Site-Directed Mutagenesis of the *Campylobacter jejuni* Fur Box and the Iron- and Oxygen-Responsive Regulation of *fumC*

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by

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Site-Directed Mutagenesis of the *Campylobacter jejuni* Fur Box and the Iron- and Oxygen-Responsive Regulation of *fumC*

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For the foodborne enteric pathogen *Campylobacter jejuni*, regulation of iron homeostasis is tightly controlled by the ferric uptake regulator Fur. Fur regulates iron-responsive gene expression by binding to the Fur box sequence and a 19 bp Fur box is positioned within the promoter region of the outer membrane haem receptor gene *chuA*. The fumarase *fumC* Fur box-like sequence shows three mismatches to the consensus sequence and this variation is predicted to be key to the contrasting iron regulation and Fur-Fur box binding affinity between *chuA* and *fumC*. The aims of this study were to determine the functionally important bases in the *C. jejuni* Fur box that are essential for Fur-Fur box interaction and to assess the interplay of iron and oxygen in the modulation of *fumC* expression.

Site-directed mutagenesis of the 1\textsuperscript{st}, 7\textsuperscript{th}, 10\textsuperscript{th}, 13\textsuperscript{th} and 19\textsuperscript{th} positions was carried out for the *chuA* and *fumC* Fur boxes and their interaction with Fur was determined \textit{in vitro} and \textit{in vivo}. Two Fur dimers were determined to bind to the Fur box, and although the 1\textsuperscript{st}, 7\textsuperscript{th}, 13\textsuperscript{th} and 19\textsuperscript{th} positions were found to facilitate the interaction of Fur with the Fur box, the architecture of the promoter region is likely to play a more significant role in Fur regulation. *fumC* encodes the only fumarase in *C. jejuni* and it is essential for cell growth and for maintaining a functional tricarboxylic acid cycle. Further characterisation of *chuA* and *fumC* expression in response to iron and oxygen indicated that both genes are controlled by Fur as well as the peroxide response regulator PerR, and the RacR-RacS two-component system. These observations illustrate the necessity for *C. jejuni* to cooperatively regulate essential gene expressions using its rather limited set of regulators thus allowing it to adapt to various conditions encountered during transmission and colonisation.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>+1 site</td>
<td>Transcriptional start site</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AHT</td>
<td>Anhydrotetracycline</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
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<td>Amp</td>
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<td>Arginine</td>
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<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bj</td>
<td>Bradyrhizobium japonicum</td>
</tr>
<tr>
<td>Bs</td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>Cap</td>
<td>Catabolite activator protein</td>
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<tr>
<td>CDT</td>
<td>Cytolethal distending toxin</td>
</tr>
<tr>
<td>CEB</td>
<td>Campylobacter electroporation buffer</td>
</tr>
<tr>
<td>Cj</td>
<td>Campylobacter jejuni</td>
</tr>
<tr>
<td>CM</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CSPD</td>
<td>Disodium 3-(4-methoxySpiro{1,2-dioxetane-3,2’-(5’)-chloro}\tricycle[3.3.1.1^{3,7}]decan} -4-yl) phenyl phosphate</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dH\textsubscript{2}O</td>
<td>Distilled water</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
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Abbreviations

DTT  Dithiothreitol
DtxR  Diphtheria toxin regulator
Ec  *Escherichia coli*
EDTA  Ethylenediamine tetraacetic acid
EMSA  Electrophoretic mobility shift assay
FAM  Fluorescein amidite
FeSO₄  Iron (II) sulphate heptahydrate
Fur  Ferric uptake regulator
Glu  Glutamine
GTG  Glycerol tolerant gel
His  Histidine
HK  Histidine kinases domain
Hp  *Helicobacter pylori*
HTH  Helix-turn-helix
IPTG  Isopropyl-β-D-1-thiogalactoside
IR  Intermediate regulator
Kan  Kanamycin
LB  Luria-Bertani
LOS  Lipooligosaccharide
MBS  Metal binding site
MH  Mueller-Hinton
MOPS  3-(N-morpholino) propanesulfonic acid
mRNA  Messenger RNA
MRS  Mur responsive sequence
MSC  Multi-cloning site
Mur  Manganese uptake regulator
Ng  *Neisseria gonorrhoeae*
Nm  *Neisseria meningitidis*
Nramp1  Natural resistance-associated macrophage protein 1
### Abbreviations

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<tr>
<td>NRPS</td>
<td>Nonribosomal peptide synthetases</td>
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<tr>
<td>Nur</td>
<td>Nickel uptake regulator</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>ONPG</td>
<td>2-nitrophenyl-β-D-galactopyranoside</td>
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<tr>
<td>Pa</td>
<td><em>Pseudomonas aeruginosa</em></td>
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<td>PBP</td>
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<td>Polymerase</td>
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<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
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<tr>
<td>Ri</td>
<td><em>Rhizobium leguminosarum</em></td>
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<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Sc</td>
<td><em>Streptomyces coelicolor</em></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Se</td>
<td><em>Salmonella enterica</em></td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
</tr>
<tr>
<td>sRNA</td>
<td>Small RNA</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>TA</td>
<td>Tris-acetate</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TAP</td>
<td>Tobacco acid pyrophosphatase</td>
</tr>
<tr>
<td>TB</td>
<td>Tris-boric acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>TCS</td>
<td>Two-component system</td>
</tr>
<tr>
<td>TMEDA</td>
<td>N,N,N',N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>Tri</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>UTR</td>
<td>Un-translated region</td>
</tr>
<tr>
<td>Va</td>
<td><em>Vibrio alginolyticus</em></td>
</tr>
<tr>
<td>VAIN</td>
<td>Variable atmosphere incubator</td>
</tr>
<tr>
<td>Van</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>Vc</td>
<td><em>Vibrio cholerae</em></td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoyl-β-D-galactoside</td>
</tr>
<tr>
<td>Zur</td>
<td>Zinc uptake regulator</td>
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*Campylobacter jejuni* is a causative agent for zoonose, and since its initial definitive association with a patient with diarrhoea in Brussels (Dekeyser *et al.*, 1972), *C. jejuni* has rapidly emerged as the most common cause of bacterial gastroenteritis and a major public health concern in the developed world. Although its unusual DNA base composition, metabolism and culture requirements have hindered early progress in understanding this organism, with the completion of the *C. jejuni* NCTC 11168 genome sequence (Parkhill *et al.*, 2000), many important aspects of epidemiology and pathophysiology of *C. jejuni* infection have been revealed in the past decade.

A key determinant of *C. jejuni* pathogenesis, and indeed of all pathogenic bacteria, is the ability to effectively regulate the expression of genes involved in host induced adaptation responses, which allows *C. jejuni* to successfully colonise the avian and human gastrointestinal tract and causes disease in the human host. Transcriptional regulation of iron metabolism genes by the ferric uptake regulator (Fur) is an example of such adaptive response regulation where the *C. jejuni* Fur (Fur$_{Cj}$) plays crucial roles in controlling cellular iron homeostasis and other cellular processes that are essential for *C. jejuni* survival (Palyada *et al.*, 2004; Holmes *et al.*, 2005). Characterisation of the Fur$_{Cj}$ binding site, the Fur$_{Cj}$ box, as well as the interaction between Fur$_{Cj}$ and the Fur$_{Cj}$ box are important for understanding the regulatory function of Fur$_{Cj}$ and are addressed in detail in this research.
1.1 REGULATION OF GENE EXPRESSION

In order to adapt to and survive in a wide range of environmental conditions as well as compete effectively with other organisms, bacteria have evolved numerous systems for sensing and responding to changes in their environment. When facing suboptimal or stressful conditions such as limitation of nutrients, temperature fluctuations and oxidative or osmotic stresses, the expression of bacterial genes participating in response to the physiological and environmental demands must be coordinated by highly sophisticated and regulated global regulatory networks. These regulatory networks are mediated through the activation or inhibition of transcription initiation by transcriptional regulators, sigma factors and corresponding signal transduction pathways. Alternatively, it is increasingly evident that elevation and repression of messenger RNA (mRNA) translation by post-transcriptional regulation are also critical determinants of gene expression. Together, these regulatory mechanisms help bacteria to adjust to the surrounding environment, modulating cellular metabolism to optimise the utilisation of limited nutrients and improving the probability of survival and establishing colonisation in various niches (Snyder and Champness, 2003).

At the heart of any transcriptional regulatory network in bacteria is the transcriptional regulator which effectively functions as a molecular switch that converts environmental input signals to output responses (Figure 1.1). A major system for bacteria to detect extracellular signals is mediated by the two-component signal transduction systems (or two-component systems, TCSs) that activate transcriptional regulators by a short, or in
transcriptional regulator
regulate gene expression
signal sensed by two-
component systems 
small molecules 
imported by 
transporters
small molecules 
synthesised by 
enzymatic reactions

Figure 1.1: A simple illustration of the bacterial transcriptional regulatory networks and the modulation of gene expressions by transcriptional regulators. Transcriptional regulators sense extracellular signals through TCSs or by binding to imported small molecules and sense intracellular signals by binding to synthetic small molecules. Input signals trigger the allosteric responses of transcriptional regulators and allow them to interact with their target promoters and control their expression according to the input signals (adapted from Sechasayee et al., 2006).
some cases, relatively long phosphorelays. Alternatively, bacteria sense extracellular or intercellular signals by the interaction of transcriptional regulators with small molecules that in most cases are either internalised by specific transport systems or synthesised internally by enzymatic reactions (Seshasayee et al., 2006). The interactions of exogenous or endogenous signals with transcriptional regulators subsequently trigger the allostERIC responses of the regulators and allow their DNA-binding domains to interact with the target promoters and either negatively or positively regulate the level of transcription (Snyder and Champness, 2003).

1.1.1 Negative gene regulation - the lactose (lac) operon

The *Escherichia coli* lac operon model originally proposed by Jacob and Monod a half century ago is the paradigm of gene regulation and allostERIC behaviour and a cogent depiction of how the concentration of metabolites in environment affects the coordinate transcription of a set of structural genes (Jacob and Monod, 1961). The first gene of the operon encodes the LacI repressor and in the absence of lactose, LacI binds with a high affinity to the operator 1 (O₁) sequence located at the 5’ end of the *lacZ* gene (Figure 1.2). This interaction dramatically compromises but does not eliminate the transcription of the downstream structural genes *lacZ*, *lacY* and *lacA*, which code for β-galactosidase, the lac permease and thiogalactoside transacetylase respectively that are required for lactose metabolism (Wilson et al., 2007).

When lactose becomes available, the inducer allolactose is synthesised by the basal
Figure 1.2: The negative regulation of the lac operon in response to lactose availability (adapted from Wilson et al., 2007). See text for details.
level of β-galactosidase in the cell and it binds to the LacI repressor to lower its affinity to the operator. However the downstream structural genes are only fully transcribed in the absence of glucose where high levels of cyclic adenosine monophosphate (cAMP) are produced by adenylyl cyclase and bound to the catabolite activator protein (Cap). The Cap-cAMP complex subsequently binds to the Cap-binding site upstream from the lacZ promoter (p1) and this in turn enhances the affinity of the RNA polymerase (pol) towards the promoter (Wilson et al., 2007).

The key component of lac operon regulation, the LacI repressor, is a tetrameric protein consisting of identical 37 kDa monomers. The three-dimensional structure of the repressor reveals that each monomer contains a C-terminal tetramerisation domain and a hinge region that connects the N-terminal headpiece with the core sugar binding domain (Figure 1.3.a). The headpiece contains a classic helix-turn-helix (HTH) motif and it flexes independently from the core domain. In the presence of the operator sequence, the hinge region undergoes coil to helix transition allowing it to make specific contacts with the minor grove of the DNA and allows the headpiece to fit into the major groove of the operator sequence. The core domain belongs to the periplasmic binding protein superfamily and consists of two structurally similar N- and C-terminal subdomains. The two subdomain’s are hinged together by three linkers that form a cleft at the subdomains interface. The effector ligand such as allolactose or isopropyl-β-D-1-thiogalactoside (IPTG) binds to this cleft through hydrogen bond formation and van de Waals contacts. The dimeric state of the repressor is held together by an extensive monomer-monomer
Figure 1.3: Ribbon illustrations of the LacI monomer (a) and the tetramer-DNA complex (b) and the illustration of the allosteric changes of the LacI dimer between the induced (left) and repressed states (c). See text for more details (taken from Lewis et al., 1996).
interface between the core domains of two monomers and it forms the functional operator binding unit. The tetrameric structure of the LacI repressor formed by the association of C-terminal tetramerisation domains does not maintain the point of group symmetry and it is essentially viewed as a V-shaped dimer of dimers where each dimer interacts with an operator and bends the DNA molecules away from the repressor (Figure 1.3.b, Lewis et al., 1996)

The allosteric change of the LacI dimer occurs primarily at the interface between two N-terminal subdomains which results in a small structural change between the N- and C-terminal subdomains of each monomer and the signal of this change is propagated to the headpieces via the hinge helices. As illustrated in Figure 1.3.c, in the induced state, the binding of an effector changes the subdomain’s interface by a small hinge motion which allows the formation of a number of electrostatic interactions across the N-terminal subdomains. This change in the dimer interface displaces the first amino acid residue of the core domain, which disrupts the interactions between the hinge helices and reduces the affinity of the DNA-binding domain for the operator. The electrostatic interactions that hold the dimer together are broken in the repressed state and the N-terminal subdomains move closer resulting in the reformation of the hinge helices interaction and the headpieces-operator complex (Lewis et al., 1996).

Aside from the primary operator sequence, the lac operon also contains two auxiliary operators located 92 bp upstream and 401 bp downstream from O₁. These three
operators share a high degree of sequence similarity and these auxiliary operators are required for the maximal level of repression. Although the allosteic transition of the LacI repressor occurs in the dimeric state, the formation of LacI tetramer allows it to interact with two operators simultaneously and creates a repression loop. The V-shaped LacI tetramer bends the operator sequence away from the repressor and creates a wrapping away loop. Alternatively, a simple loop also forms when the tetramer adapts to a conformation where the DNA-binding domain of each dimer is located at the opposite ends of the LacI tetramer (Friedman et al., 1995). Both repression loop formations mediate supercoiling of the promoter region and further enhance the repression of the structural genes in the operon (Wilson et al., 2007).

1.1.2 Positive gene regulation - the L-arabinose (ara) operon

Another well studied regulatory system in *E. coli* is the *ara* operon where the structural genes involved in the utilisation of L-arabinose, a five-carbon sugar, are positively (and negatively) regulated by AraC. The four-gene operon consists of the activator coding gene *araC* which is transcribed from its own promoter pC and followed by the structural genes *araB*, *araA* and *araD* which are divergently transcribed from *araC* by the pBAD promoter. *araB*, *araA* and *araD* encode the L-bibulose kinase, L-arabinose isomerise and D-xylulose-5-phosphate epimerase respectively. These enzymes are involved in enzymatic reactions to convert L-arabinose to D-xylulose-5-phosphate, which can be subsequently fed into the pentose phosphate pathway (Englesberg, 1961).
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The AraC protein contains a C-terminal DNA-binding domain and is linked to the N-terminal dimerisation and L-arabinose-binding domain by a linker. The N-terminal domain also contains an N-terminal arm which plays a key role in determining the ligand-dependent DNA-binding properties of the protein and renders the AraC protein with both the ability to induce and repress transcription (Soisson et al., 1997). The negative and positive regulation of the araBAD genes by AraC is explained by the switch mechanism illustrated in Figure 1.4.

In the absence of the inducer, L-arabinose, the two N-terminal arms of the AraC dimer interact with the corresponding DNA-binding domains and orientate them to form contact with the two widely separated O₂ and I₁ binding sites located upstream from pC and pBAD respectively. This allows the formation of a repression loop which prevents the interaction of RNA pol with this region and hence represses the transcription of araBAD as well as the araC gene. The negative autoregulation of araC is also mediated by the direct binding of AraC dimer to the O₁ pair of binding sites that partially overlap the pC RNA pol binding site. In the presence of L-arabinose, the N-terminal arms are repositioned by the binding of the inducer with the L-arabinose binding domain and the arms are held over the L-arabinose binding pockets by the bound sugar molecules. In this structure, the DNA-binding domains are less constrained and the repositioning of the arms provides the DNA-binding domains with the freedom to reorient and bind to the direct repeat I₁ and I₂ binding sites (Saviola et al., 1998). The binding of the ArcA protein stimulates both the interactions of RNA pol to pBAD and pC and the formation
Figure 1.4: An illustration of the regulation of the *ara* operon in the absence and presence of L-arabinose by AraC and the domains of the AraC monomer. See text for more details (taken from Schleif, 2003).
of open complexes in the promoters (Zhang et al., 1996).

Like in the lac operon, the transcription of the structural genes in the ara operon is only fully induced in the absence of glucose by the Cap-cAMP complex. The Cap-binding site is located between I$_1$ and O$_1$ binding sites and serves the divergently oriented pC and pBAD promoters. The binding of Cap-cAMP complex dimer facilitates the opening of the repression loop and the Cap dimer also interacts with the C-terminal domain of the RNA pol α subunit and stimulates the binding of RNA pol to the promoters (Zhang and Schleif, 1998).

Due to the insolubility and the inability to yield crystals, the full structure of the AraC protein is still unknown, however the crystal structure of the N-terminal domain in the presence and absence of L-arabinose (Soisson et al., 1997; Weldon et al., 2007) and the solution structure of the C-terminal domain have been determined (Rodgers and Schleif, 2009). The N-terminal domain consists of nine β strands which form an antiparallel β barrel and two α helices that are packed against the barrel (Figure 1.5.a). A single L-arabinose molecule binds into the barrel and is stabilised by hydrogen bonds formed between the side chains of the barrel and the sugar hydroxyl groups. A 12 residue-long N-terminal arm forms direct and indirect contact with the sugar molecule and completely encloses the L-arabinose in the β barrel (Soisson et al., 1997). The N-terminal domain of AraC also contains 10 amino acid residues which form part of the mutable and flexible linker that links the N-terminal domain with the DNA-binding
Figure 1.5: Ribbon illustrations of the AraC monomer N-terminal domain in the presence of L-arabinose (a, taken from Soisson et al., 1997) and the representation of the C-terminal DNA-binding domain in explicit water (b, taken from Rodgers and Schleif, 2009).
domain (Eustance et al., 1994).

In the induced form the two N-terminal domain monomers are associated by an antiparallel coiled-coil form between the α helices of each monomer and are held together by a network of hydrogen bonds formed by leucine residues anchored at the end of the coiled-coil. In the apo form, the core of the N-terminal domain monomer is virtually identical to the induced form but the N-terminal arm is unstructured in this state due to the absence of the bound sugar molecule. This distortion of the arm exposes the β barrel surface and allows it to act as an oligomerisation interface. The binding of two apo N-terminal monomers at the oligomerisation interface allows the side chain of the tyrosine (Tyr) 31 residue to occupy the β barrel of the adjacent monomers (Soisson et al., 1997). This protein aggregation however does not occur at physiological concentrations of AraC and mutation of the Tyr31 does not affect the regulatory function of AraC (Weldon et al., 2006).

The DNA-binding domain of AraC is a well folded seven-α helix structure in the absence of DNA (Figure 1.5.b) and contains two subdomains (α 2-3 and α 5-6) bearing the HTH motif. The two subdomains are weakly interconnected that allows each subdomain to rotate significantly from each other (Rodgers and Schleif, 2009). Mutational studies of the I₁ half binding site demonstrated that the two DNA-recognition helices of the subdomains bind specifically to the adjacent major grooves but not to the middle minor groove (Niland et al., 1996) and the formation of
this AraC-DNA complex significantly distorts the AraC DNA-binding domain and the operator sequence (Rodgers and Schleif, 2009).

1.1.3 TCS - the EnvZ-OmpR osmolarity regulatory system

As shown in Figure 1.1, TCSs are one of the major mechanisms for environmental signal recognition and signal responsive gene regulation in bacteria. A typical simple TCS consists of a signal sensing transmembrane histidine (His) kinase and a cytoplasmic response regulator, usually a transcriptional regulator that is activated by the histidine kinase through a signal phospho-transfer event. Bacteria and some lower eukaryotes also possess more elaborate versions of TCSs that contain additional phosphotransferases and phosphate receivers. These systems sense and respond to environmental signals through multiple phospho-transfer events known as phosphorrelays to enable inter connection and amplification between (different) signalling pathways (Stock et al., 2000).

The prototypical example of the simple TCSs is the E. coli EnvZ-OmpR regulatory system which regulates outer membrane (OM) pore expression in response to changing osmolarity. Effective osmolarity sensing and response allows bacteria to maintain an optimal osmotic pressure inside the cell and changing osmolarity also serves as an environmental cue for pathogenic bacteria to differentially express genes on entering the host (Snyder and Champness, 2003). E. coli expresses two porin proteins, OmpF and OmpC, which form pores with different size cutoffs and allow small hydrophilic
molecules to diffuse across the membrane at different rates according to the surrounding osmolarity. The regulation of porin expression occurs at the transcriptional level by a connected signal transduction pathway which consist of the osmosensing histidine kinase EnvZ and its cognate response regulator OmpR (Figure 1.6). EnvZ senses the surrounding osmolarity by the N-terminal periplasmic sensor domain and transduces the information across the inner membrane to the C-terminal cytoplasmic domain which is joined to the N-terminal by two transmembrane segments. The C-terminal domain can be further divided into a dimerisation subdomain which contains a conserved His residue and a catalytic subdomain that contains the adenosine triphosphate (ATP)-binding site (Pratt and Silhavy, 1995).

Under high osmolarity, EnvZ first undergoes autophosphorylation where the $\gamma$-phosphoryl group of the bound ATP is transferred to the His residue by the catalytic subdomain. EnvZ then transfers this high energy phosphoryl group from the His residue to the conserved aspartate (Asp) residue on the N-terminal regulatory domain of OmpR. The phosphorylated N-terminal domain enhances the DNA-binding ability of the OmpR C-terminal effector domain and allows it to activate the transcription of $ompC$ while down regulating the expression of $ompF$. OmpC trimers form smaller pores than OmpF and hence significantly reduce the rate of diffusion under high osmolarity. EnvZ also possesses phosphatase activity that promotes dephosphorylation of OmpR under low osmolarity and allows OmpR to function as a transcriptional activator of $ompF$ (Pratt and Silhavy, 1995). In addition, the expression of $ompF$, but not $ompC$, is also
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Figure 1.6: An illustration of the E. coli EnvZ-OmpR two-component system and the transcriptional regulation of *ompF* and *ompR* under different osmolarity. See text for more details (taken from http://www.uic.edu/labs/kenneyl/).
post-transcriptionally regulated by MicF in response to environmental and internal stress stimuli. Transcribed divergently upstream from *ompC, micF* encodes a non-translated small RNA (sRNA, Beisel and Storz, 2010) that binds to *ompF* mRNA and regulates *ompF* expression by inducing RNA degradation (Delichas and Forst, 2001).

In a typical TCS, the ATP-dependent autophosphorylation of the histidine kinase is a bimolecular reaction between homodimers where phosphorylation of the conserved His residue from one histidine kinase monomer is catalysed by the second monomer (Stock *et al.*, 2000). The dimerisation subdomains of an EnvZ homodimer are comprised of two identical antiparallel HTH subunits which interact along the helical axis to form a four-helix bundle structure called the H box (Figure 1.7.a). The outer helix of each subunit contains several conserved amino acid residues including the essential His243 residue which is the functional site of autophosphorylation and phosphate transfer reactions. The His side chain protrudes out from the H box that allows access of the catalytic subdomain and the response regulator OmpR. Several other essential residues are located in the inter-subunit surfaces of the four-helix bundle which indicates that dimer formation is the functional state of the histidine kinase (Tomomori *et al.*, 1999).

The EnvZ catalytic subdomain monomer has an $\alpha/\beta$ sandwich fold core consisting of five antiparallel $\beta$ sheets (stands B, D, E, G and F) and three $\alpha$ helices (helices 1, 2 and 4) followed by a polypeptide loop extending away from the core structure which forms part of the ATP-binding site (Figure 1.7.b). The bound AMP-PNP molecule is
Figure 1.7: Ribbon illustrations of the EnvZ dimerisation subdomain (a, taken from Tomomori et al., 1999), the EnvZ catalytic subdomain bound to an ATP analogue AMP-PNP (b, taken from Tanaka et al., 1998) and the OmpR effector domain (c, taken from Kondo et al., 1997).
surrounded by α helix 3 and the polypeptide loop and its triphosphate side chain is exposed to the protein surface that allows the transfer of γ-phosphoryl group to the His residue in the dimersation subdomain. The ATP-binding site encloses several highly conserved regions termed the N, G1, F and G2 boxes, in which the G1 and G2 boxes and N box are essential for the kinase and ATP-dependent autophosphorylation activities of EnvZ respectively (Tanaka et al., 1998).

The N-terminal regulatory domain of OmpR contains the conserved Asp55 residue and the phosphorylation states of this residue affect the activity of the C-terminal effector domain. The OmpR effector domain contains a four-strand β sheet connected to a β hairpin by three α helices (Figure 1.7.c). α helices 2 and 3 are joined by a long ten-residue loop (the α loop) that together forms a winged HTH DNA-binding motif. Mutational studies indicate that α helix 3 and the loop connecting β strands 5 and 6 contribute to the DNA-binding activities of OmpR, whereas the α loop interacts with the α subunit of RNA pol and effectively regulate transcription (Martínez-Hackert, 1997). Under high osmolarity, phosphorylated OmpR dimer binds in a hierarchical manner to multiple binding sites called F and C sites located in the ompF and ompR promoters respectively and functions as either a transcriptional activator or a repressor depending on the sites of interaction (Maeda and Mizuno, 1990; Rampersaud et al., 1994). Each binding site consists of a tandemly arranged 10 bp half binding sites and evidence from DNA affinity cleaving experiments indicate that phosphorylated OmpR asymmetrically interacts with ompF’s F1 site with each recognition helix forming contacts with the
major groove of each half binding site (Harrison-McMonagle, 1999).

1.2 IRON ACQUISITION, FUR AND THE FUR REGULON

The ability to effectively regulate cellular gene expression in a coordinated manner is essential for pathogenic bacteria to adapt and survive in the various hostile environments encountered during transmission and colonisation. Sensing and adjusting to the animal internal environment requires global regulatory systems that simultaneously regulate the expression of an array of virulence genes that allow pathogenic bacteria to adapt to the host and cause disease (Snyder and Champness, 2003). Regulation of iron homeostasis and other cellular processes by the global regulator Fur is an example of such adaptive responses and Fur-mediated gene regulation as well as the mechanisms involved in iron acquisition are closely linked to bacterial virulence and pathogenesis (Litwin and Calderwood, 1993; Wooldridge and Williams, 1993; Carpenter et al., 2009b).

1.2.1 Iron and iron limitation in host

Iron is a versatile transition metal which has a great redox potential span ranging from -300 to + 700 mV between the reduced ferrous (Fe$^{2+}$) state and the oxidised ferric (Fe$^{3+}$) state (Andrews et al., 2003). When incorporated into proteins, iron, either alone or in a more complex iron-sulphur cluster or haem group, serves as a biocatalyst for a broad spectrum of redox and electron transfer reactions. Many iron-containing proteins participate in major biological processes such as electron transport, energy metabolism,
peroxide reduction, respiration, amino acid and DNA synthesis, nitrogen fixation and photosynthesis, therefore iron is an essential micronutrient for virtually all living organisms (Andrews et al., 2003; Wandersman and Delepelaire, 2004). The exceptions to this absolute iron requirement are *Lactobacillus plantarum*, which uses manganese and cobalt instead of iron for cellular functions (Weinberg, 1997), as well as pathogenic bacteria *Borrelia burgdorferi* and *Treponema pallidum* (Posey and Gherardini, 2000).

Although iron is one of the most abundant elements in nature, the availability of the biologically relevant ferrous form is limited in the natural environment. Under aerobic conditions, ferrous iron is oxidised to the ferric form and produces reactive oxygen species such as superoxide anions and hydroxyl radicals through the Haber-Weiss and Fenton reactions (Wandersman and Delepelaire, 2004). Ferric iron is insoluble under aerobic, aqueous and physiological pH conditions and reactive oxygen species especially hydroxyl radicals are biotoxic, leading to cellular compound damage such as DNA breaks, lipid peroxidation and protein denaturation (Schaible and Kaufmann, 2004). In the animal host, the majority of free iron is complexed within metalloproteins which protect the host from iron induced oxidative damage and also provide a non-specific innate defence mechanism that limits the availability of iron sources to invading pathogenic bacteria (Ratledge and Dover, 2000).

Human and animal hosts obtain iron directly through their diet and it is absorbed in the ferrous form by divalent metal ion transporter 1 in the gut (Fleming et al., 1998). When
circulating in the bloodstream, extracellular ferric iron is bound to a high affinity iron-chelating glycoprotein transferrin which contains two homologous lobes, each with single ferric iron binding capacity (Wandersman and Delepelaire, 2004). Transferrin in humans is only 30% saturated with allows it to effectively scavenge any surplus iron present in the blood during infection. Cellular iron uptake is achieved by transferrin receptor 1 and 2 which each bind to holo-transferrin and the resulting complex is then internalised by endocytosis. The acidic environment in the endosome reduces the transferrin-bound ferric iron and the released ferrous iron is then transported to the cytoplasm where it is stored in ferritin in its ferric form (Hentze et al., 2004). When acquired with a sufficient amount of iron, ferritin reforms into haemosiderin, which is an insoluble amalgam of degraded protein and ferric hydroxide (Weinberg, 2009).

Lactoferrin is another iron-binding protein that is commonly found in lymph and mucosal secretions such as tears and saliva and it is structurally and functionally related to transferrin. However unlike transferrin, lactoferrin retains its high affinity for iron under the low pH conditions that usually occur at the site of infections (Wooldridge and Williams, 1993). Lactoferrin also releases an N-terminal peptide lactoferricin by acidic proteolysis, which has a broad range of antimicrobial activities (Bellamy et al., 1992).

Haem is the most abundant iron source in the animal host and dietary haem is transported to the cytosol by the haem carrier protein 1 and the gene product of haem responsive gene 1 (Yanatori et al., 2010). Haem is mostly bound intracellularly by haemoproteins such as haemoglobin and smyoglobin (Wooldridge and Williams, 1993).
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Haemoglobin released into the plasma as a result of erythrocyte lysis forms a complex with haptoglobin, which is then taken up by reticuloendothelial macrophages through haemoglobin scavenger receptor CD163-mediated endocytosis (Hentze et al., 2004). Haem dissociated from haemoglobin is sequestered due to its toxicity by haemopexin in the plasma and it is then transported to the liver where haem is removed from haemopexin by hepatic parenchymal cells. Serum albumin also binds haem though with a low affinity and haem complexed with albumin is removed from the circulation by apo-haemopexin (Wooldridge and Williams, 1993).

Under the influence of cytokines, the level of free iron in the mammalian host is further reduced during microbial invasion in a set of reactions collectively known as hypoferremia. Synthesis of transferrin, lactoferrin and haemopexin elevates during inflammation, removing any free iron and haem liberated into plasma as a result of infection. Degranulation of leukocytes also occurs at the site of infection which further increases the local concentration of lactoferrin to chelate iron and remove iron from transferrin which has a reduced affinity to iron at low pH (Litwin and Calderwood, 1993). During extracellular microbial invasion, interleukin-6 promotes the secretion of peptide hormone hepcidin which diminishes iron release from reticuloendothelial macrophages and duodenal enterocytes by inactive cellular iron exporter protein ferroportin (Weinberg, 2009). By contrast, during intracellular infection, interferon-γ enhances the function of ferroportin and represses the expression of transferrin receptor 1 in macrophages which reduces the intracellular iron level. Interferon-γ also induces
the expression of natural resistance-associated macrophage protein 1 (Nramp1) which depletes iron from the phagosome and withholds iron from invading microorganisms (Ganz, 2009).

1.2.2 Mechanisms of bacterial iron acquisition and iron storage

Despite facing the elaborate iron withdrawal mechanisms in human and animal hosts as well as the poor solubility and toxicity nature of iron, bacteria have evolved highly specific and adaptive iron acquisition systems. These systems are capable of effectively obtaining various forms of environmental iron such as ferrous iron and ferric iron as well as iron from host iron-containing proteins under iron restricted conditions (Figure 1.8).

1.2.2.1 Ferrous iron uptake

Although iron is predominately present in its ferric state under aerobic conditions, ferrous iron can be directly utilised by bacteria grown under anaerobic and microaerobic conditions. In addition, many bacteria such as E. coli (Cowart, 2002), Helicobacter pylori (Worst et al., 1988), Listeria monocytogenes (Cowart and Foster, 1986) and Pseudomonas aeruginosa (Cox, 1986) are capable of synthesising extracellular ferric iron reductases which facilitate the solubilisation of extracellular ferric iron or ferric iron bound to transferrin. In general, ferrous iron passively diffuses through the OM porins of Gram-negative bacteria and is then actively transported through the cytoplasmic membrane (CM) by the Feo system (Figure 1.8.a, Cartron et al., 2006). The
Figure 1.8: An illustration of iron acquisition systems of Gram-negative bacteria including: ferrous iron uptake (a), siderophore-mediated ferric iron uptake (b), iron uptake from transferrin/lactoferrin (c) and direct haem uptake and haem uptake from haemoproteins (d). The energy required for iron and haem uptake through the outer membrane receptor is transduced from the cytoplasmic membrane by the TonB/ExbB/ExbD protein complex (adapted from Krewulak and Vogel, 2008).
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_E. coli_ three-gene operon _feoABC_ encodes an example of such systems which are iron repressed and anaerobically induced (Hantke, 1987; Kammler _et al._, 1993).

FeoB is the cytoplasmic ferrous iron permease which consists of a C-terminal transmembrane domain and a hydrophilic N-terminal domain. The C-terminal domain contains two opposite orientated Gate motifs with conserved residues such as cysteine (Cys) that are predicted to be involved in metal binding during iron transport (Cartron _et al._, 2006). The C-terminal domain contains a G-protein region that possesses GTPase activity and is essential for FeoB transport activity (Marlovits _et al._, 2002). In _H. pylori_, Feo activity is ATP-dependent which indicates FeoB may possess GTPase as well as ATPase activity (Velayudhan _et al._, 2000). FeoA is a small hydrophilic protein probably located in the cytosol and it weakly resembles the SH$_3$ domain of the diphtheria toxin regulator protein DtxR (see 1.2.3.7). Commonly found in eukaryotic signalling proteins, the SH$_3$ domain plays a role in protein-protein interactions and therefore by analogy, FeoA potentially interacts with FeoB and facilitates FeoB-dependent ferrous iron uptake (Cartron _et al._, 2006). FeoC is a small protein that has only been found in the Feo system of γ-proteobacteria. It contains a winged HTH DNA-binding motif in the N-terminal and C-terminal iron-sulphur binding residues which indicates a potential role for FeoC as a transcriptional regulator that controls the expression the _feoABC_ operon (Cartron _et al._, 2006).

The iron transport activity of FeoB is particularly important for facultative anaerobic
pathogens and is an essential requirement for their adaptation under host oxygen limited conditions. The ability to colonise the mouse intestine was severely impeded in *E. coli* and *Salmonella enterica feo* mutants (Stojiljkovic *et al*., 1993; Tsolis *et al*., 1996). In microaerophilic *H. pylori*, FeoB-mediated ferrous iron uptake is the major iron acquisition pathway and FeoB is required for colonisation of the mouse gastric mucosa as well as for normal growth (Velayudhan *et al*., 2000).

### 1.2.2.2 Siderophore-mediated ferric iron uptake

The most common strategies for iron uptake in bacteria involves the utilisation of low molecular weight extracellular ferric chelators called siderophores which are elaborated and secreted by bacteria and fungi in response to iron restriction (Neilands, 1995). More than 500 siderophores have been characterised and they usually consist of a peptide backbone with incorporated metal-chelating functional groups such as α-hydroxycarboxylate, catecholate and hydroxamate groups (Figure 1.9.a-c) that together form hexadentate octahedral complexes with ferric iron (Andrews *et al*., 2003). Each functional group donates two oxygen ligands which are capable of forming strong ionic interactions with ferric iron and allow siderophores to chelate ferric iron with high affinity (Miethke and Marahiel, 2007). Siderophores are classified by their functional groups and structures of a fungal hydroxamate siderophore ferrichrome and an *E. coli* catecholate siderophore enterobactin are shown in Figure 1.9.d-e.

Siderophores are synthesised from common precursors such as citrate, amino acids and
Figure 1.9: Structures of siderophore metal-chelating functional groups: α-hydroxycarboxylate (a), catecholate (b) and hydroxamate (c) and structures of the siderophores ferrichrome (d) and enterobactin (e, taken from Krewulak and Vogel, 2008).
dihydroxybenzoate and the biosynthetic processes are either catalysed by nonribosomal peptide synthetases (NRPS) or by other NRPS-independent enzymatic reactions (Miethke and Marahiel, 2007). Genes encoding the siderophore biosynthetic enzymes are often clustered with genes involved in siderophore uptake and are together induced by iron limitation (Wandersman and Delepelaire, 2004). Once synthesised, siderophores are secreted from the cell through specific transporters such as the *E. coli* membrane protein Ent that belongs to a superfamily of proton motive force (PMF)-dependent membrane efflux pumps and mediates the secretion of enterobactin (Furrer et al., 2002). In addition, many bacteria such as *E. coli* are capable of utilising exogenous siderophores such as ferrichrome as well as enterobactin and related compounds synthesised endogenously by themselves (Chu et al., 2010).

Ferri-siderophores are too large to diffuse freely across the bacterial OM and therefore for Gram-negative bacteria, OM receptors are required to recognise and transport ferri-siderophores into the periplasmic space. The energy required for this process is harnessed from the electrochemical charge gradient across the CM which is delivered to the OM by the energy transducing TonB/ExbB/ExbD protein complex (Figure 1.8.b). Gram-positive bacteria on the other hand do not require TonB-dependent OM receptors for ferri-siderophore uptake due to the lack of the OM (Andrews et al., 2003). Many bacteria possess multiple OM receptors, each displaying a high ligand affinity and specificity. *E. coli* K-12, for example, possesses at least five OM receptors, FepA, CirA, FecA, FhuA and FhuE, which allow the uptake of ferric-bound enterobactin,
enterobactin degraded products, citrate, ferrichrome and rhodotorulic acid respectively (Miethke and Marahiel, 2007). All OM receptors share the same overall structure which consists of a C-terminal transmembrane barrel domain composed of 22 antiparallel β strands connected by 10 periplasmic and 11 extracellular loops (Figure 1.10.a). The N-terminal cork domain has a four-strand β sheet structure with surrounding loops and helices and is kept inside the periplasmic end of the barrel by hydrogen bounds and salt bridges (Krewulak and Vogel, 2008). The cork domain also contains apices that function in siderophore binding (Krewulak and Vogel, 2008) and the periplasmic located N-terminal TonB box essential for TonB-dependent ferri-siderophore uptake (Postle, 1993). In the case of E. coli FecA, an additional periplasmic signalling domain transmits a ferric dicitrate-binding signal to the cytoplasmic sigma factor FecI which in turn facilitates the transcription of the ferric dicitrate uptake operon fecABCDE (Braun et al., 2003).

The mechanisms of ferri-siderophore transport have been revealed by comparative structural analyses of the ligand free and ferri-siderophore bound FecA and FhuA. Conformational changes of extracellular loops 7 and 8 of the FecA barrel domain have been determined which fold inward over the siderophore binding pocket in the presence of ferric dicitrate (Sauter and Braun, 2004). Similar confirmation changes has also been observed for extracellular loops 3 and 11 of the FhuA barrel domain in the presence of ferrichrome (Ferguson et al., 1998) and deletion these loops complete abolished the ferri-siderophore binding and transporting abilities of FecA and FhuA (Sauter and
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Figure 1.10: Ribbon illustrations of the *E. coli* ferric citrate OM receptor FecA (a), the *E. coli* siderophore PBP FhuD bound to ferrichrome coloured in yellow (b) and the *E. coli* vitamin B$_{12}$ ABC permease complex BtuCD (c). β-strands that form the front section of the FecA barrel domain have been removed for easy visualisation (taken from Krewulak and Vogel, 2008).
Braun, 2004; Endriss and Braun, 2004). A switch helix in the FhuA cork domain also unwinds when bound to ferrichrome and a similar helix partial unwinding is also observed in the ferric dicitrate bound FecA cork domain (Ferguson et al., 1998; Ferguson et al., 2002). The unwinding of the switch helix leads to a conformational and positional change of the TonB box which signals the binding of a ferri-siderophore to the TonB/ExbB/ExbD protein complex (Noinaj et al., 2010).

ExbB and ExbD are both CM proteins with three and one transmembrane domains respectively and together they transduce the PMF generated at the CM and deliver the energy required for ferri-siderophore transport to the OM receptor via the TonB protein (Figure 1.8). TonB has a single N-terminal transmembrane domain anchored in the CM, a proline rich central domain that spans across the periplasmic space and a periplasmic C-terminal domain consists of one α helix and a three-strand β sheet (Krewulak and Vogel, 2008; Chang et al., 2001). TonB forms a complex with both ExbB and ExbD in a ratio of 1 TobB: 7 ExbB: 2 ExbD (Higgs et al., 2002) and this transmembrane domain-dependent complex formation is essential for TonB activity (Jaskula et al., 1994). Many bacteria have more than one TonB/ExbB/ExbD system. For instance Vibrio cholerae has two tonB genes associated with a set of exbBD genes whereas five potential TonB/ExbB/ExbD coding regions have been identified in Pseudomonas syringae (Wandersman and Delepelaire, 2004).

Structural analyses of the TonB-FhuA complex reveal a physical interaction between the
three-strand $\beta$ sheet of the TonB C-terminal domain and the FhuA TonB box which together forms an interprotein $\beta$ sheet structure. This interaction leads to a reposition of the TonB C-terminal $\alpha$ helix and allows the conserved arginine (Arg) residue 166 of TonB to form an electrostatic interaction with the FhuA cork domain. The Arg166 residue is proposed to facilitate the energy transfer from TonB to the cork domain, leading to a structural movement of the cork domain that is necessary for ferri-siderophore translocation to the periplasmic space (Pawelek et al., 2006). The exact movement of the cork domain remains uncertain and it has been postulated that the cork domain undergoes a conformational change that creates a small channel between the cork and the inner barrel wall where the translocation may occur or alternatively the cork domain may partially or completely exit the barrel during ferri-siderophore transport (Noinaj et al., 2010).

Transport of the ferri-siderophore across the periplasmic space is mediated by the siderophore periplasmic binding protein (PBP) which collects and delivers the ferri-siderophore to a cognate ATP-binding cassette (ABC) permease complex in the CM. The ferri-siderophore uptake system in Gram-positive bacteria closely resembles the ABC permease systems found in the CM of Gram-negative bacteria with the exception that the siderophore-binding protein is presented as a lipoprotein tethered to the external surface of the CM (Chu et al., 2010). Unlike OM receptors which have high specificity to individual siderophores, siderophore PBP and cognate ABC permeases are capable of transporting individual classes of siderophores. _E. coli_ K-12, for example,
possesses the three siderophore PBPs FepB, FhuD and FecB, which specifically recognise ferric-bound catecholates, hydroxamates and citrates, respectively (Miethke and Marahiel, 2007). The crystal structure of *E. coli* FhuD bound to various hydroxamate type siderophores has been determined and reveals a two-domain structure with each domain composed of a five-strand β sheet sandwiched between layers of α helices (Figure 1.10.b) and the overall structure moves in a Venus flytrap like motion upon ligand binding and releasing (Clarke et al., 2000; 2002). The ligand-binding site is located in the hydrophobic domain interface and ligand specificity is achieved by hydrogen bonding between the hydroxamate functional groups with conserved residues on the binding site. The two domains of FhuD are connected by a long α helix which imposes rigidity on the structure and abolishes the broad ligand associated conformational changes usually seen with other PBP involved in haem or sugar transport (Köster, 2001). Molecular dynamics simulation of FhuD reveals a 60° closure of the C-terminal upon ferrichrome release and this motion is speculated to allow the ferrichrome CM permease FhuB to distinguish between the ligand free and ferrichrome-bound FhuD (Krewulak et al., 2005).

