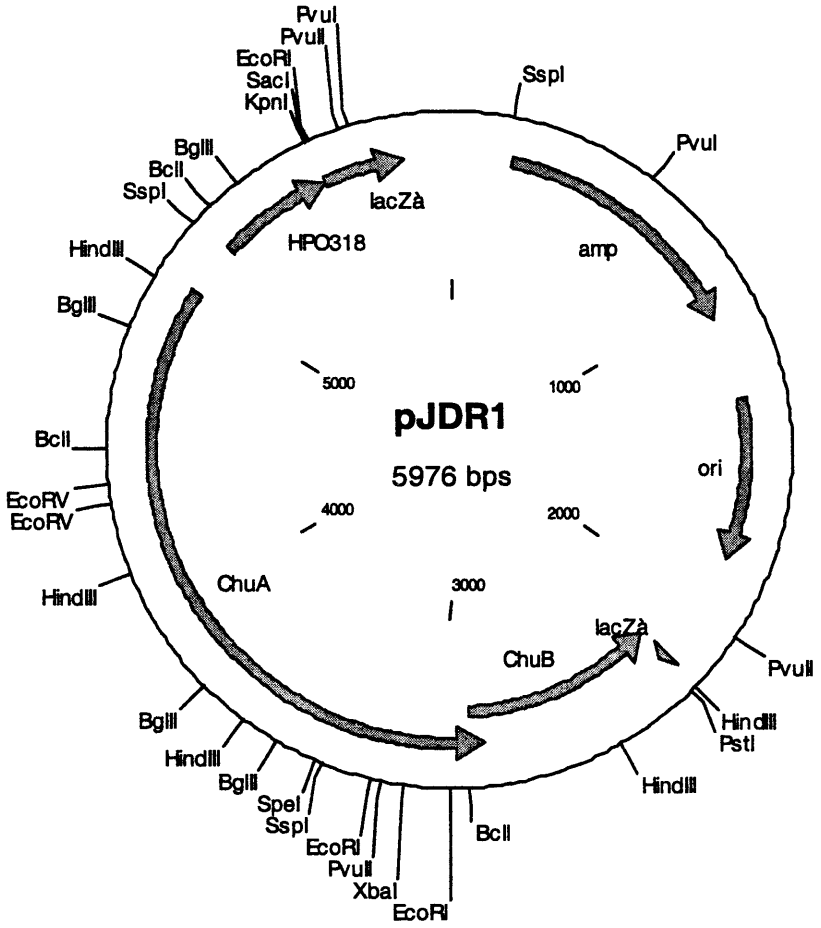
Chapter 7. General discussion.

To date it has been demonstrated that *C. jejuni* can utilise a number of molecules as iron sources, including host molecules such as transferrin, lactoferrin haem, haemoglobin, haemogobin-haptoglobin (this study), haem-haemopexin (Pickett *et al.*, 1992) and siderophores (Field *et al.*, 1986; Park and Richardson., 1995). Ability to acquire iron from a number of iron-containing molecules is vital to the transmission (colonisation of poultry and survival in the environment), and pathogenesis of *C. jejuni* in humans. The ability of *C. jejuni* to respond to environmental stimuli remains central to its survival in the range of environments it encounters during transmission. This includes regulation of protein expression in response to iron limitation and oxidative stress by the Fur and PerR proteins. Future characterisation of iron-acquisition systems in *C. jejuni* will allow further analysis of transcriptional gene regulation by Fur, PerR and other transcriptional regulators in response to environmental signals, which in turn allows prediction of genes that are critical for host colonisation and environmental survival. Conversely, comprehensive analysis of Fur and PerR binding sites will allow prediction of genes involved in iron uptake and metabolism by demonstration of such sequences within promoters by bio-informatics. Analysis of genes transcribed under iron limited conditions and demonstration of Fur/promoter binding may also allow further prediction of genes involved in iron uptake and metabolism. What is apparent from the data presented in this study is that genetic function may not be predicted by transcriptional organisation of genes with genes of known function. The organisation of *chuB*, *C* and *D* in a predicted operon with the *chuA* outer membrane haem-receptor gene and organisation of

Chapter 7. General discussion.

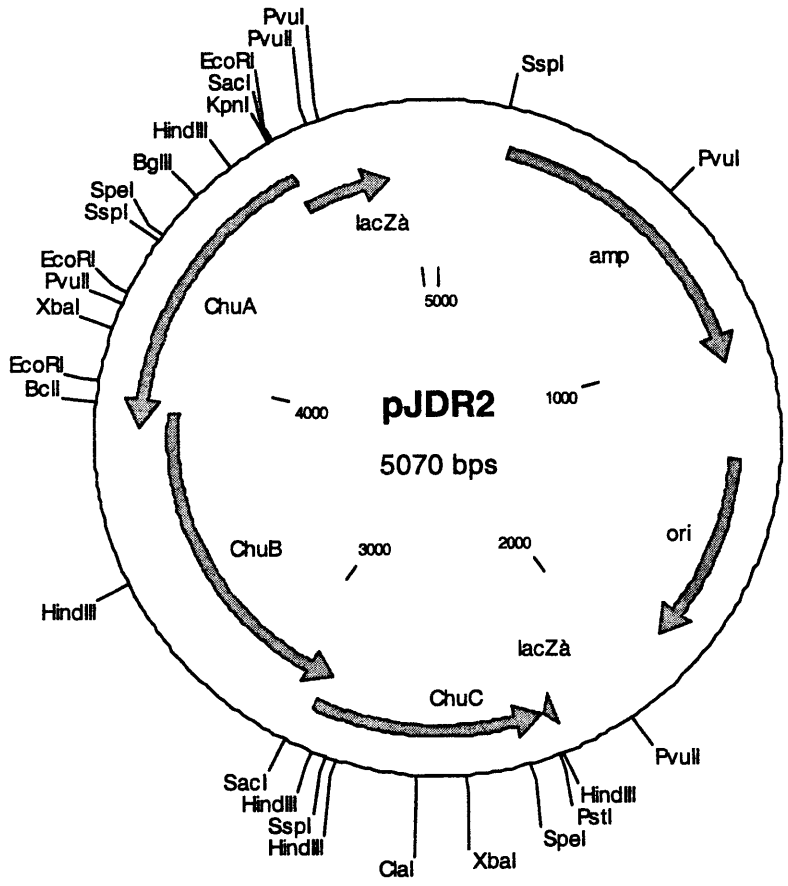
Fur in an operon with housekeeping genes are just two examples of extensive mixed function operon structure in *C. jejuni* (Parkhill *et al.*, 2000). The role of particular iron-acquisition systems and their expression during transmission of *C. jejuni* and colonisation of humans and livestock is central to the success of *C. jejuni* as a pathogen. Future research into iron-acquisition and iron-responsive gene regulation may allow identification of future targets for chemotherapeutic intervention during *C. jejuni* infection.

Appendix 1. Plasmid maps.



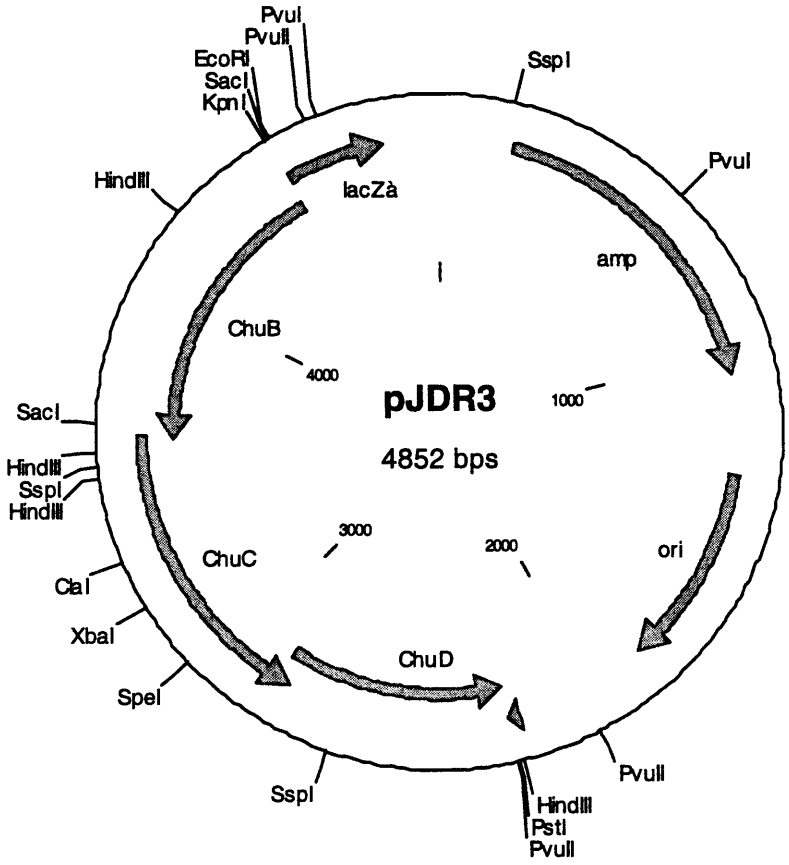
pJDR1: pUC19 containing cloned *C.jejuni* NCTC11168 *chuA* gene.