The ABC permease complex is a four-domain structure consisting of two transmembrane domains that form a ferri-siderophore transport channel on the CM and two cytoplasmic nucleotide-binding domains which provide the energy for the transporting process by hydrolysing ATP (Figure 1.8). ABC permease complexes are general assembled from separate subunits and the stoichiometry of these subunits varies.
The permease domains for the *E. coli* ferri-enterobactin transporter for instance are composed of two independent subunits FepD and FepG whereas the ferri-hydroxamate permease is formed by a large two-domain subunit FhuB. The ATP-binding domains for these permeases are assembled by a dimer of FepC and FhuC subunits respectively (Krewulak and Vogel *et al.*, 2008). In addition, the *E. coli* vitamin B\(_{12}\) permease domains are composed of two copies of the same BtuC subunits and the BtuCD complex has shown sequence, design and functional similarities to the siderophore ABC permease complexes (Lewinson *et al.*, 2010). Each BtuC subunit is composed of ten transmembrane helices where helix 5 and 10 from each subunit form a cavity that opens up to the periplasmic space and the cavity is closed from the cytoplasm by helix 4 and 5 (Figure 1.8.a). The ATP-binding protein BtuD contains conserved Walker motifs and Q-loop that are essential for ATP-binding and hydrolysis. The mechanism of ligand transport is proposed by direct contact of the ligand-bound PBP BtuF with the BtuC dimer which facilitates ligand release and BtuD ATP binding. Subsequent ATP hydrolysis triggers a conformational change of the permease that allows ligand transport into the cytoplasm (Locher *et al.*, 2002).

On entering the cytoplasm, siderophore-bound iron is liberated from the complex by one of two general mechanisms. The first method comprises of the reduction of ferric iron resulting in spontaneous release of the reduced iron due to the relative low affinity of siderophore functional groups for ferrous iron (Miethke and Marahiel, 2007). *E. coli* cytoplasmic protein FhuF is an example of a ferric reductase that has substrate
specificity for a set of ferri-hydroxamates. FhuF is iron-regulated and contains an unusual iron-sulphur cluster at the C-terminal that mediates the electron transfer of the ferri-hydroxamate substrates (Matzanke et al., 2004). The alternative iron release mechanism involves intracellular hydrolysis of the ferri-siderophore leading to complex instability and subsequent release of the bound iron, usually by ferric reductase or other iron-binding proteins (Miethke and Marahiel, 2007). An example of such an enzyme with siderophore catabolic function is the *E. coli* esterase Fes, which is encoded by the enterobactin uptake gene cluster and regulated by iron. Fes has a high specificity for ferri-enterobactin and hydrolyses the trilactone backbone of enterobactin into linear trimers, dimers and monomers of 2,3-dihydroxybenzoylserine (Brickman and McIntosh, 1992). In *E. coli* Fes is required for ferri-enterobactin utilisation and it also functions as a ferri-enterobactin-specific ferric reductase (Andrews et al., 2003).

1.2.2.3 Ferric iron uptake from transferrin/lactoferrin

Many pathogenic bacteria such as *Neisseria* species (spp) and *Haemophilus* spp are also able to extract ferric iron from host glycoprotein transferrin and lactoferrin at the cell surface and transport it across the OM using TbpA/TbpB and LbpA/LbpB receptor complexes respectively (Figure 1.8.c, Cornelissen, 2003; Beddek and Schryvers, 2010). Both TbpA and TbpB are surface exposed, iron regulated proteins that are capable of independently binding transferrin (Cornelissen, 2003). TbpA is an integral OM protein structurally related to previously described TonB-dependent ferri-siderophore OM receptors in 1.2.2.2 and its ability to transport ferric iron across the OM is supported by
the inability of TbpA-deficient *Neisseria gonorrhoeae* strains to grow with transferrin as a sole iron source both *in vitro* and *in vivo* (Cornelissen *et al.*, 1992; 1998). The topology model of *N. gonorrhoeae* TbpA reveals large surface loops that are essential for transferrin binding and subsequent iron extraction (Boulton *et al.*, 2000). TbpB is a bi-lobed lipoprotein that contains high-affinity transferrin binding residues at the N-terminal lobe (Moraes *et al.*, 2009). TbpB has a higher preference for *holo-* over *apo-*transferrin due to its ability to discriminate conformational differences between the two ligand forms and this ability has been suggested to enhance the affinity of TbpA for *holo-*transferrin and the rate of *apo-*transferrin release (Cornelissen, 2003). Although TbpB is not essential for transferrin utilisation in *Neisseria meningitidis* and *N. gonorrhoeae* (Irwin *et al.*, 1993; Anderson *et al.*, 1994), TbpB-defective strain of *Actinobacillus pleuropneumoniae*, a causative agent of pneumonia in pig, is avirulent and ineffective in colonisation (Baltes *et al.*, 2002).

The lactoferrin OM receptor complex LbpA/LbpB is functionally and structurally homologous to the TbpA/TbpB complex and is found in several species of the *Neisseriaceae* and *Moraxellaceae* families (Gary-Owen and Schryvers, 1996). In addition, LbpB also protects *N. meningitidis* from the bactericidal effects of lactoferricin and this function is mediated by the clusters of negatively charged residues in the C-terminal lobe (Beddek and Schryvers, 2010). Transport of ferric iron released from transferrin and lactoferrin across the periplasmic space and into the cytoplasm is dependent on the PBP FbpA and the cognate ABC permease complex FbpBC.
respectively (Cornelissen, 2003; Beddek and Schryvers, 2010). Structural analysis of *Haemophilus influenzae* FbpA reveals a typical two-domain structure linked by two antiparallel β stands and a 20° domain closure upon ferric iron binding (Bruns *et al.*, 1997; 2001). FbpA also bears resemblance to a single transferrin lobe (Bruns *et al.*, 1997) and possesses similar ferric iron coordination residues and ligand binding affinity that have seen with transferrin (Nowalk *et al.*, 1994).

**1.2.2.4 Direct haem uptake and haem uptake from haemoproteins**

Another alternative mechanism to siderophores for microbial iron acquisition *in vivo* is the direct utilisation of host haem and haemoproteins as iron and haem sources (Genco and Dixon, 2001). Exotoxins such as haemolysins, cytolysins and proteases are secreted by extracellular pathogens which liberate haem and haemoglobin from erythrocytes and the free haem and haem complexed with host haemoproteins can then be transported by bacteria using receptor- or haemophore-mediated uptake systems (Figure 1.8.d). The receptor-mediated systems in Gram-negative bacteria involve direct binding of haem or haemoproteins by TonB-dependent OM receptors and the haem is then transported into the cell by haem-specific PBP and ABC permease complexes (Tong and Guo, 2009). Many ligand specific OM receptors have been identified and some examples including haem-specific receptor HutA of *V. cholerae* (Occhino *et al.*, 1998) and HumR of *Yersinia pestis* (Thompson *et al.*, 1999); haemoglobin-specific receptor HmbR of *N. meningitidis* (Stojilkovic *et al.*, 1996); haem and haemoglobin receptor ChuA of *E. coli* (Torres and Payne, 1997) and PhuR of *P. aeruginosa* (Ochsner *et al.*, 2000) and
haemoglobin and haemoglobin-haptoglobin receptors HgpA, B and C of *H. influenzae* (Morton *et al.*, 1999). *N. gonorrhoeae* and *N. meningitidis* also possess haemoglobin and haemoglobin-haptoglobin receptor HpuB with associated OM lipoprotein HpuA and the HpuBA complex is an analogue to the bipartite transferrin receptor TbpA/TbpB complex described in 1.2.2.3 (Chen *et al.*, 1998; Lewis *et al.*, 1998). Gram-positive bacteria also possess receptor-mediated haem uptake systems such as the *Staphylococcus aureus* cell surface receptors IsdB and IsdH which bind haemoglobin and haemoglobin-haptoglobin respectively. Haem is removed from the bound haemoglobin by surface protein IsdA and transferred to the cell wall protein IsdC prior to passing through the membrane mediated by the translocation protein complex IsdDEF (Mazmanian *et al.*, 2003).

Several Gram-negative bacteria also secrete extracellular haemophores which bind haem or extract haem from haemoproteins and shuttle them back to the OM for uptake by specific TonB-dependent OM haemophore receptors (Tong and Guo, 2009). Two types of such systems have been reported, one is the *Serratia marcescens* haemophore HasA and its cognate receptor HasR. Secreted by the HasDEF protein complex, HasA has a high affinity to haem and functions to capture haem or extract haem from haemoglobin and present it to HasR. HasR also acts as a low affinity OM receptor for haem and haemoglobin and the energy required for haem transport through HasR is depended on HasB, a TonB-like protein (Létoffé *et al.*, 2004). HxuA of *H. influenzae* is another type of haemophore which is secreted by HxB and forms a complex with
haem-haemopexin. HxuC is the cognate OM receptor for HxuA and is also required for haem and haem-albumin uptake processes (Morton et al., 2007). The majority of haem transported into the cytoplasm is rapidly catabolised by haem oxygenase which catalyses the oxidation of haem into ferrous iron, biliverdin and carbon monoxide (Li and Stocker, 2009). Haem oxygenase has been isolated from both Gram-negative and positive bacteria such as ChuS of E. coli (Suits et al., 2005), HemO of N. meningitidis (Zhu et al., 2000) and IsdG/I of S. aureus (Skaar et al., 2004).

1.2.2.5 Iron and haem storage

When acquired by specific iron uptake systems and liberated from siderophore and haem, ferrous iron is either incorporated directly into metalloenzymes to fulfil their biological roles, or if in excess, iron is deposited in the ferric form in iron storage proteins which can then be used as intracellular iron sources when exogenous iron supplies are restricted. Iron storage proteins also protect the bacterial cell against oxidative stress induced by iron overload (Smith, 2004). Three iron storage proteins have been characterised in bacteria including the bacterial ferritin (referring to as ferritin from here onward), bacterioferritin and Dps. Both ferritin and bacterioferritin are tetracosameric spheres usually assembled by identical subunits surrounding a central iron storage cavity. Ferroxidase residues are found in the centre of each subunit which act as iron-binding ligands and use oxygen to catalyse the oxidation of bound ferrous iron into diferric intermediates for subsequent storage in the central cavity (Le Brun et al., 2010). The ferroxidase centre of ferritin closely resembles that of the eukaryotic
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ferritin H-chain centre and it functions as a gated iron pore. Bacterioferritin ferroxidase centre on the other hand is quite distinct from the H-chain eukaryotic ferritin and it acts as a true catalytic centre (Le Brun et al., 2010). In addition, all known bacterioferritins also contain additional haem groups located at the subunit interfaces near the central cavity that play an essential role in facilitating iron release from the bacterioferritin central cavity (Yasmin et al., 2011). Dps is a stress-induced non-specific DNA-binding protein with a dodecameric structure and contains non-conserved ferroxidase residues (Smith, 2004).

Inactivation of the *E. coli* ferritin encoding gene *ftnA* results in a reduction of stationary phase cellular iron content and a reduced growth rate under iron-restricted conditions which demonstrates the iron storage function of FtnA and the ability for *E. coli* to use FtnA stored iron as an intracellular iron source (Abdul-Tehrani et al., 1999). Iron stored in *H. pylori* ferritin Pfr on the other hand does not serve as an iron source, Pfr rather functions in protecting the cell from iron overload and is essential for the colonisation of gastric mucosa in animal models (Waidner et al., 2002). The *E. coli* bacterioferritin Bfr catalyses the oxidation of bound ferrous iron using hydrogen peroxide and hence reduces the formation of hydroxyl radicals, though no phenotypes have been associated with the *bfr* mutant (Bou-abdallah et al., 2001). Unlike the *E. coli* Bfr, the *N. gonorrhoeae* bacterioferritin is assembled by non-identical A and B subunits. The exact ratio of subunit A and B is unknown, however the subunit B is essential for protecting the cell against oxidative stress and iron stored in the *N. gonorrhoeae* bacterioferritin
can be utilised by the cell during iron deprivation (Chen and Morse, 1999). The synthesis of *E. coli* Dps is induced during stationary phase by nutrient starvation or oxidative stress. Dps utilises hydrogen peroxide as the ferrous oxidant during iron binding and therefore protects Dps-bound DNA from hydroxyl radicals (Zhao *et al.*, 2002). The Dps of *Porphyromonas gingivalis* is another example of an iron- and DNA-binding protein which increases bacterial survival during infection of endothelial cells. The mechanism Dps uses to protect the cell against hydroxyl radicals is not mediated by DNA-binding, but rather by decomposing hydrogen peroxide during the oxidation of ferrous iron, a mechanism also used by *E. coli* Bfr. The significance of *P. gingivalis* Dps-DNA interaction is unclear and Dps-bound iron cannot be used as an iron source (Ueshima *et al.*, 2003).

Although not indentified in most organisms, haem storage proteins have also been indentified in bacteria that acquire exogenous haem and these proteins were originally considered as haem oxygenases (Wandersman and Delepelaire, 2004). The oligomeric protein ShuS of *Shigella dysenteriae* is an experimentally characterised haem storage protein that consists of 18 identical subunits each with a single haem molecule binding capacity. ShuS also functions as a DNA-binding protein that protects DNA from haem-induced oxidative damage (Wilks, 2001). The haem-transporting protein HemS of *Yersinia enterocolitica* shares 64% sequence identity with ShuS, it protects the cell from haem toxicity effects and also has been proposed as a haem storage protein (Stojiljkovic and Hantke, 1994). HmuS of *Y. pestis* and PhuS of *P. aeruginosa* are other candidates.
that have been hypothesised to involve in haem storage (Tong and Guo, 2009).

1.2.3 Fur and the functional role of Fur as a global transcriptional regulator

Due to the necessity and toxicity of iron, many bacteria effectively regulate their iron acquisition and storage genes in response to iron availability. This enables bacteria to maintain a balanced level of intracellular iron required for biological functions and at the same time avoid unnecessary stress and damage to the cell due to excess iron. In most Gram-negative bacteria and AT-rich Gram-positive bacteria, iron homeostasis is regulated by the Fur protein. Fur also functions as a global regulator in controlling genes involved in metabolism, oxidative stress, acid tolerance and virulence factors (Hantke, 2001; Lee and Helmann, 2007; Carpenter et al., 2009b).

1.2.3.1 Fur as an iron-dependent repressor and its role in iron homeostasis

The first connection between iron homeostasis and Fur was noted back in 1978 when constitutive expression of ferri-enterobactin and ferrichrome uptake systems were observed for a *S. enterica* mutant grown in iron-rich media (Ernst et al., 1978). This strain was named *fur*, for iron uptake regulation (Ernst et al., 1978). The *E. coli fur* (*fur*Ec*) mutant isolated three years later showed constitutive expression of the OM iron-acquisition receptor genes *cir, fhuA* and *fecA* which were otherwise strongly repressed by iron in the wild-type strain (Hantke, 1981). The *fur*Ec mutant was subsequently complemented (Hantke, 1982), the *fur*Ec gene cloned (Hantke, 1984) and sequenced (Schäffer et al., 1985) and the FurEc protein purified (Wee et al., 1988).
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Fur\textsubscript{Ec} is a 17 kDa His-rich metalloprotein (Schäffer et al., 1985) consisting of an N-terminal DNA-binding domain with a winged HTH motif and a C-terminal dimerisation domain (Stojiljkovic and Hantke, 1995). Fur\textsubscript{Ec} functions as a homodimer (Bagg and Neilands, 1987b) where each subunit binds to a single ferrous iron atom (Bagg and Neilands, 1987a). Other divalent cations such as cobalt, manganese and zinc also activate Fur\textsubscript{Ec} in vitro, although the level of cobalt and manganese present in vivo are usually insufficient to be physiologically relevant for Fur\textsubscript{Ec} function (Bagg and Neilands, 1987b; de Lorenzo et al., 1987; Mills and Marletta, 2005) whereas the intercellular zinc concentration is tightly regulated to prevent it from displacing native metals in Fur\textsubscript{Ec} (Waldron and Robinson, 2009). Binding of the iron corepressor induces a conformational change in the Fur\textsubscript{Ec} protein (Coy and Neilands, 1991) which allows the N-terminal domain to recognise and bind to a 19 bp operator sequence termed the Fur\textsubscript{Ec} box (de Lorenzo et al., 1987). The Fur\textsubscript{Ec} box is found in the promoter region of Fur\textsubscript{Ec}-repressed genes, often overlapping the -10 and -35 regions of the promoter (de Lorenzo et al., 1987), thus interaction of Fur\textsubscript{Ec} with the Fur\textsubscript{Ec} box prevents the access of RNA pol to the promoter resulting in transcriptional repression of the target genes (Figure 1.11.a, Bagg and Neilands, 1987a).

The affinity of Fur\textsubscript{Ec} for ferrous iron is approximately 10 μM (Bagg and Neilands, 1987a) which is comparable to the estimated amount of free iron in the cytosol (Keyer and Imlay, 1996) and iron-binding leads to an approximate 1000-fold increase in the
**Figure 1.11: The regulatory potential of Fur in response to iron availability.** In most cases, Fur dimer in its iron-bound form binds to the Fur box that overlaps the Fur-regulated promoter and inhibits transcription initiation by blocking the access of RNA pol to the promoter (a). Iron-bound Fur dimer also activates gene expression either indirectly by repressing the intermediate sRNA synthesis which in turn allows the expression of sRNA-repressed genes (b) or directly by binding to the Fur box upstream of the Fur-regulated promoter which recruits and/or enhances RNA pol binding to the promoter (c). Both transcriptional gene repression and activation by apo-Fur have been characterised in *H. pylori* (d and e respectively). Whether apo-Fur$_{Hp}$ functions as a monomer or a dimer is uncertain and it is illustrated as a dimer in here for simplicity (adapted from Carpenter et al., 2009).
affinity of Fur\textsubscript{Ec} for the Fur\textsubscript{Ec} box (Andrews \textit{et al.}, 2003). These characteristics make Fur\textsubscript{Ec} an ideal candidate for monitoring and responding to physiologically relevant fluctuations in intracellular free iron levels (Andrews \textit{et al.}, 2003). Under iron-restricted conditions, the affinity of Fur\textsubscript{Ec} for iron is insufficient to populate the Fur\textsubscript{Ec} iron-binding site, which causes derepression of iron acquisition genes and allows accumulation of intracellular iron. However when the iron level exceeds cellular demands, Fur\textsubscript{Ec} coupled with iron prevents further iron intake by down regulating these iron acquisition genes thus protecting the cell from iron-induced oxidative stress (Andrews \textit{et al.}, 2003; Lee and Helmann \textit{et al.}, 2007).

Not surprisingly, all known \textit{E. coli} iron acquisition systems are Fur\textsubscript{Ec} and iron-repressed and some examples include enterobactin biosynthesis genes \textit{entABCDEFG} (Brickman \textit{et al.}, 1990; McHugh \textit{et al.}, 2003), ferri-enterobactin uptake genes \textit{fepABCDEFG} (Brickman \textit{et al.}, 1990; Hantke, 1981), the ferri-enterobactin esterase gene \textit{fes} (Hunt \textit{et al.}, 1994), aerobactin biosynthesis genes \textit{iucABCD} (de Lorenzo \textit{et al.}, 1987), the ferri-aerobactin uptake gene \textit{iutA} (de Lorenzo \textit{et al.}, 1987), ferrichrome uptake genes \textit{fhuABCD} (Hantke, 1981), ferric dicitrate uptake genes \textit{fecABCDE} (Enz \textit{et al.}, 2000), \textit{fec} operon regulator genes \textit{fecI} and \textit{fecR} (Hantke, 1981; Enz \textit{et al.}, 2000; Braun \textit{et al.}, 2003), the ferri-rhodotorulic acid uptake gene \textit{fluE} (McHugh \textit{et al.}, 2003), the TonB/ExbB/ExbD system coding genes (McHugh \textit{et al.}, 2003) as well as ferrous iron uptake genes \textit{feoABC} (Kammler \textit{et al.}, 1993).
Fur\textsubscript{Ec} also represses its own expression (Figure 1.12.a) under iron-rich conditions as indicated by a direct binding of Fur\textsubscript{Ec} to the \textit{fur\textsubscript{Ec}} promoter located in the intergenic region between \textit{fur\textsubscript{Ec}} and the upstream flavodoxin coding gene \textit{fldA} (de Lorenzo \textit{et al.}, 1988b). Fur autoregulation is the most common mechanism of \textit{fur} regulation which allows Fur to effectively regulate its expression according to intracellular iron levels (Carpenter \textit{et al.}, 2009b). In addition to autoregulation, Fur\textsubscript{Ec} expression is positively influenced by peroxide-sensing regulator OxyR and superoxide regulators SoxRS in response to oxidative stress (Zheng \textit{et al.}, 1999). OxyR activates the \textit{fur\textsubscript{Ec}} promoter in response to hydrogen peroxide and when exposed to superoxide-mediated stress, \textit{fur\textsubscript{Ec}} can also be co-transcribed with \textit{fldA} from the SoxRS-regulated \textit{fldA} promoter (Zheng \textit{et al.}, 1999). In fact, the estimated number of Fur\textsubscript{Ec} molecules per cell doubles under oxidative stress and this link between \textit{fur\textsubscript{Ec}} expression and oxidative stress further illustrates the essential role of Fur\textsubscript{Ec} in iron homeostasis (Zheng \textit{et al.}, 1999). Furthermore, a putative Cap-binding site has been identified in the \textit{fldA-fur\textsubscript{Ec}} intergenic region indicating potential cAMP responsive \textit{fur\textsubscript{Ec}} regulation (de Lorenzo \textit{et al.}, 1988b).

Since the discovery of Fur\textsubscript{Ec}, Fur orthologues have been identified in many Gram-negative bacteria such as \textit{H. pylori} (Bereswill \textit{et al.}, 1998), \textit{N. meningitidis} (Thomas and Sparling, 1994), \textit{P. aeruginosa} (Prince \textit{et al.}, 1993), \textit{V. cholerae} (Litwin \textit{et al.}, 1992) and \textit{Y. pestis} (Staggs and Perry, 1991), and in AT-rich Gram-positive bacteria such as \textit{Bacillus subtilis} (Bsat \textit{et al.}, 1998) and \textit{L. monocytogenes} (Ledala \textit{et al.}, 2007). All these proteins show a high degree of homology with Fur\textsubscript{Ec}. Like in \textit{E. coli} (McHugh
**Figure 1.12:** The expression of *fur* in *E. coli* (a) and *H. pylori* (b). *furEc* is repressed by its own gene product under iron-rich conditions but it is activated by Cap and OxyR in response to cAMP and exposure to hydrogen peroxide respectively. Under superoxide-mediated stress, *furEc* is activated by SoxS from a distal promoter, resulting in the production of a multicistronic mRNA also contains transcript of the upstream *fldA* gene. *furHp* is repressed by FurHp binding to the FurHp boxes 1 and 2 under iron-rich conditions and activated by apo-FurHp binding to FurHp boxes 1 and 3 under iron-restricted conditions. For easy visualisation, the length of Fur genes and Fur promoter regions are not drawn in proportion and apo-FurHp is illustrated as a dimer for simplicity (adapted from Gilbreath et al., 2011).
et al., 2003), genetic analyses of these Fur regulons indicate the majority of Fur- and iron-repressed genes in these bacteria are associated with iron homeostasis (Ochsner and Vasil, 1996; Baichoo et al., 2002; Grifantini et al., 2003; Mey et al., 2005; Danielli et al., 2006; Gao et al., 2008; Ledala et al., 2010).

1.2.3.2 Structure-function relationships of Fur

Initial characterisation of the tertiary structure of FurEc illustrated a rigid, α helix-rich structure (Saito and Williams, 1991) where each monomer possesses a regulatory iron site (Saito et al., 1991a; 1991b) and a C-terminal structural zinc site (Jacquamet et al., 1998; Althaus et al., 1999). Binding of iron or other divalent cations to the regulatory site is essential for allosteric activation of FurEc (Coy and Neilands, 1991) while zinc binding to the structural site induces C-terminal domain structural stability and dimerisation (Pecqueur et al., 2006). Spectroscopic and molecular modelling analyses reveal that iron is bound to the regulatory site in a hexa-coordinated environment (Adrait et al., 1999; Jabour and Hamed, 2009) whereas zinc is coordinated in the structural site in a tetrahedral geometry (Jacquamet et al., 1998). FurEc contains four Cys residues that are commonly associated with metal ligands in other proteins (O’Halloran, 1993) and are grouped in Cys92-Xₙ-Cys95 (Xₙ where n represents the number of amino acids) and Cys134-Xₙ-Cys139 motifs (Schäffer et al., 1985). Mutational and chemical modification studies demonstrate that Cys92 and Cys95 are essential for FurEc activity and structural zinc coordination, whereas Cys134 and Cys139 are dispensable and potentially involved in oxidative stress sensing (Coy et al.,
1994; Gonzalez de Peredo et al., 1999; Pecqueur et al., 2006). The *B. subtilis* Fur (*Fur*<sub>*Bs*</sub>) also possesses two metal-binding sites and contains four Cys residues that are all required for *Fur*<sub>*Bs*</sub> activity, stability (Bast and Helmann, 1999) and potentially function as zinc ligands (Lee and Helmann, 2007).

Early binding studies of both Fur<sub>*Ec*</sub> and Fur<sub>*Bs*</sub> have indicated that both proteins, either in the metal-bound form or in the *apo* form (in this report, *apo*-Fur is referred to as Fur protein complexed with a structural zinc but not with any bound regulatory metals) were able to bind DNA with high affinities (Althaus et al., 1999; Bsat and Helmann, 1999). In both cases, *apo*-Fur binding was likely to be caused by metal contamination (Lee and Helmann, 2007) and in fact, the ability for *apo*-Fur<sub>*Ec*</sub> to bind DNA was abolished when possible contaminating metals were removed by EDTA (Mills and Marletta, 2005). *apo*-Fur regulation is however physiologically relevant in some bacteria such as *H. pylori* and is described in detail in 1.2.3.5.

The first crystal structure of the Fur complexed with two zinc atoms per monomer was resolved in *P. aeruginosa* (*Fur*<sub>*Pa*</sub>) which confirmed the originally predicted dimeric protein structure (Figure 1.13.a, Pohl et al., 2003). Each monomer consists of four α helices (helix 1-4) followed by a two-stranded antiparallel β sheet that together form the N-terminal DNA-binding domain, while the C-terminal dimerisation domain is composed of three antiparallel β strands (strand 3-5) with a distal terminal α helix (helix 5, Phol et al., 2003). Strand 3-5 from both monomers form a six-strand β sheet that
Figure 1.13: The ribbon illustration of the Fur$_{Pa}$ dimer with bound zinc atoms and $\alpha$ helices 1 and 4 indicated (a) and stereo views of the electron density surrounded both zinc atoms (b and c, taken from Pohl et al., 2003).
connects the two monomers together and the dimeric structure is further strengthened by numerous hydrophobic and hydrophilic interactions between helix 5 of each monomer (Phol et al., 2003). Asp103 and Arg109 of each monomer also form salt bridges that facilitate dimerisation (Phol et al., 2003) as reduced DNA-binding affinity has been observed with an Asp103 mutant (Barton, 1997).

The N-terminal domain exhibits a typical winged HTH motif and helix 4 of each FurPa dimer are proposed to come in contact with two major grooves on the DNA molecule separated by a single helix turn (Phol et al., 2003). In addition, helix 1 is also involved in DNA recognition as mutation of alanine (Ala) residue 10 in helix 1 caused a partial unfolding of the N-terminal domain and the DNA-binding activity of FurPa was subsequently abolished (Barton et al., 1996). In a comparative analysis of apo-FurEc, FurEc monomer and the N-terminal domain, the folding of helix 2, 3 and 4 were unaffected by the state of the C-terminal whereas helix 1 was unstructured in apo-FurEc (Pecqueur et al., 2006). This observation led to the conclusion that helix 1 is associated with the activation state of the FurEc protein and, like FurPa, helix 1 formation is triggered by binding of the iron cofactor and it functions in facilitating DNA recognition and interaction (Pecqueur et al., 2006).

The structure of FurPa reveals the presence of two metal binding sites (MBSs) per monomer (Figure 1.13): MBS1Pa is located in the dimerisation domain and coordinates the zinc atom 1 in a distorted octahedral geometry with the side chains of His86, Asp88
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(a symmetrical bidentate), glutamine (Glu) residue 107 and His124 and a water molecule; MBS2
\textsubscript{Pa} connects the two domains and the zinc atom 2 is coordinated by the side chains of His32, Glu80, His89 and Glu100 in a tetrahedral geometry. Fur\textsubscript{Pa} only possesses one of the four Cys residues seen with Fur\textsubscript{Ec}, and it does not function as a metal ligand (Pohl \textit{et al.}, 2003). Iron substitution analysis of the zinc-bound Fur\textsubscript{Pa} revealed that zinc atom at MBS1\textsubscript{Pa} but not MBS2\textsubscript{Pa} could be replaced by iron and thus MBS1\textsubscript{Pa} and MBS2\textsubscript{Pa} were proposed to function as regulatory iron and structural zinc sites respectively (Pohl \textit{et al.}, 2003). This proposal however was contradicted with the observation of several mutational studies. For instance, the iron-responsive ability of Fur\textsubscript{Pa} was retained for a His86 and Asp88 (MBS1\textsubscript{Pa} ligands) mutant but not for a His98 (an MBS2\textsubscript{Pa} ligand) mutant (Lewin \textit{et al.}, 2002) and the iron-responsive ability was also abolished for a fur\textsubscript{Bs} mutant containing altered His97 (corresponds to His98 of Fur\textsubscript{Pa}, Bsat and Helmann, 1999). The MBS2\textsubscript{Pa} site proposed by Pohl \textit{et al.} (2003) is therefore currently reassigned by Lee and Helmann (2007) as the regulatory iron site, while MBS1\textsubscript{Pa} plays a subsidiary role that remains to be elucidated and unlike Fur\textsubscript{Ec}, Fur\textsubscript{Pa} may lack a structural zinc site (Lewin \textit{et al.}, 2002). This view is further supported by free energy calculations of Fur\textsubscript{Ec} and Fur\textsubscript{Pa} which proposes the coordination of iron in MBS2\textsubscript{Pa} in an octahedral environment with His87 as an additional putative iron ligand (Ahmad \textit{et al.}, 2009), and also supported by the crystal structure of the \textit{V. cholerae} Fur (Fur\textsubscript{Vc}, Sheikh and Taylor, 2009).

Purified Fur\textsubscript{Vc} complexed with zinc atoms also possesses two MBSs per monomer
The zinc atom 1 in MBS1\textsubscript{Vc} is tetra-coordinated by the side chains of His87, Asp89, Glu108 and His125 which are the equivalent of ligands found in MBS1\textsubscript{Pa} (Sheikh and Taylor, 2009). Mutation of His87 and His125 of \textit{Vibrio alginolyticus} Fur (Fur\textsubscript{Va}, which shares 94\% sequence identity to Fur\textsubscript{Vc}) resulted in a two-fold reduction of Fur\textsubscript{Va} activity (Liu \textit{et al.}, 2007) which by analogy, suggests the auxiliary role of MBS1\textsubscript{Vc} in Fur\textsubscript{Vc} activity (Sheikh and Taylor, 2009). MBS2\textsubscript{Vc} coordinates the zinc atom 2 in a tetrahedral environment by the side chains of His33, Glu81, His88 and His90 where His88 is substituted by Glu100 (equivalent of Glu101 in Fur\textsubscript{Vc}) in MBS2\textsubscript{Pa}. A His90 mutant of Fur\textsubscript{Vc} demonstrated that His90 is essential for iron sensing (Lam \textit{et al.}, 1994) and Fur\textsubscript{Va} activity was also abolished in His33 and His90 mutants (Liu \textit{et al.}, 2007), which together indicates the crucial role of MBS2\textsubscript{Vc} as the regulatory iron site (Sheikh and Taylor, 2009). In addition, Glu101 of Fur\textsubscript{Vc} is also proposed to function as the fifth metal ligand in coordinating iron in an octahedral environment in MBS2\textsubscript{Vc} (Sheikh and Taylor, 2009).

Fur\textsubscript{Vc} contains four conserved Cys residues grouped in two Cys-X\textsubscript{2}-Cys motifs, and interestingly unlike Fur\textsubscript{Ec}, they are not involved in metal coordination but instead Cys93 and Cys133 form a disulphide bridge that stabilises the dimeric protein structure (Sheikh and Taylor, 2009). Comparison of the overall structure between Fur\textsubscript{Vc} and Fur\textsubscript{Pa}, which share 50\% sequence identity, revealed a 30\degree ration of the DNA-binding domain relative to the C-terminal domain of zinc-bound Fur\textsubscript{Vc} such that the distance between the two helix 4s in the Fur\textsubscript{Vc} dimer is closer than that seen with Fur\textsubscript{Pa} (Sheikh and Taylor,
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Figure 1.14: The ribbon illustration of the Fur\textsubscript{Vc} dimer with bound zinc atoms and the \( \alpha \) helix 4 indicated (a) and stereo views of the electron density surrounded both zinc atoms (b and c, taken from Sheikh and Taylor, 2009).
The authors suggested that binding of zinc to MBS2\textsubscript{Vc} is inadequate for the N-terminal domain to fold into an optimal position for DNA-binding and an efficient DNA-binding is only achieved by coordinating iron in MBS2\textsubscript{Vc} (Sheikh and Taylor, 2009).

The crystal structure of the \textit{H. pylori} Fur (Fur\textsubscript{Hp}) containing two amino acid substitutions has been recently resolved which shows several unique features when compared with Fur\textsubscript{Pa} and Fur\textsubscript{Vc}. Each Fur\textsubscript{Hp} monomer (Figure 1.15) possesses a ten-residue extension at the start of the N-terminal domain, an additional \(\alpha\) helix 6 at the end of the C-terminal domain and three MBSs (Dian \textit{et al.}, 2011). MBS1\textsubscript{Hp} is located in the C-terminal and it tetra-coordinates the zinc atom 1 by the side chains of two Cys-X\textsubscript{2}-Cys motifs consisting Cys102, Cys105, Cys142 and Cys145. Binding of zinc to MBS1\textsubscript{Hp} stabilises the dimeric protein structure which reflects the role of MBS1\textsubscript{Hp} as the structural zinc site (Dian \textit{et al.}, 2011). MBS2\textsubscript{Hp} has equivalent location to MBS2\textsubscript{Pa} and MBS2\textsubscript{Vc}, but unlike in these bacteria, coordination of the zinc atom 2 in MBS2\textsubscript{Hp} adopts different geometries between each Fur\textsubscript{Hp} monomer. In one monomer, the zinc atom 2 is coordinated in a tetrahedral environment by the side chains of His42, Glu90, His97 and His99, whereas the zinc atom 2 in the second monomer is octa-coordinated by the additional side chain of Glu110. MBS3\textsubscript{Hp} is locationally equivalent to MBS1\textsubscript{Pa} and MBS1\textsubscript{Vc} and it coordinates the zinc atom 3 in a tetrahedral environment by the side chains His96, Asp98, Glu117 and His134 (Dain \textit{et al.}, 2011).
Figure 1.15: The ribbon illustration of the FurHp dimer with bound zinc atoms indicated (a) and the geometry of the three zinc-binding sites (b, taken from Dian et al., 2011).
Functional analyses of MBS2$_{Hp}$ and MBS3$_{Hp}$ demonstrated that MBS2$_{Hp}$ is essential for DNA-binding activity and the metal-induced allostERIC activation of Fur$_{Hp}$ whereas MBS3$_{Hp}$ is dispensable for DNA-binding, though its disruption significantly reduces the affinity of DNA-binding. Based on these observations, MBS2$_{Hp}$ is proposed to function as a primary regulatory iron site where metallation of this site mediates Fur$_{Hp}$-DNA interaction and this DNA-binding is further strengthened by the binding of a second metal ion to MBS3$_{Hp}$ under excess metal conditions (Dian et al., 2011). The assigned roles of MBS2$_{Hp}$ and MBS3$_{Hp}$ are further supported by an independent mutation study, which also demonstrated the subsidiary role of both sites involved in maintaining Fur$_{Hp}$ oligomerisation (Carpenter et al., 2010). The unique structural features observed for Fur$_{Hp}$ are likely to reflect its diverse regulatory potential (see 1.2.3.4 and 1.2.3.5) and the distinct metal coordination schemes adopted by Fur$_{Ec}$, Fur$_{Pa}$, Fur$_{Vc}$ and Fur$_{Hp}$ may lead to variations in the mechanisms of metal-sensing and gene regulation among these Fur orthologues (Danielli and Scarlato, 2010).

### 1.2.3.3 The Fur box and models of Fur-Fur box interaction

A 31 bp Fur$_{Ec}$ protected region containing a dyad symmetrical element was first observed in a DNase I footprinting analysis of the iron-regulated E. coli iucA promoter and a 19 bp Fur$_{Ec}$ box consensus sequence was derived from the alignment of this symmetrical element with the fluA and fepA promoter regions (de Lorenzo et al., 1987). This sequence was subsequently observed in an alignment of more than 30 iron-regulated promoters (Stojilkovic et al., 1994) and when cloned downstream of a
heterologous promoter, it was sufficient to induce iron regulation in vivo (Calderwood and Mekalanos, 1988). Although the FurEc box has since been used as the gold standard for identification of Fur-binding sites in many bacterial species, several models of Fur-Fur box interaction have been proposed based on this sequence (Table 1.1).

Due to the dimeric nature of FurEc, it was originally proposed that each FurEc monomer recognises and binds to each half of the palindromic sequence (the 9-1-9 model) resembling the mechanism of classical bacterial repressors (Ptashne, 2004). This model however was not compatible with the observation that after the initial FurEc binding to the iucA FurEc box, multiple FurEc dimers polymerised along regions that did not resemble the consensus FurEc box sequence (de Lorenzo et al., 1987; Escolar et al., 2000). Despite the rigidity of the FurEc protein, the unique protection pattern obtained from hydroxyl radical footprinting studies also indicated that FurEc wraps the iucA promoter region in a helical manner (de Lorenzo et al., 1988a) and this phenomenon has also been directly observed by electron and atomic force microscopy (Fréchon and Le Cam, 1994; Le Cam et al., 1994).

Attempts to resolve these contrasting features, have led to the reinterpretation of the palindromic FurEc box as tandem hexameric repeats arranged in a head to head to tail motif (the 6-6-1-6 model, Escolar et al., 1998; Escolar et al., 1999). High affinity FurEc binding was achieved using a minimum of three synthetic hexamers and the AT nucleotides in each hexamer were predicted to be important FurEc recognition sites.
### Table 1.1: Different interpretations of the consensus Fur box and proposed modes of Fur-DNA interaction.

The numbers 6, 7 and 9 used in model names represent the number of nucleotides in each tandem repeat recognised by a Fur monomer and the actual nucleotide sequences are underlined by orange arrows. The number 1 represents the single nucleotide separating two repeats while subscripted number 2 represents the presence of tandem repeats on both strands of the Fur-binding site.

<table>
<thead>
<tr>
<th>model name</th>
<th>interpretation of the Fur box</th>
<th>model of Fur-DNA interaction</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-1-9 model</td>
<td>GATAATGATAATCATTATC CTATTACTATTAGTAATAG</td>
<td></td>
<td>de Lorenzo et al., 1987</td>
</tr>
<tr>
<td>6-6-1-6 model</td>
<td>GATAATGATAATCATTATC CTATTACTATTAGTAATAG</td>
<td>undetermined</td>
<td>Escolar et al., 1998</td>
</tr>
<tr>
<td>(6-1-6)₂ model</td>
<td>GATAATGATAATCATTATC CTATTACTATTAGTAATAG</td>
<td></td>
<td>Lavarra et al., 2002</td>
</tr>
<tr>
<td>(7-1-7)₂ model</td>
<td>tGATAATGATAATCATTATCa aCTATTACTATTAGTAATAg t</td>
<td>Fur dimer</td>
<td>Baichoo and Helmann, 2002</td>
</tr>
<tr>
<td>(9-1-9)₂ model</td>
<td>aatGATAATGATAATCATTATCatt ttaCTATTACTATTAGTAATAGtaa</td>
<td></td>
<td>Chen et al., 2007</td>
</tr>
</tbody>
</table>
(Escolar et al., 1998). An array of such repeats was also indentified in the natural iucA promoter where the ability of FurEc to extend outside the initial binding site was dependent on these short repeats (Escolar et al., 2000). Although this model could possibly explain the phenomenon of FurEc polymerisation along otherwise functionally unrelated DNA, the mechanism of how each FurEc dimer binds to the DNA and how many repeats are needed to interact with a FurEc dimer remain difficult to explain.

During an electrophoretic mobility shift assay (EMSA) investigation of the E. coli febD-entS promoter region which contains two overlapping FurEc boxes, a hierarchy of distinct shifts was detected and the sequence determined from each shift has revealed a new model of FurEc-DNA interaction [the (6-1-6)₂ model] that combines features of the two previous models (Lavrrar et al., 2002). The consensus sequence was reinterpreted as two overlapping inverted hexameric repeats in which each set of repeats interacts with one FurEc dimer on each face of the DNA helix (Lavrrar et al., 2002). The addition of consecutive hexameric sets then allows more dimers to polymerise along the DNA molecule and the protein-protein interaction between each dimer also facilitates FurEc to form complexes with less conserved regions outside the FurEc box (Lavrrar et al., 2002; Lavrrar and McIntosh, 2003).

A similar reinterpretation was also made for the Fur Bs box and a 7-1-7 heptamer motif rather than the (6-1-6)₂ model was concluded to be the minimum recognition site for Fur Bs (Baichoo and Helmann, 2002). DNA target site comparisons have also indicated
the presence of this core 7-1-7 repeat in the recognition site of the Fur\textsubscript{Bs} homologues PerR\textsubscript{Bs} (peroxide regulon regulator, see 1.2.3.7) and Zur\textsubscript{Bs} (zinc uptake regulator, see 1.2.3.7), and a single change in each heptamer was sufficient to alter the recognition pattern between Fur\textsubscript{Bs}, PerR\textsubscript{Bs} and Zur\textsubscript{Bs} (Fuangthong and Helmann, 2003). Despite minor base differences, both (6-1-6)\textsubscript{2} and (7-1-7)\textsubscript{2} interpretations revealed the same method of Fur binding that has close resemblance to the mechanism of DtxR-DNA interaction (White et al., 1998; Pohl et al., 1999) and is also in agreement with the model predicted from the crystal structure of Fur\textsubscript{Pa} (Pohl et al., 2003).

A recent re-examination of Fur binding site clusters using information theory models constructed from experimentally confirmed Fur\textsubscript{Ec} binding sites has led to the proposal of yet another interpretation of the consensus Fur\textsubscript{Ec} box sequence, the (9-1-9)\textsubscript{2} model (Chen et al., 2007). A 9-1-9 motif was predicated as the minimum Fur\textsubscript{Ec} dimer binding site and \textit{E. coli} genome scans with this model have revealed all known Fur\textsubscript{Ec}-repressed genes involved in iron metabolism (Chen et al., 2007). This predicted model also correlates with confirmed Fur binding sites in \textit{P. aeruginosa} and \textit{B. subtilis} indicating a conserved Fur-DNA recognition mechanism between these bacteria and \textit{E. coli} (Chen et al., 2007).

Despite different views on each interpretation of the Fur box, a good correlation between the presence of the consensus sequence and the corresponding iron-responsive gene regulation (predominately gene repression) is generally accepted and observed in
numerous bacteria (Panina et al., 2001; Rodionov et al., 2006). Genome searching with a consensus Fur box sequence has been an effective approach to identify candidates for Fur-regulated genes in B. Subtilis (Baichoo et al., 2002), N. meningitidis (Grifantini et al., 2003) and Y. pestis (Gao et al., 2008). However it is also important to know that not all Fur-regulated genes bearing a Fur binding site resemble the conventional FurEc box consensus sequence as a genome scan with the FurEc box failed to indentify candidate Fur boxes in several members of the δ-proteobacteria such as Desulfovibrio spp, Desulfuromonas spp and Geobacter spp (Rodionov et al., 2004). The Bradyrhizobium japonicum Fur (FurBj) binding site is another such example in that it contains three imperfect direct hexameric repeats that poorly match the FurEc box and just two of the three repeats were sufficient to both bind a FurBj dimer and repress transcription (Freidman and O’Brian, 2003). These observations suggest that the consensus FurEc box sequence may not completely represent the target site for bacterial Fur as a whole and the basis for target set specificity among different bacteria is likely to reflect variation in their genomic base composition, Fur structure and the mechanisms of Fur regulation (Lee and Helmann, 2007).

1.2.3.4 Fur as a transcriptional activator

Although FurEc was originally described as a transcriptional repressor, several iron-induced FurEc-activated genes have also been identified including the aconitase coding gene acnA, bfr, ftnA, succinate dehydrogenase coding operon sdhCDAB and sodB which encodes a iron-dependent superoxide dismutase (Hantke, 2001; McHugh et
The exact involvement of Fur Ec in the regulation of these genes was initially puzzling but was later supported by the discovery of a non-coding regulatory RNA RhyB (Massé and Gottesman, 2002). RhyB is a 90-nucleotide long Fur Ec-repressed sRNA transcribed under iron-limiting conditions and part of RhyB is complementary to these Fur Ec-activated transcripts. RhyB forms complexes with these target transcripts in a RNA chaperone Hfq-dependent manner and subsequently triggers mRNA degradation by recruiting RNase E (Figure 1.11.b, Massé and Gottesman, 2002; Massé et al., 2003). RhyB homologues have also been indentified in other bacterial species such as *Shigella* spp, *Yersinia* spp and *Salmonella* spp (Massé and Gottesman, 2002) and *P. aeruginosa* possesses two alternative sRNAs PrrF1 and PrrF2 which are functional analogues to RhyB, but share no sequence homology with RhyB (Wilderman et al., 2004).