Appendix 1. Plasmid maps.



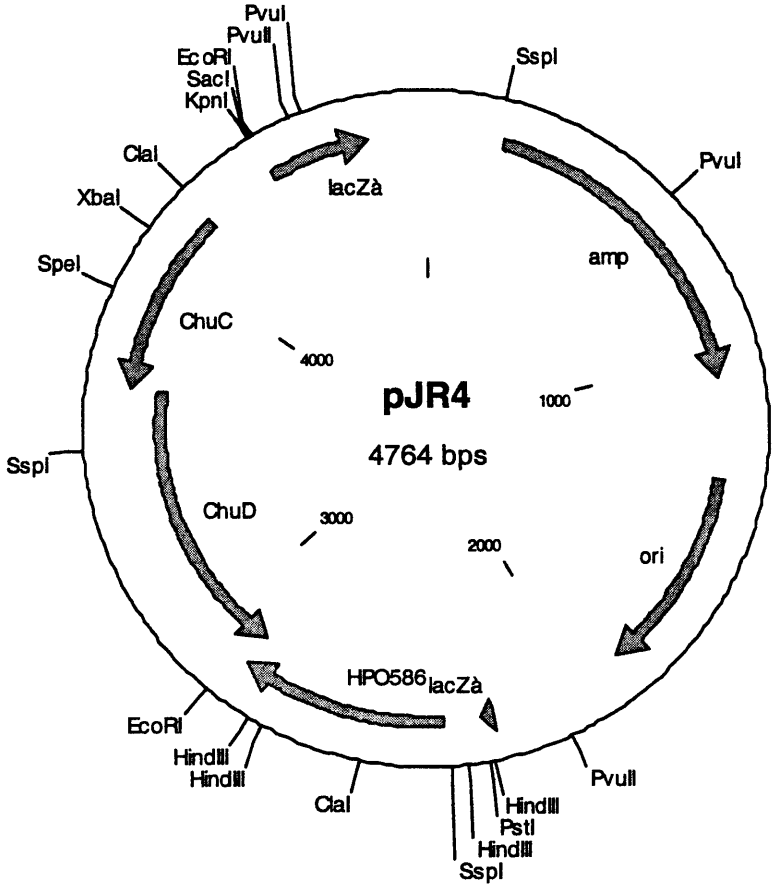
pJDR2: pUC19 containing cloned *C.jejuni* NCTC11168 *chuB* gene.

Appendix 1. Plasmid maps.



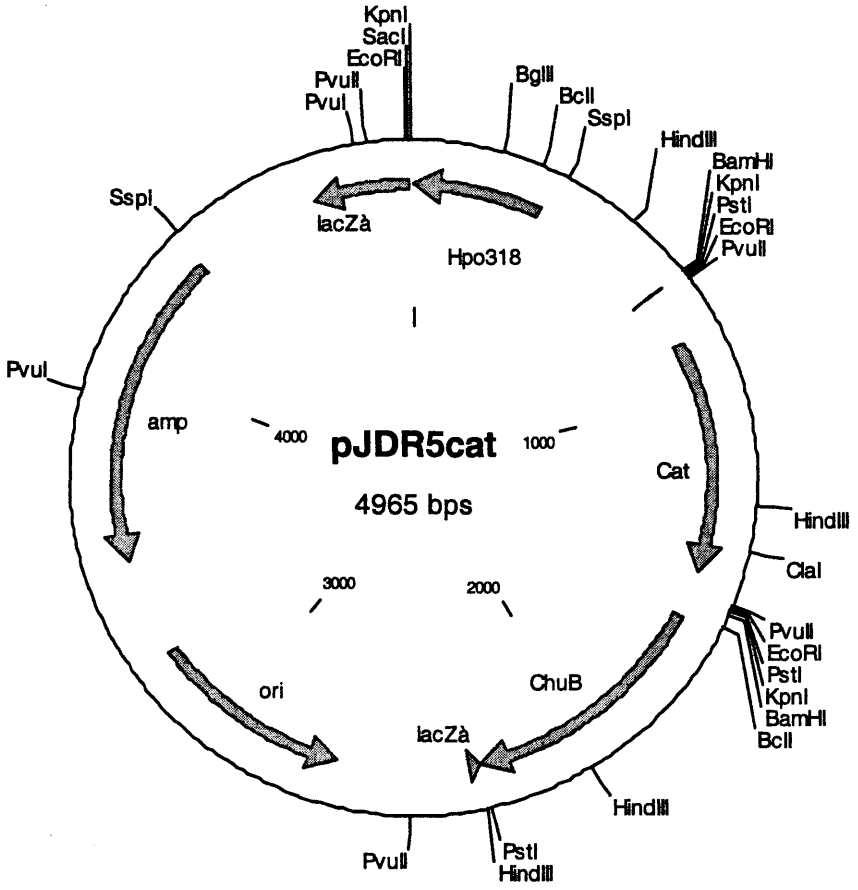
pJDR3: pUC19 containing cloned *C.jejuni* NCTC11168 *chuC* gene.

Appendix 1. Plasmid maps.



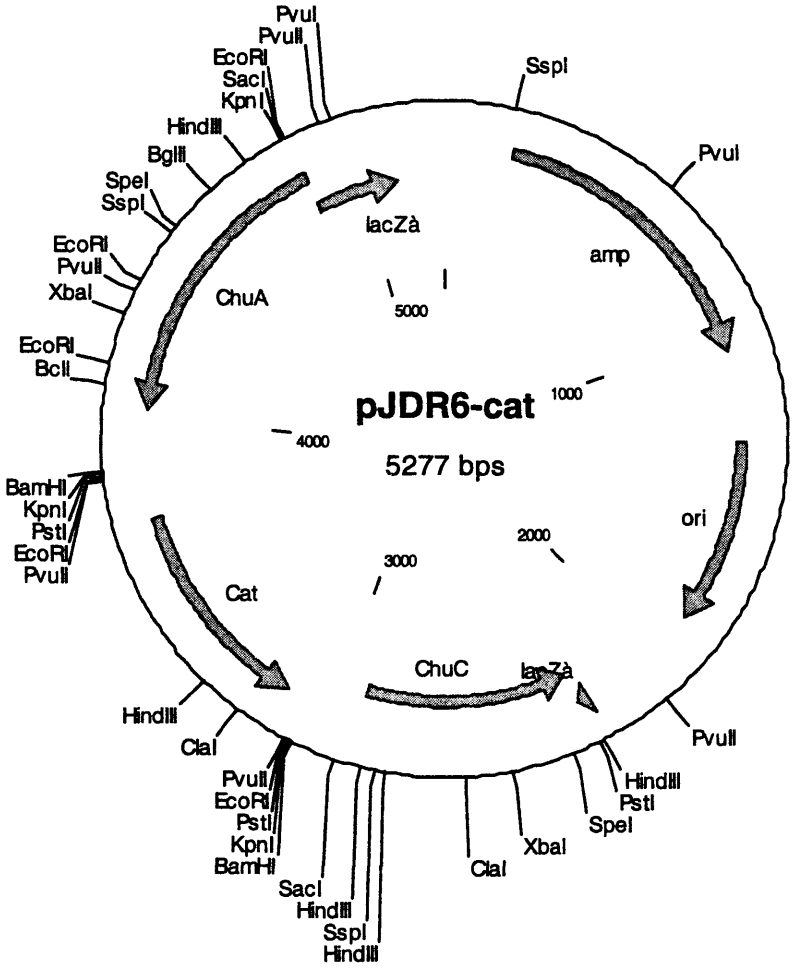
pJDR4: pUC19 containing cloned *C.jejuni* NCTC11168 *chuD* gene

Appendix 1. Plasmid maps.