Global analysis of the *E. coli* RhyB regulon revealed that the expression of 58 proteins either participating in iron storage or requiring iron for their function are directly regulated by RhyB (Massé et al., 2005). This illustrates the involvement of RhyB, along with Fur Ec, in a cellular adaptive response called iron sparing (Figure 1.16, Massé and Arguin, 2005). When the intracellular free iron level is sufficiently high, Fur Ec directly down regulates extracellular iron acquisition and indirectly, via ryhB repression, promotes intracellular iron sequestration which together protects the cell from iron-induced toxic effects. However when the cellular iron content falls below a critical level, derepressed RyhB prevents the synthesis of nonessential iron-containing and storage proteins, thus “spares” the available iron for essential cellular functions (Massé
Figure 1.1: An illustration of iron sparing response mediated by the interplay between Fur and RyhB. When the intracellular iron level is sufficiently high, Fur prevents further iron uptake by directly repressing iron acquisition genes while at the same time promotes intracellular iron sequestration by indirectly activating the expression of iron storage and usage proteins via RhyB. However when the amount of intracellular iron falls back to a critical level, derepressed RyhB down regulates iron storage and non-essential iron-containing proteins thus allowing the available iron to be utilised more effectively (taken from Massé and Arguin, 2005).
and Arguin, 2005). The ability for $\text{Fur}_{\text{Ec}}$ to effectively regulate iron acquisition
and storage genes through iron sparing further emphasises its role in iron homeostasis (Lee
and Helmann, 2009) and also explains the 70% reduction in iron content previously
seen with a $\text{fur}_{\text{Ec}}$ mutant (Abdul-Tehrani et al., 1999).

As well as indirect Fur activation, evidence has suggested that some Fur proteins can
also function as a direct transcriptional activator (Figure 1.11.c). In $\text{N. meningitidis}$, the
promoters of three metalloprotein coding genes $\text{pan1, norA}$ and $\text{norB}$ are positively
regulated by $\text{Fur}_{\text{Nm}}$ in a iron-dependent manner. Transcriptional activation is mediated
by direct binding of iron-bound $\text{Fur}_{\text{Nm}}$ to the operator sequences located upstream of
these iron regulated promoters. $\text{Fur}_{\text{Nm}}$ binding presumably facilitates RNA pol activity
and deletion of the operator sequence in the $\text{norB}$ promoter abolished this iron-
and $\text{Fur}_{\text{Nm}}$-responsive gene regulation both in vitro and in vivo (Delany et al., 2004).
Similarly in $\text{H. pylori}$, iron-bound $\text{Fur}_{\text{Hp}}$ was shown to activate the transcription of
iron-sulphur cluster synthase coding gene $\text{nifS}$ by binding to an operator sequence 300
bp upstream from the $\text{nifS}$ start codon (Alamuri et al., 2006). Most recently, the
expression of a type III secretion system regulator HilD in $\text{S. enterica}$ has also been
linked to $\text{Fur}_{\text{Se}}$ activation in an operator sequence-binding-dependent manner (Teixidó et
al., 2011).

1.2.3.5 Transcriptional regulation by apo-Fur

Although Fur has been generally considered as a transcriptional regulator that represses
or activates gene expression in its iron-bound form, characterisation of Fur regulation in *H. pylori* indicated that Fur<sub>Hp</sub> functions beyond this classic paradigm (Figure 1.11.d-e, Danielli and Scarlato, 2010). DNase I footprinting assay and EMSA studies of the *pfr* and *sodB* promoters revealed that apo-Fur<sub>Hp</sub> binds directly to the operator sequences overlapping the -10 and -35 regions of these promoters and iron decreases the efficiency of apo-Fur<sub>Hp</sub> binding (Delany *et al.*, 2001; Ernst *et al.*, 2005). The concept of direct DNA-binding by apo-Fur<sub>Hp</sub> *in vitro* is controversial as it is questionable whether or not Fur could actually be found in an experimental setting without its metal co-factor (Carpenter *et al.*, 2009b). However transcriptional analyses of both *pfr* and *sodB* promoters in a wild-type background have shown that both genes were induced by iron but repressed under iron-depleted conditions. Furthermore, both genes were also constitutively and iron-independently expressed in a fur<sub>Hp</sub> mutant, which in combination with the data from DNA-binding analyses indicates Fur<sub>Hp</sub> directly represses the expression of *pfr* and *sodB* in its apo form in response to iron limitation (Delany *et al.*, 2001; Ernst *et al.*, 2005).

apo-Fur<sub>Hp</sub> can also function as a direct transcriptional activator in a operator sequence-binding-dependent manner and the only example to date involves the autoregulation of *fur<sub>Hp</sub>* (Figure 1.12.b, Delany *et al.*, 2002; 2003). *fur<sub>Hp</sub>* is transcribed from its own promoter which consists of three operator sequences. Operator 1 overlaps the -35 region and displays the highest binding affinity for both Fur<sub>Hp</sub> and apo-Fur<sub>Hp</sub>. Operator 2 overlaps the -10 region and operator 3 is located upstream from the other
operators (Delany et al., 2002; 2003). Under iron-rich conditions, FurHp binds to both operators 1 and 2 and classically represses the expression of furHp. When iron becomes scarce, FurHp bound to operator 1 is replaced by apo-FurHp, which also binds to operator 3 and together facilitates the expression of furHp. In addition, when the FurHp concentration falls below a certain level, operator 1 is freed from FurHp regulation. This allows operator 1 to function as an UP element for RNA pol thus enhancing transcription from the furHp promoter (Delany et al., 2002; 2003).

To date, the ability of Fur to regulate gene expression in its apo form has only been studied in detail in H. pylori, thus relatively little is known about the apo-FurHp recognition site and it is currently unknown whether apo-FurHp functions as a dimer or a monomer (Carpenter et al., 2009b). Sequence comparison between pfr and sodB promoters has failed to identify a consensus apo-FurHp box sequence (Delany et al., 2001; Ernst et al., 2005) and homology to the proposed FurHp box was also not evident (Merrell et al., 2003). However, a single base substitution of the apo-FurHp binding site in the sodB promoter resulted in altered apo-FurHp-dependent regulation indicating apo-FurHp binds to its recognition site in a base-specific manner (Carpenter et al., 2009a). In an in vivo complementation study, the ability for E. coli, P. aeruginosa and V. cholerae fur genes to complement FurHp and apo-FurHp regulation when presented in trans in a furHp mutant was investigated. Although FurEc and FurVe were able to complement the FurHp regulation of the aliphatic amidase coding gene amiE, apo-FurHp regulation of pfr was unable to be complemented by any of the Fur orthologues.
indicating \text{Fur}_{\text{Hp}} contains unique structural and functional features (Miles et al., 2010a).

The crystal structure of \text{Fur}_{\text{Hp}} described in 1.2.3.2 reveals an additional N-terminal extension that is only conserved among \textit{Helicobacter} spp and an additional MBS when compared to \text{Fur}_{\text{Pa}} and \text{Fur}_{\text{Vc}} (Carpenter et al., 2010; Dian et al., 2011). \text{Fur}_{\text{Hp}} displays a dense hydrogen bonding network that connects the N-terminal extension with $\alpha$ helices 2 and 3. The authors suggested that in the absence of a metal cofactor, this hydrogen bonding network stabilises the DNA-binding domain in its active conformation thus allowing \textit{apo-}\text{Fur}_{\text{Hp}} to bind to its target operator sites (Dian et al., 2011). In addition, mutagenesis of conserved amino acids of \text{Fur}_{\text{Hp}} revealed that Glu90 and His134, which function as metal ligands in MBS2$_{\text{Hp}}$ and MBS3$_{\text{Hp}}$ respectively, are also required for \textit{apo-}\text{Fur}_{\text{Hp}} regulation (Carpenter et al., 2010) conferring the link between the unique \text{Fur}_{\text{Hp}} structure and its regulatory potential.

\subsection*{1.2.3.6 Fur as a global regulator}
In addition to its role in controlling iron homeostasis, analyses of Fur regulons in many bacteria demonstrate the role of Fur as a global regulator in controlling genes involved in the production of toxins and virulence factors, oxidative stress responses, acid tolerance responses, chemotaxis and cellular metabolic pathways (Ochsner and Vasil, 1996; Baichoo et al., 2002; Grifantini et al., 2003; McHugh et al., 2003; Mey et al., 2005; Danielli et al., 2006; Gao et al., 2008; Ledala et al., 2010). While these are too numerous to be discussed exhaustively here, a few well studied examples are briefly
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illustrated.

In many pathogenic bacteria, Fur regulates virulence genes (aside from iron-acquisition genes) that are essential for bacterial pathogenesis. In *E. coli* for example, Fur<sub>Ec</sub> negatively controls the production of haemolysin Hly (Fréchon and Le Cam, 1994), Shiga-like toxins SltA and SltB (Calderwood and Mekalanos, 1987), adhesin Iha (Rashid *et al.*, 2006) and fimbrial adhesin CFA/I (Karjalainen *et al.*, 1991). In *V. cholerae*, the production of haemolysin and an OM virulence determinant IrgA (an Iha homologue) is also negatively regulated by Fur<sub>Vc</sub> (Stoebner and Payne, 1988; Litwin and Calderwood, 1994). In *P. aeruginosa*, Fur<sub>Pa</sub> affects biofilm formation (Banin *et al.*, 2005) and it indirectly regulates the synthesis of exotoxin ToxA (Ochsner *et al.*, 1995) and enzymes involved in quorum sensing (Oglesby *et al.*, 2008). In *N. gonorrhoeae*, Fur<sub>Ng</sub> regulates the repression of 11 *opa* genes which encode OM proteins involved in host cell adherence and invasion (Sebastian *et al.*, 2002), and the production of vacuolating cytotoxin VacA in *H. pylori* is also indirectly regulated by Fur<sub>Hp</sub> (Gancz *et al.*, 2006). The essential role of Fur in controlling bacterial pathogenesis is also demonstrated by colonisation studies of *fur* mutants. The *H. pylori* fur<sub>Hp</sub> mutant for instance is less effective in mouse colonisation (Bury-Mone *et al.*, 2004) and it is easily out-competed by the wild-type strain in a gerbil infection model (Gancz *et al.*, 2006; Miles *et al.*, 2010b). Additionally, *fur* mutants of *S. aureus* (Horsburgh *et al.*, 2001), *L. monocytogenes* (Rea *et al.*, 2004) and *V. cholerae* (Mey *et al.*, 2005) also exhibit significant defects or attenuation in colonisation using murine infection models.
Another functional role for Fur is to regulate cellular processes that are essential for bacterial survival in sub-optimal conditions such as under oxidative and acidic stresses. Under oxidative stress, many bacteria produce the catalase Kat and superoxide dismutase Sod to neutralise reactive oxygen species (Touati, 2000). In *E. coli*, the expression of manganese-containing SodA is negatively regulated by Fur\textsubscript{Ec} (Privalle and Fridovich, 1993) while *sodB* is indirectly activated by Fur\textsubscript{Ec} via RhyB (Massé and Gottesman, 2002). In *N. gonorrhoeae*, Fur\textsubscript{Ng} directly activates the expression of *sod* (Sebastian \textit{et al.}, 2002) and in *H. pylori*, *sodB* is repressed by apo-Fur\textsubscript{Hp} (Ernst \textit{et al.}, 2005). *E. coli* produces two hydroperoxidases encoded by *katG* and *katK* and their expression is activated by Fur\textsubscript{Ec} (Hoerter \textit{et al.}, 2005). The expression of *kat* in *P. aeruginosa* (Hassett \textit{et al.}, 1996), *S. aureus* (Horsburgh \textit{et al.}, 2001) and *Y. pestis* (Gao \textit{et al.}, 2008) is also activated by Fur whereas the catalase-peroxidase coding gene *katG* is repressed by Fur in all *Mycobacterium* spp (Zahrt \textit{et al.}, 2001). In addition, Fur\textsubscript{Se} regulates the expression of the flavohaemoglobin coding gene *hmp* and the Nramp1 homologue coding gene *mntH* in *S. enterica* which the gene products protect the bacteria from nitric oxide- and hydrogen peroxide-induced stresses respectively (Crawfold and Goldberg, 1998; Kehres \textit{et al.}, 2002).

The involvement of Fur in acid tolerance responses are best studied in *S. enterica* and *H. pylori*. In *S. enterica*, Fur\textsubscript{Se} controls the expression of several acid tolerance response genes at pH 5.8 which mainly protect the cell from organic acid stress but only play a
minor role in inorganic acid stress (Foster, 1991; Bearson et al., 1998). The fur_{Se} mutant is unable to induce these acid tolerance response genes at pH 5.8 and is more sensitive to acid when compared to the wild-type (Foster, 1991). Interestingly, iron availability does not affect the acid tolerance response and an iron-blind Fur_{Se} mutant still exhibits an acid tolerance phenotype (Hall and Foster, 1996). When H. pylori is exposed to low pH, the number of Fur_{Hp} regulated genes increases significantly. Such genes include the glucose transporter coding gene gluP, the Holliday junction endodeoxyribonuclease coding gene rnvC, the flagellar biosynthetic protein coding gene fliP and amiE (Gancz et al., 2006). AmiE in particular has been shown to contribute to acid resistance by producing ammonia from aliphatic amides (van Vliet et al., 2003).

1.2.3.7 Fur homologues and DtxR

The Fur family of proteins is widespread within the bacterial world, as approximately 800 Fur-like proteins have been identified and most of these proteins are likely to function as metal-dependent transcriptional regulators (Lee and Helmann, 2007). However it is important to appreciate that metal-dependence and metal-specificity varies markedly for these Fur-like proteins and many proteins originally annotated as ferric uptake regulators may sense cellular or environmental signals other than metal ions (Lee and Helmann, 2007).

Like iron, zinc is also an essential but toxic trace element required for many bacteria and bacterial zinc homeostasis is usually achieved by Zur (Hantke, 2005). In E. coli,
Zur \textsubscript{Ec} represses the zinc uptake system ZnuACB in response to zinc sufficiency by binding to the bidirectional promoter region of \textit{znuA} and \textit{znuBC} (Patzer and Hantke, 2000). Binding of Zur \textsubscript{Ec} to the \textit{znuC} promoter is dependent on the occupancy of both the structural and regulatory zinc sites of Zur \textsubscript{Ec} with zinc atoms and Zur \textsubscript{Ec} binds zinc with a high affinity such that at equilibrium, there is no free zinc in the cell (Outten and O’Halloran, 2001; Outten \textit{et al.}, 2001). In \textit{B. subtilis}, Zur \textsubscript{Bs} represses the zinc uptake system operon \textit{ycdHI-yceI} (Gaballa \textit{et al.}, 2002) and DNA-binding and sequence alignment investigations indicated that Zur \textsubscript{Ec} and Zur \textsubscript{Bs} recognise palindromic operator sequences that have resembles to the consensus Fur\textsubscript{Ec} box sequence (Gaballa and Helmann, 2002; Panina \textit{et al.}, 2003). Zur \textsubscript{Bs} also represses the expression of a ribosomal protein YtiA (Akanuma \textit{et al.}, 2006) and a folate biosynthesis protein YciA (Lee and Helmann, 2007) under zinc-rich conditions where the alternative zinc-dependent versions of these proteins are expressed. Additionally in a recent characterisation of the Zur regulon in \textit{Y. pestis}, 154 Zur\textsubscript{Ye} regulated genes were identified suggesting a role for Zur\textsubscript{Ye} as a global regulator (Li \textit{et al.}, 2009).

In \textit{Rhizobium leguminosarum}, the manganese-uptake operon \textit{sitABCD} is repressed in response to manganese sufficiency by the manganese uptake regulator Mur (Diaz-Mireles \textit{et al.}, 2004). Mur\textsubscript{Rl} does not contain a structural zinc site, but instead it complexes with two manganese atoms per dimer (Bellini and Hemmings, 2006) and binds to a unique Mur\textsubscript{Rl} responsive sequence (MRS) in the \textit{sitABCD} operon promoter (Diaz-Mireles \textit{et al.}, 2005). MRS is significantly different from the consensus Fur\textsubscript{Ec} box sequence.
sequence and it is also found in the *sitABCD* operon promoter regions of many other members of the Rhizobiales and Rhodobacterales (Johnston *et al.*, 2007). Interestingly, Mur$_{Ri}$ is able to functionally complement the iron-responsive regulation of the *E. coli* bacterioferritin-associated ferredoxin in a *fur$_{Ec}$* mutant background (Wexler *et al.*, 2003) and recognises a synthetic Fur$_{Ec}$ box suggesting that Mur and Fur may share an overlapping DNA-recognition specificity (Bellini and Hemmings, 2006).

In several member of the α-proteobacteria such as *R. leguminosarum*, Fur is functionally replaced by other global iron regulators such as RirA and Irr (Johnston *et al.*, 2007). RirA is a member of the Rrf2 protein family that functions as a repressor under high iron conditions by interacts with an iron-sulphur cluster (Todd *et al.*, 2002). Irr on the other hand belongs to the Fur superfamily though it represses gene expression in its *apo* form and the repressive effect is released when Irr interacts with haem under high iron conditions (Todd *et al.*, 2006). Although RirA and Irr collectively control the expression of genes that would otherwise be regulated by a Fur orthologue and iron, RirA and Irr sense iron as functions of the intercellular concentration of iron-sulphur cluster and haem respectively (Jonston *et al.*, 2007).

Under nickel-rich conditions, the *Streptomyces coelicolor* nickel transport operon *nikABCDE* is negatively regulated by the nickel uptake regulator Nur (Ahn *et al.*, 2006). Nur$_{Sc}$ also represses the expression of the iron-containing Sod coding gene *sodF* and indirectly regulates the expression of the nickel-containing Sod gene *sodN* (Ahn *et al.*, 2006).
Nur$_{Sc}$ exhibits 27% sequence identity with Fur$_{E_{c}}$ and contains a structural zinc site and a regulatory metal site that has high specificity for nickel both in vitro and in vivo (Ahn et al., 2006). Recent resolved crystal structure of Nur$_{Sc}$ revealed that unlike Fur, Nur$_{Sc}$ possesses two horizontal $\beta$ sheets 1 and 2 in the DNA-binding domain that do not interact with DNA and this unique structure allows Nur$_{Sc}$ to recognise and bind to a palindromic operator sequence consisting of two hexameric repeats separated by 5 bp (An et al., 2009).

Another well studied Fur-like protein is PerR, and unlike many metal-sensing Fur proteins, the regulatory metal site of PerR has evolved to function as a metal-based sensor for peroxide (Lee and Helmann, 2007). First characterised in B. subtilis, PerR$_{Bs}$ is a major peroxide stress response regulator and functions as a functional replacement of the E. coli peroxide sensor OxyR (Bsat et al., 1998). Examples of the PerR$_{Bs}$-repressed genes include the Dps-like protein coding gene mrgA (Chen and Helmann, 1995), katA (Chen et al., 1995), alkyl hydroperoxidase coding genes ahpCF (Bsat et al., 1996), haem biosynthesis coding genes hemAXCDBL (Chen et al., 1995) as well as fur$_{Bs}$ and perR$_{Bs}$ (Fuangthong et al., 2002). All these PerR$_{Bs}$-regulated genes are derepressed under hydrogen peroxide-induced stress or under both iron and manganese limitation (Fuangthong et al., 2002), and the perR$_{Bs}$ mutant is highly resistant to peroxides (Bsat et al., 1998).

PerR$_{Bs}$ is a homodimer that contains a structural zinc site and it is activated by binding
of iron or manganese to the regulatory metal site thus creating two forms of PerR<sub> Bs </sub> with different sensitivities to peroxide (Herbig and Helmann, 2001). The zinc site coordinates a zinc atom by the side chains of four Cys residues in the dimerisation domain and this site plays a structural role by locking together three β strands that are involved in dimeric protein formation (Traoré et al., 2006). The metal site connects the dimerisation domain with the DNA-binding domain and it penta-coordinates an iron atom by the side chains of His37, Asp85, His91, His93 and Asp104 (Lee and Helmann, 2006). Interaction of His37 with the bound iron allows the two domains to adopt a caliper-like conformation that is essential for operator sequence-binding (Jacquamet et al., 2009). Oxidative stress sensing is also mediated by His37 and His91 where hydrogen peroxide causes rapid oxidation of both residues. Oxidation leads to 2-oxo-His formation which weakens the iron affinity to the regulator site thus resulting in protein inactivation (Lee and Helmann, 2006). This type of metal-catalysed protein oxidation seen with PerR<sub> Bs </sub> is unique and differs from the mechanism used by OxyR where protein inactivation is induced by disulphide bond formation as a direct result of oxidation of the Cys thiolates (Zheng et al., 1998).

Finally, in many GC-rich Gram-positive bacteria such as members of Brevibacterium spp (Oguiza et al., 1995), Streptomyces spp (Günther-Seeboth and Schupp, 1995) and Mycobacterium spp (Doukhan et al., 1995), global iron regulation is mediated by another family of iron-dependent regulators collectively named as DtxR after the first member to be characterised (Fourel et al., 1989). DtxR shares many functional and
structural similarities with Fur. For instance, DtxR regulates a spectrum of cellular processes similar to those regulated by Fur including siderophore biosynthesis, iron acquisition, oxidative stress responses as well as toxin production (i.e. diphtheria toxin, Hantke, 2001). Also like Fur, DtxR is a dimeric metal-dependent repressor that consists of a DNA-binding domain and a dimerisation domain and it negatively regulates gene expression by a mechanism consisting of two DtxR dimers interacting with the opposite faces of a palindromic operator sequence (White et al., 1998; Pohl et al., 1999). However, DtxR is not a Fur-like protein as both proteins display no sequence similarity and DtxR also possesses a third β sheet-rich SH3-like domain that modulates metal binding and protein activation (Pohl et al., 1999).

1.3 THE BIOLOGY OF C. JEJUNI

As stated in 1.2.3, Fur plays crucial roles in controlling cellular iron homeostasis and the expression of other virulence factors that are essential for pathogenic bacteria to establish colonisation and cause disease in their hosts. This is also true for C. jejuni, an important and prevalent enteric pathogen. Effective gene regulation by Fur$_{Cj}$ and indeed other transcriptional and translational regulators allow C. jejuni to successfully thrive during the commensal relationship with avian species, within the disease-susceptible human host and in natural environments encountered during transmission (Wösten et al., 2008).

1.3.1 Historic perspective and the genus Campylobacter
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The first possible description of *Campylobacter* was in 1886 when a noncultureable *Vibrio*-like spiral organism was identified in the colon of diarrhoeic infants (Escherich, 1886; Butzler, 2004). Several early recognised isolations of campylobacters were associated with ovine (McFadyean and Stockman, 1913; Skirrow, 2006) and bovine epizootic abortions (Smith and Taylor, 1919) and the name *Vibrio fetus* was initially proposed (Smith and Taylor, 1919). *V. fetus* and other emerging *Vibrio*-like organisms (Jones et al., 1931; Doyle, 1948; Florent, 1953; Bryans et al., 1960; Debruyne et al., 2008) were subsequently re-grouped into the new genus of *Campylobacter* due to their low GC content, microaerobic growth requirement and nonfermentative metabolism (Sebald and Véron, 1963; Véron and Chatelain, 1973). Despite several incidences of human associated infections (Levy, 1946; Vinzent et al., 1947; King, 1957), understanding of this new found genus was limited due to the lack of sufficiently selective culture techniques and therefore *Campylobacter* had largely remained as a cause of veterinary disease. Advances in epidemiological research became possible when a faecal filtration method (Dekeyser et al., 1972; Butzler et al., 1973) and appropriate growth supplements (Skirrow, 1977) were developed. Several new species have since been discovered and *C. jejuni* has rapidly been recognised as one of the most important human enteropathogens.

Phylogenetic and molecular characterisation of the *Campylobacter coli* 23S rRNA gene has positioned *Campylobacter* in the epsilon subdivision of the *Proteobacteria* (Trust et al., 1994). Within the 18 species that have been validated in this genus (Debruyne et al.,
2008), *C. jejuni* and *C. coli* are the most important human enteric pathogens and *C. jejuni* alone accounts for 80-90% of the *Campylobacter* enteric infections (Ketley 1997). The genus type species *Campylobacter fetus* on the other hand is primarily linked to veterinary infections causing ovine and bovine infectious infertility and abortions (Debruyne *et al.*, 2008). Although infrequent, *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter hyointestinalis* have also been associated with human and animal intestinal infections or similar disease manifestations (Ketley 1997; Debruyne *et al.*, 2008). *Campylobacter sputorum*, *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter rectus*, *Campylobacter gracilis*, *Campylobacter showae* and *Campylobacter hominis* are mostly isolated from the human oral cavity and require hydrogen for active growth (On, 2001; Debruyne *et al.*, 2008). *Campylobacter lanienae*, *Campylobacter insulaenigrae*, *Campylobacter canadensis* and *Campylobacter helveticus* are rarely isolated from human and animal sources and their pathogenic properties remind uncharacterised (Debruyne *et al.*, 2008).

1.3.2 Physical and genetic characteristics of *C. jejuni*

Just 0.2-0.8 μm wide and 0.5-5 μm long, *C. jejuni* is a relatively small, rod-shaped Gram-negative microorganism. It is highly motile with a corkscrew-like motion that is facilitated by its spirally curved morphology and a polar unsheathed flagellum situated at one or both ends of the cell (Ketley, 1997). *C. jejuni* is catalase, oxidase and hippurate hydrolysis positive, but lipase and lecithinase negative. Unable to oxidase or ferment carbohydrates, *C. jejuni* utilises amino acids and tricarboxylic acid (TCA) cycle
intermediates instead as its primary energy sources (Snelling et al., 2005). The microaerophilic *C. jejuni* requires an oxygen concentration of 3-15%, a carbon dioxide concentration of 3-5% and 42 °C for active growth. The thermophilic characteristic of *C. jejuni* reflects the adoption of the avian intestinal tract as its natural niche (Ketley, 1997). Upon exposure to oxidative, temperature and acidic stresses, *Campylobacter* is able to change from its normal spiral shape into a viable non-culturable coccoid form (Figure 1.17). This dormant state allows the cell to survive under prolonged sub-optimal growth conditions encountered during transmission between host organisms (Rollins and Colwell, 1986; Chaveerach et al., 2003).

The circular chromosomes of *C. coli* and *C. jejuni* are approximately 1.6-1.7 Mb in length, which is relatively small compared to other enteropathogens such as *E. coli* (van Vliet and Ketley, 2001). The GC ratio of *Campylobacter* DNA averages 30% and this high AT content coupled with differences in base methylation and codon usage compared to *E. coli* make molecular genetic studies of *Campylobacter* sometimes difficult (Ketley, 1997). The first sequenced *C. jejuni* strain NCTC 11168 does not contain any plasmid and has a 1.64 Mb long genome encoding for 1643 genes which make it amongst the densest prokaryotic genomes sequenced to date (Parkhill et al., 2000; Gundogdu et al., 2007). The NCTC 11168 genome has virtually no insertion or phage-associated sequences, has very few repeat sequences and contains several hypervariable tracts. Most of these hypervariable tracts are found in genes responsible for the biosynthesis of surface structures such as the capsule, lipooligosaccharide (LOS)
Figure 1.17: Electron micrographs of *C. jejuni* exhibiting the spiral rod-shape (a) and coccoid forms (b, taken from Rollins and Colwell, 1986). The white scale bar in each photo represents 1μm.
and flagellum and they may play important roles in *Campylobacter* survival and generating genetic diversity (Parkhill *et al*., 2000). Other sequenced *C. jejuni* strains such as 81-176 (Hofreuter *et al*., 2006) and 81116 (Pearson *et al*., 2007) also share similar genomic characteristics with NCTC 11168 with the exception that 81-176 contains two plasmids pVir and pTet that have roles in pathogenesis (Bacon *et al*., 2002; Batchelor *et al*., 2004).

### 1.3.3 Transmission and epidemiology

*C. jejuni* frequently colonises the avian gastrointestinal tract and is also considered to be part of the normal intestinal flora of a broad range of other domestic and wild animals (van Vliet and Ketley, 2001). Although a commensal in animal hosts, when present in humans, *C. jejuni* may cause acute gastroenteritis and is the most common isolated cause of bacterial gastroenteritis in the world with the numbers of recorded cases much higher than those due to any other enteric pathogen, including the frequently reported *Salmonella* spp and *E. coli* (Wooldridge and Ketley, 1997). Human infection is usually acquired by consumption of faecally contaminated or undercooked meat, unpasteurised milk or contaminated water (Figure 1.18, Wilson *et al*., 2008). *C. jejuni* infects approximately 1% of the population in the UK and USA annually, causing substantial clinical costs and loss of working hours, and is therefore considered a major public health and economic burden (Snelling *et al*., 2005).

The infectious dose of *C. jejuni*-induced gastroenteritis is around 500-800 bacteria with
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Figure 1.1: The routes and outcomes of *C. jejuni* infection. *C. jejuni* is a commensal bacterium for chicken and it frequently colonises the mucosal layer of the chicken gastrointestinal tract. Human infection is often acquired through consumption of faecally contaminated meat as well as directly from contaminated water and unpasteurised milk. When present in humans, *C. jejuni* invades the intestinal epithelial layer and causes diarrhoea (taken from Young et al., 2007).
an average incubation period of 3.2 days and the disease manifestations are dependent on socio-economic status (Blaser and Engberg, 2008). In industrialised countries, infection with *C. jejuni* is seasonal and often leads to inflammatory diarrhoea with severe cramping amongst young adults with low asymptomatic carriage rates. By contrast, infection in developing countries is not seasonal and is mostly restricted to children. The clinical symptoms are usually milder watery, non-inflammatory diarrhoea with a high rate of asymptomatic carriage. The differences in these symptoms are due to higher rates of exposure to the bacteria in developing countries and early infection in childhood may result in different immune responses (Ketley, 1997; van Vliet and Ketley, 2001).

The disease is usually self-limiting and symptoms usually last for about a week, although bacterial shedding can persist once clinical symptoms have ceased. Complications following infection are uncommon, but previous infections with *C. jejuni* have been linked to the development of a serious neuromuscular paralysis known as Guillian-Barré syndrome (GBS) or the related Miller-Fisher syndrome (Ketley, 1997; van Vliet and Ketley, 2001; Blaser and Engberg, 2008). GBS is the most common cause of acute, temporary paralysis due to autoimmune inflammatory polyradiculoneuropathy and is commonly preceded by *C. jejuni* enteritis. GBS pathogenesis is believed to be caused by the sialylated *C. jejuni* cell surface LOS that mimic human gangliosides and trigger demyelination of gangliosides by host auto-antibodies produced in response to infection (Jacobs *et al.*, 2008).
1.3.4 Pathogenesis and virulence factors

The symptoms of *C. jejuni* infection directly result in epithelial cell damage caused by several putative virulence factors (Figure 1.19) expressed by the bacteria when establishing colonisation of the host intestinal tract. Some of these essential factors include motility, chemotaxis, adhesion, invasion and toxin production (Ketley, 1997).

1.3.4.1. Motility

Motility is achieved by the expression of polar flagella that endow *C. jejuni* with a characteristic darting motility, which is required for penetrating the intestinal mucus layer on colonisation (van Vliet and Ketley, 2001). The *C. jejuni* flagellar filament is composed of two different flagellins FlaA and FlaB (Logan *et al.*, 1987). Both *flaA* and *flaB* genes have been shown to be essential for flagellar production and a reduction in mobility and colonisation was observed in a *flaA* mutant (Wassenaar *et al.*, 1991; 1993; 1994). The flagellar filament is heavily glycosylated (Guerry *et al.*, 2006) and this glycol-modification allows *C. jejuni* to escape attacks from the host immune system (Szymanski and Wren, 2005). In addition, two other proteins, FlgP and FlgQ, have been identified to be essential for flagellar motility though their functional roles are currently unknown (Sommerlad and Hendrixson, 2007). Motility and flagellar expression are also important for subsequent adhesion and invasion as a reduction in mobility resulting from paralysed flagella led to decreased adhesion and no invasion (Yao *et al.*, 1994).
A: motility
B: chemotaxis
C: adhesion
D: invasion
E: toxin production

Figure 1.19: An overview of the different stages of *C. jejuni* colonisation and virulence factors expressed at each stage (adapted from van Vliet and Ketley, 2001).
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1.3.4.2 Chemotaxis

Chemotaxis is the ability of bacteria to detect and direct their movement according to certain chemical gradients and chemotactic movement has been shown to be essential for \( C. \textit{jejuni} \) colonisation (Takata \textit{et al.}, 1992). \( C. \textit{jejuni} \) is attracted to mucins, L-serine and L-fucose, but repelled by bile acids (Hugdahl \textit{et al.}, 1988). The chemotactic protein \( cheY \) was identified from motile, non-adherent and non-invasive \( C. \textit{jejuni} \) mutants that showed duplication in \( cheY \) and colonisation of mice (Yao \textit{et al.}, 1997). A hyperadherent and hyperinvasive phenotype was observed for \( cheY \) mutant though the ability to establish colonisation and cause disease was abolished (Yao \textit{et al.}, 1997). Several other components of the chemotaxis system have been identified in \( C. \textit{jejuni} \) including \( cheA \), \( cheB \), \( cheR \), \( cheV \), \( cheW \) and \( cheZ \), which together with \( cheY \) control the flagellar rotation in response to environmental stimuli by transduction of signals from specific transmembrane chemoreceptors to flagellar motors (Marchant \textit{et al.}, 2002; Korolik and Ketley, 2008).

1.3.4.3 Adhesion

Upon infection, \( C. \textit{jejuni} \) must cross the mucus layer on the intestinal cell surface and attach to the epithelial cells (Ketley, 1997). Adhesion by bacterial pathogens is usually mediated by fimbrial structures, however fimbrial-associated adhesins have not been identified in \( C. \textit{jejuni} \) (Wassenaar and Blaser, 1999) and adhesive properties have instead been attributed to other proteins such as PEB1 and CadF (Larson \textit{et al.}, 2008). PEB1 is a putative binding component of an ABC transport system expressed from
peb1A. Inactivation of peb1A led to a significant reduction of in vitro adherence and invasion of HeLa cells and in vivo colonisation, which marked its role in C. jejuni adherence to epithelial cells (Pei et al., 1998). Many bacteria are also capable of binding to extracellular matrix components such as fibronectin during the initial phase of infection and in C. jejuni, this is mediated by CadF (Konkel et al., 1997). Mutational studies on cadF have showed a reduction in fibronectin binding and invasion of INT 407 cells (Monteville and Konkel, 2002; Monteville et al., 2003), and the ability to colonise chickens was abolished (Ziprin et al., 1999). Apart from CadF, fibronectin binding has also been proposed for C. jejuni flagellin and LOS (Moser and Schröder, 1997).

1.3.4.4 Invasion

Invasion is an important pathogenic mechanism for C. jejuni as the invasion of epithelial cells would be expected to disrupt the normal absorptive capacity of the host intestine and induce inflammation, which together leads to diarrhoea (Wassenaar and Blaser, 1999). Interaction with host cells via biochemical crosstalk is a common strategy that facilitates invasive bacterial pathogens to stimulate signalling cascades in both the bacteria and the host, which trigger rearrangements of the host cytoskeleton and cause internalisation of the pathogen (Kopecko et al., 2001). Invasive properties and mechanisms of C. jejuni have been characterised in a variety of tissue culture cell lines, in particular with INT-407, HEp-2 and Caco-2 and invasiveness was shown to be strain-dependent (Wooldridge and Ketley, 1997). Two distinct mechanisms of C. jejuni
invasion have been proposed, which either employ a microtubule-dependent mechanism seen with highly invasive *C. jejuni* 81-176 strain or in a microfilament-dependent fashion that is demonstrated by most of the *C. jejuni* strains tested (Crushell *et al.*, 2004). *C. jejuni* secretes several type III secretion Cia proteins, where CiaB in particular is required for the invasion of cultured epithelial cells (Konkel *et al.*, 1999a; 1999b). A *ciaB* mutant is motile (Konkel *et al.*, 2004) but exhibits reduced chick colonisation levels (Ziprin *et al.*, 2001) and inactivation of *ciaB* prevents the secretion of other Cia proteins (Konkel *et al.*, 1999a). *C. jejuni* does not encode a type III secretion system (Parkhill *et al.*, 2000) and Cia proteins are secreted through the flagellar secretion apparatus instead in a *C. jejuni*-host cell contact- or mucin-dependent manner (Konkel *et al.*, 2004; Rivera-Amill *et al.*, 2001). Aside from Cia proteins, the flagellar secretion apparatus also secretes FlaC, which shares limited homology with FlaA and FlaB (Song *et al.*, 2004). FlaC is not required for flagella formation or motility but it is required for invasion (Song *et al.*, 2004).

1.3.4.5 Toxin production

Despite the importance of invasion, the level of invasion detected *in vitro* is normally low and invasion itself might not be the sole response to the cytopathic effects associated with *C. jejuni* infection (Wooldridge and Ketley, 1997). Toxin production is a common virulence factor employed by many enteric pathogens and has been proposed as another important determinant for *C. jejuni* pathogenesis (van Vliet and Ketley, 2001). A variety of toxic activities have been characterised in *C. jejuni* including: a
70-kDa toxin active only on HeLa cells; a cytotoxin active on both HeLa and Vero cells; a second cytotoxin showing hemolytic effects; a shiga-like toxin; a hepatotoxin and the best characterised cytolethal distending toxin CDT (Wassenaar, 1997). *C. jejuni* CDT is a tripartite toxin encoded by the *cdtABC* operon, which is required for CDT cytotoxicity (Whitehouse *et al.*, 1998). CDT is composed of CdtB, as the enzymatically active subunit, and CdtA and CdtC, as the heterodimeric subunit that is required for translocation of CdtB (Lara-Tejero and Galán, 2001). The nuclease activities of CdtB effectively cause the target cells to undergo cytodistension and arrest at the G₂-phase of the cell cycle, which ultimately results in cell death (Lara-Tejero and Galán, 2001).

### 1.3.5 Gene regulation in *C. jejuni*

*C. jejuni* lacks many responsive regulators such as the sigma factor RpoS (σ³⁸) which regulates stress responses in many Gram-negative bacteria (Parkhill *et al.*, 2000). However considerable variation in gene expression have been observed by transcription profiling of *C. jejuni* cultured under a range of sub-optimal growth conditions indicating that regulation of genes involved in adaptive responses is essential for the lifestyle of *C. jejuni* (Wösten *et al.*, 2008). The *C. jejuni* genome contains approximately 650-750 putative promoters and they are regulated by at least 37 transcriptional regulators including three sigma factors and 34 specific regulators (Wösten *et al.*, 2008).

#### 1.3.5.1 Sigma factors

The main sigma factor in *C. jejuni* is RpoD, which belongs to the σ⁷⁰ family of
housekeeping sigma factors and it regulates most *C. jejuni* promoters (Wösten *et al.*, 2008). RpoD contains two regions, the 2.4 and 4.2 segments, which recognise the -10 and -35 promoter regions respectively (Dombroski *et al.*, 1992). The 2.4 segment is highly conserved among *E. coli*, *B. subtilis* and *C. jejuni*, and an alignment of 175 *C. jejuni* promoter regions revealed that RpoD recognises a hexameric -10 region with the consensus sequence TATAAT. The 4.2 segment on the other hand is less conserved thus resulting in the absence of a consensus -35 promoter region in *C. jejuni* (Wösten *et al.*, 1998; Petersen *et al.*, 2003). However, *C. jejuni* does possess a consensus sequence for the -16 region (Wösten *et al.*, 1998; Petersen *et al.*, 2003), which is an essential transcriptional element for many promoters in Gram-positive bacteria and for *E. coli* promoters lacking a -35 region (Voskuil *et al.*, 1995).

FliA is another alternative sigma factor belonging to the σ\textsuperscript{70} family and it regulates 14 genes (Carrillo *et al.*, 2004) encoding for proteins involved in flagella assembly and flagellin subunit glycosylation (Logan *et al.*, 2002). Consistent with its function, a fliA mutant is nonmotile and FliA activity is tightly controlled by the anti-sigma factor FlgM (Wösten *et al.*, 2008). The third *C. jejuni* sigma factor is RpoN, which belongs to the well conserved σ\textsuperscript{54} family and a rpoN mutant is nonmotile and without flagella indicating the essential role of RpoN in motility (Wösten *et al.*, 2008). In fact, the protein products of 15 out of 23 genes regulated by RpoN are involved in flagella assembly which includes components of the basal body, the flagellar hook protein FlgE, FlaB as well as FlgM (Carrillo *et al.*, 2004; Wösten *et al.*, 2004).
1.3.5.2 Specific transcriptional regulators

Ten of the specific transcriptional regulators in \( C. jejuni \) are response regulators of TCSs and seven of their cognate kinase sensor proteins have also been identified. All but one of the response regulators belong to the OmpR family (see 1.1.3) and well studied examples include PhosR, DccR and RacR (Wösten et al., 2008). PhosR forms a TCS with the cognate kinase sensor PhosS which senses the availability of phosphate in the environment. Phosphorylated PhosR regulates gene expression by binding to an operator sequence called the \( pho \) box located at the -35 region of twelve PhosR regulated genes. Examples of these genes include an alkaline phosphatase coding gene \( phoA \) and a \( P_{i} \) transport system coding operon \( pstSCAB \) (Wösten et al., 2006). The DccR-DccS system regulates a putative type I secretion system in response to an unknown stimulus. An 81-167 strain defective in this system is viable but shows a reduced ability to colonise immunocompetent mice and 1-day-old chicks (MacKichan et al., 2004).

Another well studied TCS is the RacR-RacS system which regulates the expression of eleven genes including \( racR \) itself. Like OmpR, RacR acts as both a repressor and an activator and these RacR regulated genes are expressed in response to changing growth temperature in a RacR-dependent manner. The 81116 \( racR \) mutant grows at a slower rate at 42 \( ^{\circ} \)C and it also shows reduced ability to colonise the alimentary tract of chickens (Brás et al., 1999). The only \( C. jejuni \) response regulator that does not belong
to the OmpR family is FlgR which instead belongs to the NtrC family. FlgR forms a TCS with the cognate kinase sensor FlgS and functions in concert with RpoN to regulate the same set of RpoN-controlled genes in response to an unknown stimulus (Wösten et al., 2004). Consistent with the rpoN mutant phenotype, inactivation of flgS or rpoN results in a nonmotile C. jejuni phenotype that lacks flagella (Jagannathan et al., 2001).

Aside from TCSs, C. jejuni also possesses several other regulators such as NssR, CmeR and two FurCj homologues (Wösten et al., 2008). NssR is a nitrosative stress response regulator that positively regulates the expression of Cgb, a single-domain protein involved in the scavenging and detoxification process (Elvers et al., 2004). The mechanism of nitrite and nitric oxide sensing by NssR is currently unknown and NssR represents the only recognised member of the Cap/Fnr regulator family in C. jejuni (Wösten et al., 2008). CmeR is the sole representation of the TetR regulator family in C. jejuni and it represses the cmeABC operon which encodes an efflux pump involved in multidrug resistance (Lin et al., 2002; Lin et al., 2005a). CmeR is essential for C. jejuni colonisation and CmeABC is required for bile resistance suggesting that bile salts are one of the substrates that activate CmeR (Lin et al., 2005b). FurCj and Fur homologue PerRcJ are the best characterised transcriptional regulators in C. jejuni and their roles in controlling iron homeostasis and oxidative stress response respectively in C. jejuni are described in 1.4.2.
1.4 IRON HOMEOSTASIS AND GENE REGULATION BY THE C. JEJUNI FUR

1.4.1 Iron acquisition and storage systems in C. jejuni

As a successful coloniser of the avian and human gastrointestinal tracts, C. jejuni have evolved a range of highly adaptive iron uptake systems (Figure 1.20) which enable the acquisition of nutritional iron from various sources in the animal host. These iron acquisition systems are essential for C. jejuni during commensal and pathogenic colonisation and therefore play importance roles in C. jejuni survival and pathogenesis (Stintzi et al., 2008; Miller et al., 2009; Gilbreath et al., 2011).

1.4.1.1 Ferrous iron uptake

Ferrous iron is an important iron source for enteric pathogens surviving in the oxygen-reduced intestinal environment and direct utilisation of ferrous iron by FeoB has been characterised in C. jejuni strains NCTC 11168, 81-176 and ATCC 43431 (Naikare et al., 2006). The NCTC 11168 feoB homologue is located upstream, in an operon, from a feoA homologue and both genes share various degrees of identity with the E. coli and H. pylori feoBA systems (Parkhill et al., 2000; Naikare et al., 2006). No feoC homologue has been identified and several sequenced strains such as C. jejuni RM1221 carry a non-functional feoB gene with frameshift mutations and premature translational stops (Fouts et al., 2005). Mutational analysis of NCTC 11168, 81-176 and ATCC 43431 feoB show reduced ferrous iron transport into the cytoplasm and an accumulation of ferrous iron in the periplasmic space. The ability to persist in intestinal cells is reduced for the 81-176 feoB mutant, whereas the NCTC 11168 mutant is outcompeted.
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Figure 1.20: Iron acquisition systems of *C. jejuni* including: ferrous iron uptake, siderophore-mediated ferric iron uptake and iron uptake from host iron-containing compounds. The energy required for iron and haem uptake through the OM receptor is transduced from the CM by one of the three TonB/ExbB/ExbD protein complexes (adapted from Miller et al., 2009).
by the wild-type for colonisation of the rabbit ileal loop (Naikare et al., 2006). Furthermore colonisation of the chick caecum and piglet intestinal tract is also significantly compromised in all three feoB mutants, which indicate the functional importance of C. jejuni FeoB in ferrous iron uptake and pathogenesis (Naikare et al., 2006). The ability to utilise ferrous iron is not affected in C. jejuni M129 and F38011 feoB mutants (Raphael and Joens, 2003) therefore implying that both strains as well as RM1221 may possess feoB homologues or alternative FeoB-independent ferrous iron acquisition systems (Miller et al., 2009).

1.4.1.2 Siderophore-mediated ferric iron uptake

Although siderophore production activity in several C. jejuni strains has been documented in an early characterisation of cellular responses to iron limitation (Field et al., 1986), no genes associated with siderophore biosynthesis have been identified from genome analysis of sequenced C. jejuni strains (Parkhill et al., 2000; Hofreuter et al., 2006). However, C. jejuni possesses specific uptake systems for several exogenous ferri-siderophores including ferri-enterobactin (Palyada et al., 2004), ferrichrome (Galindo et al., 2001) and ferri-rhodotorulic acid (Stintzi et al., 2008). In addition, stress hormone noradrenaline also facilitates C. jejuni iron uptake in a siderophore-like and OM receptor-dependent manner (Cogan et al., 2007).

An analogue to the E. coli Fep system, the C. jejuni ferri-enterobactin uptake system consists of an OM receptor CfrA (Palyada et al., 2004), a PBP CeuE (Park and
Richardson, 1995) and an ABC permease complex CeuBCD (Parkhill et al., 2000). NCTC 11168 cfrA is transcribed independently from the downstream ceuBCDE locus which forms a transcriptional unit (Parkhill et al., 2000). When compared with FepA of E. coli, NCTC 11168 CfrA contains many conserved structural motifs but also shows substantial sequence variation in the putative ligand binding site which could affect the ligand binding affinity of CfrA (Carswell et al., 2008). CeuE is an atypical siderophore PBP which contains a signal sequence resembling that of a lipoprotein (Park and Richardson, 1995) and compared to FhuD of E. coli, the ligand binding site of CeuE is more hydrophilic and positively charged (Müller et al., 2006). The unusual structural characteristics of CfrA and CeuE would potentially provide C. jejuni with a broader range of siderophore binding than that provided by orthologous E. coli siderophore transporters (Gilbreath et al., 2011). Mutational studies of NCTC 11168 cfrA and ceuE revealed that both genes are required to establish chick intestinal colonisation and although CfrA is essential for enterobactin-mediated iron acquisition, the ability to utilise ferri-enterobactin as a sole iron source is only partially affected by the CeuE mutant, indicating a degree of functional redundancy shared among iron transporters in C. jejuni (Palyada et al., 2004; Ridley et al., 2006). In addition, noradrenaline also facilitates cellular iron uptake in a CfrA-dependent manner as noradrenaline-mediated growth in iron restricted conditions is abolished in NCTC 11168 cfrA mutant (R. Haigh, personal communication). By contrast, both CfrA and CeuE of C. coli are dispensable in ferri-enterobactin utilisation (Richardson and Park, 1996; Guerry et al., 1997) and the ability to colonise the chick intestinal tract is not compromised in a ceuE mutant
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(Cawthraw et al., 1996). CfrA is conserved amongst a large subset of C. jejuni isolates with the exception of strains such as 81-176 and 81116. Both strains however encode a functional TonB-dependent siderophore OM receptor Cj0444 for which the gene homologue in NCTC 11168 is present as a pseudogene (Parkhill et al., 2000; Hofreuter et al., 2006; Pearson et al., 2007). Recently, the NCTC 11168 Cj0444 homologue has been characterised in several C. coli and C. jejuni isolates as a second ferri-enterobactin OM receptor and consequently named CfrB (Xu et al., 2010). The ability to utilise ferri-enterobactin is greatly impaired in several C. coli strains and abolished in C. jejuni JL11 carrying a cfrB mutation. In addition, cfrB presented in trans is able to complement the ability to utilise ferri-enterobactin in a NCTC 11168 cfrA mutant and mutation of cfrB greatly reduces colonisation in the chick infection model (Xu et al., 2010). Interestingly, 81-176 carrying a functional copy of cfrB and ceuBCD is unable to utilise ferri-enterobactin (Zeng et al., 2009), suggesting the potential presence of a ceuBCD homologue or a novel ABC permease complex in C. jejuni that facilitates CfrB-mediated iron acquisition (Xu et al., 2010).

A comparative study of C. jejuni RM129 OM receptor profiles identified the expression of a three-gene operon cfhuABD in the presence of Hep-2 epithelial cells, which was proposed to encode a putative ferrichrome uptake system (Galindo et al., 2001). The iron regulated cfhuABD operon has an unusually high GC content compared with the surrounding genome and encodes an OM receptor CfhuA, a PBP CfhuD and a CM permease CfhuB, where each protein displays sequence homology with its counterpart
in the *E. coli* ferrichrome system Fhu (Galindo *et al.*, 2001). Although in this study *cfhuA* homologues were identified by PCR in six of the eleven *C. jejuni* isolates, the *cfhuABD* operon has not been identified in any of the sequenced *C. jejuni* strains (Parkhill *et al.*, 2000; Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Pearson *et al.*, 2007). Therefore given the lack of presence in *C. jejuni* and the unusual GC content, the functional importance of CfhuABD in iron uptake and the identity of the cognate ATP-binding component require further characterisations.

Many *C. jejuni* strains contain a highly conserved *cj1658-cj1663* locus which the NCTC 11168 homologue encodes proteins functioning in ferri-rhodotorulic acid utilisation (Miller *et al.*, 2009). The CM permease coding gene *cj1658* is located upstream from *cj1659* which the gene product P19 is a PBP (Janvier *et al.*, 1998) and possesses both ferric iron- and copper-binding capacities (Chan *et al.*, 2010). Although the involvement of P19 in copper uptake is not fully characterised, neither NCTC 11168 *cj1658* nor *p19* mutants are able to utilise ferri-rhodotorutic acid to support cellular growth (Stintzi *et al.*, 2008). Furthermore, an 81-176 *p19* mutant survives poorly in iron restricted conditions when compared with the wild-type strain, which suggests that P19 is able to acquire iron from other sources and the *p19* mutant cannot be fully compensated by other *C. jejuni* iron transporters (Chen *et al.*, 2010). The *cj1660-cj1663* locus encodes a putative ABC permease complex and putative CM proteins and no dedicated ferri-rhodotorotic acid OM receptor has been identified to date (Miller *et al.*, 2009).
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1.4.1.3 Iron acquisition from host iron-containing compounds

*C. jejuni* was once considered incapable of obtaining iron from host ferri-glycoproteins (Pickett *et al.*, 1992), however the function of a putative OM receptor Cj0178 and a PBP-dependent transport system consisting of a PBP Cj0175c, a CM permease Cj0174c and an ATP-binding protein Cj0173c have been linked with the utilisation of iron-bound human transferrin, lactoferrin, and avian ovotransferrin in NCTC 11168 (Miller *et al.*, 2008). NCTC 11168 *cj0178* is divergently transcribed from the *cj0173c-cj0175c* locus which forms an operon with *cj0176c*, though no *cj0178* homologue has been identified from the genomes of 81-176 and 81116 (Hofreuter *et al.*, 2006; Pearson *et al.*, 2007; Miller *et al.*, 2008). Unlike the TbpA and LbpA systems in *Neisseria* spp, Cj0178 is a multispecific OM receptor for both transferrin and lactoferrin as mutation of *cj0178* showed reduced growth in the presence of ferri-lactoferrin and reduced ability to bind and acquire iron from ferri-transferrin (Miller *et al.*, 2008). However the ability to acquire iron from ferri-lactoferrin in the *cj0178* mutant is only partially abrogated and together with a small growth defect seen for the *cj0174c* mutant with ferri-transferrin suggests that *C. jejuni* may deploy alternative ferri-glycoprotein uptake systems (Miller *et al.*, 2008; Gilbreath *et al.*, 2010). In addition, the *cj0178* mutant also resulted in reduced colonisation potential in both the chick cecum and rabbit ileal loop models which demonstrates the importance of Cj0178 and ferri-glycoprotein-mediated iron acquisition *in vivo* (Palyada *et al.*, 2004; Stintzi *et al.*, 2005).

Under iron-restricted conditions, many *C. jejuni* strains are able to acquire iron from
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haem, haemoglobin, haem-haemopexin and haemoglobin-haptoglobin and an OM receptor ChuA has been identified and associated with haem utilisation in 81-176 and NCTC 11168 (Pickett et al., 1992; Ridley et al., 2006). NCTC 11168 chuA is the leading gene of a four gene operon that also encodes a PBP ChuD, a CM permease ChuB and an ATP-binding protein ChuC, and this operon is divergently transcribed from chuZ the gene product of which functions as a haem oxygenase (Ridley et al., 2006). Cellular growth in the presence of haem or haemoglobin as a sole iron source is compromised in both the chuA and chuZ mutants whereas ChuBCD appear to be unessential and partially redundant in haem utilisation (Ridley et al., 2006). Although the expression of NCTC 11168 chuA and chuB are elevated in the chick cecum model (Woodall et al., 2005), preliminary data has suggested that the 81-178 chuA is not required for optimal chick colonisation (Haigh et al., 2010). Other putative proteins associated with haem utilisation include Cj0177 and Cj0178 which both bind haem in vitro (Chan et al., 2006), and also CfrA which displays haemolytic activity in an E. coli background (Park and Richardson, 1995). However haem utilisation is not affected by the cj0178 mutant (Miller et al., 2008) and the cfrA mutant (R. Haigh, personal communication) and further investigation is required to establish conclusive roles for Cj0177 in haem uptake.

1.4.1.4 TonB and iron storage proteins

A search of the NCTC 11168 genome reveals the presence of three tonB homologues, in which both tonB1 and tonB2 are transcriptionally coupled with their cognate exbB and
exbD pairs and are located near the coding regions of ferri-glycoprotein and haem transporters respectively. *tonB3* on the other hand is not adjacent to *exbB/D3* and instead it is divergently transcribed from *cfrA* (Parkhill *et al.*, 2000). The ability for NCTC 11168 to utilise ferri-enterobactin is dependent on TonB3 whereas ChuA can only be energised by either TonB1 or TonB2, and these observations indicate some degree of functional specificity among the three TonB proteins in *C. jejuni* (Stintzi *et al.*, 2008). Unlike NCTC 11168, many strains like 81-178 and 81116 for instance only possess the *tonB2* homologue (Hofreuter *et al.*, 2006; Pearson *et al.*, 2007) and the 81-178 TonB is required to achieve optimal chick colonisation (Haigh *et al.*, 2010). Likewise, only one *tonB3* homologue has been identified so far in *C. coli* and the gene product is essential for the utilisation of haem, ferri-enterobactin and ferrichrome as iron sources (Guerry *et al.*, 1997).

Intracellular iron acquired through various iron uptake systems in *C. jejuni* is generally stored in Cft, a ferritin-like protein that shares a high degree of similarity with *H. pylori* Pfr (Wai *et al.*, 1996). The iron-containing Cft provides an intracellular iron source during iron deprivation as the growth of a cft mutant is inhibited under iron restricted conditions. A cft mutant also shows increased susceptibility to oxidative damage which indicates an additional role of Cft in oxidative stress response (Wai *et al.*, 1996). 81-176 produces an additional iron-binding protein Dps which lacks the DNA-binding ability seen with the *E. coli* Dps. 81-176 dps is constitutively expressed and the dps mutant is more sensitive to hydrogen peroxide which suggests a primary role for Dps in cellular
defence against iron-induced oxidative stress (Ishikawa et al., 2003).

1.4.2 *C. jejuni* Fur homologues and their function in gene regulation

*C. jejuni* possess two Fur homologues: the Fur\(_{Cj}\) protein which regulates the expression of iron metabolism genes (Wooldridge et al., 1994; van Vliet et al., 1998) and PerR\(_{Cj}\) which functions in oxidative stress defence (van Vliet et al., 1999). Recent transcriptomic and proteomic analysis of Fur\(_{Cj}\) and PerR\(_{Cj}\) reveal the functional role of Fur\(_{Cj}\) and PerR\(_{Cj}\) as global transcriptional regulators and their importance of coordinating iron homeostasis and oxidative stress responses in *C. jejuni* colonisation and survival (Palyada et al., 2004; 2009; Holmes et al., 2005). In addition, the *C. jejuni* zinc uptake system coding locus *znuABC* is not regulated by Fur\(_{Cj}\) or PerR\(_{Cj}\) (Davis et al., 2009) potentially indicating the presence of a putative Zur homologue or a novel member of the Fur family in *C. jejuni*.

1.4.2.1 Fur\(_{Cj}\) and fur\(_{Cj}\) regulation

The *fur\(_{Cj}\)* gene was originally identified by reporter assays in 81116 (Wooldridge et al., 1994) and independently by direct sequencing upstream of the ATCC 43431 *lysS* gene (Chan et al., 1995). The 17.9 kDa Fur\(_{Cj}\) displays 40% identity to Fur\(_{Ec}\), particularly at the C-terminal domain (van Vliet et al., 2002), and shows sequence homology with orthologues from other bacterial species (Wooldridge et al., 1994; Chan et al., 1995). Despite the sequence similarity between Fur\(_{Cj}\) and Fur\(_{Ec}\), Fur\(_{Cj}\) is only able to partially repress a Fur\(_{Ec}\)-regulated promoter in a fur\(_{Ec}\) mutant and it is not recognised by Fur\(_{Ec}\).
antiserum, which together indicates that Fur$_{Cj}$ differs significantly from Fur$_{Ec}$ and further structural and functional characterisation of Fur$_{Cj}$ are required (Wooldridge et al., 1994). The fur$_{Cj}$ mutant grows poorly under both iron-restricted and repleted conditions when compared with the wild-type strain, though some of this growth defect may be attributed to altered transcription of the downstream lysS and glyA genes (van Vliet et al., 1998). The fur$_{Cj}$ mutant is more resistant to hydrogen peroxide, but more sensitive to cumene hydroperoxide and menadione when compared with the wild-type, suggesting the role of Fur$_{Cj}$ in oxidative stress defence (Palyada et al., 2009). fur$_{Cj}$ mutation also affects chick colonisation which indicates the importance of Fur$_{Cj}$ regulation in vivo and the role of Fur$_{Cj}$ in C. jejuni virulence (Palyada et al., 2004; 2009).

The genomic organisation of fur$_{Cj}$ is rather unusual compared to fur$_{Ec}$ and fur$_{Hp}$ as it is located in an operon which also contains the downstream housekeeping genes lysS and glyA and upstream genes gatC and Cj0399. fur$_{Cj}$ also does not have its own promoter and it is likely to be co-transcribed as a multicistronic mRNA by two distal promoters located upstream of gatC and Cj0399 respectively. These promoters are not iron regulated, which suggests the absence of iron-responsive autoregulation of fur$_{Cj}$ (van Vliet et al., 2000). This iron and Fur-independent regulation of fur has also been observed for P. aeruginosa (Ochsner et al., 1999) and the unique promoter configuration of fur$_{Cj}$ would allow C. jejuni to regulate Fur$_{Cj}$ in response to unknown environmental stimuli other than just iron (van Vliet et al., 2000).
1.4.2.2 Iron and FurCj-mediated gene regulation in C. jejuni

In order to understand iron homeostasis and the role of FurCj in iron regulation, comparative analysis of protein expression profiles of the NCTC 11168 wild-type and a furCj mutant in response to iron availability were initially carried out to identify members of the iron and FurCj regulon (van Vliet et al., 1998). As expected, derepressed expression of several iron transport proteins including CfrA, CeuE, P19, Cj0178, ChuA and ChuD were observed under iron-restricted conditions in the wild-type strain and in the furCj mutant demonstrating the classic role of FurCj as a repressor in controlling iron homeostasis. By contrast, oxidative stress defence proteins KatA and AhpC, which function as a catalase and an alkyl hydroperoxide reductase respectively (Grant and Park, 1995; Baillon et al., 1999) were still iron-repressed in the furCj mutant, suggesting the involvement of FurCj-independent iron regulation (van Vliet et al., 1998).

In a more comprehensive global transcriptomic analysis of NCTC 11168 in response to iron, expressions of 208 genes were significantly altered between iron-limited and iron-rich growth conditions during steady state. In addition, 460 genes, representing 27% of the C. jejuni genome, were differently transcribed within 15 minutes of iron supplementation illustrating a global adaption in C. jejuni to increased iron (Palyada et al., 2004). Genes encoding proteins involved in iron acquisition and storage systems including cfrA, ceuC, p19, cj1658, cj1661-1664, cj0173c-0175c, chuABCD, chuZ, tonB1, tonB3, exbB1, exbB2, exbD1, exbD2 and cft were up-regulated in iron limited
conditions. Iron-repressed transcription was also observed for components of the oxidative stress response system such as \textit{perR}_{Cj}, \textit{katA}, \textit{ahpC}, \textit{sodB} and \textit{tpx}, which encodes a thiol peroxidase. By contrast, genes induced after the addition of iron, such as ferredoxin coding gene \textit{fdxA} and oxidoreductase coding genes \textit{oorABCD}, are mostly associated with energy metabolism, and not surprisingly, these genes encode enzymes that require iron for their function (Palyada \textit{et al.}, 2004). When the transcriptome profiles of wild-type cells grew at steady state under iron-limitation and iron-rich conditions were compared with the \textit{furCj} mutant, 53 genes were found to be \textit{FurCj} regulated and in particular, 29 of these \textit{FurCj} regulated genes have their expression directly repressed by iron and \textit{FurCj}. Indeed, 17 iron-repressed iron acquisition genes reported in this study were directly repressed by \textit{FurCj}, once again illustrating the primary regulatory role of \textit{FurCj} in iron homeostasis (Palyada \textit{et al.}, 2004).

In another independent transcriptomic and also proteomic investigation of the NCTC 11168 iron and \textit{FurCj} regulon, the transcription of 147 genes were altered with respect to iron availability and the expression of 43 iron-regulated genes were also altered in the \textit{furCj} mutant compared to the wild-type in iron-rich conditions (Holmes \textit{et al.}, 2005). Many iron-repressed genes identified previously by Palyada \textit{et al.} (2004), especially genes associated with iron acquisition and oxidative stress defence were consistently observed in this study with the addition of \textit{tonB2}, \textit{cj0444} and \textit{cj0178}, where \textit{cj0178} was also repressed by \textit{FurCj} (Holmes \textit{et al.}, 2004). Interestingly, unlike other iron acquisition genes, iron and \textit{FurCj}-dependent regulation was not detected for \textit{feoB} in both studies.
(Palyada et al., 2004; Holmes et al., 2005). Many genes associated with general cellular physiology were identified as iron-responsive in Palyada et al.’s study (2004) but not by Holmes et al. (2005) and in fact, only 65 iron-responsive genes were reported in both studies. The discrepancies between these two studies are likely reflected by the different growth media used which would alter, to a certain degree, the cellular physiology and consequently alter the transcriptome in response to iron availability (Stintzi et al., 2008). The temperature variations between the two studies are also likely to influence the experimental outcomes as the expression of approximately 20% of the NCTC 11168 genome is responsive to temperature elevation from 37 °C to 42 °C (Stintzi et al., 2003).

Despite slight variations in experimental design and outcomes, both studies provide a genome wide picture of C. jejuni responses to iron availability and the regulatory role of FurCj not only in controlling iron acquisition genes, but also the function of FurCj as a global regulator (Palyada et al., 2004; Holmes et al., 2005). Additionally in both studies, several members of the FurCj regulon were non-classically regulated by FurCj such as fdxA, several flagellum biosynthesis genes (Palyada et al., 2004) and fumC (Holmes et al., 2005). fumC codes for a fumarase, a key enzyme of the TCA cycle (Guest and Roberts, 1983) and the expression of fumC was found to be iron-induced and FurCj-activated (Holmes et al., 2005). As transcriptomic analysis cannot distinguish between a direct and indirect effect of FurCj on gene expression, the exact involvement of FurCj in fumC regulation and indeed for all other non-classically FurCj regulated genes in C. jejuni are currently unknown.
In a recent transcriptional analysis of the 81-176 dsb genes which have a role in intramolecular disulfide bridge formation, *apo*-Fur*$_Cj$ repression was demonstrated for the *dsbA2* promoter (Grabowska *et al.*, 2011). Prior to Grabowska *et al.*’s investigation (2011), *apo*-Fur repression has been exclusively demonstrated in *H. pylori* (Delany *et al.*, 2001; Ernst *et al.*, 2005), and this type of regulation was believed to be unconserved across bacterial species (Miles *et al.*, 2010a). Fur*$_Hp$ possesses an N-terminal extension which is proposed to stabilise the active DNA-binding domain in the absence of a metal cofactor (Dian *et al.*, 2011) and a similar extension has also been observed in Fur*$_Cj$ by sequence analysis (Carpenter *et al.*, 2010). However, *fur$_Cj$* when presented in *trans* was unable to complement *apo*-Fur*$_Hp$ regulation in a *H. pylori fur$_Hp$* mutant (Miles *et al.*, 2010a) indicating subtle structural and sequence differences between both proteins in their *apo* forms and their corresponding binding sites respectively.

1.4.2.3 Characterisation of the Fur*$_Cj$* box

The Fur*$_Cj$* box was first described in two *fur$_Cj$* characterisation studies where several putative Fur*$_Cj$*-binding sites resembling the *E. coli* consensus sequence were identified closely upstream of the *fur$_Cj$* gene (Wooldridge *et al.*, 1994; Chan *et al.*, 1995). The 81116 Fur*$_Cj$* was demonstrated to partially recognise the consensus Fur*$_Ec$* box (Wooldridge *et al.*, 1994) and purified ATCC 43431 Fur*$_Cj$* was able to bind to the four Fur*$_Cj$* box-like sequences associated with the *fur$_Cj$* gene (Chan *et al.*, 1995), both results therefore indicated that Fur*$_Cj$* potentially deploys a similar recognition sequence when
compared with *E. coli* (van Vliet *et al.*, 2002). However these Fur\textsubscript{Cj} box-like sites were later demonstrated to be functionally irrelevant as Fur\textsubscript{Cj} does not have its own promoter (van Vliet *et al.*, 2000). Fur\textsubscript{Cj} also shows a degree of variation to Fur\textsubscript{Ec} in the N-terminal DNA recognition and binding domain (van Vliet *et al.*, 2002), which suggests that the Fur recognition sequence may have diverged between *C. jejuni* and *E. coli* (Wooldridge *et al.*, 1994).

The initial search for the Fur\textsubscript{Cj} binding site failed to identify any good matches in the genome when the *E. coli* three consecutive hexamer model (Escolar *et al.*, 1999) was applied, however further refined searches with six NAT (N represents any nucleotide) in the promoter region of iron acquisition genes, including *chuA*, *cfrA*, *p19*, *feoB*, *ceuB*, *cj0177*, *cfhuA* and *exbB* have enabled the verification of this trimer repeat as the putative Fur\textsubscript{Cj} recognition site (van Vliet *et al.*, 2002). In a more comprehensive transcriptomic analysis of the Fur\textsubscript{Cj} regulon, a 19 bp consensus sequence was derived from the promoter regions of 16 iron and Fur\textsubscript{Cj}-regulated operons by computational analysis (Palyada *et al.*, 2004). This Fur\textsubscript{Cj} binding site however poorly matches the *E. coli* consensus sequence and does not resemble any known Fur-recognition motifs (Table 1.2).

Direct Fur\textsubscript{Cj} binding with various affinities have been observed for several *C. jejuni* promoters, including the *cfrA* and *ceuB* promoters and the *p19* operon promoter (Holmes *et al.*, 2005, Berg, 2007), the *chuA* and *ahpC* promoters (Li, 2005), the
Table 1.2: Sequence alignment of the Fur$_{Ec}$ box and the putative Fur$_{Cj}$ box. The sequence logo of the Fur$_{Cj}$ box is redrawn from Palyada et al. (2004) using Weblogo ([http://www.bio.cam.ac.uk/seqlogo](http://www.bio.cam.ac.uk/seqlogo)). The height of each letter in the Fur$_{Cj}$ box indicates the relative frequency of each base occurring at the indicated base position. Sequence differences between the *E. coli* and *C. jejuni* Fur boxes are highlighted in yellow.
Chapter 1: Introduction

*chuA-chuZ* intergenic region (Ridley et al., 2006), the *cj0176c* and *cj777* promoters (Miller et al., 2008), and the *tonB3-cfrA* intergenic region (Shearer et al., 2009). Nevertheless, the putative Fur$_{Cj}$ box proposed by Palyada et al. (2004) has not been verified with detailed mutational and DNase I footprinting analysis.

1.4.2.4 PerR$_{Cj}$ and oxidative stress response

As a microaerophilic bacterium, *C. jejuni* requires protection against reactive oxygen species generated during aerobic metabolism, iron acquisition and by the host immune system. Several oxidative stress response proteins have been characterised in *C. jejuni*, including SodB which catalyses the dismutation of superoxide radicals into hydrogen peroxide and oxygen (Pesci et al., 1994), KatA which converts hydrogen peroxide into water and oxygen (Grant and Park, 1995) and AhpC which converts alkyl peroxide into alcohol (Baillon et al., 1999). A *sodB* mutant shows impaired ability to survive hydrogen peroxide, cumene hydroperoxide and menadione whereas *katA* and *ahpC* mutants are more sensitive to hydrogen peroxide and cumene hydroperoxide respectively (Palyada et al., 2009). Furthermore, both *sodB* and *katA* mutants are unable to colonise the chick cecum and the colonisation ability of an *ahpC* mutant is also significantly reduced when compared to the wild-type (Palyada et al., 2009). These observations indicate the direct role of SodB, KatA and AhpC in oxidative stress responses as well as in *C. jejuni* pathogenesis. Other less well characterised oxidative stress defence proteins in *C. jejuni*, include the thioredoxin reductase TrxB, which is predicted to function in AhpC recycling (Stintzi et al., 2008), and FdxA, which in its
absence significantly reduces aerotolerance of *C. jejuni* (van Vliet *et al.*, 2001).

Regulation of oxidative stress response genes in many Gram-negative bacteria is usually mediated by SoxRS and OxyR (Imlay, 2008), both regulators however are absent from the *C. jejuni* genome and instead they are functionally substituted by PerR*<sub>Cj</sub>* (van Vliet *et al.*, 1999; Palyada *et al.*, 2009). The 15.9 kDa PerR*<sub>Cj</sub>* shares 32% and 37% identity with PerR*<sub> Bs</sub>* and Fur*<sub>Cj</sub>* respectively and contains a conserved HTH domain and two metal-binding domains. Unlike fur*<sub>Cj</sub>*, perR*<sub>Cj</sub>* has its own promoter and is transcribed as a monocistronic mRNA (van Vliet *et al.*, 1999). A protein profiling analysis of the perR*<sub>Cj</sub>* mutant shows derepressed expression of both KatA and AhpC in levels much higher when compared with the wild-type and in addition, the iron-responsive repression of KatA is only fully abolished in the perR*<sub>Cj</sub>* fur*<sub>Cj</sub>* double mutant indicating co-regulation of katA by PerR*<sub>Cj</sub>* and Fur*<sub>Cj</sub>* (van Vliet *et al.*, 1999). A perR*<sub>Cj</sub>* mutant exhibits reduced motility but is more resistant to hydrogen peroxide, cumene hydroperoxide and menadione (van Vliet *et al.*, 1999; Palyada *et al.*, 2009). The perR*<sub>Cj</sub>* mutant also shows attenuated colonisation of the chick cecum and the colonisation ability is abolished in the perR*<sub>Cj</sub>* fur*<sub>Cj</sub>* double mutant demonstrating the importance of oxidative stress regulation by PerR*<sub>Cj</sub>* in vivo (Palyada *et al.*, 2009). Putative PerR*<sub>Cj</sub>* boxes have been identified for the katA and ahpC promoters, however due to the lack of experimental support and the potential overlap between the PerR*<sub>Cj</sub>* and Fur*<sub>Cj</sub>* boxes, the PerR*<sub>Cj</sub>* box remains poorly characterised (van Vliet *et al.*, 2002).
In a recent transcriptome comparison between wild-type and a \( \textit{perR}_{Cj} \) mutant strain in response to iron availability and exposure of hydrogen peroxide, cumene hydroperoxide and menadione, 104 genes were identified to belong to the PerR\(_{Cj} \) regulon. Those PerR\(_{Cj} \) regulated genes found were associated with a variety of biological functions ranging from oxidative stress defence, iron acquisition and flagellar and fatty acid biosynthesis illustrating the regulatory role of PerR\(_{Cj} \) beyond its classic function in controlling oxidative stress response genes (Palyada \textit{et al.}, 2009). Within 82 iron- and PerR\(_{Cj} \)-repressed genes identified in this study, the transcript levels of oxidative stress genes such as \( \textit{katA} \) and \( \textit{ahpC} \) and iron storage genes \( \textit{cft} \) and \( \textit{dps} \) were increased in response to one of the three oxidants, whereas the expression of 11 flagellar biosynthesis genes were found to be nonresponsive to oxidant exposures. In addition, many previously identified Fur\(_{Cj} \)-regulated iron acquisition genes (Palyada \textit{et al.}, 2004; Holmes \textit{et al.}, 2005) were also PerR\(_{Cj} \) repressed and down-regulated in response to iron-limitation and oxidant exposure indicating a tight link between iron homeostasis and oxidative stress (Palyada \textit{et al.}, 2009). The regulatory potential of PerR\(_{Cj} \) as a transcriptional activator was also observed in this study with or without the presence of iron. The level of \( \textit{sodB} \) transcript for instance was PerR\(_{Cj} \)-activated under the conditions of iron restriction, whereas the expression of \( \textit{perR}_{Cj} \) itself was auto-activated by PerR\(_{Cj} \) in the presence of iron (Palyada \textit{et al.}, 2009). However like in the case of Fur\(_{Cj} \), the exact mechanism of direct or indirect transcriptional activation by PerR\(_{Cj} \) is currently unknown.
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1.5 Research aims

Recognition of the Fur box by the Fur protein and the subsequent protein-DNA interaction are key steps in Fur-mediated gene regulation (Carpenter et al., 2009b). Despite recent advances in the understanding of the global regulatory role of FurCj in controlling iron homeostasis and other cellular processes (Palyada et al., 2004; Holmes et al., 2005), the mechanism of FurCj-FurCj box interaction and the FurCj box itself (van Vliet et al., 2002; Palyada et al., 2004) are poorly studied when compared with other bacteria such as E. coli and are proposed to be different from E. coli as seen by the Fur and Fur box sequence variations between the two bacteria (Wooldridge et al., 1998; Palyada et al., 2004). The primary aim of this research was therefore to determine the functionally important bases in the FurCj box that are essential for FurCj-FurCj box interaction by mutational analysis of FurCj boxes from differently FurCj-regulated promoters. The outcome of such an investigation will allow a better understanding of the basis of sequence-specific recognition of the FurCj box by FurCj and provide a model of FurCj-FurCj box interaction as well as an experimental verification of the proposed FurCj box consensus sequence.

In addition, although Fur has long been considered as a transcriptional repressor, transcriptional activation by Fur or by a Fur-regulated sRNA intermediate(s) have been characterised in many bacteria (Carpenter et al., 2009b). Iron-induced and FurCj-activated genes such as fumC have also been identified in C. jejuni (Holmes et al., 2005), however without the detailed characterisation of FurCj-regulated sRNA
intermediate or evidence of direct binding of Fur$_{Cj}$ to the $fumC$ promoter ($pfumC$, Li, 2005), the involvement of Fur$_{Cj}$ in iron-induced regulation of $fumC$ is unclear. The second research aim was therefore to verify the iron- and Fur$_{Cj}$-responsive regulation of $fumC$ and to determine any additional regulators or stimuli that are potentially involved, either directly or indirectly, in controlling $fumC$ expression. The research outcome will reveal any additional regulatory potential of Fur$_{Cj}$ and the functional interplay between Fur$_{Cj}$ and other regulators in iron-responsive gene regulation in $C. jejuni$. 
Chapter 2: Material and methods

2.1 Microbial analysis

2.1.1 Bacterial growth media, antibiotics and supplements

All media were purchased from Oxoid and sterilised before use in an Omega™ (Prestige Medical) autoclave at 121 °C, under 1.5 bar pressure for 15 minutes. Luria-Bertani (LB) broth was prepared with 1% (weight/volume, w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. LB agar was prepared by the addition of 1.5% (w/v) agar to LB broth. Mueller-Hinton (MH) broth was prepared with 30% (w/v) beef dehydrated infusion, 1.75% (w/v) casein hydrolysate and 0.15% (w/v) starch. MH agar was prepared by the addition of 1.7% (w/v) agar to MH broth.

All chemicals were purchased from Fisher Scientific and all reagents were dissolved in distilled water (dH₂O) unless specified otherwise. Antibiotics and supplements were added to media for selective and supportive microbial growth and their concentrations were: 100 μg/ml ampicillin (Amp) sodium salt (Melford), 20 μg/ml chloramphenicol (Cm, Sigma-Aldrich, dissolved in 100% ethanol), 50 μg/ml kanamycin (Kan) monosulphate (Melford), 5 μg/ml trimethoprim (Tri, Sigma-Aldrich, dissolved in 50% ethanol), 10 μg/ml vancomycin (Van, Duchefa Biochemie), 6% (volume/volume, v/v) pre-warmed defibrinated house blood (Oxoid), 20 μM deferoxamine mesylate salt (Desferal, Sigma-Aldrich,), 40 μM iron (II) sulphate heptahydrate (FeSO₄, Sigma-Aldrich), 4 mM malic acid (Malate, Sigma-Aldrich), 0.2 mM IPTG (Melford) and 40 μg/ml 5-bromo-4- chloro-3-indoyl-β-D-galactoside [X-gal (Melford), dissolved
in dimethylformamide (DMF). Antibiotics and supplements were filter sterilised with a Plastipak™ syringe (BD) and an Acrodisc® 2.5 mm syringe filter (Pall) and stored at 4 °C. Antibiotics and supplements were added to room temperature broth before microbial inoculation. Molten agar was cooled to 55 °C before supplementation with antibiotics and supplements and poured into Petri dishes (Sterilin) prior to storage at 4 °C for up to a month.

2.1.2 Bacterial strains used

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α™</td>
<td>F φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(ek− mK+) phoA supE44 thi-1 gyrA96 relA1 λ−</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Rosetta™(DE3) pLysS</td>
<td>F ompT hsdS8(rB− mB+) gal dcm (DE3) pLysSRARE (CmR)</td>
<td>Novagen</td>
</tr>
<tr>
<td>Top10</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacFΔ ZΔM15 Tn10 (TetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>Wild-type clinical isolate</td>
<td>National Collection of Type Culture, Colindal, London</td>
</tr>
<tr>
<td>480 (NCTC 12744)</td>
<td>Clinical isolate for maintaining shuttle vector pMW10</td>
<td>King et al., 1991</td>
</tr>
<tr>
<td>81-176</td>
<td>Clinical isolate</td>
<td>Korrath et al., 1985</td>
</tr>
<tr>
<td>81116 (NCTC 11828)</td>
<td>Clinical isolate</td>
<td>Manning et al., 2001</td>
</tr>
<tr>
<td>AB3</td>
<td>NCTC 11168 ΔracR::aphA-3 (KanR)</td>
<td>A. M. Brás</td>
</tr>
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<td>AV17</td>
<td>NCTC 11168 Δfur::aphA-3 (KanR)</td>
<td>van Vliet et al., 1998</td>
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<td>AV41</td>
<td>NCTC 11168 Δfur::cat (CmR)</td>
<td>van Vliet et al., 1998</td>
</tr>
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<td>AV63</td>
<td>NCTC 11168 ΔperR::aphA-3 (KanR)</td>
<td>van Vliet et al., 1999</td>
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<td>AV67</td>
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<td>van Vliet et al., 1999</td>
</tr>
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<td>RR1</td>
<td>480 Δfur::aphA-3 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>RR2</td>
<td>NCTC 11168 ΔfumC::aphA-3 (KanR)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2.1: Bacterial stains used in this study.
2.1.3 Bacterial growth conditions

*E. coli* strains were cultured aerobically at 37 °C in LB media for 16 hours (overnight). A liquid culture was set up in a 5 ml volume in a 30 ml universal tube (Sterilin) and incubated with shaking on a G10 Gyrotory® shaker (New Brunswick Scientific) at 200 revolutions per minute (rpm), whereas for plate culture, cells were spread or streaked onto an agar plate. LB media were supplemented with appropriate antibiotics for selection of a desired recombinant plasmids and LB agar was also supplemented with IPTG and X-gal for blue/white screening.

*C. jejuni* strains were cultured at 42 °C in MH media in a variable atmosphere incubator (VAIN, Don Whitely Scientific Limited), which maintains an atmosphere of 84% nitrogen, 10% carbon dioxide and 6% oxygen. Each liquid culture was set up in a 5 ml volume in a 15 ml centrifuge tube (Corning) with a loosened cap and incubated with shaking on an Orbit 300 (Labnet) shaker at 50 rpm for up to 16 hours. To achieve large scale liquid growth, 50 ml cultures were set up in 250 ml Cellstar® cell culture flasks (Greiner Bio-One) and incubated with shaking at 70 rpm. For plate culture, cells were spread on agar plates with a Eurotubo® collection swab (Beltalab) and incubated for up to a week. MH media were supplemented with Van and Tri to reduce the chance of contamination and other appropriate antibiotics for mutant selection. Media supplemented with FeSO₄ or Desferal were used to achieve high or low iron growth conditions respectively and malate was supplemented to promote growth of RR2. MH
agar supplemented with defibrinated horse blood was used to recover poorly growing mutants.

2.1.4 Strain maintenance and recovery

For long-term storage, *E. coli* overnight liquid cultures or *C. jejuni* harvested from plate cultures using MH broth were mixed with an equal amount of 25% (v/v) glycerol in a 1.5 ml screw cap tube (Sarstedt) and stored at -80 °C. For *E. coli* short-term storage, liquid and plate cultures were stored at 4 °C for up to a month. For *C. jejuni*, short-term storage was not possible and strains were maintained by sub-culture onto fresh media and incubated for up to a week.

*E. coli* strains were recovered from glycerol stocks by streaking onto LB agar plates and single colonies obtained were used for subsequent liquid inoculations. *C. jejuni* strains were recovered from glycerol stocks by swabbing onto MH agar plates and incubating for 2 days prior to sub-culture onto fresh plates and incubating for further 2 days. The cells were harvested and used for subsequent liquid inoculations or assays.

2.1.5 Preparation of electrocompetent *E. coli*

LB broth (200 ml) was inoculated with 2 ml of overnight *E. coli* liquid culture and was incubated at 37 °C with shaking until the optical density at 600 nm (OD$_{600}$) reached 0.5. The OD was measured in an Ultrospec 10 cell density spectrometer (Amersham Bioscience) using 1.5 ml semi-micro disposable cuvettes (Kartell). The cells were
chilled on ice for 30 minutes and pelleted by centrifugation at 3220 x g at 4°C for 15 minutes in a centrifuge 5810 R (Eppendorf). The cell pellet was then washed with 200 ml of ice-cold dH2O followed by subsequent washes with 100 ml (once) and 50 ml (twice) of ice-cold dH2O. The cell pellet was resuspended in 2 ml of ice-cold 10% (v/v) glycerol and aliquoted into 40 μl volumes in ice-cold microcentrifuge tubes (Eppendorf). The aliquots were flash frozen on dry ice and stored at -80 °C.

2.1.6 Preparation of electrocompetent C. jejuni

*C. jejuni* plate cultures were harvested with 10 ml of ice-cold *Campylobacter* electroporation buffer [CEB, 272 mM sucrose, 15% (v/v) glycerol and autoclaved] and centrifuged at 3220 x g at 4 °C for 20 minutes. The cell pellet was then washed twice with 10 ml of ice-cold CEB and resuspended in 1 ml of ice-cold CEB prior to aliquoting into 50 μl volumes in ice-cold microcentrifuge tubes. The aliquots were flash frozen on dry ice and stored at -80 °C.

2.1.7 C. jejuni growth assay

*C. jejuni* plate cultures were harvested and used to inoculate 5 ml of MH broth containing appropriate antibiotics to an OD$_{600}$ of 0.1. The culture was incubated overnight and was then used to inoculate three 5 ml volumes of MH broth (triplicate samplings) containing appropriate antibiotics and 40 μM FeSO$_4$ or 20 μM Desferal to an OD$_{600}$ of 0.025. The cultures were incubated and the OD$_{600}$ of each culture was measured every 5 hours for up to a total of 30 hours.
2.2 DNA analysis

2.2.1 Extraction and purification of *C. jejuni* chromosomal DNA

*C. jejuni* plate cultures were harvested with 10 ml of MH broth and centrifuged at 3220 x g for 20 minutes. The cells were lysed in 600 μl of buffer 1 [40 mM tris-acetate (TA) pH 7.8, 20 mM sodium acetate, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1% (w/v) sodium dodecyl sulphate (SDS)] and neutralised with the addition of 200 μl of 5 M NaCl. The cell lysate was centrifuged at 15700 x g for 5 minutes in a centrifuge 5415 D (Eppendorf) and the supernatant was thoroughly mixed with 600 μl of chloroform/iso-amyl alcohol (24:1 v/v) in a fresh microcentrifuge tube by inverting the tube 100 times. The mixture was centrifuged at 15700 x g for 1 minute and the aqueous layer containing the DNA was transferred to a fresh microcentrifuge tube. The chloroform/iso-amyl alcohol extraction step was repeated at least twice. The aqueous layer was then transferred to a microcentrifuge tube containing 1 ml of 100% ethanol and thoroughly mixed to allow precipitation of the chromosomal DNA. The precipitated DNA was transferred to a microcentrifuge tube containing 800 μl of 70% (v/v) ethanol and pelleted at 15700 x g for 2 minutes. The ethanol was removed by aspiration and the DNA was hydrated in 200 μl of dH₂O at 4 °C overnight.

2.2.2 *C. jejuni* natural transformation

*C. jejuni* natural transformation was carried out using a biphasic system (Wang and Taylor, 1990). *C. jejuni* plate cultures were harvested with MH broth and the cell
concentration was adjusted to an OD<sub>600</sub> of 0.5 with MH broth. 0.5 ml of the cell suspension was added to a 15 ml centrifuge tube containing 1 ml of solidified MH agar and incubated for 5 hours. 1 to 5 μg of <i>C. jejuni</i> chromosomal DNA was then mixed with the cell culture and incubated for 4 hours prior to plating onto MH agar containing appropriate antibiotics and incubated for up to a week.

2.2.3 Extraction and purification of plasmid DNA

Plasmid DNA from 5 ml of <i>E. coli</i> or <i>C. jejuni</i> overnight cultures were extracted and purified using the E.Z.N.A.® plasmid mini kit I (Omega Bio-Tek) in accordance with the manufacturer’s instructions. <i>E. coli</i>-derived plasmids were routinely eluted in 50 μl of dH<sub>2</sub>O and 30 μl for <i>C. jejuni</i>-derived plasmids and were stored at -20 °C.