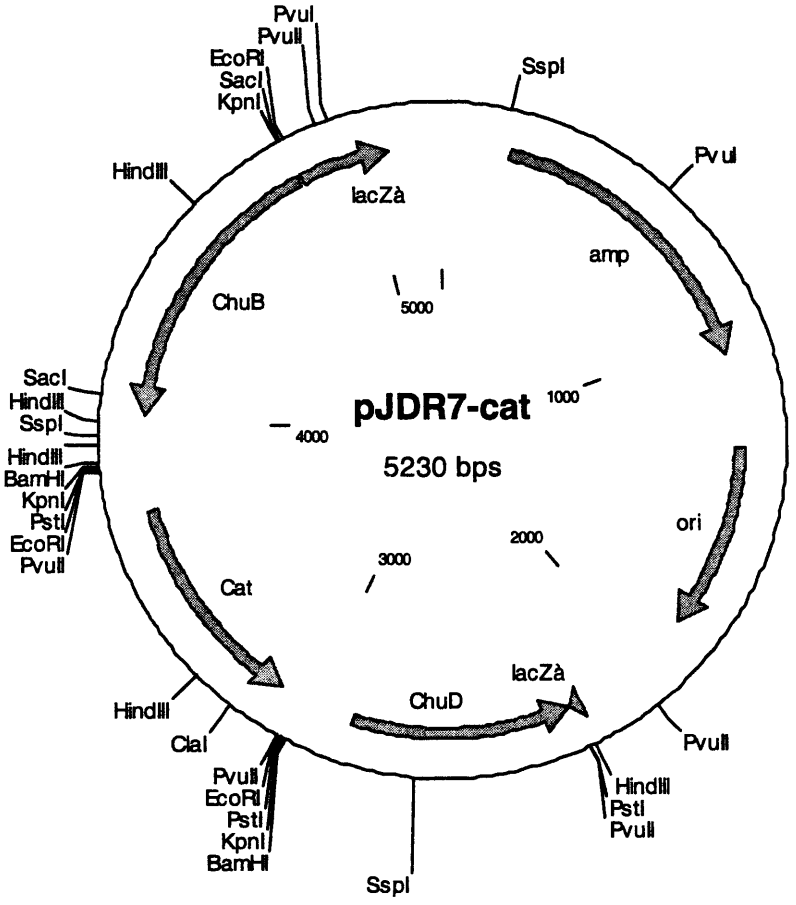
pJDR5:pJDR1 with deletion in *chuA* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



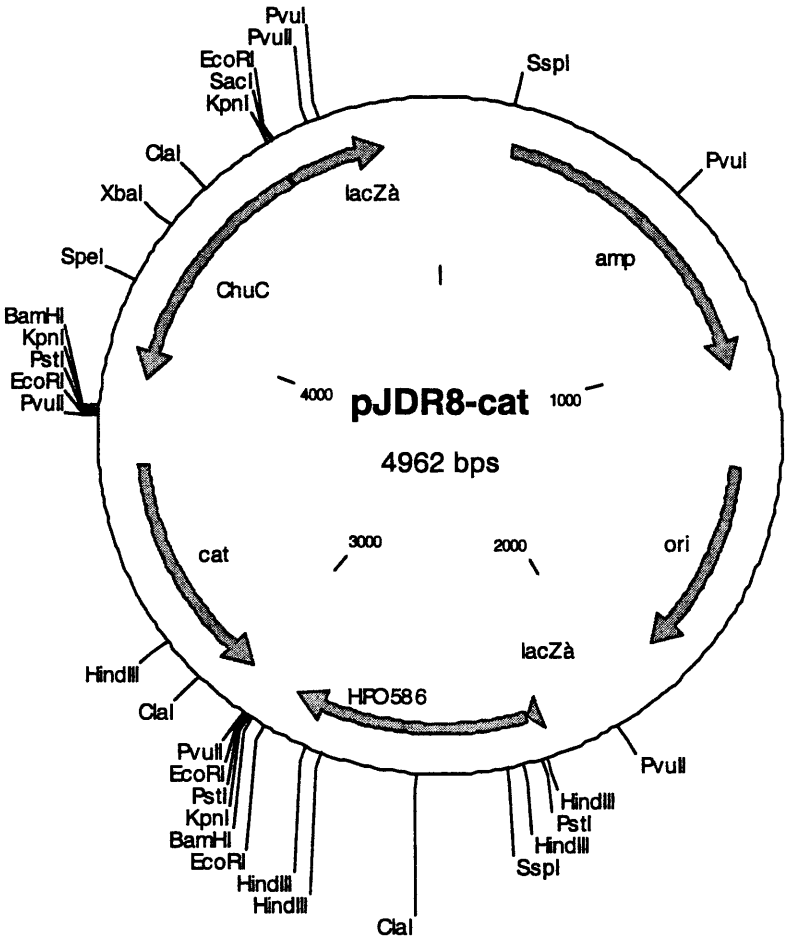
pJDR6: pJDR2 with deletion in *chuB* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



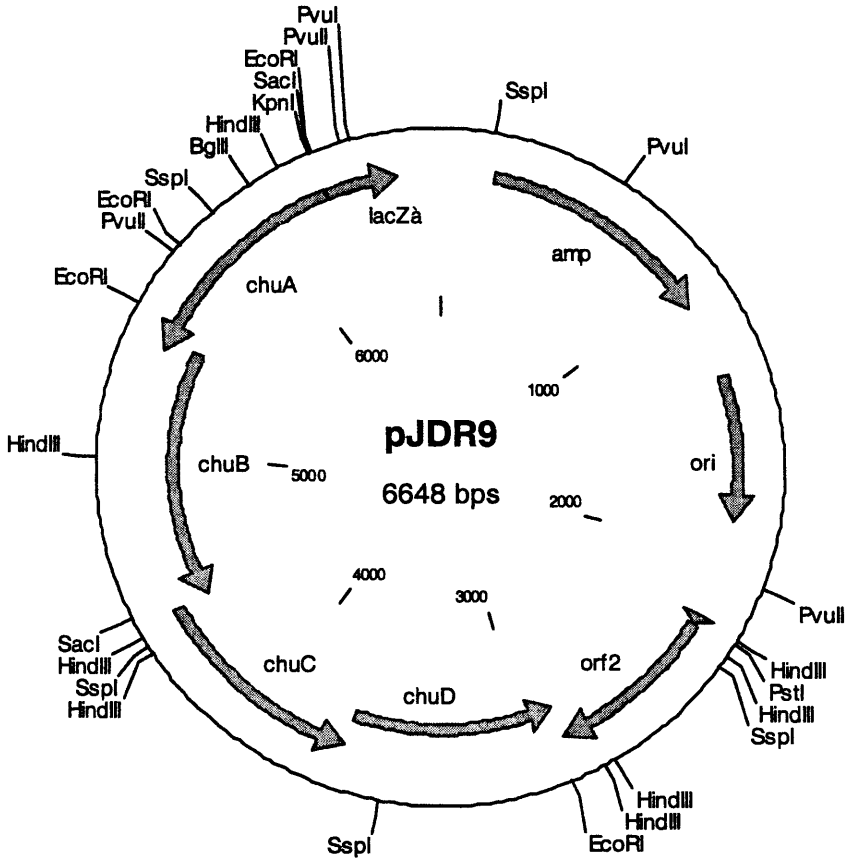
pJDR7: pJDR3 with deletion in *chuC* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



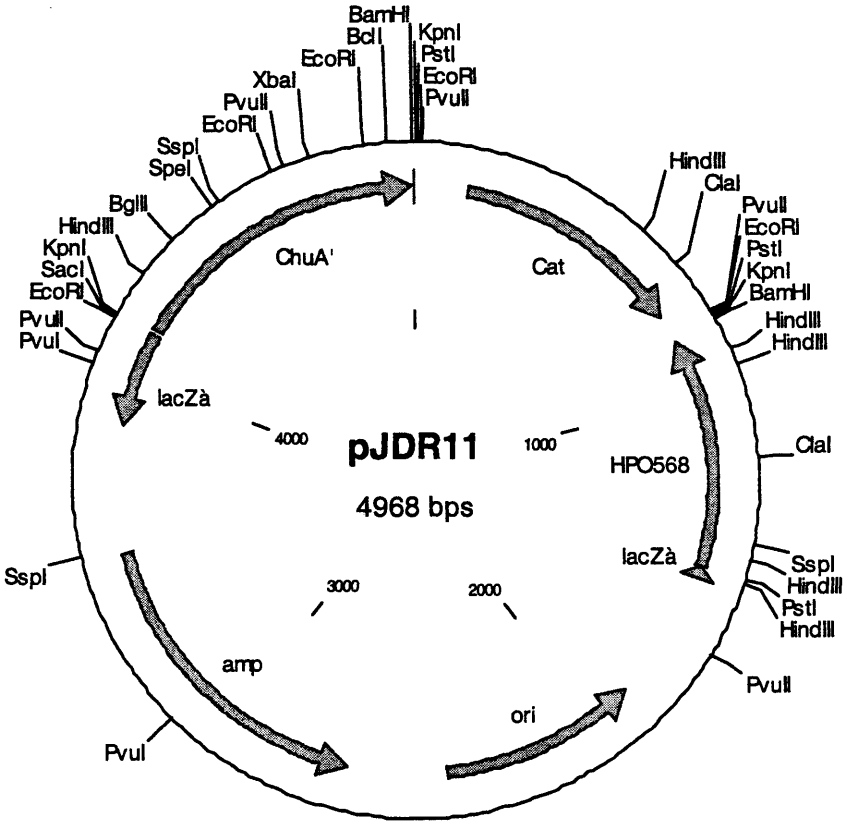
pJDR8: pJDR4 with deletion in *chuD* reading frame. Cm^r resistance cassette ligated in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



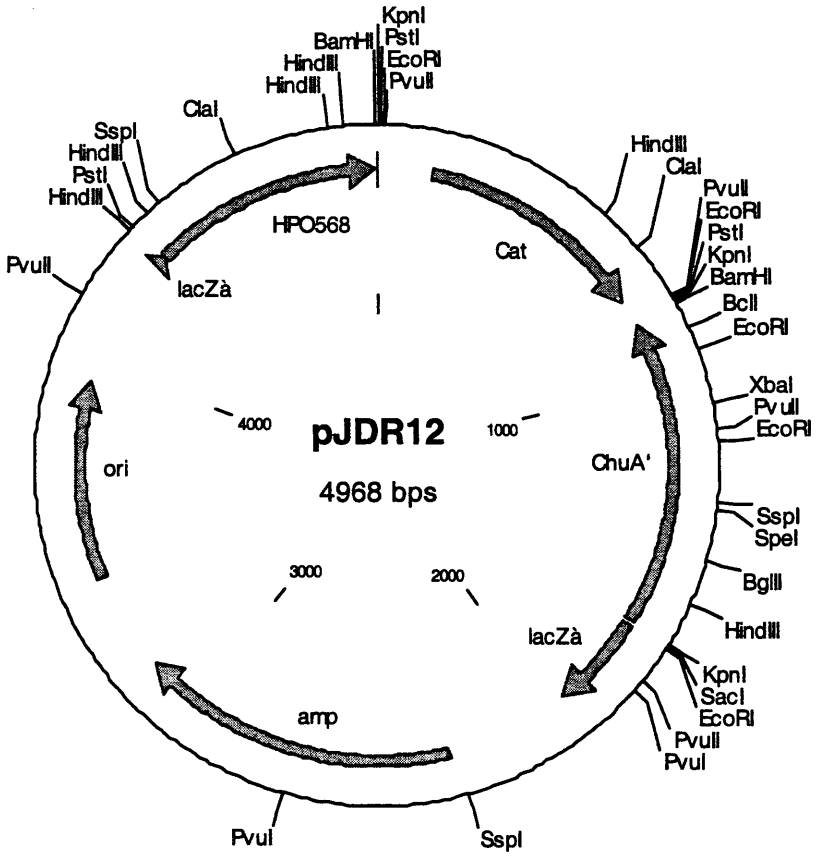
pJDR9: pUC19 containing cloned *C.jejuni* NCTC11168 *chuB, C* and *D* genes.

Appendix 1. Plasmid maps.



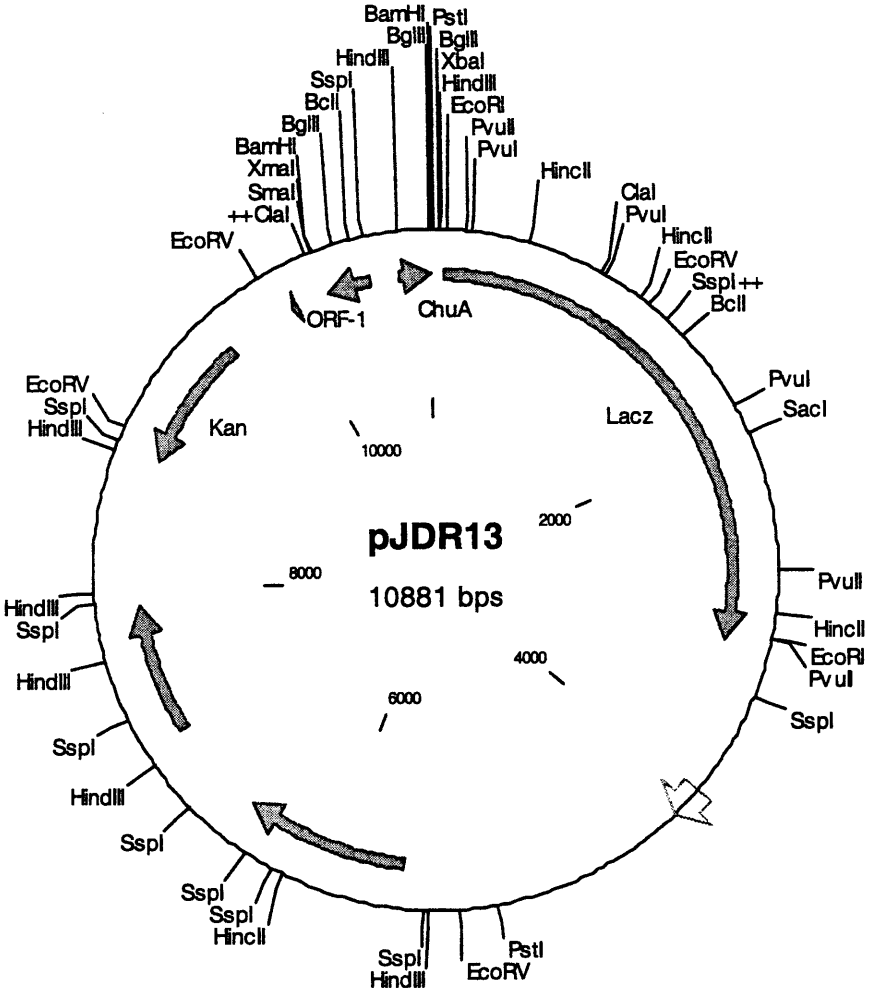
pJDR11: pJDR9 with deletion in *chuB, C* and *D* open reading frames. Cm^r resistance cassette ligated (in the same orientation as the deleted genes) in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



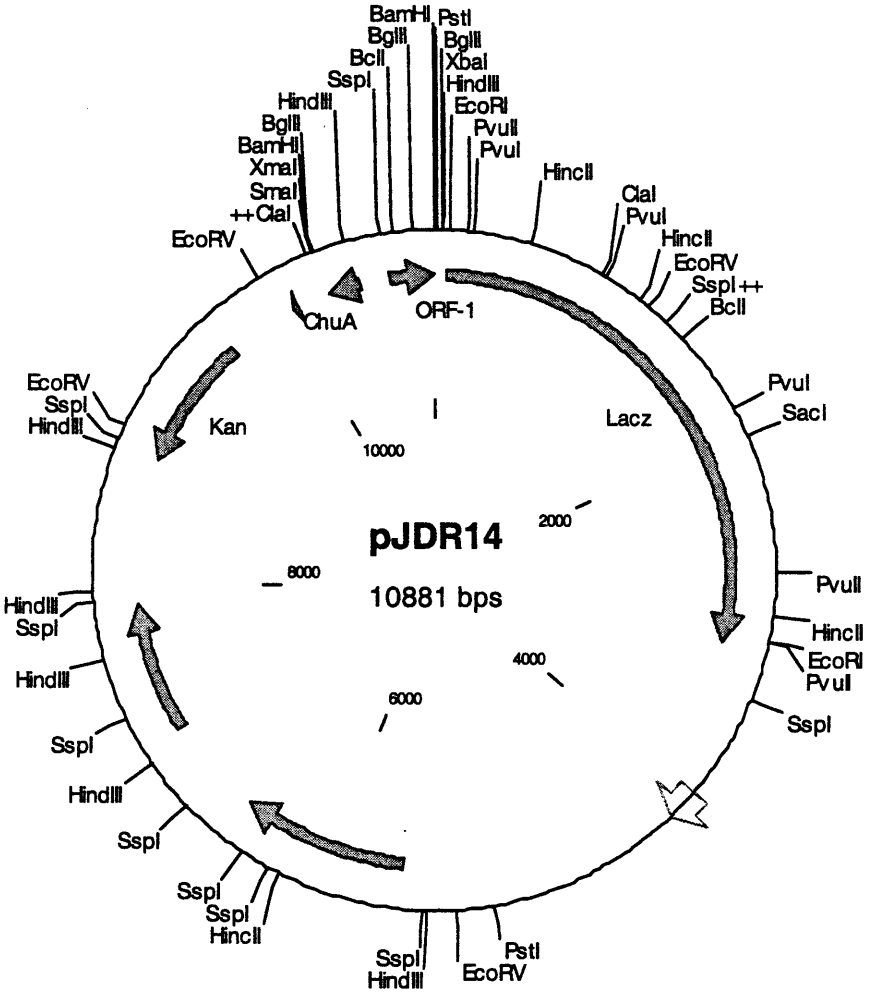
pJDR12: pJDR9 with deletion in *chuB, C* and *D* reading frames. Cm^r resistance cassette ligated (in the opposite orientation to the deleted genes) in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