2.2.4 Plasmids used

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Description</th>
<th>Source/Reference</th>
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<tr>
<td>p23E5</td>
<td>&lt;i&gt;C. jejuni&lt;/i&gt; 129108 pmetK cloned into the BglII site of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Wösten et al., 1998</td>
</tr>
<tr>
<td>pAV32</td>
<td>&lt;i&gt;C. coli&lt;/i&gt; aphA-3 cloned into the BclI site of fur in pAV25 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>van Vliet et al., 1998</td>
</tr>
<tr>
<td>pAV35</td>
<td>&lt;i&gt;C. coli&lt;/i&gt; cat cloned into pBluescript (Amp&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>van Vliet et al., 1998</td>
</tr>
<tr>
<td>pAV80</td>
<td>cat cloned into the BglII site of fur in pAV57 (Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>van Vliet et al., 1998</td>
</tr>
<tr>
<td>pGEMCWH01</td>
<td>NCTC 11168 cj0752 with internal multi-cloning sites (MCSs) cloned into pGEM&lt;sup&gt;®&lt;/sup&gt;-T (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Elvers et al., 2005</td>
</tr>
<tr>
<td>pGEM&lt;sup&gt;®&lt;/sup&gt;-T Easy</td>
<td>General cloning vector (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Promega</td>
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<tr>
<td>pJDR13</td>
<td>NCTC 11168 chuA promoter (pchuA, genome position 1540370-1540988) cloned into the BamHI site of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Ridley et al., 2006</td>
</tr>
<tr>
<td>pJMck1</td>
<td>NCTC 11168 fur cloned into the BsaI site of pASK-IBA7 (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Holmes et al., 2005</td>
</tr>
<tr>
<td>pJMck6</td>
<td>NCTC 11168 pchuA (genome position 1540722-1540995) cloned into the EcoRI site of pUC19 (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>J. McNicholl-Kennedy</td>
</tr>
<tr>
<td>Construct</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>pJMCK30</td>
<td><em>C. coli</em> <em>aphA</em>-3 cloned into pUC19 (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>van Vliet <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>pLEICES-01</td>
<td>Protein expression vector containing N-His&lt;sub&gt;6&lt;/sub&gt; tag (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Protex, University of Leicester</td>
</tr>
<tr>
<td>pMW10</td>
<td><em>E. coli-C. jejuni</em> shuttle <em>lacZ</em> reporter vector (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Wösten <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>pRRE</td>
<td><em>ermC</em> cloned into pRR [([Karlyshev and Wren, 2005], Amp&lt;sup&gt;R&lt;/sup&gt; Ery&lt;sup&gt;R&lt;/sup&gt;] O. Bridle</td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>General cloning vector (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>pYL1</td>
<td>NCTC 11168 <em>pfumC</em> (genome position 1297511-1297743) cloned between <em>BamHI</em> and <em>EcoRI</em> sites of pCR&lt;sup&gt;R&lt;/sup&gt;®2.1-TOPO&lt;sup&gt;R&lt;/sup&gt; (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Li, 2005</td>
</tr>
<tr>
<td>pRR1</td>
<td>pJMck6 containing the 10&lt;sup&gt;th&lt;/sup&gt; base mutation of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR2</td>
<td>pJMck6 containing the 13&lt;sup&gt;th&lt;/sup&gt; base mutation of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR3</td>
<td>pJMck6 containing the 19&lt;sup&gt;th&lt;/sup&gt; base mutation of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pRR4</td>
<td>pJMck6 containing 10&lt;sup&gt;th&lt;/sup&gt; and 13&lt;sup&gt;th&lt;/sup&gt; base mutations of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR5</td>
<td>pJMck6 containing 10&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR6</td>
<td>pJMck6 containing 13&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR7</td>
<td>pJMck6 containing 10&lt;sup&gt;th&lt;/sup&gt; 13&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the chuA Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR8</td>
<td>pYL1 containing the 10&lt;sup&gt;th&lt;/sup&gt; base mutation of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR9</td>
<td>pYL1 containing the 13&lt;sup&gt;th&lt;/sup&gt; base mutation of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR10</td>
<td>pYL1 containing the 19&lt;sup&gt;th&lt;/sup&gt; base mutation of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR11</td>
<td>pYL1 containing 10&lt;sup&gt;th&lt;/sup&gt; and 13&lt;sup&gt;th&lt;/sup&gt; base mutations of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR12</td>
<td>pYL1 containing 10&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pRR13</td>
<td>pYL1 containing 13&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR14</td>
<td>pYL1 containing 10&lt;sup&gt;th&lt;/sup&gt; and 13&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the fumC Fur box (Amp&lt;sup&gt;R&lt;/sup&gt; Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<td>pRR15</td>
<td>pchuA amplified from pJMck6 cloned between <em>BamHI</em> and <em>XbaI</em> sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>pRR16</td>
<td>pchuA from amplified pRR1 cloned between <em>BamHI</em> and <em>XbaI</em> sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
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<td>pRR17</td>
<td>pchuA amplified from pRR2 cloned between <em>BamHI</em> and <em>XbaI</em> sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR18</td>
<td>pchuA amplified from pRR3 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
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<tr>
<td>pRR19</td>
<td>pchuA amplified from pRR4 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR20</td>
<td>pchuA amplified from pRR5 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR21</td>
<td>pchuA amplified from pRR6 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR22</td>
<td>pchuA amplified from pRR7 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR23</td>
<td>pfumC amplified from pYL1 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR24</td>
<td>pfumC amplified from pRR8 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
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</tr>
<tr>
<td>pRR25</td>
<td>pfumC amplified from pRR9 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR26</td>
<td>pfumC amplified from pRR10 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
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<tr>
<td>pRR27</td>
<td>pfumC amplified from pRR11 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
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<td>pRR28</td>
<td>pfumC amplified from pRR12 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
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</tr>
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<td>pRR29</td>
<td>pfumC amplified from pRR13 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR30</td>
<td>pfumC amplified from pRR14 cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
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<tr>
<td>pRR31</td>
<td>pchuA from pJMck6 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR32</td>
<td>pchuA from pRR1 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR33</td>
<td>pchuA from pRR2 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
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<tr>
<td>pRR34</td>
<td>pchuA from pRR3 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
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<tr>
<td>pRR35</td>
<td>pchuA from pRR4 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
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<td>pRR36</td>
<td>pchuA from pRR5 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (KanR)</td>
<td>This study</td>
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<tr>
<td>pRR37</td>
<td>pchuA from pRR6 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (Kan^R)</td>
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<tr>
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</tr>
<tr>
<td>pRR38</td>
<td>pchuA from pRR7 extended by 61 bp upstream and cloned between BamHI and XbaI sites of pMW10 (Kan^R)</td>
<td>This study</td>
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<tr>
<td>pRR39</td>
<td>PolyG-tailed NCTC 11168 chuA cDNA cloned between BamHI and XbaI sites of pUC19 (Amp^R)</td>
<td>This study</td>
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<tr>
<td>pRR40</td>
<td>PolyG-tailed NCTC 11168 waaC cDNA cloned between BamHI and XbaI sites of pUC19 (Amp^R)</td>
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<tr>
<td>pRR41</td>
<td>cat and partial fur amplified from pAV80 and cloned between SalI and BamHI sites of pUC19 (Amp^R Cm^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR42</td>
<td>pfumC from pYL1 extended by 217 bp upstream and cloned between BamHI and XbaI sites of pMW10 (Kan^R)</td>
<td>This study</td>
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<tr>
<td>pRR43</td>
<td>5’ oligonucleotide tagged NCTC 11168 chuA cDNA TA cloned into pGEM®-T Easy (Amp^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR44</td>
<td>5’ oligonucleotide tagged NCTC 11168 fumC cDNA 1 TA cloned into pGEM®-T Easy (Amp^R)</td>
<td>This study</td>
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<tr>
<td>pRR45</td>
<td>5’ oligonucleotide tagged NCTC 11168 fumC cDNA 2 TA cloned into pGEM®-T Easy (Amp^R)</td>
<td>This study</td>
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<tr>
<td>pRR46</td>
<td>NCTC 11168 racR (genome position 1191788-1192459) cloned into pLEICES-01 by recombination (Amp^R)</td>
<td>This study (by Protex, University of Leicester)</td>
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<td>pRR47</td>
<td>NCTC 11168 pchuA (genome position 1540661-1540995) cloned between BamHI and XbaI sites of pUC19 (Amp^R)</td>
<td>This study</td>
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<tr>
<td>pRR48</td>
<td>pRR47 containing the 1st base A to C mutation of the chuA Fur box (Amp^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR49</td>
<td>335 bp pchuA amplified from pRR48 cloned between BamHI and XbaI sites of pMW10 (Kan^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR50</td>
<td>274 bp pchuA amplified from pRR48 cloned between BamHI and XbaI sites of pMW10 (Kan^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR51</td>
<td>NCTC 11168 fumC and flank regions (genome position 1295731-1298155) cloned between BamHI and XbaI sites of pUC19 (Amp^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR52</td>
<td>1067 bp internal deletion of fumC in pRR51 by insertion of aphA-3 into the BglII site created by inverse PCR (Amp^R Kan^R)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR53</td>
<td>NCTC 11168 racS histidine kinases domain (racS-HK genome position 1192948-1193691) cloned into pLEICES-01 by recombination (Amp^R)</td>
<td>This study (by Protex, University of Leicester)</td>
</tr>
<tr>
<td>pRR54</td>
<td>pRR47 containing the 1st base A to T mutation of the chuA Fur box (Amp^R)</td>
<td>This study (by T.</td>
</tr>
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</table>
### Table 2.2: Plasmids used in this study.

All pRR plasmids were maintained in *E. coli* Top 10 with the exception of pRR46 and pRR53 which were maintained in *E. coli* Rosetta™(DE3) pLysS. All pMW10 based plasmids were also maintained in *C. jejuni* 480. pJMcK1 was maintained in *E. coli* XL1-Blue and all other plasmids were maintained in *E. coli* DH5™.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRR55</td>
<td>335 bp <em>chuA</em> amplified from pRR54 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR56</td>
<td>274 bp <em>chuA</em> amplified from pRR54 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR57</td>
<td>pRR47 containing the 7&lt;sup&gt;th&lt;/sup&gt; base mutation of the <em>chuA</em> Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR58</td>
<td>335 bp <em>chuA</em> amplified from pRR57 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR59</td>
<td>274 bp <em>chuA</em> amplified from pRR57 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR60</td>
<td>pRR47 containing 1&lt;sup&gt;st&lt;/sup&gt; and 7&lt;sup&gt;th&lt;/sup&gt; base mutations of the <em>chuA</em> Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR61</td>
<td>335 bp <em>chuA</em> amplified from pRR60 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR62</td>
<td>274 bp <em>chuA</em> amplified from pRR60 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR63</td>
<td>pRR47 containing 1&lt;sup&gt;st&lt;/sup&gt;, 7&lt;sup&gt;th&lt;/sup&gt;, 13&lt;sup&gt;th&lt;/sup&gt; and 19&lt;sup&gt;th&lt;/sup&gt; base mutations of the <em>chuA</em> Fur box (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR64</td>
<td>335 bp <em>chuA</em> amplified from pRR63 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR65</td>
<td>274 bp <em>chuA</em> amplified from pRR63 cloned between <em>BamH</em>I and <em>Xba</em>I sites of pMW10 (Kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study (by T. Caudle)</td>
</tr>
<tr>
<td>pRR66</td>
<td>NCTC 11168 <em>fumC</em> and flank regions (genome position 1296244-1297960) cloned into the <em>Kpn</em>I site of pGEMCWH01 (Amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pRR67</td>
<td><em>cat</em> cloned into the <em>BamH</em>I site of pRR66 (Amp&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 2.2.5 Nucleic acid quantification

DNA and RNA concentrations were quantified by Nanodrop® ND-1000 spectrophotometer (Thermo Scientific) which converts 260 nm absorbance into ng/μl.
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2.2.6 Transformation of electrocompetent E. coli

Plasmid DNA (0.5 μg) purified from a bacterial culture (2.2.3) or a whole ligation reaction (2.2.17) was routinely introduced into E. coli by electroporation. Plasmid DNA was dialysed against dH₂O for 30 minutes on a 0.025 μm MF™ membrane filter (Millipore) prior to electroporation to remove any traces of salt. Desalted DNA was then mixed with 40 μl of thawed electrocompetent E. coli cells (2.1.7) in a pre-cooled 2 mm electroporation cuvette (Cell Projects) and a pulse of 2.5 kV was applied through the cuvette using a Gene Pulser™ (Bio-Rad) electroporator set at 200 Ω and 125 μFD. The cells were recovered by the addition of 1 ml of LB broth and incubated for an hour. The cells were then plated onto LB agar plates with appropriate antibiotic selection, 0.2 mM IPTG and 40 μg/ml X-gal and incubated overnight.

2.2.7 Transformation of electrocompetent C. jejuni

Desalted plasmid DNA (5 μg) was electroporated into 50 μl of electrocompetent C. jejuni cells (2.1.8) in the same manner as for E. coli (2.2.6). The cells were recovered by the addition of 100 μl of MH broth and incubated on MH agar overnight. The cells were then sub-cultured onto MH agar plates with appropriate antibiotic selection and supplements, and incubated for up to a week.

2.2.8 DNA purification by ethanol precipitation

DNA was mixed thoroughly with 0.1 volumes of 3 M sodium acetate, 1 μl of glycogen (Roche) and 2 volumes of 100% ethanol and incubated at -80 °C or in an ethanol/dry ice
bath for 30 minutes. The DNA was pelleted at 15700 x g for 30 minutes and was washed twice with 70% (v/v) ethanol. The ethanol was removed by aspiration and the DNA was hydrated in an appropriate amount of dH₂O.

2.2.9 DNA purification by phenol/chloroform extraction

Equal amounts of DNA sample and phenol/chloroform/iso-amyl alcohol (25:24:1) were mixed thoroughly in a Phase Lock Gel™ tube (Eppendorf) by shaking vigorously for 30 seconds. The mixture was centrifuged at 15700 x g for 15 minutes and the aqueous layer was transferred to a fresh microcentrifuge tube prior to purification by ethanol precipitation (2.2.8). For DNA samples contaminated with small amounts of protein, chloroform/iso-amyl alcohol (24:1) was used instead of phenol/chloroform/iso-amyl alcohol.

2.2.10 Agarose gel electrophoresis

DNA samples were visually analysed by electrophoresis on 1% (w/v) agarose gels which were prepared by dissolving Seakem® LE agarose (Lonza) in 1x tris-acetate-EDTA (TAE, 40 mM TA and 1 mM EDTA, pH 7.8) at 55 °C. Ethidium bromide (0.5 μg/ml) was added to pre-cooled molten agarose and the agarose was poured into a casting tray. DNA samples were mixed with 6x DNA loading buffer [50x TAE pH 7.8, 15% (v/v) glycerol and 0.3% (w/v) Orange G (Sigma-Aldrich)] and were loaded into the wells on the gel (formed using appropriate size combs) along with 5 μl of DNA size markers [λ DNA HindIII-digest/ΦX174 DNA HaeIII-digest (2.5:1, New
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England Biolabs), 75 mM NaCl and 30% (v/v) 6x DNA loading buffer]. The gel was run at 110 V in 1x TAE for an appropriate amount of time and visualised in a GeneGenius bio imaging system (Syngene).

2.2.11 DNA purification from agarose gel

DNA samples excised from an agarose gel on a Chromato-Vue™ transilluminator (UVP) were purified using the Zymoclean™ gel DNA recovery kit (Zymo Reaserch) in accordance with the manufacturer’s instructions. DNA samples were routinely eluted in 15 μl of dH2O.

2.2.12 Standard polymerase chain reaction (PCR)

Standard PCR was used to amplify a specific DNA fragment or to screen for the presence of a specific gene or plasmid. A full list of reagents and cycle conditions used are listed in Table 2.3 and 2.4 respectively. In general, PCR was routinely carried out in 20 μl or 50 μl reaction volumes in a 0.2 ml Thermo-Tube (Thermo Scientific) and consisted of high fidelity Phusion® DNA polymerase (Finnzymes) and 5x Phusion® HF buffer (Finnzymes) for cloning purpose. KAPA Taq (KAPA Biosystems) DNA polymerase and 10x high fidelity buffer (Eppendorf) were used for other purposes. 10 mM of pre-mixed deoxynucleotide triphosphates [dNTPs, from 100 mM dATP, dTTP, dGTP and dCTP stocks (Promega)] as well as an appropriate primer set (2.2.11) and a template such as linear, plasmid or chromosomal DNA were also included. DNA extracted from bacterial colonies were also used as DNA templates and in each case, a
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Table 2.3: Reagents used in various types and volumes of PCR. Primers used in all the reactions were diluted to 2 pmol with the exception of primers used in 20 μl Phusion® PCR. They were diluted to 10 pmol in order to fit into the 20 μl reaction volume. Plasmids and linear DNA fragments used in Taq PCR were diluted to 1 μg/μl whereas DNA used in Phusion® PCR was diluted to 10 μg/μl. The concentration of chromosomal DNA was not usually measured.

<table>
<thead>
<tr>
<th>Reagents (μl)</th>
<th>20 μl Taq PCR with DNA extracted from a colony</th>
<th>Chromosomal DNA</th>
<th>Plasmid or linear DNA</th>
<th>20 μl Phusion® PCR with Chromosomal DNA</th>
<th>Plasmid or linear DNA</th>
<th>50 μl Phusion® PCR with Chromosomal DNA</th>
<th>Plasmid or linear DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Forward primer</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Template</td>
<td>2</td>
<td>2</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>dH₂O</td>
<td>11.35</td>
<td>13.35</td>
<td>15.1</td>
<td>12.4</td>
<td>12.4</td>
<td>11.5</td>
<td>12.5</td>
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</table>

Table 2.4: Cycle conditions used in various types of PCR. The appropriate annealing temperature for each set of primers was determined by gradient PCR. Gradient PCR was carried out in the same manner as a standard PCR, except the annealing temperature was set to 55 °C and the gradient G value was set to 10 °C. In this way, 12 temperatures ranged from 45 to 65 °C were applied simultaneity. For inverse PCR, the standard PCR principle and conditions were applied except overlapping primers facing outward from each other were used.

<table>
<thead>
<tr>
<th>Cycle name</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>1</td>
<td>30</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle temperature and time for Taq PCR</td>
<td>94 °C, 2 minutes</td>
<td>94 °C, 30 seconds</td>
<td>45-65 °C, 30 seconds</td>
<td>72 °C, 1 minute per 1 kb product</td>
<td>72 °C, 5 minutes</td>
</tr>
<tr>
<td>Cycle temperature and time for Phusion® PCR</td>
<td>98 °C, 30 seconds</td>
<td>98 °C, 10 seconds</td>
<td>45-65 °C, 10 seconds</td>
<td>72 °C, 1 minute per 1 kb product</td>
<td>72 °C, 5 minutes</td>
</tr>
</tbody>
</table>
single colony was boiled in 20 μl of dH₂O at 96 °C for 5 minutes and centrifuged at 15700 x g for 2 minutes prior to using the supernatant in PCR reactions. The amplification step was performed in a GS1 (G-Strom) thermal cycler with an initial denaturation step followed by 30 cycles of denaturation, annealing and extension and finished with a final extension step.

### 2.2.13 Primers used

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence 5’-3’</th>
<th>Primary Target DNA</th>
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<tr>
<td>chuA10F</td>
<td>GAAATTATCATTATTTTTATTTAAG</td>
<td>pJMcK6</td>
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<td>chuA10R</td>
<td>TGATAATTCTATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
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<td>chuA13F</td>
<td>TAAATTATCATTATTTTTATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA13R</td>
<td>TGATAAAATTATATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA19F</td>
<td>TAAATTATCTTTTTATTTTTATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA19R</td>
<td>AGATAAATTATATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA10-13F</td>
<td>GAATTTATCATTATTTTATTTAAG</td>
<td>pJMcK6</td>
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<tr>
<td>chuA10-13R</td>
<td>TGATAAAATTCTATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA10-19F</td>
<td>GAAATTATCTTTTTATTTTTATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA10-19R</td>
<td>AGATAAATTATATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA13-19F</td>
<td>TAATTTATCTTTTTATTTTTATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA13-19R</td>
<td>AGATTTATATATCATATATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA10-13-19F</td>
<td>GAATTTATCTTTTTATTTTTATTTAAG</td>
<td>pJMcK6</td>
</tr>
<tr>
<td>chuA10-13-19R</td>
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<td>pJMcK6</td>
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<td>Gene</td>
<td>Sequence (Strand)</td>
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<tr>
<td><em>chuA</em>1CF</td>
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<tr>
<td><em>chuA</em>1CR</td>
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<td><em>chuA</em>1TF</td>
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<td><em>chuA</em>1TR</td>
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<td><em>chuA</em>7TF</td>
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<td><em>chuA</em>1-7TF</td>
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<td>pRR47</td>
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<tr>
<td><em>chuA</em>5 (BamHI)</td>
<td>ATGCAGATCCTTAGATTAAGTTAAATAGG</td>
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<tr>
<td><em>chuA</em>3 (XbaI)</td>
<td>ATGCCTAGAATTCTTGAATCTTTGCC</td>
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<tr>
<td><em>chuAFUSION5’</em></td>
<td>CTAGATTAAGTTATATTAAAGG</td>
<td>pJMcK6, pRR1-7</td>
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<td><em>chuANEW5</em>(BamHI)</td>
<td>ATGCAGATCAATTATCAATTAAATGC</td>
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<td><em>chuAFUSION3’</em></td>
<td>CTTATGATATAAATTATCATTATTTATTTATTAAG</td>
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<td><em>fumC</em>10F</td>
<td>TTATTTATTTTTTAAATTATA</td>
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<td><em>fumC</em>10R</td>
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<td>pYL1</td>
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<td><em>fumC</em>19F</td>
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<td>pYL1</td>
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<td><em>fumC</em>19R</td>
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<td><em>fumC</em>10-13F</td>
<td>TTTATTTTTTTTAGCTTTATAATAATA</td>
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### Primer extension and rapid amplification of cDNA ends (RACE)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Genome Position</th>
</tr>
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<tbody>
<tr>
<td>fumC10-13R</td>
<td>AAATAATTTTCAAAAAAC GTTTTCCCTAC</td>
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<tr>
<td>fumC10-19F</td>
<td>TTATATTATTAGCTTTATA ATAAAATA</td>
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<td>fumC10-19R</td>
<td>TAATAAAATTCTCTCTCTCTCTCTCTCTAC</td>
<td>pYL1</td>
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<tr>
<td>fumC13-19F</td>
<td>GTAATTATTATTAGCTTTATA ATAAAATA</td>
<td>pYL1</td>
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<tr>
<td>fumC13-19R</td>
<td>TAATAATTACTTCTCTCTCTCTCTCTAC</td>
<td>pYL1</td>
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<tr>
<td>fumC10-13-19F</td>
<td>TTAATTATTATTAGCTTTATA ATAAAATA</td>
<td>pYL1</td>
</tr>
<tr>
<td>fumC10-13-19R</td>
<td>TAATAATTACTTCTCTCTCTCTCTCTAC</td>
<td>pYL1</td>
</tr>
<tr>
<td>fumC5(BamHI)</td>
<td>ATGCCTAGATATCTCTATGATTGAATTTGC</td>
<td>pYL1, pRR8-14</td>
</tr>
<tr>
<td>fumC3(XbaI)</td>
<td>ATGC[CTAGAC]CTTCTGTGGCAT TTTCTCACAACC</td>
<td>pYL1, pRR8-14</td>
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<tr>
<td>fumCFUSION5'</td>
<td>CTCTTTCTCTGTCTCTATGCATGAA</td>
<td>pYL1</td>
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<tr>
<td>fumCNEW5'(BamHI)</td>
<td>CTAAAGATCTTTCAAGAAGCAAGAAGCATATTCAAGC</td>
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<td>fumCFUSION3'</td>
<td>GCAATTCTAGGCATTATAG</td>
<td>NCTC 11168 genome position 1297723-1297743</td>
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<tr>
<td>chuA PE+FAM (fluorescein amidite)</td>
<td>[6FAM]GATCTTTTGCTTTCTATGCTGATTACATTGC</td>
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<td>chuA3'(XbaI) short</td>
<td>GATCTTTTGCTTTCTATGCTGATTACATTGC</td>
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<td>chuA RT</td>
<td>CCGCTTGTCACGCAATTAC</td>
<td>NCTC 11168 genome position 1541077-1541093</td>
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<tr>
<td>chuA nested</td>
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<td>NCTC 11168 genome position 1540970-1540987</td>
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<tr>
<td>fumC PE+FAM</td>
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<td>waaC PE+FAM</td>
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<tr>
<td>BamHI + poly(C)</td>
<td>ATGCCTAGATACCTCCCTCCCTCCCTCCCTCCCTCCCTCC</td>
<td>Poly(G) tailed cDNA</td>
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## Chapter 2: Material and methods

### 5’-adapter-specific DNA oligonucleotide B6

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>RNase oligonucleotide A3 (Wagner and Vogel, 2009)</th>
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<tr>
<td>GCCGGAATTCCTGTAGA</td>
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### fur mutagenesis

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<tr>
<th>Fur3’ BamHI</th>
<th>GTCGAGATCCATCAAGGCTTGCTGTC</th>
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### DNase I footprinting

<table>
<thead>
<tr>
<th>chuA+DIG (digoxigenin)</th>
<th>[DIG]ATGC[GAATTC]ATTTTGTAAGATCTTTGCC</th>
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<tbody>
<tr>
<td>chuA3’ label</td>
<td>GCTTTATTTGATTTGTGTC</td>
<td>pRR15-22</td>
</tr>
<tr>
<td>chuA3’ label long</td>
<td>CTATGCTGATTACATTGCG</td>
<td>pRR15-22</td>
</tr>
<tr>
<td>fumCR+DIG</td>
<td>[DIG]GAATTCCTTTGCGCAATCTCAACACC</td>
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<td>fumC3’ label</td>
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<td>pMW105’ non-label</td>
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### Probe amplification for northern blot

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<tbody>
<tr>
<td>chuA NB F</td>
<td>AGATATGCGTGGAAGAAGAATTG</td>
<td>1541076-1541093</td>
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<tr>
<td>chuA NB R</td>
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<td>fumC NB F</td>
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<td>fumC NB R</td>
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<td>1296731-1296752</td>
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<tr>
<td>chuZ3’</td>
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<td>racRF</td>
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<td>1191905-1191923</td>
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<td>racRR</td>
<td>CGACTTACAGGCTGTTC</td>
<td>1192302-1192320</td>
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### RacR and RacS expression and purification

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<td>N-ter racR</td>
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<tr>
<td>C-ter racR</td>
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<td>N-ter racS</td>
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<tr>
<td>C-ter racS</td>
<td>TACCTCAATCCATGAGAAGACAAGTTGCGGAAG</td>
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### aspA gel shift

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<tr>
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<tr>
<td>aspAR</td>
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### fumC mutagenesis and complementation

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<td>NCTC 11168 genome position 1297941-1297960</td>
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<tr>
<td>fumCinvF</td>
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<td>fumCinvR</td>
<td>ATGCCAGTGTAAGCTGCTAA</td>
<td>pRR51</td>
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#### fumCC5(KpnI)

| CTAAAGATCAGGAGAAGGATGATTATAG | NCTC 11168 genome position 1298136-1298155 |

#### fumCC3(KpnI)

| GTTGGGCTAGCTGCTTATC | NCTC 11168 genome position 1296244-1296262 |

### Sequencing and screening

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<th>pUC19 based plasmid</th>
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<td>pUC19 based plasmid</td>
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<tr>
<td>STM invKan-F</td>
<td>GAGAAGCTATATGACCATGATCC</td>
<td>pUC19 based plasmid</td>
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<td>ATATCACGCAATTAACCTTGG</td>
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<td>catR_KpnI</td>
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<td>cat (chloramphenicol resistance cassette)</td>
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<tr>
<td>catInvF</td>
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<td>cat (chloramphenicol resistance cassette)</td>
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<tr>
<td>furR long</td>
<td>ATATCACGCAATTAACCTTGG</td>
<td>cat (chloramphenicol resistance cassette)</td>
</tr>
<tr>
<td>Cj81-176 fur5’</td>
<td>ATATCACGCAATTAACCTTGG</td>
<td>cat (chloramphenicol resistance cassette)</td>
</tr>
<tr>
<td>Cj81-176 Fur3’</td>
<td>ATATCACGCAATTAACCTTGG</td>
<td>cat (chloramphenicol resistance cassette)</td>
</tr>
</tbody>
</table>
Table 2.5: Primers used in this study. All primers were designed based on the genetic information obtained from CampyDB website (http://xbase.bham.ac.uk/campydb/) or commercial plasmid providers using Clone Manager Professional 9 (Scientific and Educational Software). Primers were purchased from Sigma-Aldrich and were diluted to 100 pmol with either dH₂O or for primers used in RNA analysis, diethyl pyrocarbonate (DEPC, Melford) treated H₂O [0.1% (v/v) DEPC resuspended in H₂O, allowed to evaporate overnight and autoclaved] prior to storage at -20 °C. Restriction sites incorporated into primers are boxed.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cj81-176 fur5'</td>
<td>GGTTATGGACAAGGTGGC</td>
<td>81-176 genome position</td>
</tr>
<tr>
<td>Cj81-176 fur3'</td>
<td>CCCTAAAGAGTGCAATCAG</td>
<td>81-176 genome position</td>
</tr>
<tr>
<td>racR5'</td>
<td>GGACACTAGAATGATTAATG</td>
<td>NCTC 11168 genome position</td>
</tr>
<tr>
<td>racR3'</td>
<td>CATCCTATCAGTTTATATCC</td>
<td>NCTC 11168 genome position</td>
</tr>
<tr>
<td>fumC flank F</td>
<td>GTAGTAGAGCAGCCAATTGAAAG</td>
<td>NCTC 11168 genome position</td>
</tr>
<tr>
<td>fumC flank R</td>
<td>GGAACAGAACTTGTATCTAGTC</td>
<td>NCTC 11168 genome position</td>
</tr>
<tr>
<td>cj0752upF</td>
<td>GGAATAATCAAGCCTACAAATC</td>
<td>pGEMCWH01, pRR66-67</td>
</tr>
<tr>
<td>cj0752downR</td>
<td>GTTTTGCGTTGTATTGTGGAATTGAC</td>
<td>pGEMCWH01, pRR66-67</td>
</tr>
</tbody>
</table>

2.2.14 PCR product purification

PCR products (as well as other DNA fragments) were purified using the E.Z.N.A.® cycle pure kit I (Omega Bio-Tek) in accordance with the manufacturer’s instructions. PCR products were routinely eluted into 30 μl of dH₂O. This kit was also used to concentrate DNA samples into smaller volumes.

2.2.15 Automated DNA sequencing

DNA sequence was determined using the BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems) in accordance with the manufacturer’s instructions. The
sequencing reaction was terminated with 0.1 volumes of 2.2% (w/v) SDS at 98 °C for 5 minutes and purified using a Performa® DTR gel filtration cartridge (EdgeBio) in accordance with the manufacturer’s instructions. The sequencing reaction was processed using a 3730 DNA analyser (Applied Biosystems) by the Protein nucleic acid chemistry laboratory (PNACL, University of Leicester) and analysed using Chromas v1.45 (Technelysium Pty Ltd) and Clone Manager Professional 9.

2.2.16 Restriction of DNA

Restriction enzymes were routinely used to allow insertion of DNA fragments into plasmids during the process of constructing recombinant plasmids (cloning). 500 ng of DNA was digested in a 50 μl reaction at 37 °C for 2 hours and the reaction was purified (terminated) by the E.Z.N.A.® cycle pure kit I in accordance with the manufacturer’s instructions. Restriction enzymes and cognate digestion buffers (New England Biolabs) were used in accordance with the manufacturer’s instructions.

2.2.17 Ligation of DNA

Ligation reactions were carried out to join DNA fragments and linearised plasmids with compatible ends in a reaction consisting of 1 μl of 400 U/μl T4 DNA ligase (New England Biolabs), 10x buffer (New England Biolabs) and 1 μl of 10 mM adenosine 5’-triphosphat disodium salt. A 3:1 molecular ratio of insert to vector DNA was usually used and the total reaction volume was kept to a minimum in order to increase ligation efficiency. The reaction was incubated at 16 °C overnight and was purified by dialysis
2.2.18 Standard mutagenesis and complementation

Standard mutagenesis was used to create a *C. jejuni* mutant strain with a specific gene deletion in the genome. A desired gene along with 400 bp flanking DNA on each side of this gene was amplified from the wild-type *C. jejuni* genome using Phusion® DNA polymerase and cloned into pUC19. The majority portion of this gene was deleted by inverse PCR (Table 2.4) and replaced with a desired antibiotic resistance gene in the same orientation as the gene. The finishing construct was finally electroporated into a desired *C. jejuni* strain (2.1.7) and the wild-type copy of this gene in the genome was replaced by the mutated copy by recombination. As well as plasmid based mutagenesis, chromosomal DNA of a *C. jejuni* strain carrying a desired mutation was naturally transformed (2.2.2) into another *C. jejuni* wild-type strain and the wild-type copy of the target gene in the recipient strain was replaced by the mutated donor copy by recombination.

Complementation was used to reverse the effect of mutagenesis by inserting a wild-type copy of the gene into the *cj0752* of the mutant genome (Elvers *et al.*, 2005). The mutagenesis principal was applied in complementation except pGEMCWH01 was used instead of pUC19.

2.2.19 Site-directed mutagenesis
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Site-directed mutagenesis adapted from the QuickChange® site-directed mutagenesis kit (Stratagene) was used to create specific single base mutations. A DNA fragment containing the target base was cloned into a cloning vector (pUC19 or pCR2.1-TOPO) and the construct was then used as the template in a 50 μl inverse PCR (Table 2.4) with Phusion® DNA polymerase and a pair of complementary primers containing the desired mutations (Table 2.5). 25 μl of the reaction was digested with DpnI (2.2.16) to remove the wild-type methylated template and the intact synthesised DNA containing the desired mutation was electroporated into E. coli Top10 (2.1.6). The synthesised DNA was finally circularised in E. coli Top10 and the resulting vector was screened by sequencing (2.2.15).

2.2.20 β-galactosidase assay

β-galactosidase assay was used to detect specific promoter activities in a pMW10 transcriptional lacZ fusion system (Wösten et al., 1998) and the β-galactosidase activity was measured by the conversion of 2-nitrophenyl-β-D-galactopyranoside (ONPG, Melford) to nitrophenol (Miller, 1972). C. jejuni strain 480 harbouring a desired promoter construct was cultured on a MH agar plate with appropriate antibiotics for 2 days and was sub-cultured onto a MH agar plate with appropriate antibiotics and 40 μM FeSO₄ or 20 μM Desferal for 2 more days. The cells were harvested and used to inoculate three 5 ml cultures in MH broth (triplicate samplings) with appropriate antibiotics and 40 μM FeSO₄ or 20 μM Desferal to an OD₆₀₀ of 0.1. The cells were incubated for 5 hours and cooled on ice for 20 minutes prior to centrifugation at 15700
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x g for 20 minutes. The cells were resuspended in 1 ml of phosphate buffered saline (PBS, Oxoid, 1 PBS tablet dissolved in 100 ml of dH2O and autoclaved) and the OD600 was measured prior to transfer of 0.05 ml of the cell suspension to a fresh microcentrifuge tube. The cell suspension was mixed with 450 μl of buffer Z [60 mM Na2HPO4·2H2O, 40 mM NaH2PO4·2H2O, 10 mM KCl, 1 mM MgSO4·7H2O and 0.39% (v/v) β-mercaptoethanol (Sigma-Aldrich)], 8 μl of 0.1% (w/v) SDS and 15 μl of chloroform and vortexed for 30 seconds. The reaction was incubated at 28 °C for 5 minutes and incubated for a further hour in the presence of 250 μl of 4 mg/ml ONPG prior to termination of the reaction by the addition of 250 μl of 1M Na2CO3. The β-galactosidase activity was measured at OD420 in an Ultrospec 200 UV/visible spectrophotometer (Pharmacia Biotech) and converted to Miller units using the equation:

Miller units = 1000 x OD420 / t x v x OD600 where t is the 60 minutes incubation time and v is the starting 0.05 ml cell volume.

2.2.21 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were used instead of agarose gel to achieve better separation and visualisation of smaller or similar sizes DNA fragments. A Mini-Protean® II electrophoresis cell (Bio-Rad) was used to run small gels and all the components were set up in accordance with the manufacturer’s instructions. An 8% (v/v) polyacrylamide gel mix was prepared with 4.1 ml of dH2O, 0.3 ml of 10x tris-boric acid (TB, 0.89 M tris and 0.89 M boric acid, pH 8), 1.6 ml of Ultra Pure ProtoGel® [National Diagnostics, 30% (w/v) acrylamide:0.8% (w/v) bis-acrylamide (37.5:1)], 100 μl of 10% (w/v)
ammonium persulphate (APS, Melford) and 5 μl of N,N,N’,N’-tetramethylethylene diamine (TMEDA, Sigma-Aldrich). The gel mix was loaded into a gel casting assembly and allowed to set. The gel was pre-run in 0.5x TB at 40 V for 15 minutes and DNA samples were electrophoresed through the gel at 80 V for an appropriate amount of time.

2.2.22 Transfer DNA from polyacrylamide gel onto membrane (Southern blot)

DIG-labelled DNA samples separated by PAGE (2.2.21) were routinely transferred onto a 0.45 micron Magna™ nylon transfer membrane (GE Healthcare). Following PAGE, each component of the Trans-Blot™ cell (Bio-Rad) blotting cassette was soaked in 0.5x TB and the cassette was set up as follows: negative side of the cassette, sponge, 2 pieces of 3 mm Chr chromatography paper (Whatman), the gel, the membrane, 2 pieces of 3 mm paper, sponge and the positive side of the cassette. The gel was blotted in 0.5x TB at 40 V for an hour and fixed onto the membrane in an ultraviolet crosslinker (Amersham Bioscience) at 70x10³ μJ/cm².

2.2.23 Detection of DNA by anti-DIG-antibodies

DIG-labelled DNA samples were blotted onto a nylon transfer membrane (2.2.22) and were detected by interaction with anti-DIG-antibody. The membrane was rinsed in 20 ml of washing solution [0.3% (v/v) Tween® 20 (Sigam-Aldrich) resuspended in maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5 and autoclaved)] and blocked in 100 ml of blocking solution [1% (w/v) blocking reagent (Roche) dissolved in maleic
acid buffer and autoclaved] for 30 minutes. The membrane was incubated with 20 ml of antibody solution [0.005% (v/v) anti-DIG-alkaline phosphatase antibody (Roche) resuspended in blocking solution] for 30 minutes and washed twice with 100 ml of washing solution for 15 minutes. The membrane was then incubated with 20 ml of detection solution (0.1 M tris and 0.1 M NaCl, pH 9.5 and autoclaved) for 5 minutes and incubated with 1 ml of disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-{(5'-chloro)tricycle[3.3.1.13.7]decan}-4-yl) phenyl phosphate (CSPD) working solution [1% (v/v) CSPD resuspended in detection solution] at 37 °C in a hybridisation bag (Roche) for 10 minutes. The membrane was placed into a fresh hybridisation bag and exposed to a Super RX Fuji medical X-ray film (Fujifilm) for an hour prior to developing in a Compact x4 automatic X-ray film processor (Xograph).

2.2.24 Electrophoretic mobility shift assay

EMSA was used to analyse specific protein-DNA interactions (Garner and Revzin, 1986). The desired DNA fragment containing the putative protein interaction site was amplified by PCR (2.2.12) and extracted from agarose gel (2.2.11). 3.85 pmol of DNA was DIG-labelled in a 20 μl reaction in accordance with the DIG gel shift kit, 2nd generation (Roche) instructions and the volume of the reaction was brought up to 25 μl with dH2O (instead of EDTA) to give a final labelled DNA concentration of 155 fmol/μl. The EMSA reaction was carried out in a total volume of 20 μl which consisted of 1 μl of 1.55 fmol labelled DNA, an appropriate amount of recombinant protein (2.4.1 and 2.4.2), 4 μl of 5x binding buffer (20 mM bis-tris, pH 7.6), 2 μl of 20 mM dithiothreitol.
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(DTT), 2 μl of 1 mg/ml bovine serum albumin (BSA), 2 μl of 1 mM MnSO₄ and 2 μl of 3 mg/ml UltraPure™ salmon sperm DNA solution (Invitrogen). The reaction was incubated at room temperature for 10 minutes and then mixed with 5 μl of 5x loading buffer [10x TB and 60% (v/v) glycerol]. The sample was run on a polyacrylamide gel (2.2.21) for 2 hours, blotted onto nylon membrane (2.2.22) and DNA detected by anti-DIG-antibody (2.2.23).

2.2.25 Sequencing PAGE

The Sequi-Gen® GT nucleic acid electrophoresis cell (Bio-Rad) was used to run large sequencing gels and all the components were set up in accordance with the manufacturer’s instructions. 60 ml of 6% (v/v) polyacrylamide gel was prepared using Pur1te select (Ondeo) purified H₂O, 30 g of urea, 7.2 ml of Long Ranger® 50% gel solution (Lonza), 3 ml of 20x glycerol tolerant gel (GTG) buffer (USB), 60 μl of 25% (w/v) APS and 60 μl of TMEDA. The gel mix was injected into a gel casting assembly, of which one side of the inner glass plate was pre-treated with Gel Slick® solution (Cambrex), and the gel was allowed to set overnight. The gel was pre-run in 1x GTG buffer at 90 W for 30 minutes and DNA samples denatured at 100 ºC for 10 minutes were loaded using a shark’s tooth comb (Bio-Rad) and then separated on the gel at 50 W until the dye front reached the end of the gel. The gel was then transferred onto 3 mm paper and vacuum dried in a Model 583 gel dryer (Bio-Rad) for 2 hours. The gel was wrapped in Saran™ film (DOW) and exposed to an X-ray film at -80 ºC for 2 days.
2.2.26 Manual DNA sequencing

Manual sequencing reaction (Sanger et al., 1977) was used in DNase I footprinting (2.2.27) and primer extension (2.3.6) to generate a DNA reference sequence for comparison. The sequencing primer was 5’ labelled with EasyTides® ATP γ-32P (PerkinElmer) and OptiKinase™ (USB) in accordance with the manufacturer’s instructions. The radio-labelled primer was then used to sequence a DNA fragment using the thermo sequenase cycle sequencing kit (USB) in accordance with the manufacturer’s instructions. 1.5 μl of each sequencing sample was denatured at 100 °C for 10 minutes and analysed by sequencing PAGE (2.2.25).

2.2.27 DNase I footprinting assay

DNase I footprinting assay was used to identify the location of protein-DNA interactions (Galas and Schmitz, 1978) and the method used was adapted from Fuangthong and Helmann (2003). DNA template was amplified in a 20 μl PCR reaction (2.2.12) with 1 μl of 2 pmol forward primer and 1 μl of radio-labelled reverse primer. 5 μl of the PCR product was then used in a 20 μl reaction containing an appropriated amount of recombinant protein (2.4.1 and 2.4.2), 4 μl of 5x binding buffer, 2 μl of 20 mM DTT, 2 μl of 1 mg/ml BSA, 2 μl of 1 mM MnSO₄ and 2 μl of 3 mg/ml UltraPure™ salmon sperm DNA solution. The reaction was incubated at room temperature for 10 minutes and mixed with 2 μl of RQ1 RNase-free DNase 10x reaction buffer (Promega) and 10 μl of CaCl₂/MgCl₂ solution (5 mM CaCl₂ and 10 mM MgCl₂). The reaction was digested with 1 μl of 0.025 U/μl RQ1 RNase-free DNase (Promega) at 37 °C for 80
seconds and terminated with 1 μl of RQI DNase stop solution (Promega) at 65 °C for 10 minutes. The reaction was ethanol precipitated (2.2.8) and hydrated in 6 μl of dH2O and 4 μl of stop solution (USB). 2.5 μl of DNase I footprinting sample along with sequencing samples (2.2.26) were denatured at 100 °C for 10 minutes and analysed by sequencing PAGE (2.2.25).

2.3 RNA analysis

2.3.1 Extraction and purification of C. jejuni total RNA

For RNA analysis, screw cap tubes, Biosphere® filter tips (Sarstedt) and solutions made from DEPC H2O were used to avoid RNase contamination. C. jejuni strains were cultured on MH agar plates with appropriate selection for 2 days and were sub-cultured onto MH agar plates with selection for 2 more days. The cells were harvested and used to inoculate 50 ml of MH broth in a cell culture flask with appropriate antibiotics to an OD600 of 0.1. The culture was incubated overnight and harvested. The cells were then used to inoculate 50 ml of MH broth in a cell culture flask with appropriate selection and 40 μM FeSO4 or 20 μM Desferal to an OD600 of 0.1 and incubated for 10 hours. The cells were harvested and resuspended in 1 ml of RNAlater® tissue collection: RNA stabilisation solution (Applied Biosystems). C. jejuni total RNA was extracted and purified using the total RNA purification kit (Norgen Biotek Corporation) in accordance with the manufacturer’s instructions. DNA contamination was removed using the TURBO DNA-free™ kit (Applied Biosystems) in accordance with the manufacturer’s instructions and RNA samples were stored at -80 °C.
2.3.2 **Formaldehyde agarose gel electrophoresis**

For RNA virtualisation, formaldehyde agarose gels adapted from the protocol in the RNeasy® mini kit (Qiagen) handbook were used. 1.8 g of agarose was first heat dissolved in 130.5 ml of DEPC H₂O and mixed with 3 μl of 10 mg/ml ethidium bromide, 4.5 ml of 37% formaldehyde solution (Sigam-Aldrich) and 15 ml of 10x 3-(N-morpholino) propanesulfonic acid (MOPS, 0.2 M MOPS pH 7, 50 mM sodium acetate and 1 mM EDTA) in a 50 ml centrifuge tube (Corning) prior to pouring into an ethanol cleaned casting tray. Each RNA sample was mixed with 5x RNA loading buffer [0.16% (v/v) saturated aqueous bromophenol blue solution, 4 mM EDTA pH 8, 2.7 % (v/v) formaldehyde, 20% (v/v) glycerol, 30.8 % (v/v) deionised formamide and 4x formaldehyde agarose gel buffer (80 mM MOPS, 12.5 mM sodium acetate and 2.5 mM EDTA, pH 7)] and the mixture as well as 10 μl of RNA size markers [40% (v/v) RNA markers 0.5-9 kbp (Lonza) and 40% (v/v) 5x RNA loading buffer] were denatured at 65 °C for 10 minutes. The sample and markers were cooled on ice for 5 minutes and loaded onto the gel prior to run at 120 V in 1x MOPS for an appropriate amount of time.

2.3.3 **Transfer RNA from agarose gel onto membrane (northern blot)**

RNA samples separated in a formaldehyde agarose gel (2.3.2) were routinely transferred onto a nylon transfer membrane by capillary action created in a saline sodium citrate (SSC) solution gradient. Following formaldehyde agarose gel electrophoresis, the blotting assembly was set up as follows: a 3 mm paper wick soaked in 20x SSC (3 M
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NaCl and 300 mM tri-sodium citrate, pH 7) and rested in a tray filled with 20x SSC, the
gel, the membrane soaked in 2x SSC, 2 pieces of 3 mm paper soaked in 2x SSC, stack
of paper towels and a weight. The gel was blotted overnight and fixed onto the
membrane at 70x10³ μJ/cm².

2.3.4 RNA detection by RNA-DNA hybridisation

RNA samples blotted onto a nylon transfer membrane (2.3.3) were detected by RNA
hybridisation with radio-labelled DNA. A membrane containing fixed RNA samples
was first prehybridised for 3 hours in a hybridisation oven (Hybaid) at 65 °C in a
hybridisation bottle (Hybaid) one third filled with Church Gilberst buffer [0.5 M
Na₂HPO₄/NaH₂PO₄ pH 7.4, 1 mM EDTA and 7% (w/v) SDS]. 30 ng of DNA fragment
complementary to the target RNA (DNA probe) was denatured at 100 °C for 5 minutes
and labelled in a 27.5 μl reaction with 5 μl of 5x oligolabelling buffer [250 mM tris pH
8, 25 mM MgCl₂, 5 mM β-mercaptoethanol, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 1
M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 6.6 and 1 mg/ml
hexadeoxyribo nucleotides], 1 μl of 10 mg/ml BSA, 1 μl of 5 U/μl DNA polymerase I
large (Klenow) fragment (New England Biolabs) and 2.5 μl of 10 μCi of EasyTides®
CTP α-³²P (PerkinElmer) at 37 °C for 2 hours. The labeled probe was purified with an
illustra NICK™ column in accordance with the manufacturer’s instructions and
denatured at 100 °C for 10 minutes. The labelled probe was then cooled on ice for 10
minutes and allowed to hybridise with the membrane at 65 °C overnight. The membrane
was rinsed with washing solution [2x SSC and 0.1% (w/v) SDS] and washed three times
for 30 minutes and once for an hour with washing solution. The membrane was wrapped in Saran™ film and exposed to an X-ray film at -80 °C for an appropriate amount of time. Radio-labelled probe was finally stripped away from the membrane with multiple 15-minute 0.1% (w/v) SDS washes at 65 °C in a hybridisation oven/shaker S1 2OH (Stuart Scientific) and the membrane was then re-probed with a radio-labelled control probe. All probes used in this study were approximately 400 bp PCR products amplified from the middle section of desired genes using gene-specific primers (Table 2.5).

2.3.5 Complementary DNA (cDNA) synthesis by reverse transcriptase (RT)

C DNA template was synthesised from total C. jejuni RNA by RT and used in primer extension (2.3.6) and RACE (2.3.7). 6 μg of total RNA was adjusted to a volume of 10 μl with DEPC H2O and denatured at 65 °C for 5 minutes prior to cooling on ice. 4 μl of 5x cDNA synthesis buffer (Invitrogen), 2 μl of 10 mM dNTPs, 1 μl of 0.1 M DTT, 0.5 μl of 20 U/μl RNasin® ribonuclease inhibitor (Promega), 1 μl of 15 U/μl ThermoScript™ RT (Invitrogen), 1 μl of 2 pmol gene specific primer and 0.5 μl of DEPC H2O were added to the RNA sample and the reaction was incubated (in a thermal cycler) at 65 °C for 5 minutes followed by 20 minutes each of incubation at 55 °C, 60 °C and 65 °C, ending with a 5-minute denaturation at 85 °C. Total RNA template was removed from the cDNA product with 1 μl of 5 U/μl RNase H (New England Biolabs) at 37 °C for 20 minutes.
2.3.6 Primer extension

Primer extension was carried out to identify the transcriptional start site (+1 site) of *C. jejuni* genes and the method was adopted from Lloyd *et al.* (2005). cDNA template was synthesised (2.3.5) using 1 μl of 2 pmol FAM-labelled primer and ethanol precipitated (2.2.8). The FAM-labelled cDNA pellet was resuspended in 9.5 μl of deionised formamide (Amresco) and 0.5 μl of Gene Scan™ 500 LIZ™ size standard (Applied Biosystems). The sample was processed using a DNA analyser by PNACL and the exact length of the FAM-labelled cDNA was determined using Peak Scanner™ software v0.1 (Applied Biosystems). The +1 site was deduced using the exact cDNA length and the position where the FAM-labelled primer binds.

Alternatively, 1.5 μl of radio-labelled primer was used to synthesise radio-labelled cDNA (2.3.5) and DNA sequencing samples (2.2.26) and the +1 site was deduced following cDNA and sequencing samples separation by sequencing PAGE (2.2.25).

2.3.7 RACE

RACE was also performed to identify the +1 site of *C. jejuni* gene. The method described by Gerhart *et al.* (2009) was used and this method was originally adapted from Bensing *et al.* (1996). 6 μg of total RNA was adjusted to 43.75 μl with DEPC H₂O and mixed with 5 μl of 10x tobacco acid pyrophosphatase (TAP) digestion buffer (Epicentre Biotechnologies), 0.25 μl of 20 U/μl ribonuclease inhibitor and 1 μl of 10 U/μl TAP (Epicentre Biotechnologies) prior to incubation at 37 °C for 30 minutes. The
reaction was then mixed with 1 μl of 500 pmol RNA oligonucleotide A3 (5'AUAGCGCGAAUUCUAGAAGAAA) and 100 μl of DEPC H2O and purified by phenol/chloroform extraction (2.2.9) prior to resuspension in 14 μl of DEPC H2O. The sample was denatured at 90 °C for 5 minutes and allowed to cool on ice for 5 minutes. 2 μl of 10x RNA ligation buffer (New England Biolabs), 2 μl of dimethyl sulfoxide, 1.8 μl of 20 U/μl T4 RNA ligase 1 (New England Biolabs) and 0.2 μl of 20 U/μl ribonuclease inhibitor were mixed with the sample and the ligation reaction was incubated at 16 °C overnight. 1 μl of 2 pmol gene specific primer was added to the ligation reaction and the volume was adjusted to 150 μl with DEPC H2O. The sample was purified by phenol/chloroform extraction and resuspended in 20 μl of DEPC H2O. 10 μl of the RNA sample was then used in a RT reaction (2.3.5) to generate cDNA template (N.B. gene specific primer was added to each RNA sample before phenol/chloroform extraction rather than in the RT reaction). 2 μl of cDNA was used as the template in a PCR reaction with 25 μl of HotStarTaq master mix (Qiagen), 1 μl of 25 pmol 5'-adapter-specific DNA oligonucleotide B6, 1 μl of 25 pmol gene specific nested primer and 21 μl of dH2O. The PCR reaction was performed with an initial denaturation cycle of 95 °C of 15 minutes, followed by 30 cycles of 40 seconds denaturation at 95 °C, annealing at 58 °C and extension at 72°C and ended with a 10 minutes final extension at 72 °C. The PCR products were analysed by agarose gel electrophoresis (2.2.10) and the desired product was purified from the gel (2.2.11) and eluted in 25 μl of dH2O. The product was finally TA cloned into pGEM®-T Easy and the +1 site was deduced by sequence analysis (2.2.15).
Alternatively, 13 μl of purified cDNA (2.3.5) was used in a G tailing reaction with 2 μl of 10 mM dGTP, 5 μl of 5x terminal transferase buffer (Roche) and 4 μl of 25 mM CoCl$_2$ (Roche). The reaction was denatured at 94 °C for 3 minutes and incubated at 37 °C for 30 minutes with the addition of 1 μl of 40 U/μl terminal transferase (Roche). The reaction was terminated at 70 °C for 10 minutes and purified (2.2.14). PolyG-tailed cDNA was then used in a PCR reaction (see above paragraph) with polyG-tail specific primer and gene specific nested primer. The product was finally cloned into pUC19 and the +1 site was deduced by sequence analysis (2.2.15).

2.4 Protein analysis

2.4.1 Expression and purification of recombinant Fur$_{Cj}$

pASK-IBA7 (IBA) harbouring NCTC 11168 fur$_{Cj}$ (pJMcK1) had been previously constructed (Holmes et al. 2005), which allowed the expression and purification of N-terminal Strep-tagged Fur$_{Cj}$ induced by anhydrotetracycline (AHT). 200 ml of LB broth supplemented with Amp was inoculated with 2 ml of overnight liquid DH5α™ culture carrying pJMcK1 and incubated at 37 °C with shaking until the OD$_{600}$ reached 0.5. The culture was induced with 20 μl of 2 mg/ml AHT (dissolved in DMF) and incubated for 3 more hours at 37 °C. The cells were harvested at 3220 x g at 4 °C for 15 minutes and resuspended in 2 ml of buffer W (100 mM tris pH 8 and 150 mM NaCl) and 10 μl of 100 mg/ml lysozyme prior to incubation at 37 °C for 2 hours. One complete mini EDTA-free protease inhibitor cocktail tablet (Roche) was added to the cell
suspension and the cells were lysed at 4 °C by 10 rounds of 30 seconds sonication in a MSE sonicator (MSE Scientific Instruments) set at 6 amplitude microns and low power. Cell debris was removed by centrifugation at 3220 x g at 4 °C for 20 minutes and the protein suspension was passed through a syringe to remove any remaining cell debris. The FurCj protein was purified using a 5 ml gravity flow Strep-Tactin® Sepharose® column (IBA) in accordance with the manufacturer’s instructions with the exception that EDTA was not included in buffer E (100 mM tris pH 8, 150 mM NaCl and 2.5 mM desthiobiotin) and buffer R (1 mM hydroxyl-azophenyl-benzoic acid resuspended in buffer W).

2.4.2 Expression and purification of recombinant C. jejuni RacR and RacS-HK

NCTC 11168 racR and racR-HK which only encodes the histidine kinase domain of RacS predicted by Prosite http://www.expasy.ch/prosite (ExPASy proteomics server, Swiss Institute of Bioinformatics) were cloned into pLEICES-01 (pRR46 and pRR53 respectively) to enable the expression and purification of N-terminal His6-tagged RacR and RacS-HK induced by IPTG. 200 ml of LB broth supplemented with Amp and Cm was inoculated with 2 ml of overnight liquid E. coli Rosetta culture carrying either pRR46 or pRR53 and incubated at 37 °C with shaking until an OD600 of 0.5 was reached. The culture was induced with 0.8 mM IPTG and incubated for 3 more hours at 37 °C. The cells were harvested at 3220 x g at 4 °C for 15 minutes and resuspended in 10 ml of binding buffer (50 mM tris, 500 mM NaCl and 80 mM imidazole, pH 7.4). One protease inhibitor tablet was added to the cell suspension and the cells were lysed by sonication
(2.4.1). RacR and RacS-HK proteins were both purified using 5 ml His Trap™ FF crude (GE Healthcare) columns in accordance with the manufacturer’s instructions with the exception that tris was used instead of sodium phosphate in the binding buffer and the elution buffer (50 mM tris, 500 mM NaCl and 500 mM imidazole, pH 7.4).

2.4.3 Protein buffer exchange

RacR and RacS-HK were buffer exchanged to buffer B [(van Mourik et al., 2009), 50 mM tris pH 7.6, 200 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol] using Zeba™ desalt spin columns (Thermo Scientific) in accordance with the manufacturer’s instructions.

2.4.4 Protein concentration

FurCj was concentrated using a 10 K Microcon® centrifugal filters (Millipore) in accordance with the manufacturer’s instructions and protein aliquots were stored at -20 °C. RacR and RacS-HK were concentrated using 10 K Amicon® Ultra-15 centrifugal filters (Millipore) in accordance with the manufacturer’s instructions and stored at 4 °C.

2.4.5 SDS-PAGE

SDS-PAGE carried out in a Mini-Protean® II electrophoresis cell was used to separate protein samples and all the components were set up in accordance with the manufacturer’s instructions. A 12% (v/v) resolving gel mix was prepared with 3.35 ml of dH₂O, 2.5 ml of resolving gel buffer (1.5 M tris pH 8.8), 0.1 ml of 10% (v/v) SDS, 4
ml of Ultra Pure ProtoGel®, 50 μl of 10% (w/v) APS and 5 μl of TMEDA. The gel mix was loaded into a gel casting assembly and overlaid with 1 ml of iso-butanol. When the gel was set, the iso-butanol was washed away and the resolving gel layer was overlaid with a 4% (v/v) stacking gel mix consisting of 2.38 ml of dH2O, 1 ml of stacking gel buffer (0.5 M tris pH 6.8), 0.62 ml of Ultra Pure ProtoGel®, 40 μl of 10% (w/v) APS and 4 μl of TMEDA. Protein samples were mixed with an equal amount of 2x SDS-PAGE sample buffer [0.15 M tris pH 6.8, 1.2% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol and 0.0018% (w/v) bromophenol blue] and denatured at 100 °C for 10 minutes. Once the gel was set, protein samples and 5 μl of PageRuler™ prestained protein ladder (Fermentas) were loaded and run at 70 V in the stacking gel layer and at 110V in the resolving gel layer in 1x SDS-PAGE running buffer [1% (w/v) SDS, 25 mM tris and 192 mM glycine] until the dye front reached the end of the gel.

2.4.6 SDS-PAGE gel visualisation and drying

For protein visualisation, SDS-PAGE gel containing protein samples (2.4.5) were stained overnight in coomassie blue staining solution [45% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) coomassie brilliant blue R-250 (Thermo Scientific)] and destained with 4 rounds of 30-minute washes with coomassie blue destaining solution [45% (v/v) methanol and 10% (v/v) acetic acid]. For long term storage, destained gels were washed with water for 30 minutes and incubated with gel drying buffer [10% (v/v) glycerol and 20% (v/v) ethanol] for 30 minutes. The gel was then sealed in between two sheets of DryEase mini cellophane (Invitrogen) and allowed to air dry for 2 days.
Chapter 2: Material and methods

2.4.7 Transfer protein from SDS-PAGE gel onto membrane (western blot)

Protein samples separated by SDS-PAGE (2.4.5) were routinely transferred onto Hybond™-C extra supported nitrocellulose membrane (Amersham Biosciences) to allow further protein detection. Following SDS-PAGE, each component of the OmniPAGE mini electroblotter (Geneflow) blotting cassette was soaked in ice-cold transfer buffer [0.037% (w/v) SDS, 47.9 mM tris and 38.6 mM glycine] and the cassette was set up in the following order: negative side of the cassette, sponge, 3 pieces of 3 mm paper, the gel, the membrane, 3 pieces of 3 mm paper, sponge and the positive side of the cassette. The gel was blotted in ice-cold transfer buffer at 150 mA for an hour.

2.4.8 Detection of protein by anti-His-antibody

His<sub>6</sub>-tagged protein samples blotted onto nitrocellulose membrane (2.4.7) were detected by anti-His-antibody. The membrane was rinsed in PBST [0.5% (v/v) Tween®, 20 resuspended in PBS] and blocked in 50 ml of blocking solution [7% (w/v) skimmed milk powder (Oxoid)] for an hour. The membrane was incubated with 20 ml of antibody solution [0.005% (v/v) anti-polyHis-peroxidase antibody (Sigma- Aldrich) resuspended in blocking solution] for an hour and washed with three times for 5 minutes, twice for 15 minutes and finally three times for 5 minutes with 20 ml of PBST. The membrane was then incubated with 4 ml of equilibrated (5 minutes at room temperature) detection solution [50% (v/v) EZ-ECL solution A and 50% (v/v) EZ-ECL solution B (Biological
industries)] for 2 minutes and wrapped in Saran™ film prior to exposure to X-ray film for 30 seconds.

2.4.9 Protein identification

Protein samples from a destained SDS-PAGE gel (2.4.6) were identified by peptide mass fingerprinting and the entire identification process was conducted by PNACL. In brief, the protein sample was excised from the gel and digested with trypsin. The sample was then analysed by MALDI-ToF mass spectrometry using a 4000 Q TRAP® LC/MS/MS system (Applied Biosystems) and the MASCOT peptide mass fingerprint searching tool http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF (Mascot search, Matrix Science) was used to determine the protein identity.

2.4.10 Protein quantification

The concentration of purified recombinant protein was estimated by Bradford assay (Bradford, 1976). A series of BSA protein standards and recombinant protein dilutions were prepared in 100 μl volumes in 0.15 M NaCl. The protein mixtures were mixed with 900 μl of Bradford reagent (Sigma-Aldrich) and their OD₅₉₅ was measured. The absorbance value of each standard was plotted against protein concentration to construct a standard curve from which the concentration of recombinant protein was determined.

2.4.11 Phosphorylation assay
Chapter 2: Material and Methods

Phosphorylation assay was used to detect the autophosphorylation of RacS-HK and the phosphate transfer from RacS-HK to RacR (van Mourik et al., 2009). 30 μl of RacS-HK was mixed with 1 μl of 10 μCi ATP γ-32P and incubated at room temperature for 15 minutes. 2 μl of the RacS-HK mixture was transferred into a screw cap tube containing 3 μl of dH2O and 5 μl of 2x SDS-PAGE sample buffer and incubated on ice to inactivate the reaction. 18 μl of RacR was then added to the RacS-HK mixture and 5 μl of the RacS-HK/RacR mixture was transferred into a screw cap tube containing 5 μl of 2x SDS-PAGE sample buffer after 15 seconds, 30 seconds, 1 minute, 2 minutes, 4 minutes, 8 minutes and 16 minutes. All the samples were denatured at 100 °C for 10 minutes and analysed by SDS-PAGE (2.4.5). The gel was then transferred onto 3 mm paper and vacuum dried for 30 minutes prior to wrapping in Saran™ film and exposed to X-ray film at -80 °C for a day.

2.4.12 Fumarase activity assay

The fumarase activity assay was used to detect the level of translation of C. jejuni fumC mRNA in wild-type and mutant backgrounds under different iron conditions. Fumarase activity was measured by the conversion of L-malate to fumarate (Kanarke and Hill, 1964) and whole cell extract was used instead of the pure protein (Hassett et al., 1997). C. jejuni cells cultured under different iron conditions (2.3.1) were harvested and resuspended in 5 ml of 50 mM sodium phosphate pH 7.3 (0.5 M Na2HPO4 and 0.5 M Na2H2PO4, pH 7.3). Whole cell extracts were obtained by sonication (2.4.1) and diluted to an OD280 of 0.5 with 50 mM sodium phosphate pH 7.3. 200 μl of each cell extract
was mixed with 800 μl of malate solution (50 mM L-malate and 50 mM sodium phosphate, pH 7.3) and the change of OD\textsubscript{280} was measured every 10 seconds at 25 °C for a minute. The fumarase activity (U/mg) was calculated by the equation: Specific activity = units of activity per ml x 0.51 / OD\textsubscript{280}. 0.51 is the extinction coefficient for crystalline fumarase at OD\textsubscript{280} and units of activity per ml is defined as the initial rate of change in OD per 10 seconds at 25 °C times a thousand (Kanarke and Hill, 1964).

2.5 Bioinformatics

Based on the Fur\textsubscript{Cj} box consensus sequence proposed by Palyada et al. (2004), the chuA and fumC Fur\textsubscript{Cj} box sequences were predicted using the CampyDB pattern search tool http://campy.bham.ac.uk/pattern/index.cgi?help=pattern&frame= (xBASE, N.B. this site is no longer available). The criteria used for this prediction were set as the following:

geno me to search: \textit{C jejuni} NCTC 11168; pattern: [at][ta][ta][tag]tga[ta][at][tag][ta][ag] [atg]ta[ta][tca]a (square brackets indicate base redundancy, i.e. [at] means a or t);

restricted to regions within: 200 bp upstream of a gene start; report matches within intergenic regions only and allow up to 3 mismatched bases.

The sequence of \textit{H. pylori} 26695 fumC was obtained from NCBI website (http://www.ncbi.nlm.nih.gov) and was compared with the sequence of \textit{C. jejuni} NCTC 11168 fumC using ALIGN Query http://xylian.igh.cnrs.fr/bin/align-guess.cgi (Genestream Search).
Other bioinformatics software and searching tools used were CampyDB website (Table 2.5), Clone Manager Professional 9 (Table 2.5), Chromas v1.45 (2.2.15), Peak Scanner™ software v0.1 (2.3.6), Prosite (2.4.2) and Mascot (2.4.9).
Chapter 3: Site-directed mutagenesis of the chuA and fumC Fur boxes

3.1 Introduction

In order to characterise the sequence specificity of binding to the FurCj box by FurCj and to verify the proposed FurCj box sequence, candidate FurCj box sequences from two differently FurCj-regulated promoters were required for subsequent comparative mutational analyses in conjunction with an EMSA based experimental design.

With a 100-fold increase in the level of transcription under iron-limiting conditions in a wild-type background and a non-iron-responsiveness in a furCj mutant background, the C. jejuni NCTC 11168 haem OM receptor gene chuA has been extensively characterised as an example of a classically iron- and Fur-repressed genes (van Vliet et al., 1998; Palyada et al., 2004; Holmes et al., 2005; Ridley et al., 2006). A computational searching of the chuA promoter region for the consensus FurCj box sequence proposed by Palyada et al. (2004) revealed a perfect matched sequence (Table 3.1) and the chuA promoter region containing this FurCj box sequence has been shown by EMSA to bind FurCj with a high affinity (Li, 2005; Ridley et al., 2006). Based on these previous observations, the chuA FurCj box was therefore chosen as the first candidate sequence for this study.

Detailed transcriptomic and proteomic analysis of the FurCj regulon have revealed many genes that are positively regulated by FurCj and iron (Palyada et al., 2004; Holmes et al., 2005). The C. jejuni fumarate hydratase gene fumC (see chapter 4 for a more detailed
Table 3.1: Sequence alignment of the FurEc box, the putative FurCj box and the predicted C. jejuni chuA and fumC FurCj boxes. The sequence logo of the FurCj box is redrawn from Palyada et al., 2004 using Weblogo (http://www.bio.cam.ac.uk/seqlogo). The height of each letter in the FurCj box indicates the relative frequency of each base occurring at the indicated base position. Sequence differences between the E. coli and C. jejuni Fur boxes are highlighted in yellow and sequence differences between the chuA and fumC FurCj boxes with respect to the FurCj box consensus sequence are highlighted in red.
description) is such an example in that its transcription decreased 2.4-fold under iron-limited conditions in a wild-type background and in a furCj mutant background (Holmes et al., 2005). A computational analysis of the fumC promoter region revealed a FurCj box-like sequence that contains three mismatches at the 10th, 13th, and 19th base positions when compared with the putative FurCj box sequence (and the chuA FurCj box, Table 3.1) and direct FurCj binding in this region was not detected (Li, 2005) indicating the iron-responsive regulation of fumC is indirectly mediated by FurCj. The variation between the fumC FurCj box-like sequence (referring to as the fumC FurCj box from this point onward) sequence and the chuA FurCj box at these positions is likely to be a key determinant for the previous observed contrasting iron regulation and FurCj-FurCj box binding affinity between chuA and fumC and the fumC FurCj box was therefore chosen as a reverse control of the chuA FurCj box.

3.2 Aims

Previous experimental and computational evidence strongly demonstrated that C. jejuni NCTC 11168 chuA and fumC are differently FurCj-regulated and their FurCj box sequences varied only at the 10th, 13th and 19th positions with respect to the consensus FurCj box sequence. It was hypothesised that by making chuA FurCj box fumC-like and vice versa at these three base positions (Table 3.2), both mutated promoters would exhibit altered iron- and FurCj-regulation and affinity to FurCj binding similar to their opposite wild-type counterparts thus illustrating the importance of these three bases (positions) of the consensus FurCj box sequence in FurCj-DNA interaction and
Table 3.2: Illustration of the mutational change at the 10\textsuperscript{th}, 13\textsuperscript{th} and 19\textsuperscript{th} positions of the \textit{chuA} and \textit{fumC} putative Fur\textsubscript{Cj} boxes. Mutants were named based on the position/s of mutational change/s. \(\leftrightarrow\) indicates base change at a particular position, i.e. for \textit{chuA} 10/19 mutant, T and A at 10\textsuperscript{th} and 19\textsuperscript{th} positions of the \textit{chuA} wild-type (wt) Fur\textsubscript{Cj} box were changed to G and T respectively and vice versa for the \textit{fumC} 10/19 mutant.
Chapter 3: Site-directed mutagenesis of the *chuA* and *fumC* Fur boxes

Fur$_{Cj}$-responsive gene regulation. By using site directed mutagenesis at the 10$^{th}$, 13$^{th}$ and 19$^{th}$ positions of the *chuA* and *fumC* Fur$_{Cj}$ boxes, we were initially aiming to determine how *chuA* and *fumC* are differentially regulated by Fur$_{Cj}$ in terms of Fur$_{Cj}$ box sequence variation by assessing the changes in the affinity of Fur$_{Cj}$ binding to the mutated Fur$_{Cj}$ boxes by EMSA; analysing the changes in promoter activity of mutated *chuA* and *fumC* promoters using *lacZ* reporter gene assay in a wild type or a *fur_{Cj}* mutant background; and determining the changes in the location of Fur$_{Cj}$-Fur$_{Cj}$ box interaction by DNAse I footprinting method (outlined in Figure 3.1).

### 3.3 results

#### 3.3.1 Site-directed mutagenesis

In order to verify the proposed theory of Fur$_{Cj}$ box sequence variation-mediated differential Fur$_{Cj}$ regulations between *chuA* and *fumC*, the 10$^{th}$, 13$^{th}$ and 19$^{th}$ positions of each Fur$_{Cj}$ box were mutated by site-directed mutagenesis (2.2.19). The mutation scheme was designed to swap the wild-type consensus matched bases at the 10$^{th}$, 13$^{th}$ and 19$^{th}$ positions of the *chuA* Fur$_{Cj}$ box with the wild-type mismatches in the *fumC* Fur$_{Cj}$ box and vice versa. In other words, it was designed to make *chuA* Fur$_{Cj}$ box *fumC*-like and *fumC* Fur$_{Cj}$ box *chuA*-like at these three base positions (Table 3.2).

An inverse PCR-based mutagenesis approach exploiting the different methylation status between the wild-type PCR template and mutated products was applied in this study (Zheng *et al.*, 2004). A 274 bp fragment containing part of the *chuZ-chuA* intergenic
Figure 3.1: A brief outline of the stages carried out in the characterisation of the putative Fur$_Cj$-binding site using chuA and fumC Fur$_Cj$ boxes as models. The actual order of each stage carried out is alphabetically listed in the figure.
region and part of the *chuA* gene and a 233 bp fragment containing the *cj365c-fumC* intergenic region and parts of the *cj1365c* and *fumC* genes (Figure 3.2) were previously cloned into pUC19 and pCR 2.1-TOPO respectively to form pJMcK6 and pYL1 (Li, 2005) and were used as templates for the inverse PCR. 5’ overlapping complementary inverse primers harbouring desired mutation(s) were used in a standard inverse PCR fashion with proof reading Phusion DNA polymerase to ensure high fidelity and the nicked circular products containing mutational change(s) were selected by the removal of methylated template using *Dpn*I. Once introduced into *E. coli* Top10 strain by electroporation, the nicks on the mutated recombinant plasmids are repaired and the putative mutants were verified by sequencing. 14 mutated recombinant plasmids named pRR1-14 were successfully obtained and each construct is essentially a pJMcK6 or pYL1 variant containing mutational change(s) in the cloned *chuA* or *fumC* Fur$_{Cj}$ boxes respectively. The combination of mutational change(s) for each Fur$_{Cj}$ box are listed in Table 3.3 and with sufficient *Dpn*I digestion, this inverse PCR-based site-directed mutagenesis method was shown in this study as a simple and efficient tool to create mutational changes in the AT rich *C. jejuni* promoter regions.

### 3.3.2 Purification of the recombinant Fur$_{Cj}$ protein

The *fur$_{Cj}$* gene was previously cloned into the pASK-IBA7 expression vector (pJMcK1) and the Strep-tagged recombinant Fur$_{Cj}$ was expressed in *E. coli* XL1-Blue host cells and purified by affinity chromatography (2.4.1, Holmes *et al.*, 2005). Two bands of 17.5 kDa and 18.6 kDa in size were typically observed for the Fur$_{Cj}$ protein when analysed
Figure 3.2: The *C. jejuni* NCTC 11168 *chuA* and *fumC* promoter regions under investigation. *chuA* is co-transcribed with *chuBCD* (not shown) as an operon in the opposite direction downstream from *chuZ* whereas *fumC* is located upstream from *cj365c* which encodes a secreted serine protease. For easy visualisation, *fumC* and *cj365c* are drawn in the opposite orientation to their natural genomic positions. The putative FurCj box and the start codon for each gene are circled and DNA sequences are coloured corresponding to the colour of each gene. The regions underlined were originally included in the pJMcK6 and pYLI1 constructs whereas regions under dash lines were extended in this study.
<table>
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<tr>
<th>Mutant name</th>
<th>Construct name</th>
<th>Sequence alignment</th>
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<td></td>
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<tr>
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</tbody>
</table>

Table 3.3: Partial sequence alignments between wild-type (top stand) and mutated Fur$_Cj$ boxes (bottom stand) performed using Clone Manager software. Base changes in respect to the wild-type Fur$_Cj$ box are highlighted in red.
by SDS-PAGE (Figure 3.3). They were the untagged and tagged version of the protein respectively as confirmed by previous western blotting experiments using anti-Strep-tag antibodies and N-terminal protein sequencing (Li, 2005). Because the Strep-tag was dissociated from a large proportion of the recombinant FurCj protein, further tag removal was not carried out.

3.3.3 EMSA

chuA and fumC promoter regions containing the wild-type FurCj boxes were first amplified from pJMcK6 and pYL1 respectively using their original cloning primers (Table 2.5) and the DIG-labelled PCR products were then subjected to EMSA (2.2.24) with purified FurCj to confirm previous observations. The FurCj concentration range used previously (Li, 2005; Holmes et al., 2005; Ridley et al., 2006) was first tested with the wild-type chuA FurCj box, however high affinity FurCj binding was not observed (results not shown). The actual FurCj protein sample used in these studies was also tested and similar results were obtained which indicated that an error had occurred when the FurCj concentration used in these studies was originally calculated. A new range of FurCj concentration was determined and applied for wild-type chuA and fumC FurCj boxes. As shown in Figure 3.4, high affinity FurCj binding was observed for wild-type chuA FurCj box with as little as 25 nM FurCj and two distinct shift species were observed. The bottom species indicated the binding of a FurCj dimer and binding of two dimers was represented by the upper shift species. When the FurCj concentration was increased, majority of the labelled DNA was bound with FurCj and predominately in the form of
Figure 3.3: A SDS-PAGE analysis of the purified recombinant Fur$_{Cj}$. Lane 1 and 10: ProSieve® color protein markers (Lonza); lane 2: pre-column sample; lane 3: flow-through sample; lane 4: mid-wash sample; lanes 4-9 and 11-15: elution samples 1-10 respectively.
Figure 3.4: Standard (a) and competitive (b) EMSA of 1.55 fmol DIG-labelled *chuA* and *fumC* promoter regions containing the wild-type and triple mutated Fur$_{Cj}$ boxes with the purified Fur$_{Cj}$ protein. For standard EMSA, lanes 1-8: labelled DNA with 0 nM, 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM and 175 nM Fur$_{Cj}$ respectively. For competitive EMSA, lanes 1-2: labelled DNA with 0 nM and 175 nM Fur$_{Cj}$ respectively; lanes 3-4: labelled DNA with 175 nM Fur$_{Cj}$ and 1500x and 2000x unlabelled competitor respectively. Unlabelled *chuA* and *fumC* wild-type promoter regions were used as competitors for *chuA* and *fumC* (both wild-type and triple mutant) competitive EMSA respectively.
two FurCj dimers. For the fumC FurCj box, no FurCj binding was detected even at 175 nM FurCj. Despite differences in FurCj concentration ranges used in this study and previous studies, the shift patterns observed for the wild type chuA and fumC FurCj boxes were consistent with previous observations indicating the purified FurCj protein in this study was functional.

To test the effects of mutational changes in the affinity of FurCj binding, chuA and fumC FurCj boxes containing triple mutational changes were first analysed as they were predicted to give the most dramatic changes in FurCj binding (Figure 3.4). For chuA10/13/19, although two shift species were still detectable, most of the FurCj-DNA complex was in the single dimer state and a large amount of unbound DNA was observed which indicated a significant decrease in the ability for at least one FurCj dimer to recognise and bind to this mutated Fur box. Two shift species were also demonstrated for fumC10/13/19 with as little as 25 nM FurCj and these species were not observed for the wild-type fumC FurCj box. The shift pattern obtained for fumC10/13/19 was comparable with chuA10/13/19, however in this case, these two shift species were created with the substitution of three mismatches in the fumC FurCj box thus the ability for FurCj to recognise and bind to the FurCj box with a relatively high affinity was partially restored.

To verify the results obtained, competitive EMSAs were performed for chuA and fumC wild-type FurCj boxes and triple mutants with 1500 and 2000 times corresponding
unlabelled wild-type promoter region as competitors (Figure 3.4). For *chuA*, specific Fur$_{Cj}$ binding was reduced for both the wild-type and mutant as most of the Fur$_{Cj}$ protein was competed away by the unlabelled DNA. The opposite effect was observed for *fumC* where the shift pattern was unchanged for the wild-type and mutant as *fumC* wild-type Fur$_{Cj}$ box does not bind Fur$_{Cj}$. A much higher affinity of Fur$_{Cj}$ binding was observed for *fumC*10/13/19 with 175 nM Fur$_{Cj}$, however this high level of Fur$_{Cj}$ binding was not observed when standard EMSA was performed therefore was likely to be caused by cross contamination. The results obtained from competitive EMSAs again demonstrated the different specificity of Fur$_{Cj}$ binding between *chuA* and *fumC* promoters and verified the shift patterns observed for standard EMSAs were caused solely by the interaction between labelled promoter regions and *C. jejuni* Fur$_{Cj}$.

To determine the importance of each individual base at the 10$^{\text{th}}$, 13$^{\text{th}}$ and 19$^{\text{th}}$ positions of the Fur$_{Cj}$ box, EMSA was subsequently conducted for all the *chuA* and *fumC* single and double mutants. For *chuA* mutants, there was a clear correlation between the unbound DNA profiles and mutational positions in the *chuA* Fur$_{Cj}$ box. An increase in the intensity of the unbound DNA in the presence of 175 nM Fur$_{Cj}$ was observed for all the *chuA* mutants and most notably with the *chuA*10/19 and *chuA*13/19 (Figure 3.5), which indicated a decrease in the affinity of Fur$_{Cj}$ binding.

For *fumC* mutants, no shift species was observed for *fumC*10 (Figure 3.6). A weak higher shift species representing a double Fur$_{Cj}$ dimers-DNA complex was detected for
Figure 3.5: Standard EMSA of 1.55 fmol labelled chuA promoter regions containing mutated FurCj boxes with the purified FurCj protein. Lanes 1-8: labelled DNA with 0 nM, 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM and 175 nM FurCj respectively.
Figure 3.6: Standard EMSA of 1.55 fmol labelled fumC promoter regions containing mutated FurCj boxes with the purified FurCj protein. Lanes 1-8: labelled DNA with 0 nM, 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM and 175 nM FurCj respectively.
fumC13 while a lower shift species was observed for fumC19 that demonstrated the forming of single FurCj dimer-DNA complexes. The reason for this higher shift species formation (but not the lower species) seen with fumC13 was unclear, however it was likely that although high affinity binding of FurCj single dimer was not achieved, the mutational change at position 13 of the FurCj box has led to a conformational change of the DNA such that allowed the formation of a weak DNA-double FurCj dimers complex. Both DNA binding patterns observed for fumC13 and fumC19 were also consistently observed in the fumC double mutants and the intensities of these two shift species were significantly increased for fumC13/19. These findings have collectively indicated that mutations introduced into the chuA and fumC FurCj boxes, in particular the 13th and 19th positions, strongly affect FurCj binding whereas the 10th position may play a subsidiary role.

3.3.4 β-galactosidase assay

To corroborate the in vitro interactions observed between chuA and fumC promoters and FurCj, β-galactosidase assays were preformed to verify the functional relevance of these interactions in vivo and to investigate the effects in promoter activities due to mutational changes in the chuA and fumC promoter regions. The goal was to amplify the wild type and mutant chuA and fumC promoters and clone into the multi-copy shuttle vector pMW10 (Wösten et al., 1998) for subsequent screening in a C. jejuni background under high and low iron conditions. pMW10 is a 10 kb shuttle vector contains ori of both C. jejuni and E. coli and a MCS situated upstream of a promoterless lacZ gene (Wösten et
Chapter 3: Site-directed mutagenesis of the chuA and fumC Fur boxes

*al.,* 1998). *C. jejuni* NCTC 11168 could not be used as a host stain as it cannot be successfully transformed by plasmids, therefore *C. jejuni* 480, a strain readily accept plasmids, was used instead and this system has been successfully used in several occasions to study specific promoter activities in *C. jejuni* (van Vliet *et al.*, 2000; Ridley *et al.*, 2006; Miller *et al.*, 2008). It should be noted that although using a difference *C. jejuni* strain was not ideal, it was essential to investigate Fur$_{Cj}$ regulation in a *C. jejuni* background as previous studies indicated that when presented in an *E. coli* background, the AT-rich *C. jejuni* sequence interact non-specifically with Fur$_{Ec}$ (Rock, 2003).

The original cloning primers for chuA and fumC wild-type promoters were not compatible with pMW10, cloning primers with 5’ BamHI and XbaI restriction sites were therefore designed and used to allow directional cloning of the promoter region into pMW10. All the wild-type and mutated promoter regions were cloned into pMW10 in this manner and the resulting constructs pRR14-30 were transformed into *C. jejuni* 480.

β-galactosidase assays (2.2.20) were first carried out with the chuA wild-type promoter construct (pRR15) along with several controls under high iron (40 μM FeSO$_4$) and low iron (20 μM Desferal) conditions. The promoter region of housekeeping gene metK cloned into pMW10 (p23E5) and pMW10 without insert were used as non-iron-regulated positive and negative controls respectively. The chuA wild-type promoter previously cloned into pMW10 (pJDR13) that contains the chuZ-chuA
intergenic region (Table 2.2) was used as an iron-regulated comparative positive control. Promoter activities previously observed for all the controls (Ridley et al., 2006) were consistently observed though no promoter activity was determined for the *chuA* wild-type promoter cloned in this study (Figure 3.7). The entire pRR15 construct was later sequenced and no mutations or deletions were found, which led to the conclusion that the length of the original *chuA* promoter region cloned in pJMck6 (Figure 3.2) and subsequently cloned in pRR15 was not sufficient to allow the promoter to function correctly.

As all the mutated promoter regions had already been cloned into pMW10, therefore instead of remaking all the mutants, each wild-type and mutated *chuA* promoter regions were PCR amplified and fused using fusion PCR with a 61 bp fragment amplified directly upstream of the *chuA* promoter region originally cloned (Figure 3.2). The extended *chuA* promoters were re-cloned back into pMW10 (pRR31-38) and promoter activities were tested along with controls (Figure 3.8).

Iron-induced gene repression was observed for the *chuA* wild-type promoter under high iron conditions and the promoter was de-repressed when the iron concentration was reduced, therefore indicating the iron responsive regulation of the *chuA* promoter was restored with the extended promoter region. Mutational change at the 10\textsuperscript{th} position of the *chuA* Fur\textsubscript{Cj} box did not alter the level of gene expression when compared to the wild-type, however three- to four-fold de-repression was observed under high iron
Figure 3.7: β-galactosidase assay of the chuA wild-type promoter region with controls under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
Figure 3.8: β-galactosidase assay of the chuA wild-type and mutated promoter regions with controls under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
conditions for mutants containing 13\textsuperscript{th} and/or 19\textsuperscript{th} base changes which indicated a decrease in the ability of \( \text{Fur}_{Cj} \) to interact with the mutated \( \text{Fur}_{Cj} \) box and hence has allowed these promoters to be partially transcribed by the RNA polymerase. These results have demonstrated the importance of the 13\textsuperscript{th} and 19\textsuperscript{th} bases of the \( \text{Fur}_{Cj} \) box in \( \text{Fur}_{Cj} \)-\( \text{Fur}_{Cj} \) box interaction and this finding was consistent with the EMSA results (Figures 3.4 and 3.5).

In addition, although similar levels of \( \text{Fur}_{Cj} \)-repression were detected for \( \text{chuA} \) wild-type and \( \text{chuA} \)\textsuperscript{10} under iron-rich conditions, clear differences in the level of derepression were observed between mutants with and without the 10\textsuperscript{th} base mutation (i.e. \( \text{chuA} \)\textsuperscript{13} was different from \( \text{chuA} \)\textsuperscript{10/13} and \( \text{chuA} \)\textsuperscript{13/19} was different from \( \text{chuA} \)\textsuperscript{10/13/19}). These differences were possibly non-statistical variations caused by inaccuracies of the \( \beta \)-galactosidase assays, but most likely, they were reflected by the important but unessential role of the 10\textsuperscript{th} position of the \( \text{Fur}_{Cj} \) box in facilitating or stabilising the \( \text{Fur}_{Cj} \)-\( \text{Fur}_{Cj} \) box interaction.

When \( \beta \)-galactosidase assays were carried out for the wild-type and mutated \( \text{fumC} \) promoters under the same experimental conditions (Figure 3.9), unexpected results were observed. Although promoter activities were observed for \( \text{fumC} \) wild-type promoter under both iron conditions, the iron-dependent gene regulation identified previously (Holmes \textit{et al.}, 2005) was not reproduced for the wild-type \( \text{fumC} \) promoter. This non-iron-responsive gene regulation was further observed for \( \text{fumC} \textsuperscript{10}, \text{fumC} \textsuperscript{19} \) and
Figure 3.9: β-galactosidase assay of the *fumC* wild-type and mutated promoter regions with controls under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
FumC10/19 which indicated that although FurCj-binding was demonstrated for mutated FumC FurCj boxes *in vitro* (Figure 3.6), these FurCj-FurCj box interactions were physiologically insignificant to alter the expression of *fumC* *in vivo*. The promoter activates for mutants containing the 13th base change (*fumC*13, *fumC*13/19 and *fumC*10/13/19) were severely reduced comparing to *fumC* wild-type, which indicated that the *fumC* FurCj box is overlapping with the recognition site of one of the importance transcription regulatory element hence the promoter activity was disrupted when the 13th base was mutated. Interestingly the expression of *fumC*10/13 was only partially affected by the 13th base mutation. In this case, the detrimental effect of the 13th base mutation was potentially compensated by the G to T change at the 10th position of the *fumC* FurCj box as T is a more favourable base generally found in the -10 and -35 regions of a bacterial promoter.

The non-iron responsive gene regulation observed for *fumC* wild-type promoter was possibly caused by the inadequate promoter length cloned into pYL1. As in the case of chuA, fusion PCR was used to extend the *fumC* promoter region by 217 bp upstream (217 bp was used for the convenience of primer design) and both the original and extended (pRR42) versions were tested in β-galactosidase assays (Figure 3.10). Iron induced activation was observed for both promoters, though the differences in promoter activity between high and low iron were marginal and no obverse difference was noticeable between the two versions of the *fumC* promoter. Iron responsive regulation of *fumC* may also have been mediated by growth phase as cells were routinely cultured to
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Figure 3.10: β-galactosidase assay of the original (pRR23) and extended (pRR42) fumC wild-type promoter regions with controls under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions for different amount of time i.e. 5 and 16 hours. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
late lag - early exponential phase in high and low iron conditions for β-galactosidase assay whereas the cells used in Holmes’s study were grown to mid - late exponential phase (Holmes et al., 2005). 16 hours incubation time was subsequently used prior to β-galactosidase assays of the fumC wild-type promoter and controls and a similar level of iron responsive gene regulation was observed when compared with the standard 5 hours incubation (Figure 3.10). Although this minor difference in fumC promoter activity between high and low iron was consistent with the 2.4-fold decrease in transcription level under iron-limited conditions previously observed (Holmes et al., 2005), however promoter activity fluctuations were commonly observed for non-iron regulated metK due to inaccuracies of the assay, therefore the genuine iron responsive regulation of fumC could not be solely substantiated by β-galactosidase assays of the wild-type promoter and controls.