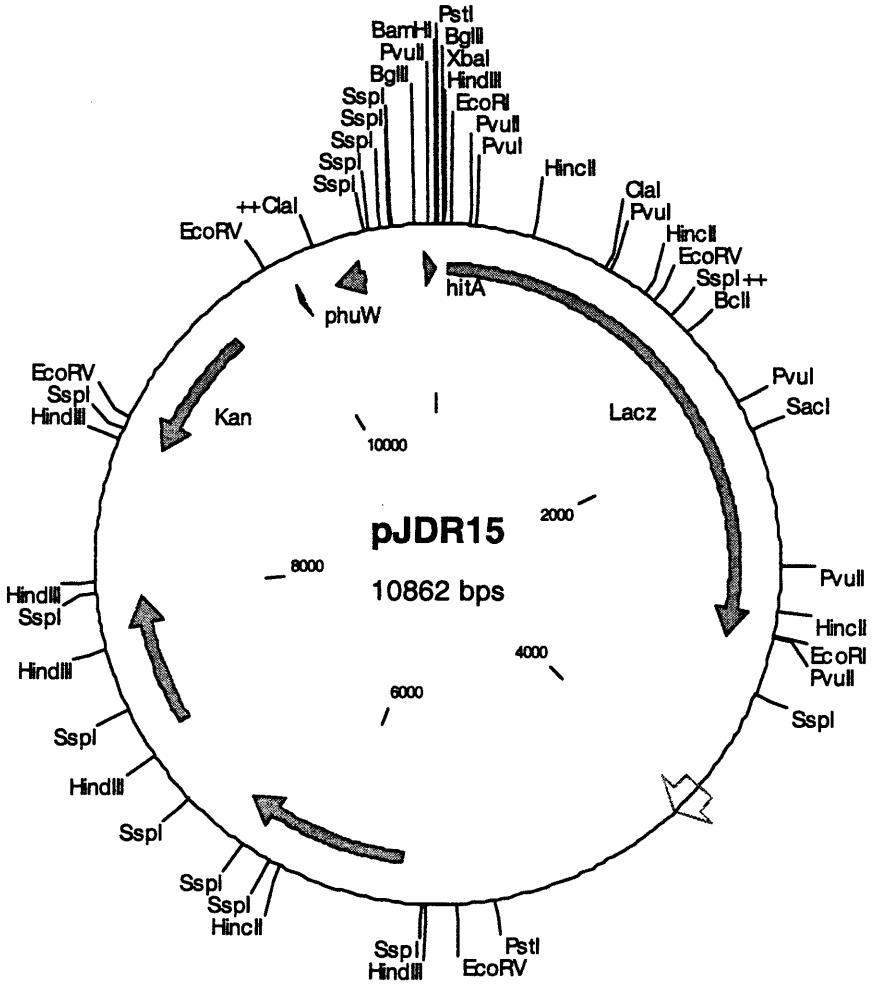
pJDR13: pMW10 containing the *chuA* promoter:lacZ fusion.

Appendix 1. Plasmid maps.



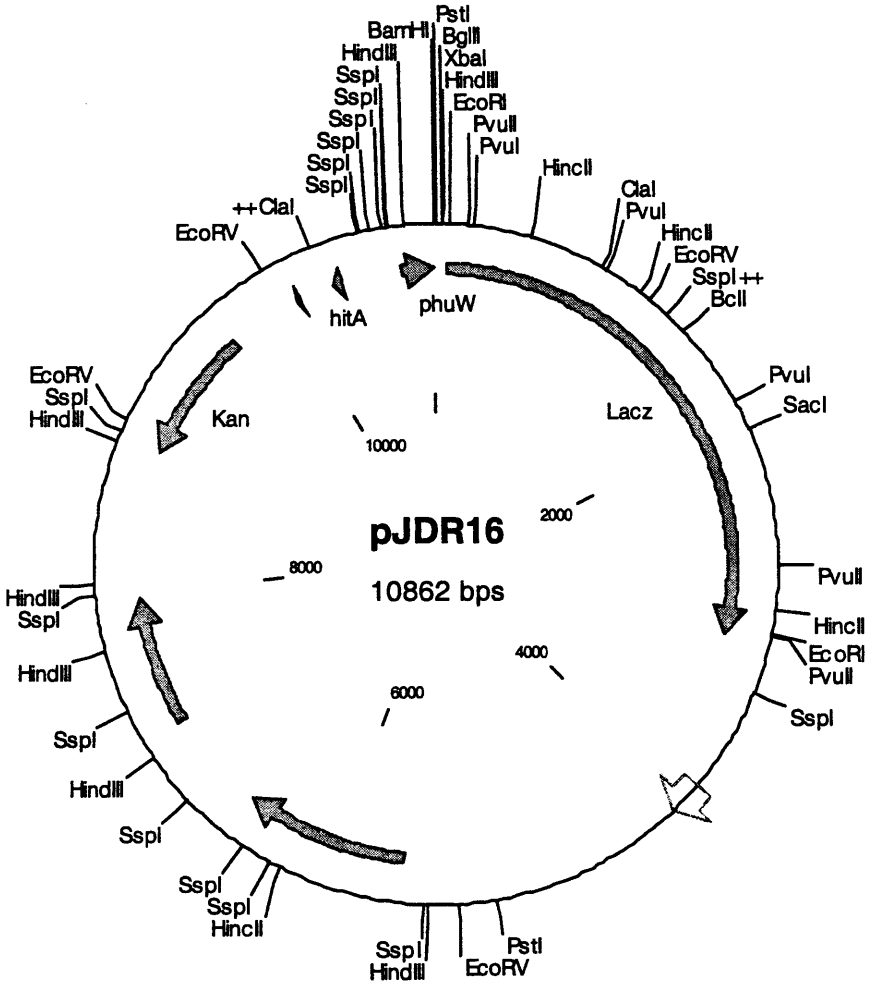
pJDR14: pMW10 containing the Cj1613 promoter:lacZ fusion.

Appendix 1. Plasmid maps.



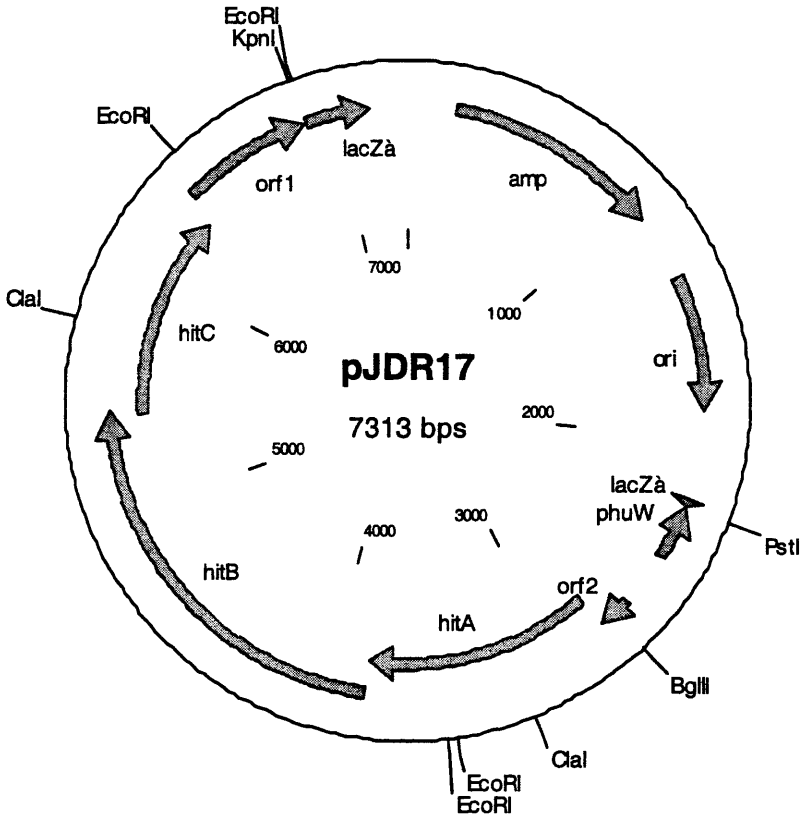
pJDR15: pMW10 containing the *hitA* promoter:lacZ fusion.

Appendix 1. Plasmid maps.



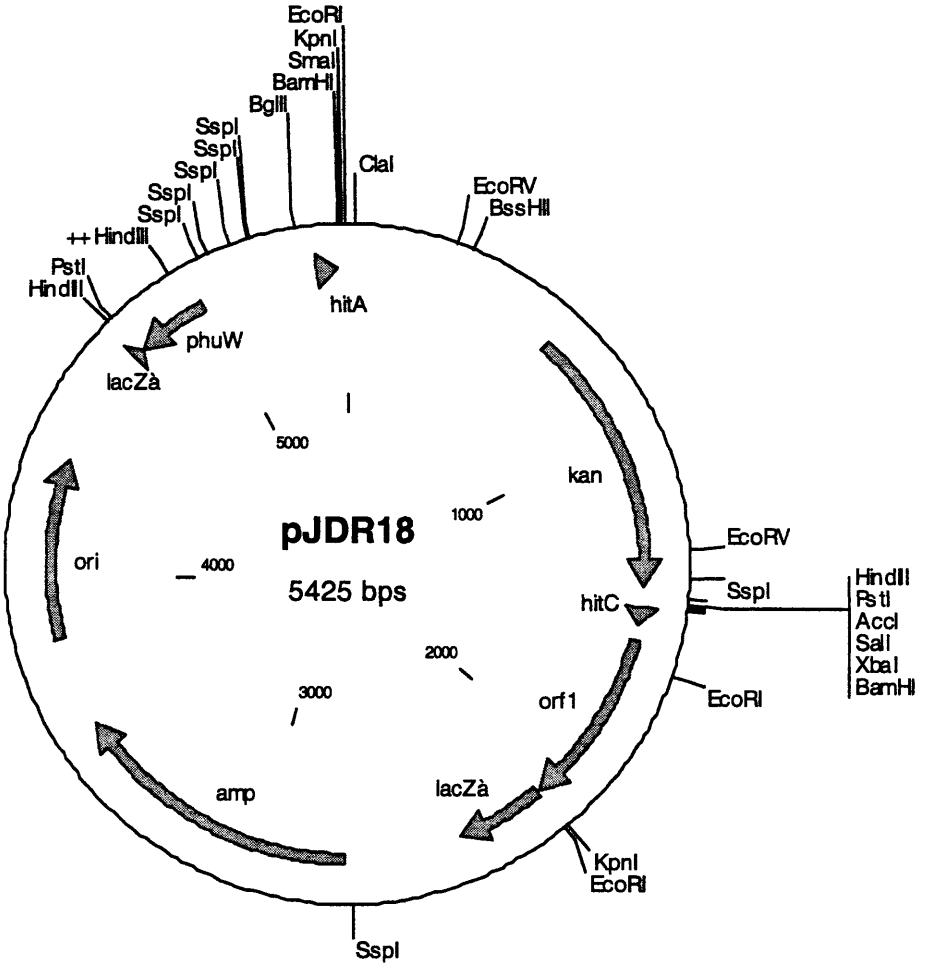
pJDR16: pMW10 containing the *phuW* promoter:lacZ fusion.