3.3.5 Construction of the C. jejuni 480 furCj mutant

To verify the minor iron responsive regulation observed for fumC promoter by β-galactosidase assays, one of the strategies applied was to construct a C. jejuni 480 furCj mutant where any FurCj-dependent iron regulation would be abolished. By using β-galactosidase assays in different FurCj backgrounds, iron-mediated differential gene regulation of fumC would be determined by a direct comparison of the promoter activity profiles.

A C. jejuni 480 furCj mutant strain has been previously constructed by Grabowska et al.
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(2011) where *C. jejuni* 81-176 *fur*\textsubscript{Cj} mutant was first created by electroporation of pAV80 (Figure 3.11) into the wild-type 81-176 strain and the genomic DNA extracted from the resulting mutant was then naturally transformed into the wild-type 480 strain to facilitate homologous recombination between the wild-type 480 *fur*\textsubscript{Cj} and the 81-176 mutated *fur*\textsubscript{Cj} (Grabowska et al., 2011). pAV80 is a mutational construct harbouring a Cm resistance cassette inserted into the 5’ of the NCTC 11168 *fur*\textsubscript{Cj} in a forward direction and was previously used to create a NCTC 11168 *fur*\textsubscript{Cj} mutant (AV41, van Vliet et al., 1998). As pMW10 based constructs used for β-galactosidase assays were Kan resistance, therefore a Cm resistance 480 *fur*\textsubscript{Cj} knockout would be an ideal strain to analyse the iron responsive regulation of *fumC*. However in order to avoid any *C. jejuni* 480 strain differences, the 81-176 *fur*\textsubscript{Cj} mutant genomic DNA purified by Grabowska et al. (2011) was obtained and was used in an attempt to create a 480 *fur*\textsubscript{Cj} mutant strain that would allow valid comparisons with the wild-type 480 strain using β-galactosidase assays.

The 81-176 *fur*\textsubscript{Cj} mutant genomic DNA and the wild-type counterpart were first PCR analysed to confirm the presence of a Cm resistance cassette. A 1798 bp fragment containing the *fur*\textsubscript{Cj} gene with the cassette was expected to be obtained using Cj81-176 *fur*5’ and 3’ long primers and the Cj81-176 *fur*5’ long and catinvR primer set was expected to confirm the orientation of the cassette (Figure 3.11). When analysed by gel electrophoresis, a 946 bp band representing the wild-type *fur*\textsubscript{Cj} was observed for 81-176 *fur*\textsubscript{Cj} mutant and no product was detected using Cj81-176 *fur*5’ long and catinvR
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Figure 3.1: The mutated fur\(_{Cj}\) genes and flanking regions of NCTC 11168 fur\(_{Cj}\) mutant strains AV17 and AV41. The corresponding regions recombined from the original mutational constructs pAV32 and pAV80 and the fragment cloned into pRR41 are indicated by dashed lines. Primers used for screening fur\(_{Cj}\) mutants and their binding sites and directions are indicated by arrows. Cj81-176 fur\(_5\)’ long and Cj81-176 fur\(_3\)’ long primers were used for C. jejuni 81-176 strain and their binding sites and directions on 81-176 are equivalent to fur\(_F\) and fur\(_R\) respectively on NTCT 11168.
primers (Figure 3.12). When a pair of Cm resistance cassette specific primers was used, a 322 bp fragment confirming the present of the cassette was observed which indicated that either the genomic DNA was amplified from the wrong strain or homologous recombination has occurred elsewhere in the 81-176 genome.

As further communication regarding to 81-176 furCJ mutant was not established, the two steps method was repeated and further simplified by introducing NCTC 11168 mutated furCJ gene (in the forms of mutational constructs or genomic DNA) straight into 480 as the 480 furCJ and flanking regions (300 bp on each side of the gene) shares 94% identity to the NCTC 11168 furCJ when compared by sequencing (result not shown). In order to explore the possibility of single recombination event, mutational construct pRR41 was also created that contained part of the Cm resistance cassette and part of the NCTC 11168 furCJ gene amplified from pAV80.

No colonies were obtained when pAV80, pRR41 and AV41 genomic DNA were introduced straight into 480 whereas for 81-176, the non-homologous recombination determined in Figure 3.12 was consistently observed for both DNA samples (Table 3.4). The mechanism of this Cm resistance cassette-associated non-homologous recombination in 81-176 was not clear and it was likely that either the 81-176 strain used by Grabowska et al. (2011) was different from the 81-176 strain used for this study or the 480 furCJ mutant they have created was incorrect. Although it’s not an ideal host for pMW10 based β-galactosidase assays, the possibility to create a Kan resistance 480
Figure 3.12: Verification of the 81-176 furCj mutant by PCR. Lanes 1 and 6: λ/Φ DNA size marker; lanes 2 and 4: PCR amplification using Cj81-176 fur5’ and 3’ long primers; lanes 3 and 5: PCR amplification using Cj81-176 fur5’ long and catinvR primers; lane 7: PCR amplification using catinvF and catR_KpnI primers.
<table>
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<tr>
<th>DNA used</th>
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<th>outcomes</th>
</tr>
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<td>pAV80</td>
<td>C. jejuni 480</td>
<td>no transformants</td>
</tr>
<tr>
<td>AV41 genomic DNA</td>
<td>C. jejuni 480</td>
<td>no transformants</td>
</tr>
<tr>
<td>pAV80</td>
<td>C. jejuni 81-176</td>
<td>non-homologous recombinants</td>
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</tr>
<tr>
<td>pRR41</td>
<td>C. jejuni 480</td>
<td>no transformants</td>
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</table>

**Table 3.4: Summary of the approaches used to create the C. jejuni 480 fur<sub>Cj</sub> mutant strain.** pAV80, pAV32 and pRR41 were introduced into the recipient strains by electroporation whereas AV41 and AV17 genomic DNA were naturally transformed.
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The *furC_J* mutant strain was also explored using pAV32 and AV17 genomic DNA (Figure 3.11). When both DNA samples were separately introduced into 480, and resulting clones screened by PCR using *furC_J* flanking primers (*furF* and *furR*), two bands indicating the mutated (2414 bp) and wild-type *furC_J* (911 bp) were detected in each clones. The presence of *furC_J* merodiploid clones was initially predicted to be caused by cross-contaminations between *furC_J* mutants and wild-type breakthroughs and the screened colonies were therefore re-streaked and re-screened by PCR.

As shown in Figure 3.13, for 480 *furC_J* mutant created using AV17 genomic DNA, only the 2414 bp band representing the mutated *furC_J* gene was detected. A band of 996 bp confirming the presence of the Kan resistance cassette in the *furC_J* gene was also obtained using *furF* and cassette specific primer SkanR which together confirming the identity of a pure 480 *fur* knockout clone. This *furC_J* mutant strain was named RR1. For the 480 *furC_J* mutant created using pAV32, the merodiploid state of *furC_J* remained and further investigation was not followed. When AV17 genomic DNA and pAV32 was separately introduced into 81-176, clones containing only wild-type *furC_J* were obtained. There were likely to be non-specific breakthroughs and the presence of the Kan resistance cassettes elsewhere in the genome was not investigated.

As pMW10 and RR1 are both resistant to Kan, the level of antibiotic resistances was tested as the wild-type 480 strain containing the multi-copy pMW10 was predicted to be more resistant to high Kan concentrations than the single copy resistance cassette on the
Figure 3.13: Verification of the 480 *furC* mutant by PCR. Lane 1: λ/Φ DNA size marker; lanes 2 and 4: PCR amplification using *furF* and *furR* primers; lanes 3 and 5: PCR amplification using *furF* and SKanR primers.
chromosome of RR1. 8- and 9-fold diluted wild-type 480 cells carrying pMW10 and RR1 cells were plated onto plate agars with increasing Kan concentrations and colonies formed were counted and compared. At a concentration of 4 mg/ml Kan, which was 80 times more concentrated than the level routinely used for Kan resistance selections, a selection against RR1 was achieved whereas colonies for pMW10 harbouring strain were able to form on agar containing up to 7 mg/ml Kan.

When pRR31 (chuA wild-type), p23E5 (metK) and pMW10 were electroporated into RR1 and plated onto agar containing 4 mg/ml Kan, large amount of breakthroughs were formed. The cultures were further selected with 7 mg/ml Kan and colonies formed were verified by PCR using pMW10 backbone- and Kan resistance cassette-specific primers. Only RR1 strain carrying pMW10 was successfully selected and further screening of RR1 strain harbouring pRR31 or p23E5 failed to identify any positive clones. When all the pRR31 and p23E5 transformants were washed off from their plates and used as templates for PCR verifications (plate PCR), the presence of positive clones were identified amongst the clone populations, through the proportion was very low as indicated by the faint bands (Figure 3.14). Higher Kan concentrations were not tested due to the poor solubility of concentrated Kan in dH2O and plate hybridisation technique for clone screening was considered unpractical due to the relative large number of mutants used in this study.

The difficulties associated with maintaining pRR31 or p23E5 in RR1 were likely caused
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Figure 3.14: Verification of the RR1 transformants by PCR using M13F and SKanR primers. Lanes 1 and 8: \(\lambda\Phi\) DNA size marker; lanes 2, 4 and 6: PCR amplification using 480 strain harbouring pRR31, p23E5 and pMW10 respectively; lanes 3, 5 and 6: PCR amplification using RR1 strain potentially harbouring pRR31, p23E5 and pMW10 respectively; lanes 9 and 10: plate PCR from re-streaked RR1 transformants potentially harbouring pRR31 and p23E5 respectively.
by the constant expression of pRR31 and p23E5’ lacZ genes which put pressures on the already weakened RR1 strain due to the lack of a functional furCj gene. pMW10 by contrast was readily maintained in RR1 due to the lack of a promoter upstream of the lacZ gene. To backup this theory, pRR15 which contains an incomplete chuA promoter was electroporated into RR1 and positive clones were identified on 7 mg/ml Kan agar (results not shown) conferring that the 480 furCj mutant was not a suitable host to study FurCj-responsive gene regulation using multi-copy plasmids system and a single copy system (i.e. single chromosomal lacZ reporter system) would be more appropriate.

3.3.6 Verification of fumC iron regulation by northern blot

The second approach used to verify the fumC iron responsive regulation was to purify the NCTC 11168 total RNA from cells cultured under high and low iron conditions and subsequently subject to northern blot analysis. In order to allow direct comparisons with the results obtained by β-galactosidase assays, cells were cultured as described in 2.2.20 with the exception that a 16-hour liquid culture incubation under high (40 μM FeSO₄) and low (20 μM Desferal) iron conditions was used to obtain sufficient amount of cells for total RNA purification. The total RNA purified from NCTC 11168 wild-type and furCj mutant (AV17) cultured under different iron conditions were separated by electrophoresis and hybridised with labelled fumC-specific probe and 16S-specific probe as a loading control (Figure 3.15). An approximate 1.4 kb single transcript slighter longer than the fumC gene was observed which indicated that fumC has its own promoter. fumC was expressed in both iron conditions though the level of expression
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Figure 3.15: Northern blot analysis of the iron- and FurGj-responsive regulation of fumC.

Figure 3.16: Northern blot analysis of the iron-, FurGj- and RacRjGj-responsive regulation of fumC.
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was significantly increased under high iron conditions and was consistent with data obtained from β-galactosidase assays. However this iron induced transcriptional activation was not mediated by Fur$_{Cj}$ as an identical transcriptional profile was observed for the *furCj* mutant strain and this result was in contradiction with previous observations (Holmes *et al.*, 2005).

The northern experiment shown in Figure 3.15 was repeated with the addition of RNA purified from a NCTC 11168 *perR$_{Cj}$* knockout strain (AV63) to explore the possibility of PerR$_{Cj}$-mediated iron regulation of *fumC*. Iron-induced transcriptional activation for *fumC* was observed though independently from Fur$_{Cj}$ and PerR$_{Cj}$ (results not shown). The same set of RNA used was re-hybridised with labelled *chuA*-specific probe as a quality control for the RNA samples and expected iron- and Fur$_{Cj}$-mediated gene regulation were observed for *chuA* indicating that in this experimental setup (i.e. the growth conditions used to culture cells prior to RNA purification), *C. jejuni* *fumC* is not regulated, or at least directly regulated by Fur$_{Cj}$ and the iron-responsive regulation of *fumC* is likely to be mediated by other unknown regulator/s.

A recent investigation of oxygen responsive metabolic gene regulation was conducted by van Mourik *et al.* (2009) in which *fumC* was demonstrated to be repressed by the two component regulator, RacR, under oxygen limited conditions in *C. jejuni* 81116. Regulation of gene expression in response to oxygen concentration is essential due to oxygen-mediated oxidative stress and oxidative stress is also intimately linked with iron
availability in *C. jejuni* (Palyada *et al.*, 2009). As van Mourik *et al.*’s study was mainly focused on the global effect of the RacR/RacS TCS in 81116 but not specifically on the regulatory effect of RacR on *fumC* expression, the involvement of NCTC 11168 RacR in the iron-responsive regulation of *fumC* was therefore assessed.

NCTC 11168 wild-type, *furCj* mutant and a previously constructed *racR* mutant (AB3, A. Brás, unpublished data) were cultured under conditions mimicking the conditions described by Holmes *et al.* (2005, 2.3.1) to allow accurate determination of gene expression mediated by iron and also to verify the FurCj-dependent *fumC* regulation previously observed. Total RNA were purified from each sample and were subjected to northern blot analysis as shown in Figure 3.16. Unexpectedly, the expression of *fumC* was found to be slightly repressed by iron and this finding was in contrast with results previously observed with β-galactosidase assays and northern blot analysis. Despite the slight loading error of RNA purified from *furCj* mutant under low iron conditions, the *fumC* transcriptional profiles in *furCj* mutant background was consistent with the data shown in Figure 3.15. However as a consistent transcriptional profile of *fumC* was not observed in Figure 3.15 and Figure 3.16, the involvement of FurCj in *fumC* regulation (if any) and the mechanism could not be substantiated. The same set of strains cultured for 5 hours in high and low iron liquid media (like for β-galactosidase assay) were also analysed, though the same transcriptional profiles were observed which indicated that the differences in iron- and FurCj-mediated *fumC* regulation was caused by the variations in the initial plate culturing stage between β-galactosidase assay and Holmes’
study and *fumC* regulation is growth condition dependent.

A derepression of *fumC* was observed under high iron conditions in the *racR* mutant background, which indicated NCTC 11168 *fumC* is repressed by RacR under iron replete conditions. A much higher level of transcription was observed for *fumC* under both iron conditions in a *racR* mutant background and under high iron conditions in the *furCj* mutant background, and a potential longer secondary transcript of *fumC* was also observed under high iron conditions in the *racR* mutant background. As contradictory results were obtained for iron- and *FurCj*-mediated *fumC* regulation and the involvements of oxygen and PerR were not determined, a detailed characterisation of *fumC* regulation by *FurCj*, RacR and PerR in response to iron and oxygen was performed and is described in chapter 4.

### 3.3.7 Identification of the transcriptional start site

As promoter activity was not determined from the *chuA* promoter originally cloned in pJMcK6 and contradictory iron-responsive regulations were observed for *fumC*, transcriptional start sites for both *chuA* and *fumC* promoters were identified. Several approaches were investigated in this study including primer extension, RACE and RNA ligation RACE methods (Figure 3.17), and primer extension was initially chosen due to its simplicity (i.e. no cloning involved) over the RACE methods.

Total RNA purified from NCTC 11168 wild-type cultured under low iron conditions
Figure 3.17: Illustration of different approaches used to identify the transcriptional start sites for *chuA* and *fumC*. a. fluorescent primer extension; b. RACE; c. RNA ligation RACE (see text for more detailed descriptions).
was used as a template for *chuA* cDNA synthesis whereas total RNA purified from a *racR* mutant cultured under high iron conditions was used for *fumC* cDNA synthesis as the maximal level of *fumC* transcription was detected. In addition, two *fumC* transcripts with different lengths were also observed in the *racR* mutant background (Figure 3.16) indicating that the *fumC* transcript repressed by RacR is transcribed from an alternative +1 site to the one used for the Fur$_{Cj}$ regulated *fumC* transcript and using total RNA purified from a *racR* mutant would allow the identification of these two +1 sites. The +1 site for the non-iron responsive lipopolysaccharide heptosyltransferase coding *waaC* gene was used as a positive control (Phongsisay and Fry, 2007) and total RNA purified from NCTC 11168 wild-type cultured under normal conditions was used for *waaC* cDNA synthesis.

A fluorescent approach (2.3.6) that had advantages over the conventional radioactive primer extension method in speed and safety was initially applied in which the +1 site could be deduced by determining the length of a FAM-labelled cDNA primer extension product using the Peak Scanner software (Lloyd *et al.*, 2005). *waaC, fumC* and *chuA* cDNA were synthesised with corresponding FEM-labelled primers and the labelled cDNA products were concentrated by ethanol precipitation and resuspended with formamide and size markers prior to analysis in a DNA sequencer (Figure 3.17.a). The length of each cDNA product represented by a peak in Peak Scanner software was measured by comparison with the size markers and the +1 site was then deduced from the primer annealing site using the measured cDNA length. No peaks were initially
detected for both samples and the waaC control and it was likely to be caused by RNA degradation or low concentration of labelled primer. These possibilities were subsequently eliminated as PCR products with the correct sizes were amplified by reverse transcriptase PCR using the same RNA samples and primer concentration. Ethanol precipitation and formamide rehydration steps were also verified using FAM-labelled PCR product and samples with correct sizes were observed with the detectable level as low as 5 ng of DNA. Both verification steps indicated that the proportion of target transcripts from the total RNA purified was too low, therefore although the concentration of the resulting cDNA product was sufficient enough to allow downstream PCR amplification, it was below the detection level by the DNA sequencer. Several cDNA enrichment steps were subsequently included during the cDNA synthesis step with additional enzyme and dNTPs and although cDNA fragments with stronger signals were detected, they were non-specific cDNA products as fragments of the same size were obtained for chuA, fumC and waaC.

Due to the low cDNA concentration encountered with the primer extension approach, RACE (2.3.7) was used instead to allow amplification of the cDNA by PCR. chuA, fumC and waaC cDNA were amplified with standard primers and tagged with a polyG tail using terminal transferase and dGTPs. The tagged cDNA was then amplified using polyG tail- and nested gene-specific primers and the PCR products were cloned into pUC19. The +1 site for each gene was then determined either by direct sequencing of the PCR products or the final constructs (Figure 3.17.b). Sequencing results were not
obtained from direct sequencing of the DNA products and \textit{fumC} DNA could not be cloned into pUC19 despite several attempts. \textit{chuA} and \textit{waaC} cDNA cloned into pUC19 were sequenced and only one putative site was determined for \textit{chuA} though this site was 4 bp downstream from the start codon. As the control \textit{waaC} +1 was not determined, the validity of the site determined for \textit{chuA} could not be substantiated and the \textit{chuA} cDNA amplified and cloned was likely to be a truncated version caused by RNA secondary structure. An additional RNA denaturation step was applied prior to cDNA synthesis but no +1 site was determined for any of the genes.

The last approach investigated was RNA ligation RACE (2.3.7) in which the primary 5’-triphosphate transcripts from the total RNA sample were hydrolysed with TAP and the resulting 5’-monophosphate transcripts were then ligated with RNA adaptors and used as templates for cDNA synthesis. The cDNA samples were amplified using RNA adaptor- and nested gene-specific primers and the PCR products were cloned and the +1 sites were determined as described by standard RACE (Figure 3.17.c.). The RNA ligation RACE has many advantages over the standard RACE as it allows a selection and enrichment of the primary transcripts from processed RNA and the RNA adaptor is more specific than the less defined homo nucleotide adaptor that allows more accurate identification of +1 site by sequencing (Gerhart \textit{et al.}, 2009). \textit{chuA} and \textit{fumC} cDNA were synthesised from the TAP treated and untreated RNA samples and were compared by electrophoresis (Figure 3.18). Enriched \textit{chuA} cDNA product was observed for TAP treated RNA when compared with untreated RNA and two bands containing enriched
Figure 3.18: Comparison of *chuA* and *fumC* cDNA synthesised from the TAP treated and untreated RNA. Lane: 100 bp log ladder (New England Biolabs); lanes 2 and 3: *chuA* cDNA; lanes 4 and 5: *fumC* cDNA. Bands containing the enriched cDNA are circled and two bands circled for *fumC* are termed L and H intensity.
fumC cDNA (L and H) were detected which indicated the presence of two transcripts with difference lengths and hence two potential corresponding +1 sites.

The enriched cDNA samples for chuA, fumC L band and fumC H band were extracted from the gel and used as templates for cDNA amplification. The PCR products were then cloned into pGEM-t easy and the +1 sites were determined by sequencing. A +1 site 51 bp upstream of the chuA start codon and a +1 site 25 bp upstream of the fumC start codon were identified from direct PCR product sequencing and the -10 region for each promoter were subsequently mapped (Figure 3.19). The +1 site identified for fumC was in agreement with previously 454 RNA sequencing data (A. van Vliet, unpublished data), however the same +1 site was determined for PCR products amplified using cDNA extracted from both the L and H bands which indicated that both cDNA samples were not completely separated during electrophoresis. The distance between the chuA +1 site and -10 region was not ideal but the +1 site was acceptable when compared with the 454 RNA sequencing data and was within the optimal range for a C. jejuni 5’ un-translated region (UTR, A. van Vliet, personal communication). The predicted -35 region for the fumC promoter overlaps with the 11th to 16th bases of the fumC FurCj box which indicated that the decreased promoter activities observed for mutated fumC FurCj boxes containing the 13th base change (Figure 3.9) were caused by the disruption of the fumC -35 region. Likewise the predicted chuA -10 region was located just downstream from the 5’ boundary of the chuA promoter originally cloned in pJMcK6 (Figure 3.11) therefore the absence of promoter activity observed for the original construct (Figure
Figure 3.19: Organisation of the \textit{chuA} and \textit{fumC} promoter regions. The +1 site for \textit{chuA} is located 4 bp upstream from the \textit{chuA} \textit{Fur}_{Cj} box while the \textit{fumC} \textit{Fur}_{Cj} box overlaps the \textit{fumC} -35 region. The -35 region, the -10 region, the +1 site and the start codon are coloured in blue, purple, red and green respectively whereas the \textit{Fur}_{Cj} box is underlined.
3.7) was caused by the absence of a functional -35 region.

When constructs containing *chuA* and *fumC* PCR products were sequenced, a second +1 site located 69 bp upstream from the *fumC* start codon was identified for PCR products amplified from the L band. The longer transcript transcribed from this second +1 site was only detected in RNA samples purified from a *racR* mutant cultured under high iron conditions and the level of this longer transcript was much less than the primary transcript detected by northern blot and cDNA synthesis (indicated by the H band) suggesting that the mRNA transcribed from this +1 site was likely to be a weaker secondary transcript repressed only by RacR. Two faults “+1 sites” were also detected for *chuA* as both sites were located downstream from the start codon, therefore they were likely to be caused by truncated cDNA products due to RNA secondary structures. RNA ligation RACE was repeated for *chuA* and the +1 site determined was in agreement with the results originally determined by direct PCR sequencing.

**3.3.8 DNase I footprinting assay**

To determine the location of Fur$_{Cj}$ binding site on the *chuA* and *fumC* promoters and to verify the proposed Fur$_{Cj}$ box sequence, DNase I footprinting assays were performed for the wild-type and mutated *chuA* and *fumC* promoter regions. A DIG-based DNase I footprinting technique was previously used to identify the Fur$_{Hp}$ binding site for *H. pylori sodB* gene though a sequencing ladder was not included in this investigation thus the relative position of the Fur$_{Hp}$ box could not be determined (Ernst *et al.*, 2005). A
possibility of generating a DIG-based sequencing ladder was therefore initially explored and although various sequencing reactions and PAGE conditions were tested, a ladder with clear separation and resolution was not achieved and the more conventional radioactive alternative was therefore used. The method described by Fuangthong and Helmann (2003) was used for this investigation and various buffer concentrations and experimental conditions were optimised (2.2.27).

The *chuA* wild-type promoter (the unextended version without promoter activity) and the *chuA*10/13/19 promoter cloned into pMW10 (pRR15 and pRR22 respectively) were first used as templates to amplify DNA fragments for subsequent manual sequencing and DNase I footprint assays. The primers used for these amplifications were designed so that the Fur$_{Cj}$ box was positioned in the centre of each amplicon that contained part of pMW10 and part of the *chuA* promoter thus allowing a better separation and resolution on the PAGE gel. However to avoid overlooking any additional Fur$_{Cj}$ binding or polymerisation sites further upstream from the *chuA* Fur$_{Cj}$ box, the extended *chuA* wild-type promoter (pRR31) containing the *chuA* and *chuZ* integeneic region up to the *chuZ* Fur$_{Cj}$ box was also tested.

A 31 bp Fur$_{Cj}$ protected region was detected for the *chuA* wild-type promoter (Figure 3.20) resulting from the binding of two Fur$_{Cj}$ dimers, and this mechanism of Fur$_{Cj}$-Fur$_{Cj}$ box interaction was in agreement with the two shift species observed by EMSAs. The Fur$_{Cj}$ protected region was positioned 2 bp downstream from the +1 site and the
Figure 3.20: DNase I footprinting assays for chuA wild-type and chuA10/13/19 promoters along with corresponding sequencing ladders. FurCj concentration used ranged from 0 to 300 nM. Regions representing the -10 region and the FurCj box and the FurCj protected region are indicated by black lines on the left and right sides of the film respectively. The DNA sequence for the FurCj protected region is indicated on the left hand side of the figure with the putative FurCj box highlighted in red.
predicted *chuA* Fur$_{Cj}$ box was completely overlapped by this region. These observations provided direct physical evidence that the iron induced *chuA* repression is mediated by the direct binding of the Fur$_{Cj}$ protein to the *chuA* Fur$_{Cj}$ box located in the promoter region. Such binding will prevent the functional interaction between the RNA pol and the -10 region or prevent the RNA pol from transcribing the DNA template. The Fur$_{Cj}$ protected region was completely abolished for *chuA*10/13/19 as a direct result from the mutational changes in the Fur$_{Cj}$ box which have led to a dramatic decrease in the affinity of Fur$_{Cj}$ binding, an observation in agreement with the corresponding EMSA data (Figure 3.4). No secondary Fur$_{Cj}$ binding or polymerisation sites were detected for the extended *chuA* promoter which indicated that the binding of two Fur$_{Cj}$ dimers is sufficient to block the transcription of *chuA* and the only Fur$_{Cj}$ binding site is associated with the predicted Fur$_{Cj}$ box.

Based on the DNase I footprinting data obtained with the unextended wild-type *chuA* promoter, DNase I footprinting assays were subsequently performed for all the unextended *chuA* mutant promoters cloned into pMW10 (pRR16-22) and are shown in Figure 3.21. The 31 bp Fur$_{Cj}$ protected region was consistently observed for the wild-type and *chuA*10 promoters and a less defined but identifiable protected region was obtained with *chuA*13 and *chuA*19 promoters. Smaller protected regions were observed for the *chuA*10/13 and *chuA*10/19 promoters. The latter case clearly demonstrated that a high affinity Fur$_{Cj}$ binding was not achieved at the 3’ end of the *chuA* Fur$_{Cj}$ box containing the 13$^{th}$ or 19$^{th}$ mutational changes and this smaller protected
Figure 3.21: DNase I footprinting assays for *chuA* wild-type and mutated promoters along with corresponding sequencing ladders. Fur*$_Cj$* concentration used ranged from 0 to 300 nM. Regions representing the -10 region and the Fur*$_Cj$* box and the Fur*$_Cj$* protected region are indicated by black lines on the left and right sides of the film respectively. A smaller protected region seen with *chuA*10/19 is indicated by a white line. The DNA sequence for the Fur*$_Cj$* protected region is indicated on the left hand side of the figure with the putative Fur*$_Cj$* box highlighted in red.
region may reflect the binding of a single dimer. No Fur$_{Cj}$ protected region was observed for chuA13/19 and chuA10/13/19 promoters and the DNase I footprinting results for chuA wild-type and mutated promoters collectively demonstrated the importance of the 13$^{\text{th}}$ and 19$^{\text{th}}$ positions of the Fur$_{Cj}$ box in Fur$_{Cj}$-Fur$_{Cj}$ box interaction and in maintaining the integrity of the Fur$_{Cj}$-Fur$_{Cj}$ box complex and this observation was also consistently observed by EMSAs and β-galactosidase assays.

For the fumC wild-type promoter, no Fur$_{Cj}$ protected region was observed (Figure 3.22). This was expected as EMSA data (Figure 3.4) indicated that Fur$_{Cj}$ protein does not bind to the fumC promoter region. A 31 bp Fur$_{Cj}$ protected region was identified for the fumC10/13/19 promoter immediately upstream from the -10 region and the mutated Fur$_{Cj}$ box was again completely overlapped by this region. When DNase I footprinting assays were carried out for all the fumC mutant promoters (Figure 3.23), a poorly defined Fur$_{Cj}$ protected region was only observed for the fumC19, fumC13/19 and fumC10/13/19 promoters with a high Fur$_{Cj}$ concentration. The clear defined Fur$_{Cj}$ protected region demonstrated for the fumC10/13/19 promoter (Figure 3.22) was not reproduced and the Fur$_{Cj}$ binding activities for fumC10 and fumC13 Fur$_{Cj}$ boxes detected by EMSA were also not observed. The intensity of the DNase I footprinting samples for the fumC wild-type promoter was very low, and this was likely to be caused by the degradation of pRR23 which was used to amplify this promoter. Several experimental repeats were performed though clearer results were not obtained possibility due to Fur$_{Cj}$ degradation. The protected regions obtained the fumC19,
Figure 3.22: DNase I footprinting assays for *fumC* wild-type and *fumC*10/13/19 promoters along with corresponding sequencing ladders. FurCj concentration used ranged from 0 to 300 nM. Regions representing the -10 region and the FurCj box and the FurCj protected region are indicated by black lines on the left and right sides of the film respectively. The DNA sequence for the FurCj protected region is indicated on the left hand side of the figure with the putative FurCj box highlighted in red. N.B. DNase I footprinting samples for both promoters with 100 nM FurCj were loaded the wrong way around due to human error.
Figure 3.23: DNase I footprinting assays for *fumC* wild-type and mutated promoters along with corresponding sequencing ladders. Fur$_{Cj}$ concentration used ranged from 0 to 300 nM. Regions representing the -10 region and the Fur$_{Cj}$ box and the Fur$_{Cj}$ protected region are indicated by black lines on the left and right sides of the film respectively. The DNA sequence for the Fur$_{Cj}$ protected region is indicated on the left hand side of the figure with the putative Fur$_{Cj}$ box highlighted in red.
fumC13/19 and fumC10/13/19 promoters nevertheless demonstrated the crucial roles of the 13th and 19th positions of the FurCj box and these observations were consistent with the increased affinity of FurCj binding toward the mutated fumC FurCj box observed with EMSAs.

3.3.9 Further characterisation of the FurCj box

Although the importance of the 13th and 19th positions of the FurCj box were clearly demonstrated in this study, however mutation of these positions only enables the investigation of the 3’ end of the FurCj box. The 1st and 7th positions of the FurCj box were subsequently analysed as they are in symmetry with the 13th and 19th bases and also contain the base adenine. To keep a consistency in the mutational schemes, A to T mutational changes were applied to these two bases (1T and 7), and as T is also an option for the 1st base of the consensus sequence, an A to C mutational change was also applied (1C). The extended wild-type chuA promoter region was cloned into pUC19 (pRR47) and was used as template for mutagenesis. Promoters containing the mutational changes were then cloned into pMW10 and were used as templates for EMSAs and for β-galactosidase assays. Mutagenesis, EMSAs and β-galactosidase assays described in this section were carried out by T. Caudle under supervision.

No shift pattern variations were observed for chuA1T when compared with the wild-type (Figure 3.24), however for chuA1C, chuA7 and chuA1T/7 mutants, the concentration of upper shift species representing the binding of two FurCj dimers was
Figure 3.24: Standard EMSA of 1.55 fmol labelled chuA promoter regions containing the wild-type and mutated FurCj boxes with the purified FurCj protein. Lanes 1-8: labelled DNA with 0 nM, 25 nM, 50 nM, 75 nM, 100 nM, 125 nM, 150 nM and 175 nM FurCj respectively.
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decreased and was only observed when the Fur$_{Cj}$ concentration was increased to 50 - 75 nM. When EMSA was performed for *chuA*$_{1T/7/13/19}$, the ability for Fur$_{Cj}$ to bind the Fur$_{Cj}$ box was completely abolished indicating the functional importance of these four positions in the Fur$_{Cj}$ box. When compared with the EMSAs data shown in Figure 3.4, the affinity of Fur$_{Cj}$ binding to the wild-type *chuA* Fur$_{Cj}$ box was decreased indicating potential Fur$_{Cj}$ degradation or person to person variation. However due to the comparative nature of this test and the clear observation of two shift species, further Fur$_{Cj}$ purification was not performed.

When the promoter activity for each mutant was tested by β-galactosidase assays (Figure 3.25), various levels of derepression were observed for *chuA$_{1C}$*, *chuA$_7$*, *chuA$_{1T/7}$* and *chuA$_{1T/7/13/19}$* which indicated a decrease in the affinity of Fur$_{Cj}$ binding and also in agreement with the EMSA results. When compared with the wild-type promoter under high iron conditions, the level of repression for *chuA$_{1T}$* mutant was higher than the wild-type which indicated that at this position of the Fur$_{Cj}$ box, T is more favourable for Fur$_{Cj}$ than A. However, a higher level of derepression was observed under high iron conditions for *chuA$_{1T/7}$* when compared with *chuA$_7$*. In additional, although the ability for Fur$_{Cj}$ to interact with the *chuA$_{1T/7/13/19}$* Fur$_{Cj}$ box was completely abolished (Figure 3.24), the *chuA$_{1T/7/13/19}$* promoter was only partially derepressed under high iron conditions (Figure 3.25). These observations were likely to be caused by inaccuracies of the β-galactosidase assays.
Figure 3.25: β-galactosidase assay of the chuA wild-type and mutated promoter regions with controls under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
3.4 Discussions

The Fur protein has been extensively characterised in many bacteria as a global regulator that controls an array of genes ranging from iron acquisition to enzyme metabolism, however the model of Fur-Fur box interaction has been controversial which led to several interpretations of the functional Fur binding site (Lee and Helmann, 2007). The transcriptional response of C. jejuni genes to iron availability and FurCj is also pleiotropic (Palyada et al., 2004; Holmes et al., 2005) and although in vitro binding of FurCj to the promoter region of several members of the FurCj regulon involved in iron acquisition have been previously demonstrated (Holmes et al., 2005; Li, 2005; Ridley et al., 2006; Berg, 2007; Miller et al., 2008; Shearer et al., 2009), the mechanism and the location of FurCj binding were not defined. An early characterisation of the promoter region of FurCj-regulated genes indicated that the FurCj binding site does not correspond well with the defined FurEc box (van Vliet et al., 2002), a fact also reflected by the recent proposed FurCj box consensus sequence (Palyada et al., 2004), which together suggested potential variation in the mechanism of DNA recognition and Fur-DNA interaction between FurCj and FurEc. In this investigation, the 19 bp FurCj box was verified using the chuA and fumC promoter regions and functionally important bases were determined, which revealed an unconserved mechanism of FurCj box recognition by FurCj.

3.4.1 FurCj-FurCj box interaction

Detailed EMSA and Ferguson analysis of natural and synthetic DNA hexamers and
heptamers with FurEc and FurBs have determined two shift species which represented the forming of a DNA-Fur complex with either single or double Fur dimers and the binding of two Fur dimers on each side of the DNA template was associated with the 19 bp Fur box (Lavrrar and McIntosh, 2003; Baichoo and Helmann, 2002). When the wild-type chuA promoter region was analysed by EMSA, two distinct shift species were observed at a low FurCj concentration and the concentration of the upper shift species was significantly increased at a higher FurCj concentration. The same two species were also observed with fumC10/13/19, and these findings indicated that in the functional state, two FurCj dimers recognise and cooperatively bind to the FurCj box and the binding of one dimer observed by EMSAs is likely to be the intermediate state rather than the functional state.

Further characterisation of the chuA promoter region by DNase I footprinting assay has revealed a 31 bp FurCj protected region that is typical of the size observed for E. coli (de Lorenzo et al., 1987). Furpa is approximately 15.2 KDa in size (Vasil, 2007) which is expected to protect 20 bp of DNA in a DNase I footprinting assay (Pohl et al., 2003). As FurCj is approximately 17.5 kDa in size, therefore the formation of a typical 31 bp protected region indicated that the binding of the two FurCj dimers are likely associated with opposite sides of the DNA molecule. FurCj polymerisation was not detected when the extended chuA promoter was analysed indicated the binding of two FurCj dimers is sufficient to repress the transcription of chuA.
Two shift species were also observed when the iron-regulated \textit{p19} operon promoter and \textit{cj0176c} and \textit{cj0177} promoters were analysed by EMSA (Berg, 2007; Miller \textit{et al.}, 2008) which suggested that potentially in \textit{C. jejuni}, Fur\textsubscript{Cj}-dependent transcriptional repression is mediated by the binding of only two Fur\textsubscript{Cj} dimers to the Fur\textsubscript{Cj} box. Fur\textsubscript{Cj} polymerisation was reported when the \textit{chuA-chuZ} intergenic region was investigated where four shift species were observed (Ridley \textit{et al.}, 2006). However as the \textit{chuA} and \textit{chuZ} Fur\textsubscript{Cj} boxes were both within the intergenic region, the multiple shift species were likely to be the various stages of Fur\textsubscript{Cj} binding to the two Fur\textsubscript{Cj} boxes rather than Fur\textsubscript{Cj} polymerisation along the DNA template. Only one shift species was observed when the \textit{cfrA} and \textit{ceuB} promoters and the \textit{p19} operon promoter were investigated (Holmes \textit{et al.}, 2005), and this was potentially caused by the different amount of the Fur\textsubscript{Cj} protein used rather than any functional difference in Fur\textsubscript{Cj} binding between these promoter regions. The relatively small Fur\textsubscript{Cj} concentration used by Holmes \textit{et al.} (2005) was not reproduced in this and Berg’s (2007) studies even when the original protein sample was used suggesting a possible miscalculation of the Fur\textsubscript{Cj} concentration in Holmes \textit{et al.’s} study.

### 3.4.2 The location of Fur\textsubscript{Cj} binding

When DNase I footprinting assays were carried out for the wild-type \textit{chuA} promoter region, a 31 bp protected region that completely overlaps the consensus Fur\textsubscript{Cj} box sequence was observed (Figure 3.20). When the consensus matching Fur\textsubscript{Cj} binding sequence was introduced into the \textit{fumC} promoter (i.e. \textit{fumC10/13/19}), a Fur\textsubscript{Cj} protection
region with the same size was also detected that overlapped the mutated Fur$_{Cj}$ box-like sequence. These observations indicated the validity of the proposed Fur$_{Cj}$ box sequence and this sequence was sufficient to induce Fur$_{Cj}$ binding to functional unrelated DNA sequence (i.e. $fumC$ Fur$_{Cj}$-box like sequence). However due to the limitation of the DNase I footprinting assay, whether the proposed 19 bp sequence is or only contains part of the Fur recognition site cannot be easily established. As this is the only verification of the Fur$_{Cj}$ binding site to date, further detailed DNase I footprinting analyses of other defined Fur$_{Cj}$-repressed promoters are required to allow direct comparisons of experimentally conferred Fur$_{Cj}$ protected regions.

To identify the location of Fur$_{Cj}$ binding with respect to the promoter elements, +1 site for $chuA$ and $fumC$ transcripts were mapped. Initial difficulties such as the low level of target transcripts and RNA secondary structure were encountered with both fluorescent primer extension and conventional RACE methodologies and the +1 site for each gene was eventually identified using RNA ligation RACE which offers high specificity and accuracy. The mapped promoter region of each gene with identified Fur$_{Cj}$ protected regions are summarised in Figure 3.26.

The $chuA$ transcript contains a 51 bp 5’ UTR and the Fur$_{Cj}$ box is located just 4 bp downstream from the +1 site. This finding combined with the Fur$_{Cj}$ protected region identified by DNase I footprint assay demonstrated the iron-repressed regulation of $chuA$ is mediated by the direct binding of Fur$_{Cj}$ to the UTR downstream of the $chuA$
Figure 3.26: chuA and fumC promoter regions investigated in this study. For chuA, the FurCj protected region is located in the 51 bp UTR region while the FurCj protected region for fumC mutant promoter overlaps the putative -35 region of the primary +1 site. In addition, fumC also possesses a secondary +1 site upstream of the primer site indicating that fumC is regulated in response to more than one regulator. The putative FurCj box and the start codon of each gene are coloured in orange and green respectively. Predicted +1, -10 and -35 regions are coloured in red, purple and blue respectively. The observed FurCj protected regions for wild-type chuA and mutated fumC promoters are boxed and the underlined regions were not originally included in pJMck6 and pYL1 constructs.
promoter region and this FurCj-DNA interaction thus prevents the RNA pol from further transcribing the downstream chuA gene. Two fumC +1 sites were identified in a racR mutant background suggesting fumC is under the control of more than one regulator. RacR as well as FurCj are likely to play important role in the regulation of fumC and this is characterised in chapter 4. The mapping of the promoter region of both genes has also provided a physical explanation of the abnormal promoter activities encountered for both genes as the -35 region was missed out from the original chuA promoter clone whereas the fumC -35 region was disrupted by the mutation introduced to the 13\textsuperscript{th} position of the fumC FurCj-box like region. As the C. jejuni genome is very AT-rich and especially in the promoter regions, therefore detailed promoter mapping should always be applied in future promoter or gene regulation studies.

3.4.3 Functional important bases of the FurCj box

The chuA FurCj box is a perfect match to the proposed FurCj box and it has a high affinity of FurCj binding and a strong functional repression in vivo. The FurCj-box like sequence for fumC on the other hand does not bind FurCj and this is likely caused by the three mismatches at the 10\textsuperscript{th}, 13\textsuperscript{th} and 19\textsuperscript{th} positions of this FurCj-box like sequence when compared to the 19 bp consensus sequence. This FurCj box sequence variation was predicted to play an important role in the contrasting FurCj and iron regulation between chuA and fumC. When the mutated chuA and fumC promoter regions were compared by a combination of EMSA, β-galactosidase and DNase I footprinting assays, a dramatic decrease in the ability of FurCj to recognise and bind to the mutated FurCj box was
observed for chuA, in particular with mutants containing 13<sup>th</sup> and/or 19<sup>th</sup> base changes. When these bases were introduced into the fumC Fur<sub>Cj</sub>-box like sequence, the ability to bind Fur<sub>Cj</sub> was partially restored which supported the functional importance of these two bases. Further characterisation of the 5′ end of the chuA Fur<sub>Cj</sub> box also indicated the functional importance of the adenine at the 1<sup>st</sup> and 7<sup>th</sup> positions of the Fur<sub>Cj</sub> box as the Fur<sub>Cj</sub>-DNA interaction was completely abolished for chuA1T/7/13/19 (Figure 3.24).

Based on missing T contact studies of the hexamer model described by Escolar <i>et al.</i> (1998), the AT motif of the GATAAT hexamer was predicted to be an essential recognition element in direct contact with Fur<sub>Ec</sub> as the Fur<sub>Ec</sub>-DNA interaction was abolished or impaired when the thymine at the 3<sup>rd</sup> and 6<sup>th</sup> and -2<sup>nd</sup> and -5<sup>th</sup> (corresponding to the second and fifth adenines on the top strand) were substituted with uracil (Escolar <i>et al.</i>, 1998). Using UV crosslinking and mass spectrometry, Tyr55 of the Fur<sub>Ec</sub> protein and the thymines at the 15<sup>th</sup> and 16<sup>th</sup> position of the Fur<sub>Ec</sub> box were demonstrated to be involved in binding and the TT motif is also presented in the <i>P. aeruginosa</i> and <i>B. subtilis</i> Fur boxes (Tiss <i>et al.</i>, 2005). Furthermore, mutational studies of the Fur<sub>Bs</sub>, PerR<sub> Bs</sub> and Zur<sub> Bs</sub> boxes have also demonstrated that the 5<sup>th</sup> and 6<sup>th</sup> bases of the heptamer model are important for the discrimination of their target site by Fur<sub>Bs</sub>, PerR<sub> Bs</sub> and Zur<sub> Bs</sub> as small changes in these sites were sufficient to alter binding preferences amongst the three proteins (Fuangthong and Helmann, 2003). When a five base AT-rich region of the Fur<sub>Ec</sub> binding site in the <i>E. coli</i> fepDGC-entS promoter region was substituted with a GC-rich sequence, the ability of Fur<sub>Ec</sub> binding was inhibited.
However when the important AT bases were introduced into the Fur$_{Ec}$ box to enhance Fur$_{Ec}$ binding, dramatic improvements in the affinity of Fur$_{Ec}$ bind to this sequence were not observed which have led to the suggestion that although certain bases in the Fur box may a play more important role than others in determining Fur binding affinity, the overall architecture of the binding site is more critical in determining how well Fur recognises the Fur box (Lavrrar et al., 2002).