Appendix 1. Plasmid maps.



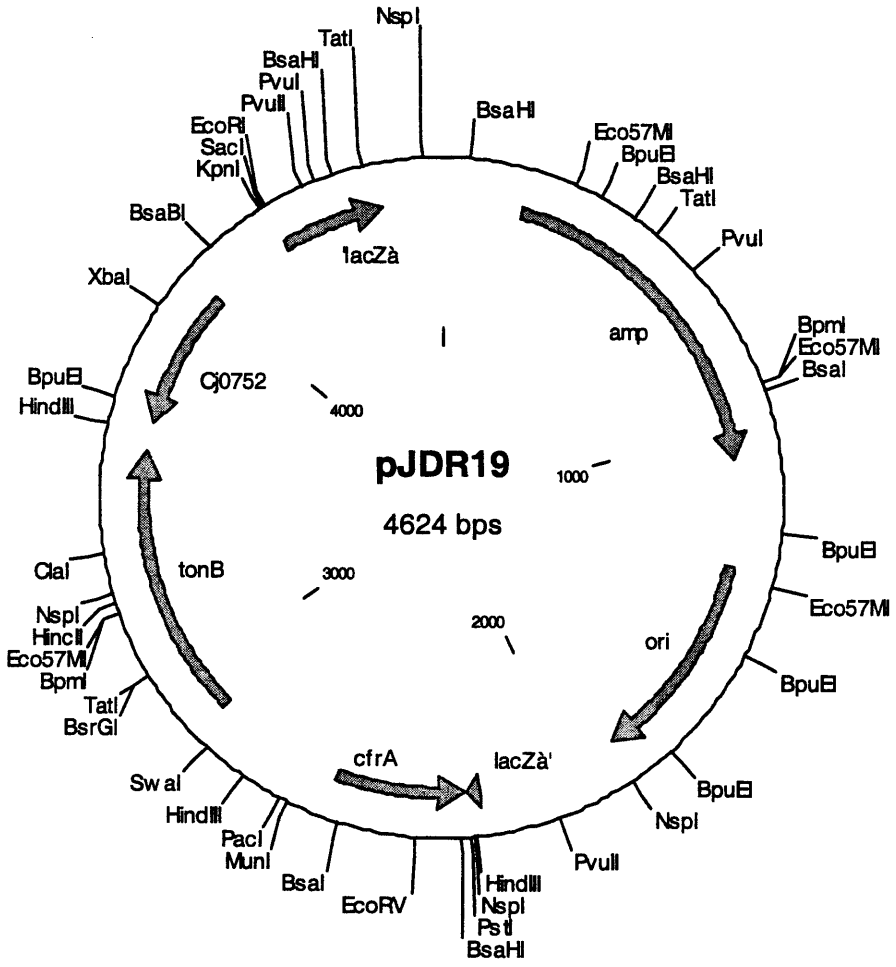
pJDR17: pUC19 containing *C.jejuni* NCTC11168 *hitA, B* and *C* genes.

Appendix 1. Plasmid maps.



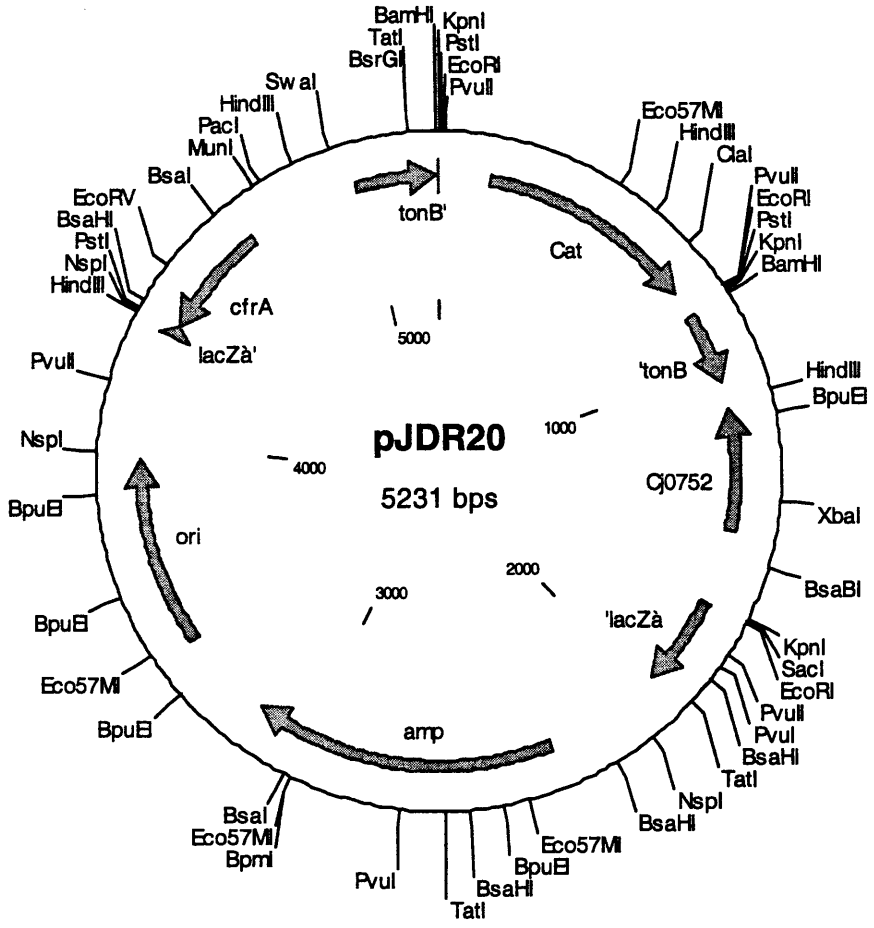
pJDR18: pJDR17 with deletion in *hitA, B* and *C* open reading frame. Kan^r resistance cassette ligated (in the same orientation as the deleted genes) in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



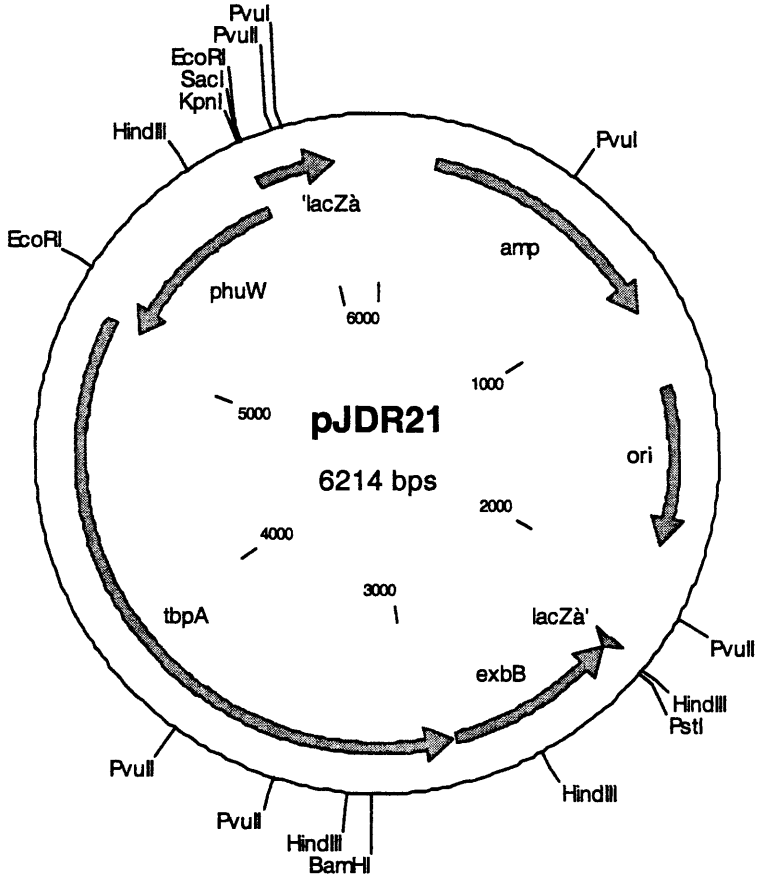
pJDR19: pUC19 containing cloned *C.jejuni tonB1* gene.

Appendix 1. Plasmid maps.



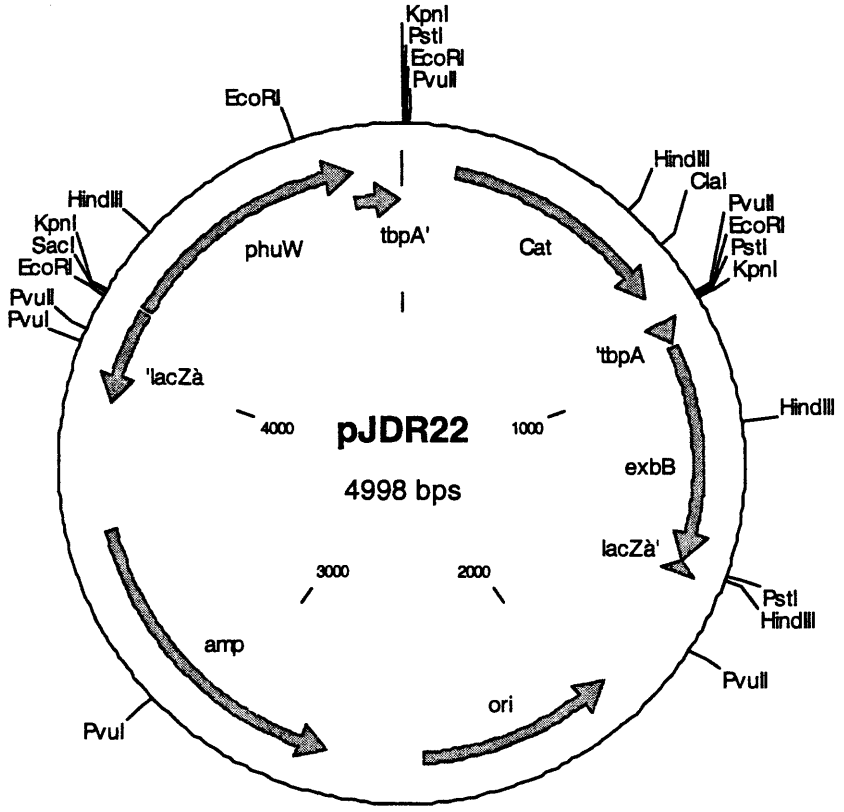
pJDR20: pJDR19 with deletion in *tonB1* reading frame. Cm^r resistance cassette ligated (in the same orientation as the deleted gene) in a *Bam*H1 site created at the point of deletion.

Appendix 1. Plasmid maps.



pJDR21: pUC19 containing cloned *C.jejuni* Cj0178(tbpA) gene.

Appendix 1. Plasmid maps.



pJDR22: pJDR21 with deletion in Cj0178 reading frame. Cm^r resistance cassette ligated (in the same orientation as the deleted gene) in a *Bam*H1 site created at the point of deletion.

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The iron-responsive regulator Fur of *Campylobacter jejuni* is expressed from two separate promoters

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Abstract

A *lacZ*-based reporter gene system was used to identify the promoter of the *Campylobacter jejuni* iron-responsive gene regulator Fur. In other Gram-negative bacteria, the *fur* promoter is usually located directly upstream of the *fur* gene and is often autoregulated in response to iron. In this study we demonstrate that expression of the *C. jejuni fur* gene is controlled from two promoters located in front of the first and second open reading frames upstream of *fur*. Neither of these promoters was iron-regulated, and the presence of both promoters in front of *fur* gives higher expression of the *lacZ* reporter than with either promoter alone. Expression from two distal promoters might be a mechanism for regulating the level of the *C. jejuni* Fur protein in response to unknown stimuli. © 2000 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Gene regulator; Fur protein; Iron-responsive regulator

1. Introduction

The ferric uptake regulator (Fur) is the major bacterial iron-responsive regulatory protein, which controls cellular iron homeostasis by regulating the expression of iron acquisition systems. Additional genes regulated by Fur include toxins, oxidative stress defence and housekeeping genes [1,2]. Fur homologues have been identified in both Gram-negative and Gram-positive bacteria [1–3].

In all *fur* genes so far characterised, *fur* is transcribed from its own promoter located directly upstream of the *fur* open reading frame [4–6]. Expression of *fur* in *Vibrio cholerae* seems to be controlled by growth phase only [7], whereas control of expression of *fur* in *Escherichia coli* is more complex. The *E. coli fur* gene is both autoregulated and regulated by the cAMP receptor protein Crp [4]. In addition, it was shown recently that the *E. coli fur* gene is not only transcribed monocistronically from its own promoter, but under conditions of superoxide stress *fur* is also transcribed from the promoter of the upstream flavodoxin gene *fldA* as a multicistronic messenger [8]; this response is regulated by the SoxRS system. The peroxide stress regulator OxyR also influences expression from the *E. coli fur* promoter under conditions of peroxide stress [8].

Environmental iron concentration is an important stimulus for *Campylobacter jejuni*, as the bacterium has two iron-responsive regulators that regulate iron uptake (through Fur [9]) and peroxide stress defence (through PerR [10]). Transcription of the *C. jejuni fur* gene has already been shown to have unusual characteristics when compared to most other Gram-negative *fur* genes. *C. jejuni fur* is located directly upstream of the housekeeping genes *lysS* and *glyA*, which seem to be co-transcribed with *fur* [9,11,12]. Putative Fur boxes and Crp-binding sequences were identified but not confirmed in the sequence directly upstream of *C. jejuni fur* [11,12], and it was hypothesised that expression of *fur* in *C. jejuni* is under similar control as described in *E. coli* [12].

In this study we used a plasmid-based *C. jejuni lacZ* reporter gene-based system [13] to identify the two *C. jejuni fur* promoters that are not located directly upstream of *fur*.

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2. Materials and methods

2.1. Bacterial strains and plasmids and growth conditions

C. jejuni 480 (NCTC 12744) [14] was grown on Mueller–Hinton (MH) medium (Oxoid, Basingstoke, UK) at 37°C in a Variable Atmosphere Incubator (VAIN; Don Whitley Scientific, Shipley, UK) in 10% CO_2, 5% O_2 and 85% N_2. Media for *C. jejuni* were routinely supplemented with 10 μg ml^{-1} vancomycin and 5 μg ml^{-1} trimethoprim. Iron-restricted and iron-replete conditions were achieved by supplementing MH medium with desferrioxamine-B (Desferal, Sigma) or ferric sulfate to concentrations of 20 μM and 40 μM, respectively [9]. *E. coli* strains DH5α (Gibco BRL) and MC4100 were grown aerobically in Luria–Bertani medium [15] at 37°C. Plasmids used were pBluescript II SK^- (Stratagene) and pMW10 [13]. Selection for plasmids in *E. coli* and *C. jejuni* was done by supplementing media with kanamycin (50 μg ml^{-1}) or ampicillin (100 μg ml^{-1}) as appropriate.

2.2. Construction of transcriptional lacZ fusions

Transcriptional *lacZ* fusions were created by using the *Campylobacter-E. coli* shuttle vector pMW10 [13]. This plasmid contains a *Campylobacter* origin of replication, a kanamycin resistance gene and a promoterless *lacZ* reporter gene preceded by a polylinker with a unique *Bam*HI site for insertion of DNA fragments containing putative promoter sequences [13]. Overlapping fragments of the *C. jejuni fur* region were amplified from *C. jejuni* 81116 (NCTC 11828) using Expand polymerase mix (Boehringer Mannheim) and cloned into the *Bam*HI site of pBluescript (pBS). Subsequently the sequence was checked for absence of incorporation errors and cloned in both orientations into the *Bam*HI site upstream of the promoterless *lacZ* gene of pMW10. Plasmid constructs were transformed into *C. jejuni* 480 by electroporation as described previously [16], and the presence and identity of the reporter plasmids were determined by PCR and by restriction enzyme analysis of plasmid isolated from *C. jejuni*. β-Galactosidase (β-gal) activity was determined from cultures grown in iron-restricted or iron-replete MH broth for 7 h at 37°C using standard protocols [17,18].

3. Results and discussion

3.1. Genomic organisation of the C. jejuni fur region

Previous studies have demonstrated that the *lacZ* reporter gene on plasmid pMW10 is a useful tool to study gene expression in *C. jejuni* [13,18]. We initially used this system to construct a transcriptional fusion of the 370-bp region directly upstream of *C. jejuni fur* (Fig. 1, fragment H) to the *lacZ* gene, to confirm the previously reported iron-regulated expression from this (promoter) region [12]. However, there was no detectable *lacZ* expression using this region, indicative of the absence of a promoter (Table 1). This suggests that the *C. jejuni fur* gene is not located 5′ in an operon, but might be located within an operon with both upstream and downstream genes.

The nucleotide sequence of the region upstream of the *C. jejuni* 81116 *fur* gene on plasmid pKG20 [11] was de-

Table 1
β-Gal activity of overlapping constructs containing the putative promoters P1–P5 in the *C. jejuni fur* region

Plasmid	Fragment[a]	Position[b]	Promoter(s)::*lacZ*[c]	β-Gal activity[d]
pLM22	A	364 033 → 364 411	P2	275 ± 65
pLM24	A_{inv}	364 411 ← 364 033	P1	803 ± 135
pLM14	B	364 033 → 364 826	P2+P3	541 ± 121
pLM12	B_{inv}	364 826 ← 364 033	P1	952 ± 100
pLM37	C	364 033 → 365 259	P2+P3+P4	358 ± 32
pLM38	C_{inv}	365 259 ← 364 033	P1	980 ± 183
pLM27	E	364 468 → 364 826	P3	164 ± 43
pLM28	E_{inv}	364 826 ← 364 468	–	3 ± 9
pLM18	F	364 468 → 365 259	P3+P4	99 ± 7
pLM20	F_{inv}	365 259 ← 364 468	–	9 ± 7
pLM31	H	364 917 → 365 259	P4	7 ± 8
pLM32	H_{inv}	365 259 ← 364 917	–	0 ± 12
pLM30	J	365 243 → 365 760	P5	0 ± 8
pLM35	J_{inv}	365 760 ← 365 243	–	0 ± 11
Controls				
pMW10[e]	–	–	none	1 ± 5
23E5[e]	–	1 030 950 → 1 030 474	*metK*	248 ± 33

[a]Fragment location as shown in Fig. 1. Inversion of the fragment relative to the *lacZ* reporter gene is indicated by the subscripted 'inv' notation.
[b]Position of the DNA fragment in the *C. jejuni* genome sequence [19]. Orientation of the fragment relative to the *lacZ* reporter gene (as shown in Fig. 1) is indicated by arrows.
[c]Promoter positions as indicated in Fig. 1.
[d]β-Gal activity represented as Miller units (± S.E.M.) [17].
[e]pMW10 and 23E5 have been described previously [13].

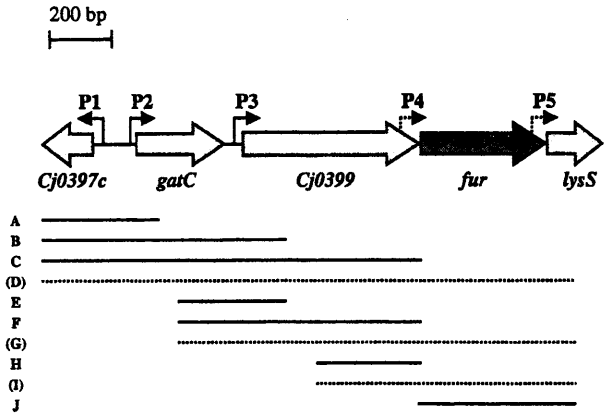

Fig. 1. Genomic organisation of the region containing the *C. jejuni fur* gene, showing the position of putative promoters and the overlapping fragments used for localisation of the *fur* promoter. Promoters not found to have activity are indicated by dashed arrows. Dashed lines with fragment indication between parentheses indicate that these fragments could not be cloned in *E. coli* without sequence errors (see text for details). ORF designations are as in the *C. jejuni* genome sequence [19].

termined and analysed for the presence of open reading frames (ORFs). This analysis showed the presence of two complete ORFs and one partial ORF missing a stop codon (Fig. 1). The two complete ORFs directly upstream of *fur* were in the same orientation as *fur*, whereas the third (incomplete) ORF was orientated divergently (Fig. 1). The organisation of this region was compared with the recently completed genome sequence of *C. jejuni* strain NCTC 11168 [19], and found to be identical. The identity between the DNA sequences from *C. jejuni* 81116 and NCTC 11168 was 99% (data not shown), and these differences resulted in one amino acid difference between each of the two deduced ORFs upstream of *fur* and *fur* itself. We have used the designation of the ORFs (*Cj0397c*, *Cj0398* (*gatC*) and *Cj0399*) as annotated in the genome sequence (Fig. 1) [19]. All three ORFs show homology to ORFs of the phylogenetically related pathogen *Helicobacter pylori* that do not map close to the *H. pylori fur* gene [20]. The product of ORF *Cj0399* shows highest homology to a protein (Hp0181), which is found directly upstream of the *H. pylori lysS* and *glyA* genes. ORF Hp0181 and Cj0399 were annotated as putative integral membrane proteins in the *H. pylori* and *C. jejuni* genomes, respectively. The product of ORF *Cj0398* is homologous to the Hp0975 protein, which is predicted to be a Glu-tRNA Gln amidotransferase, subunit C (*gatC*). Finally, the product of the *Cj0397c* ORF is homologous to the Hp0864 protein, and both were annotated as hypothetical proteins [19,20].

3.2. Localisation of promoters in the C. jejuni fur region

The *lacZ*-based reporter gene system of plasmid pMW10 [13] was utilised for identification of promoters and their iron regulation in the *fur* region. Given the organisation of the *fur* region, the *fur* promoter could be expected to be in front of *gatC* (designated P2), *Cj0399* (designated P3) or, as speculated previously [14], in front of *fur* (P4). Other possible promoter positions in this region were in front of *lysS* (P5) and the divergent promoter in front of *Cj0397c* (P1). Overlapping fragments were amplified which contained all putative promoters alone or in combinations (Fig. 1), cloned in pBS and checked for incorporation errors. Despite several attempts using a DNA polymerase with proofreading activity, we were unable to clone the fragments D, G and I without incorporation errors, and interestingly, the errors were always in the *fur* coding sequence (data not shown). We were able to clone fragment J, which contains the full *fur* coding sequence, but not the upstream region (fragment I). This indicates that the *C. jejuni* Fur protein might interfere with *E. coli* metabolism when expressed from a high copy number plasmid. All other fragments were cloned in front of the promoterless *lacZ* gene of pMW10 in both orientations. These constructs were transformed into *C. jejuni* 480 and their β-gal activity was determined in both iron-restricted and iron-replete conditions. As a control for iron-responsive expression of the *lacZ* reporter we used the *C. jejuni katA::lacZ* fusion described earlier [18] (data not shown).

The reporter gene constructs clearly showed that only P1, P2 and P3 have promoter activity, as fragments containing only P4 or P5 did not show any β-gal activity. P1 is divergently orientated to *fur* and thus is not expected to influence expression of *fur*. Interestingly, expression of the *fur::lacZ* fusion was higher when both P2 and P3 were included in the tested fragment (fragment C; 358 ± 32 β-gal units) compared to P3 alone (fragment F; 99 ± 7 β-gal units). This indicates that both P2 and P3 might be involved in the expression of *fur* in *C. jejuni*. None of the promoters identified in the *fur* region was iron-regulated (data not shown), indicating the absence of iron-responsive autoregulation or regulation through PerR [10]. Although the reporter system used here is located on a plasmid with a medium copy number (ca. 20 [13]), it was used successfully to demonstrate iron-regulated expression from the *C. jejuni ahpC* and *katA* promoters [18]. We also determined the β-gal activity of the different promoters in an *E. coli* background. Interestingly, P4, which shows no activity in *C. jejuni*, promotes expression in *E. coli* (data not shown). This could explain the successful complementation of an *E. coli fur* mutant that was observed when the *C. jejuni fur* gene was initially identified [11].

Previous studies using Northern hybridisation indicated that there are multicistronic mRNAs containing *fur* in *C. jejuni* strain TGH9011 [12]. Three *fur* transcripts were identified, but were not further characterised. Analysis of the region downstream of the *glyA* gene showed three more ORFs that are likely to be co-expressed with the *fur* gene, given that each ORF in the putative operon is

directly adjacent or overlaps [19]. The two long transcripts identified by Chan et al. [12] are likely to be transcripts initiated from the two promoters identified in this study. The third, short transcript could be the result of RNA processing [12].

Expression of *fur* from two promoters has been recently described in *E. coli* [8], and results in the regulation of expression of *fur* in response to several stimuli, including superoxide and peroxide stress. The *C. jejuni fur* gene is unique, as it does not have its own promoter but is located in an operon that includes the downstream housekeeping genes *lysS* and *glyA*. However, there are two possible multicistronic messengers that may be formed, one initiated from the P2 promoter upstream of *gatC* and one from the (weaker) P3 promoter upstream of *Cj0399*. Although neither promoter is regulated in response to iron, differential promoter expression would allow for regulation of *fur* in response to different environmental stimuli; the nature of these signals is currently under investigation.

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