As each study has its own interpretation of the Fur box, a consensus model of base-dependent sequence recognition by Fur and subsequent protein-DNA interaction could not be easily determined, therefore it’s difficult to conclude the functional importance of the adenine at the 1st, 7th, 13th and 19th bases of the Fur$_{Cj}$ box by correlating with previous observations in other organisms. When EMSAs were carried out for chuA13 and chuA19 (Figure 3.5), although the concentration of unbound DNA was slightly increased at a higher Fur$_{Cj}$ concentration, two shift species were clearly observed with as little as 25 nM Fur$_{Cj}$. On the other hand, the higher shift species was only clearly observed for chuA1C and chuA7 at a Fur$_{Cj}$ concentration between 50-75 nM therefore potentially suggesting that adenine at the 13th and 19th positions may play important roles in stabilising the Fur$_{Cj}$-Fur$_{Cj}$ box complex or maintaining the important overall binding site architecture that allows high affinity Fur$_{Cj}$ binding. The 1st and 7th bases on the other hand are likely to play roles in initial Fur$_{Cj}$ recognition. In addition, because the Fur$_{Cj}$ protected region determined for chuA has suggested that Fur$_{Cj}$ binds to the Fur$_{Cj}$ box on both sides of the DNA, therefore some mutational effects observed for
the adenine bases may be caused by the indirect alteration of the four thymine bases on
the bottom stand of the FurCj box and as the precise model of FurCj box recognition by
FurCj was not determined, the potential importance of these bases could not be
substantiated. In order to understand the precise mechanism of FurCj-FurCj box
interaction, it would require the determination of a FurCj-DNA complex crystal structure,
however such study has not yet been performed for any of the Fur orthologues.

3.4.4 Interpretation of the FurCj box

Since the identification of the 19 bp FurEc box consensus sequence (de Lorenzo et al.,
1987), the interpretation of a functional pattern of the Fur box has been the centre of
attention rather than the consensus sequence itself. However it should be noted that an
exact match to the consensus sequence has not been identified in E. coli to date, 14 to
15 bp matches out of the 19 bp are rather more typical and as little as 11 bp matches has
been observed for the tonB FurEc box (Newman and Shapiro, 1999). Therefore
considering FurCj only shares 40% identity with FurEc, it’s not all too surprising that the
putative FurCj box proposed by van Vliet et al. (2002) and Palyada et al. (2004) matched
poorly with the FurEc box.

The NAT trimer motif used to identify a FurCj binding site for C. jejuni (van Vliet et al.,
2002) has also been previously used to successfully determine FurHp binding site for the
closely related H. pylori (A. van Vliet, unpublished data). A consensus sequence of
AATAATNNTNA has also been proposed as the FurHp binding site using a
bioinformatic approach though further experimental analysis of the proposed site were not conducted (Merrell et al., 2003). This divergence in Fur box sequence between the closely related *C. jejuni* and *H. pylori* and *E. coli* is also extended to bacteria from other taxonomic groups. The Fur$_{BJ}$ binding site of *B. japonicum* for example contains three direct hexamer repeats (Friedman and O’Brain, 2003) whereas a consensus of WTGAAAATNATTTTCAW (W represents A or T) was observed for members of the δ-proteobacteria group (Rodionov et al., 2004). Although bioinformatic tools have been generally used to determine the functional Fur binding site that resembles the Fur$_{Ec}$ box, most of the experimental studies with detailed DNase I footprinting analysis were only limited to *E. coli*, *B. subtilis* and *P. aeruginosa*, therefore the Fur-DNA recognition mechanism described for these organisms may not represent all the bacteria.

The Fur$_{Cj}$ box is such an example that although the mechanism of Fur$_{Cj}$-DNA interaction determined in this study closely resembles of that described for *E. coli* and *B. subtilis*, none of the Fur box interpretations proposed fits well with this sequence. A similar mutational study was carried out for the *C. jejuni* p19 operon Fur$_{Cj}$ box where several bases were altered in order to determine bases that are functionally important in Fur$_{Cj}$-Fur$_{Cj}$ box interaction (Berg, 2007). When the 1$^{st}$, 5$^{th}$, 6$^{th}$, 7$^{th}$, 9$^{th}$, and 16$^{th}$ base positions that contained the match bases were mutated and analysed by EMSAs, no significant decreases in the affinity of Fur$_{Cj}$ binding were determined. Surprisingly when the 19$^{th}$ base that contains a mismatch thymine was mutated to adenine to resemble the consensus sequence, a decrease in the ability of Fur$_{Cj}$ to bind the p19
operon $\text{Fur}_{CJ}$ box was observed that was in contrast to this current investigation (Berg, 2007). As DNase I footprinting assays were not performed in Berg’s study, the result variations in particular at the 1$^{\text{st}}$, 7$^{\text{th}}$ and 19$^{\text{th}}$ positions of the $\text{Fur}_{CJ}$ box were likely due to the inaccurate prediction of the $p19$ operon $\text{Fur}_{CJ}$ box. However both investigations have indicated that most of the bases in the $C.\text{jejuni}$ $\text{Fur}_{CJ}$ box may not play important roles in direct $\text{Fur}_{CJ}$-$\text{Fur}_{CJ}$ box interaction and rather is the AT-rich $\text{Fur}_{CJ}$ box sequence that maintains the overall architecture of the $\text{Fur}_{CJ}$ binding site that allows the $\text{Fur}_{CJ}$ protein to bind the target site with various affinities. In another words, the proposed 19 bp sequence might not be the consensus $\text{Fur}_{CJ}$ box sequence per se, but rather allows the promoter region to form an overall architecture that is in favour of high affinity $\text{Fur}_{CJ}$ binding. Further DNase I footprinting analysis of defined $\text{Fur}_{CJ}$ regulated promoters are needed to support this hypothesis and to refine or redefine the consensus $\text{Fur}_{CJ}$ box sequence.

The importance of individual bases in the $\text{Fur}_{CJ}$ box also should not be overlooked as important bases are likely required for $\text{Fur}_{CJ}$ to discriminate the true $\text{Fur}_{CJ}$ regulatory site from the overall AT-rich genome. When the $\text{chuA10/19}$ was analysed with DNase I footprinting assay, a much smaller $\text{Fur}_{CJ}$ protected region of ATTATGA was observed (Figure 3.21). This region is probably too small to be caused by the binding of one $\text{Fur}_{CJ}$ dimer, but this sequence is likely to play an important role in $\text{Fur}_{CJ}$ recognition. Interestingly this region is enclosed by the 1$^{\text{st}}$ and 7$^{\text{th}}$ adenine that are also determined to play critical roles in $\text{Fur}_{CJ}$-DNA recognition, therefore the region enclosed by the 13$^{\text{th}}$
and 19th adenine may also be an important FurCj binding site perhaps for the second FurCj dimer that binds the bottom strand of the FurCj box. Further investigation of the exact role of each important adenine and the region enclosed by them are needed by mutational studies on the refined C. jejuni FurCj box.

In addition, although a clear correlation between the in vitro FurCj binding and the in vivo transcriptional FurCj-repression was observed for the chuA wild-type promoter. The altered affinity of FurCj binding determined in vitro for each mutated promoter may not be accurately reflected or even physiological relevant in vivo. For example, although FurCj-binding (despite a relative low affinity compared to chuA) has been clearly observed for fumC10/19 by EMSAs and DNase I footprinting assays (Figures 3.6 and 3.23), its expressions was not affected under high iron conditions in β-galactosidase assays. The ability for FurCj to bind the chuA1T/7/13/19 promoter was complete abolished (Figure 3.24), however its expression was only partially derepressed under iron-rich conditions. These result discrepancies were largely due to the drawbacks of the multi-copy plasmid reporter system used in this study. The large pMW10 copy number meant that in vivo, the FurCj binding site cloned into each plasmid was essentially diluting the intercellular FurCj level, thus the expression of the promoter on each plasmid was not reflected accurately by the overall outcome. Furthermore although the sequences of the NCTC 11168 furCj and the 480 furCj are almost identical, both FurCj proteins may not behave in an identical manner. As the 480 furCj mutant constructed (RR1) was also concluded not suitable for expression studies of strong FurCj-regulated
promoters (such as chuA), single copy chromosomal reporter systems in the NCTC 11168 wild-type and furCj mutant backgrounds are therefore more appropriate and accurate, and should be applied for future studies of C. jejuni gene regulation.
Chapter 4: The iron- and oxygen-responsive regulation of \textit{fumC}

4.1 Introduction

4.1.1 Regulation of fumarase genes in \textit{E. coli}

Fumarase is a key component of the TCA cycle that catalyses the interconversion of fumarate to L-malate and it also participates in the reductive conversion of oxaloacetate to succinate during anaerobic growth. \textit{E. coli} contains three fumarase genes, \textit{fumA} (Guest and Roberts, 1983), \textit{fumB} and \textit{fumC} (Guest \textit{et al.}, 1985), which encode biochemically distinct enzymes. \textit{fumA} and \textit{fumB} share a high degree of homology and both express class I fumarase that form thermolabile dimers. \textit{fumC} is transcribed from its own and upstream \textit{fumA} promoters (Park and Gunsalus, 1995), however it does not show any homology with either \textit{fumA} and \textit{fumB} and the gene product is a class II fumarase that forms thermostable tetramers (Woods \textit{et al.}, 1988). Both FumA and FumB are iron-dependent hydrolases whereas FumC does not require iron for its function (Ueda \textit{et al.}, 1991). The expression of each fumarase gene has been demonstrated to respond to an array of environmental stimuli such as oxygen, iron and carbon sources in a hierarchical fashion and is controlled in overlapping regulatory networks by several global regulators.

The oxygen responsive regulation of fumarase genes is mediated by the global oxygen limitation and oxidative stress regulators Fnr, ArcA and SoxS proteins (Park and Gunsalus, 1995; Tseng, 1997). FumA is the major fumarase under microaerophilic conditions as FumA activity peaks between 1-2% oxygen, but is constitutively
synthesised at a basal level under anaerobic conditions; the partial repression of \(fumA\) during anaerobic growth is mediated by ArcA (Park and Gunsalus, 1995; Tseng \textit{et al.}, 2001). FumB activity is predominantly detected during anaerobic growth where the \(fumB\) gene is positively regulated by both the ArcA and Fnr proteins (Tseng, 1997; Tseng \textit{et al.}, 2001). FumB acts as an alternative fumarase for FumA under anaerobic conditions as it has a higher affinity for L-malate than for fumarate which allows it to mediate the conversion of L-malate to fumarate during anaerobic respiration (Woods and Guest, 1988). FumC activity remains at a basal level under anaerobic and microaerophilic conditions and this partial repression is facilitated by the interaction of ArcA with both the \(fumA\) and \(fumC\) promoters and also by Fnr through the \(fumA\) promoter (Park and Gunsalus, 1995; Tseng \textit{et al.}, 2001). Under high oxygen concentrations where iron-dependent FumA and FumB are inactivated due to oxidative stress, \(fumC\) is activated by SoxS to allow an elevation of FumC activity that maintains the TCA cycle flux (Park and Gunsalus, 1995; Tseng \textit{et al.}, 2001). In addition, \(fumC\) is also directly activated by Rob, a transcriptional regulator functionally related to SoxS and the multiple antibiotic resistance regulator MarA (Ariza \textit{et al.}, 1995; Jair \textit{et al.}, 1996).

The expression of \(fumA\) and \(fumB\) that encode iron-dependent fumarases is activated by iron and Fur\(_{Ec}\) (Park and Gunsalus, 1995; Tseng, 1997). Although the mechanism of \(fumB\) activation by Fur\(_{Ec}\) is unclear, the positive regulation of \(fumA\) by Fur\(_{Ec}\) is mediated by the intermediate sRNA RhyB (Massé and Gottesman, 2002). The iron-independent
fumarase gene *fumC* is induced under iron limitation and oxidative stress conditions and the activation of the *fumC* promoter is mediated by SoxS (Park and Gunsalus, 1995). Interestingly in a *hemA* mutant strain which is defective in haem synthesis, the expression of all the fumarase genes is down regulated though the regulatory mechanism is unclear (Park and Gunsalus, 1995; Tseng, 1997).

The expression of *fumA* and *fumC* is also affected by the available carbon substrates as the expression of both genes is repressed when glucose is used as the sole carbon source (Park and Gunsalus, 1995) and FumA and FumC activity is elevated when acetate is used instead of glucose (Tseng *et al.*, 2001). The catabolite regulation of *fumA* and *fumC* occurs primarily from the *fumA* promoter (Park and Gunsalus, 1995) and involves Cap-cAMP complexes as Cap binding sites have been proposed within the *fumA* promoter region (Woods and Guest, 1988). Cap-cAMP complexes are also involved in the lower growth rate induction of *fumA* and *fumC* as the growth rate-dependent fumarase activity is abolished in a *cyA* mutant strain which lacks the ability to synthesise cAMP, however this growth rate-dependent catabolite control of both genes is independent from carbon source utilisation (Tseng *et al.*, 2001).

The expression of both *fumA* and *fumC* is also affected by the cell growth phase where an increase expression of *fumC* through different growth phases is correlated with the growth phase-dependent expression of the global regulator gene rpoS and RpoS is predicated to play more significant role than SoxR in the regulation of *fumC* when cells
enter stationary growth phase (Rahman et al., 2008). DNA superhelicity has also been reported to affect fumB expression as fumB is negatively regulated in a topA mutant strain that contains highly negatively supercoiled DNA (Tseng, 1997).

4.1.2 \textit{fumC} regulation and the function of \textit{FumC} in other bacteria

\textit{P. aeruginosa} processes at least two fumarase genes and the level of fumarase activity is greater in mucoid bacteria than nonmucoid cells (Hassett et al., 1997b). The \textit{fumC} gene is located in a four-gene operon that also contains the \textit{sodA} gene which encodes a manganese-cofactored superoxide dismutase that protects the cell under oxidative stress (Hassett et al., 1997a). \textit{fumC} is co-transcribed with \textit{sodA} and the operon is negatively regulated by iron and Fur \textit{Pa}. Two overlapping Fur\textit{Pa} boxes were identified upstream from the first gene in the operon and interaction with both \textit{P. aeruginosa} and \textit{E. coli} Fur has been demonstrated within this region (Polack et al., 1996; Hassett et al., 1997a; Ochsner et al., 2002). The production of aliginate, a viscous exopolysaccharide, is greatly reduced in a \textit{fumC} mutant strain, which indicates the importance of FumC in \textit{P. aeruginosa} pathogenicity as the production of aliginate leads to the deterioration of the condition of cystic fibrosis patients (Hassett et al., 1997b). In \textit{Pseudomonas fluorescens}, the enzymatic activity of FumA is severely inhibited by the toxicities of aluminium and gallium while FumC displays an increase in expression and activity under these metal induced stress conditions (Chenier et al., 2008).

In \textit{V. cholerae}, \textit{fumC} as well as \textit{sodA} is repressed by iron and Fur\textit{Vc} and the putative
Fur$_{Vc}$ box sequence has been identified for both genes (Mey et al., 2005). sRNA IG-524 is encoded in the opposite strand of the *H. pylori* *fumC* gene and is proposed to participate in *fumC* regulation in a sequence complementary manner (Xiao, et al., 2009). A significant reduction in virulence for mice was demonstrated in a *fumC* defective strain of *L. monocytogenes* and the ability for the mutant to grow in cultured mouse phagocytes is abolished (Gahan and Hill, 2000). Phylogenetic analysis of the *N. meningitides* *fumC* indicates that it belongs to a unique cluster that is separated from those of other genera (Goh et al., 2005) and a virulent clone of *N. meningitides* serotype 2a carrying a mutation in the *fumC* gene was responsible for the outbreak and spreading of meningococcal group C disease in Canada during the late 1980s (Ashton et al., 1991).

Both *fumA* and *fumC* genes in *Brucella abortus* are functionally redundant and the FumC protein co-localises at the old pole of *B. abortus* with an essential cytoplasmic histidine kinase PdhS (Mignolet et al., 2010).

### 4.1.3 Regulation of *fumC* in *C. jejuni*

The *C. jejuni* cj1364 gene has been identified through genome analysis as a homologue to *fumC* of *E. coli* and *H. pylori*. Fumarase activity in *C. jejuni* is not affected by oxygen concentration and cell age, and as no gene orthologous to *fumA* and *fumC* are identified, FumC is proposed to be the only fumarase in *C. jejuni* (Smith et al., 1999). Transcriptomic analysis of the *C. jejuni* NCTC 11168 Fur$_{Cj}$ regulon indicated that unlike *P. aeruginosa* and *V. cholerae*, *C. jejuni* *fumC* is positively regulated by iron and Fur$_{Cj}$ (Holmes et al., 2005) and although a Fur$_{Cj}$ box-like sequence was identified upstream of
the gene, the *fumC* promoter does not bind Fur<sub>Cj</sub> and therefore if it’s Fur<sub>Cj</sub>-responsive this is likely to be indirect.

The transcriptional regulation of *fumC* by iron and Fur<sub>Cj</sub> was investigated using β-galactosidase and northern blot assays as part of this study to characterise the *C. jejuni* Fur box<sub>Cj</sub>. Under the growth conditions routinely used for *C. jejuni* based β-galactosidase assays (2.2.20), which involve the setting up of *C. jejuni* cultures on high or low iron MHA plates prior to inoculating into MHB supplemented with 40 μM FeSO<sub>4</sub> or 20 μM Desferal respectively and incubated for 16 hours, the expression of *fumC* at a basal level was detected under iron limited conditions and the level of transcription was significantly elevated in the presence of additional iron but independent of Fur<sub>Cj</sub> (Figure 3.10 and 3.15). A *C. jejuni* 480 *fur<sub>Cj</sub>* mutant was also constructed in order to verify the iron and Fur<sub>Cj</sub> responsive regulation of *fumC*, though the *fur<sub>Cj</sub>* mutant was determined as an unsuitable host for the pMW10-based reporter system due to the constant expression of the lacZ gene and a high antibiotic concentration required for maintaining the reporter construct.

During the course of this study, a microarray based investigation of the *C. jejuni* strain 81116 has been published which demonstrated that the expression of several energy metabolism genes are altered by the RacR-RacS TCS in response to oxygen (van Mourik *et al.*, 2009). The response regulator RacR has been previously demonstrated in 81116 to affect the cell growth in a temperature-dependent manner and the inactivation
of racR has led to a reduced ability for the bacteria to colonise the alimentary tract in chickens (Brás et al., 1999), which is presumably caused by the inability for the mutant to alter its energy metabolism in response to the low oxygen environment encountered in the chicken gut (van Mourik et al., 2009). The expression of 81116 fumC as well as other metabolic genes such as the aspartase gene aspA was up regulated in the RacR mutant under oxygen limited conditions and a direct binding of the purified RacR protein in the presence of RacS-HK and ATP was demonstrated for the aspA promoter (van Mourik et al., 2009). In E. coli, fumC and aspA genes share a high degree of homology and their gene products are also structurally related and are predicated to use analogous chemical mechanisms to fulfil their biological functions (Woods et al., 1986).

The involvement of FurCj and RacR in the iron responsive regulation of NCTC 11168 fumC was reevaluated by northern blot assays using growth conditions described by Holmes et al. (2005, 2.3.1), which involve the setting up of C. jejuni cultures on plain MHA plates prior to inoculating into MHB supplemented with 40 μM FeSO₄ or 20 μM Desferal respectively and incubated for 10 hours. Under these conditions, fumC was unexpectedly repressed by iron and the repression was mediated by RacR. The repression of fumC by iron was also facilitated by FurCj and PerR_Cj though the exact mechanism was unclear (Figure 3.16).

4.2 Aims

Like E. coli, the fumC regulatory system in C. jejuni is complex and involves
overlapping regulatory networks that respond to changes in environmental iron and oxygen concentrations. However the preliminary data illustrated in chapter 3 suggested that unlike in *E. coli* where iron and oxygen responsive regulation is mediated by separate global regulators, RacR and Fur*{Cj}* and possibly PerR*{Cj}* are likely to play important roles in the co-regulation of *C. jejuni* *fumC* in response to both stimuli. To confirm this hypothesis, the first aim was therefore to comprehensively re-examine the expression of NCTC 11168 *fumC* in response to iron and oxygen concentrations and the co-involvement of Fur*{Cj}, RacR and PerR*{Cj}* in this regulatory network using northern blot analyses. In addition, although the preliminary data from this investigation and previous microarray study (van Mourik *et al.*, 2009) indicated that the expression of *fumC* in both NCTC 11168 and 81116 is negatively regulated by RacR, the exact mechanism of RacR regulation is unknown. Thus the second aim was to investigate the *in vitro* interaction between the purified recombinant RacR and the *fumC* promoter by EMSAs and DNase I footprinting analyses. Lastly, unlike *E. coli*, which possesses three fumarases, the *C. jejuni* *fumC* gene product was predicated to be the only functional fumarase in *C. jejuni* (Smith *et al.*, 1999). To test this prediction, the last aim was to construct a NCTC 11168 *fumC* mutant and to determine the functional importance of *fumC* by investigating the impact of this gene deletion on the mutant’s *in vitro* growth and cellular fumarase activities.

**4.3 Results**

*4.3.1 Iron-responsive regulation of 81116 racR*
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Using 2-D gel electrophoresis, previous work (Brás et al., 1999) compared the expression of 81116 RacR under different iron conditions and found that RacR does not respond to changes in iron levels. However as the expression of fumC is repressed by RacR and potentially by iron, the expression of 81116 racR in response to changing iron concentrations was first examined by northern blot analysis in order to reveal a clear picture of this regulatory process.

In other experiments, C. jejuni liquid cultures were routinely cultured in 5 ml volumes in 15 ml centrifuge tubes and incubated under 50 rpm agitation. However due to the large volume of cells required to extract a sufficient amount of total RNA for the northern blot analysis, multiple culture tubes were required for each sample; this was determined to be both inaccurate and impractical. To achieve the maximum cell recovery while ensuring that all samples have reached to the same growth phase, C. jejuni liquid cultures were scaled up and grown in 250 ml tissue culture flasks with gas permeable caps. A growth level difference between using the tubes and flasks was observed though this variation was corrected with 70 rpm agitation (results not shown) and the total RNA purified from cells grown in tissue culture flasks were used for all the northern blot assays described in this chapter.

Total RNA purified from wild-type 81116 cultured under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions were analysed by northern blot analysis (Figure 4.1) using 81116 racR- and 16S-specific probes (2.3.4). A transcript of approximately
Figure 4.1: Northern blot analysis of the iron-responsive regulation of 81116 racR. Total RNA samples were purified from 81116 wild-type strain cultured under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions and were hybridised with 81116 racR- and NCTC 11168 16S-specific probes.
1.6 kb was detected indicating 81116 racR and racS are co-transcribed. A dramatic increase in the level of racR/racS transcript was observed under high iron conditions which demonstrated that although the cellular 81116 RacR levels are iron independent, the transcription of racR and indeed racS is iron-induced.

4.3.2 Iron- and oxygen-responsive gene regulation

To comprehensively characterise the iron- and oxygen-responsive regulation of NCTC 11168 fumC and racR and the involvement of FurCj, PerRcj and RacR, total RNA purified from NCTC 11168, AV17 (furCj mutant), AV63 (perRcj mutant) and AB3 (racR mutant) cultured under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions under 3%, 7% and 11% oxygen were examined by northern blot analysis using fumC- and racR-specific probes. The 7% oxygen was routinely used for culturing C. jejuni cells in this study, and the 3% and 11% oxygen were chosen to study the effect of changing oxygen concentrations on gene expression while maintaining a viable cell growth. A fur perR double mutant strain (AV67) was initially tested, however this strain was not included in the northern blot analyses due to poor growth and RNA recovery rate. All the RNA samples used to determine the expression of fumC and perR were re-hybridised with chuA- and chuZ-specific probes respectively as positive controls to demonstrate response to iron.

Under 3% oxygen (Figure 4.2), both chuA and chuZ were repressed by iron and FurCj and the expression profiles of both genes were consistent with the previous
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![Northern blot analysis of the iron-responsive regulation of *fumC*, *racR*, *chuA* and *chuZ* under 3% oxygen.](image)

Figure 4.2: Northern blot analysis of the iron-responsive regulation of *fumC*, *racR*, *chuA* and *chuZ* under 3% oxygen. Total RNA samples were purified from NCTC 11168 wild-type, AV17, AV63 and AB3 strains cultured under 3% oxygen under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions and were hybridised with *fumC*-*, racR*-*, chuA*-*, chuZ*- and 16S-specific probes.
characterisation of iron and Fur\textsubscript{Cj} regulation of \textit{chuA} and \textit{chuZ} (Palyada et al., 2004; Holmes et al., 2005; Ridley et al., 2006). The expression of \textit{chuA} and \textit{chuZ} however were noticeably decreased in AV63 under iron-limited conditions suggesting the potential involvement of PerR\textsubscript{Cj} in the regulation of both genes. Interestingly, the size of the \textit{chuA} transcript determined was approximately 1.6 kb indicating that \textit{chuA} is primarily transcribed as a monocistronic mRNA rather than a multicistonic RNA which also contains the RNA of downstream \textit{chuBCD} genes. \textit{racR} was induced by iron and this observation was consistent with 81116 \textit{racR}, however this iron-responsive regulation was independent from Fur\textsubscript{Cj} and PerR\textsubscript{Cj}. \textit{fumC} was expressed under both iron conditions, though the difference in the level of expression between both conditions was small and could not be substantiated without quantitative analysis. \textit{fumC} was derepressed under high iron conditions in AV17 and AB3, however as \textit{racR} was not regulated by Fur\textsubscript{Cj} and Fur\textsubscript{Cj} binding to the \textit{fumC} promoter region was not observed (Figure 3.4), the exact involvement of Fur\textsubscript{Cj} in the iron-induced \textit{fumC} repression under a low oxygen concentration was unclear.

Under 7% oxygen (Figure 4.3), the expression profiles for \textit{chuA} and \textit{chuZ} showed same patterns with 3% oxygen which indicated that under microaerobic conditions, the expression of \textit{chuA} and \textit{chuZ} was not affected by changing oxygen conditions from 3% to 7%. The Fur\textsubscript{Cj} and PerR\textsubscript{Cj}-independent iron responsive regulation of \textit{racR} was also observed though the level of \textit{racR} expression was much lower when compared to that observed with 3% oxygen. The up regulation of \textit{racR} under low oxygen conditions is in
Figure 4.3: Northern blot analysis of the iron-responsive regulation of fumC, racR, chuA and chuZ under 7% oxygen. Total RNA samples were purified from NCTC 11168 wild-type, AV17, AV63 and AB3 strains cultured under 3% oxygen under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions and were hybridised with fumC-, racR-, chuA-, chuZ- and 16S-specific probes.
agreement with van Mourik et al. (2009) and supports the proposal that RacR is an important regulator controlling low oxygen induced adaptive gene expression.

The level of *fumC* expression at 7% oxygen under both iron limited and replete conditions were consistent with 3% oxygen. A derepression of *fumC* was observed in AB3 and a higher level of *fumC* derepression was also detected in AV17 under high iron conditions when compared with the same conditions at 3% oxygen. These observations suggest that Fur<sub>Cj</sub> plays a more significant role in *fumC* repression under 7% oxygen (an optimal level for cell growth) than RacR, which itself is down regulated. Interestingly, the level of *fumC* transcription was reduced under low iron conditions in both AV17 and the AV63 which suggests that both Fur<sub>Cj</sub> and PerR<sub>Cj</sub> also directly or indirectly influence the expression *fumC* under low iron and optimal oxygen conditions. Additionally, the expression profile of *fumC* was consistent with the northern blot analysis using cells cultured in tubes (Figure 3.16) therefore indicated that the expression of at least *fumC*, *racR*, *chuA* and *chuZ* were not altered by using tissue culture flasks under the increased agitation.

Under 11% oxygen (Figure 4.4), the expression profiles of *chuZ* were unaffected by the elevated oxygen concentration, however the iron-responsive regulation of *racR* was completely abolished in AV63. Derepression of *chuA* was also observed under high iron conditions in AV63 and AB3 which suggests that under elevated oxygen and iron concentrations PerR positively regulates the expression of *racR* and the gene product of
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**Figure 4.4: Northern blot analysis of the iron-responsive regulation of *fumC*, *racR*, *chuA* and *chuZ* under 11% oxygen.** Total RNA samples were purified from NCTC 11168 wild-type, AV17, AV63 and AB3 strains cultured under 3% oxygen under high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions and were hybridised with *fumC*, *racR*, *chuA*, *chuZ* and *16S*-specific probes. N.B. Due to the relative shorter half-life of the *chuA* transcribes, the expression of *chuA* under low iron conditions in both AV17 and AV63 were detected in the form of smears on the bottom half of the blot (results not shown).
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which acts in turn as a repressor for *chuA*.

The level and patterns of *fumC* expression in the wild-type and AB3 in 11% oxygen were similar to with 3% and 7% oxygen indicating that although *fumC* is differently regulated by several regulators in response to oxygen and potentially iron, the overall level of *fumC* transcript remains constant. An increased level of *fumC* derepression was observed in the AV63 under high iron conditions that presumably was caused by the abolished *racR* expression. A greater decline of *fumC* expression was detected in both AV17 and AV63 under iron limited conditions which further demonstrates that *fumC* is also positively influenced under low iron conditions by FurCj and PerR_Cj, especially under oxidative stress conditions or indirectly regulated by another regulator.

4.3.3 Expression and purification of recombinant NCTC 11168 RacR and RacS-HK

In order to analysis the interaction between RacR and the *fumC* promoter *in vitro*, His6-tagged NCTC 11168 RacR and RacS-HK were expressed and purified in *E. coli*. The entire *racR* gene was cloned into an IPTG-induced expression vector. In order to avoid inclusion body formations during the protein purification stage of RacS, only a section of the *racS* gene coding the predicted histidine kinase domain but not the transmembrane sensor domain was cloned (Figure 4.5). The resulting constructs pRR46 and pRR53 were electroporated in the *E. coli* expression strain Rosetta and various parameters in the expression and purification processes for both proteins were optimised. Optimisation of the expression conditions found that 3 hours induction by 0.8 mM
Figure 4.5: The predicted transmembrane domains (red) and the histidine kinase domain (blue) of NCTC 11168 RacS. Sequence encoding the underlined regions with an added start codon was cloned into the expression vector pLEICES-01 to form pRR53.
IPTG at 37 °C were sufficient to allow expression of both proteins while 80 mM imidazole in the binding (washing) buffer was used in the purification stage to allow the maximum recovery of both proteins with minimum nonspecific protein contaminations (2.4.2).

Single bands were observed when column purified RacR and RacS-HK were analysed by SDS-PAGE (Figure 4.6) and their sizes were consistent with the predicted sizes of tagged RacR (25.6 KDa) and the histidine kinase domain of RacS (28.3 KDa). High levels of protein expression were observed for both proteins during induction trials (results not shown) however the concentration of column purified protein was relative low, particularly with RacR. This was caused by the high imidazole concentration in the column binding buffer used during column purification in order to eliminate co-purification of nonspecific host proteins. A host ribosomal small subunit protein (verified by peptide mass fingerprinting) was co-purified in small quantities with both RacR and RacS-HK. Higher concentrations of imidazole to eliminate the contaminating protein were not used to avoid compromising the protein yield of RacR and RacS-HK.

When both proteins were concentrated and buffer exchanged to buffer B (van Mourik et al., 2009) to allow subsequent analysis in phosphorylation and EMSA assays, the nonspecific host protein was eliminated from both samples presumably due to degradation. The identities of both proteins were verified by peptide mass fingerprinting and western blot analysis; no protein aggregation or degradation was detected for either
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Figure 4.6: SDS-PAGE analysis of the purified recombinant RacR (25.6 KDa, a) and RacS-HK (28.3 KDa, b) proteins. Lane 1: PageRuler prestained protein ladder; lane 2: pre-column sample; lane 3: flow-through sample; lane 4: mid-wash sample; lanes 5-13: elution samples 1-9 respectively.
4.3.4 Phosphorylation of RacR by RacS-HK

Autophosphorylation of 81116 RacS-HK and the in vitro transfer of phosphate between RacS-HK and RacR proteins have been previously demonstrated (van Mourik et al., 2009), therefore phosphorylation assays (2.4.11) were performed to verify the functionalities of the newly purified NCTC 11168 RacR and RacS-HK. RacR was added to a RacS-HK/ATP γ-32P solution and aliquots of the mixture were taken at the time indicated in Figure 4.8 and analysed by SDS-PAGE. At time 0 where only RacS-HK and ATP γ-32P were present in the reaction, autophosphorylation of NCTC 11168 RacS-HK was detected which indicated the histidine kinase domain predicted was functional. Due to high radioactivity levels and the marginal size difference, RacR and RacS-HK were not separated very well and a band doublet containing the two proteins was formed between 0.25 and 4 minutes which demonstrated the phosphorylation of RacR by RacS-HK. From 8 minutes onward, all the γ-32P has been transferred onto RacR as indicated by the single band (RacR) that was smaller in size than RacS-HK. The in vitro transfer of phosphate between NCTC 11168 RacR and RacS observed indicates both purified proteins are functional and the NCTC 11168 RacR and RacS form a true TCS.

4.3.5 Interaction of RacR and the fumC and aspA promoters

To demonstrate the direct repression of the fumC promoter region by RacR, EMSA and
Figure 4.7: SDS-PAGE and western blot analysis of the concentrated recombinant RacR and RacS-HK. Lane 1: PageRuler prestained protein ladder; lanes 2 and 4: RacR; lanes 3 and 5: RacS-HK.
**Figure 4.8:** *in vitro* phosphorylation of NCTC 1168 RacR and RacS-HK analysed by SDS-PAGE. The presence or absence of RacS-HK/ATP γ-32P and RacR in each reaction is indicated by ✔ or ✗ respectively while the time stated for each reaction represents the time elapsed since the addition of RacR to the RacS-HK/ATP γ-32P mixture.

<table>
<thead>
<tr>
<th>Reaction Time (mins)</th>
<th>Presence of RacS-HK</th>
<th>Presence of RacR</th>
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<tr>
<td>0</td>
<td>✔</td>
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<tr>
<td>0.25</td>
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<tr>
<td>16</td>
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RacS-HK 28.3 KDa

RacR 25.6 KDa
DNase I footprinting assays were carried out for the NCTC 11168 *fumC* promoter. The 81116 *aspA* promoter has been demonstrated to interact with the phosphorylated 81116 RacR (van Mourik *et al.*, 2009) and was therefore included in this study as a functional control. A DNA fragment containing 149 bp of the 81116 AspA coding region and 284 bp upstream from the start codon was amplified for *aspA* while for *fumC*, the same promoter region used to characterise the Fur$_{Cj}$ box in 3.3.3 was used.

For the *aspA* promoter (Figure 4.9), two shift species were observed with 124 nM RacR in the presence of RacS-HK and ATP which indicated high affinity protein-DNA interactions. The intensity of the shift species was increased with the elevated RacR concentration and at 496 nM RacR, a super shift was detected where all the *aspA* promoter fragments were bound to RacR. The super shift was either caused by an aggregation of protein-DNA complex in the gel well due to the presence of two proteins at high concentration or by the polymerisation of RacR along the DNA molecule. Although NCTC 11168 RacR was used in this study to interact with the 81116 *aspA* promoter, the shift patterns were in full agreement with van Mourik *et al.* (2009).

Interestingly a relatively strong shift was observed with *aspA* promoter and the RacR protein alone. This shift was not detected with DNA and RacS-HK alone and the intensity of this shift was significantly reduced when both RacR and RacS-HK were presented but without ATP. Although the physiological relevance of this interaction is uncertain, it nevertheless suggests that RacR, in its unphosphorylated form, may form
Figure 4.9: Standard EMSA of 1.55 fmol labelled *fumC* (a) and *aspA* (b) promoter regions with purified *C. jejuni* NCTC 11168 RacR, RacS-HK and ATP. Lane 1: labelled DNA; lane 2: labelled DNA with 496 nM RacR; lane 3: labelled DNA with 391 nM RacS-HK; lane 4: labelled DNA with 496 nM RacR and 391 nM RacS-HK; lanes 5-8: labelled DNA with 1 mM ATP, 391 nM RacS-HK and 124 nM, 248 nM, 372 nM and 496 nM RacR respectively.
weak interaction with its target prompter regions and this interaction is negatively influenced by the unphosphorylated RacS protein. The interaction between \textit{aspA} promoter and unphosphorylated RacR and RacS-HK was not investigated by van Mourik \textit{et al.} (2009).

A less intensive shift species was formed for \textit{fumC} with an increasing RacR concentration which demonstrated that the repression of \textit{fumC} by RacR was caused by direct protein-DNA interaction. The affinity of RacR binding was low, a conclusion consistent with the partial repression of \textit{fumC} by RacR detected by northern blot analysis (Figures 4.2-4.4). Nonspecific DNA interaction by unphosphorylated RacR was not observed, presumably due to the low affinity of RacR toward the \textit{fumC} promoter or a different regulatory mechanism involved.

Once the interaction of RacR and the \textit{fumC} and \textit{aspA} promoters was demonstrated by EMSAs, the location of the RacR with respect to the promoter region was subsequently analysed for both genes by DNase I footprinting assays. The highest RacR concentration used for EMSA (496 nM) was used as the starting concentration and due to the large reaction volume, the RacR concentration was also doubled and quadrupled. RacS-HK was kept at 391 nM due to its high phosphorylation activities determined in 4.3.4. However when the \textit{fumC} and \textit{aspA} promoters were analysed, no protected region was observed (Figure 4.10). The starting base of the \textit{aspA} gene is indicated in Figure 4.10 and a protected region is likely to be detected upstream from it while RacR is most
Figure 4.10: DNase I footprinting assays for the *fumC* and *aspA* promoters along with corresponding sequencing ladders. 391 nM RacS-HK and 1 mM ATP were used in each reaction and the RacR concentration ranged from 0 to 1488 nM. Regions representing the two *fumC* -10 regions are indicated by black lines on the left side of the film and the first base of the *aspA* gene indicated by the white line. N.B. no protected region was found.
certainly to interact with \textit{fumC} in the region between or upstream of the two -10 regions.

\textbf{4.3.6 Construction of the \textit{C. jejuni NCTC 11168 fumC mutant}}

\textit{C. jejuni fumC} was previously proposed to encode the only fumarase in the cell (Smith \textit{et al.}, 1999), therefore in order to test this hypothesis and to determine the functional importance of this gene, the NCTC 11168 \textit{fumC} gene was mutated by insertion of an antibiotic resistance cassette (Figure 4.11). The entire \textit{fumC} gene plus 400 bp flanking region on each side were amplified and cloned into pUC19 (pRR51). A 1067 bp internal deletion of \textit{fumC} in pRR51 was carried out by inverse PCR and was ligated with a terminator-less \textit{aphA-3} with its own promoter (a Kan resistance cassette) to form pRR52. pRR52 was then transformed into NCTC 11168 by electroporation. Transformants were recovered on MH agar with selection as well as MH agar supplemented with 6\% horse blood or 4 mM malate at pH 7.3 which was previously determined to enhance the wild-type \textit{C. jejuni} growth in liquid cultures (Hinton, 2006; S. Hardy, personal communication). Horse blood and malate were used to provide essential TCA cycle components for the \textit{fumC} mutant thus allowing the recovery of the mutant if \textit{fumC} was the only fumarase coding gene in NCTC 11168. The \textit{fumC} mutant (RR2) was only able to recovery from blood agar and the identity of the mutant was verified by PCR using gene specific primers (\textit{fumCF} and \textit{fumCR}) and \textit{aphA-3} specific inverse primers (SkanR and STM invKan-F).
Figure 4.11: Illustration of the mutagenesis strategies used to create a NCTC 11168 fumC mutant (RR2). A DNA fragment amplified by fumCF and fumCR containing the wild-type fumC and flanking regions (a) was cloned into pUC19 to form pRR51 (b). To make an internal deletion of fumC in pRR51, an inverse PCR was carried out using fumCinvR and fumCinvF primers and pRR51 (as the template), and the PCR product was ligated with aphA-3 (a Kan resistance cassette) to form pRR52 (c). pRR52 was then electroporated into NCTC 11168 to allow homologous recombination between the flanking regions of aphA-3 in pRR52 (that contains C. jejuni DNA) and their corresponding flanks in the genome of NCTC 11168 wild-type (d). This led to a replacement of the middle section of the wild-type fumC gene with aphA-3 thus creating a fumC mutant (RR2, e). Primers used for mutant construction and verification are indicated by blue arrows and genes, plasmids and genomes are not drawn in proportion to their actual sizes.
Chapter 4: The iron- and oxygen-responsive regulation of \( fumC \)

As shown in Figure 4.12, a single band of 2445 bp in lane 2 containing \( fumC \) gene and flanking regions was observed for the wild-type whereas a slight larger 2882 bp band in lane 5 was detected for the mutant. Bands with the expected sizes were also observed with the \( aphA-3 \) specific primers which demonstrated that the majority of the \( fumC \) gene was replaced by the Kan resistance cassette and the cassette was placed in the same orientation as the gene. The \( fumC \) mutant was also sequenced using a combination of primers indicated in Figure 4.11 and no nonspecific mutations in the 400 bp flanking regions of the mutated \( fumC \) gene were detected (results not shown).

For constructing a \( fumC \) complemented strain, the method described by Elvers et al. (2005) was applied where the functional copy of the mutated gene was recombined into the mutant strain and inserted into the pseudogene \( cj0752 \). The \( fumC \) gene plus approximately 300 bp of flanking regions on each side of the gene were amplified and cloned into the \( cj0752 \) gene in pGEMCWH01 to form pRR66. The \( cat \) gene (a Cm resistance cassette) was amplified from pAV35 and cloned into pRR66 in the same orientation downstream from the \( fumC \) gene. The resulting vector pRR67 was verified by sequencing and the construct was electroporated into RR2 prior to recovery on MH agar with selection as well as agar supplemented with blood or malate. No transformants were formed during two transformation attempts and further investigations were not carried out due to time limitations.

\[ 4.3.7 \text{ The effects of the fumC mutant on growth rate and fumarase activities} \]
Figure 4.12: Verification of the NCTC 11168 *fumC* mutant (RR2) by PCR. Lane 1: λ/Φ DNA size marker; lanes 2 and 5: PCR amplification using *fumCF* and *fumCR* primers; lanes 3 and 6: PCR amplification using *fumCF* and SKanR primers; lanes 4 and 7: PCR amplification using STM invKan-F and *fumCR*. RR2 and primer positions are shown in Figure 4.11.
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The effects of the *fumC* mutation on cell growth were assessed using both plate and liquid cultures. No cell growth was detected when RR2 was plated straight from glycerol stock onto MH agar. The mutant grew poorly on the MH agar plate supplemented with 4 mM malate and a notable increase in cell growth was observed when RR2 was plated onto the blood MH agar. When RR2 grown on blood MH agar was subcultured onto fresh media, marginal cell growth was detected on MH agar. An increase in cell growth compared to cells plated straight from glycerol stocks was also observed on MH agar supplemented with either malate or blood, though the level of growth was notably less when compared with the wild-type.

When the growth rate of the mutant strain was assessed under different iron conditions in MH broth, a significant growth defect was observed in both iron conditions compared with the wild-type strain (Figures 4.13 and 4.14). The reduced growth rate observed for RR2 was consistent in both solid and liquid media and indicated that *C. jejuni fumC* is essential for maintaining active cell growth. When the growth media was supplemented with 4 mM malate, the growth rate of the wild-type cells was improved under both iron conditions. The growth level for the mutant was also approximately doubled though the level and the rate of growth for RR2, especially under high iron conditions were not restored back to the level seen with the wild-type without the malate supplement. When a range of malate concentrations was tested to improve the level of mutant growth, a growth level of RR2 similar to the level shown in Figures 4.13 and 4.14 was observed between 4 to 8 mM malate and a decline in the growth rate for RR2 was detected outside this concentration range (results not shown).
Figure 4.13: Growth assays of the wild-type NCTC 11168 and RR2 (fumC mutant). MH broth was used and each assay was supplemented either with 40 μM FeSO₄ (+ Fe) or 20 μM Desferal (-Fe) and with or without 4 mM malate. Data presented are the means of triplicate sampling from two independent experiments with the standard error.
Figure 4.14: Growth assay of RR2 (fumC mutant). MH broth was used and each assay was supplemented either with 40 μM FeSO$_4$ (+ Fe) or 20 μM Desferal (-Fe) and with or without 4 mM malate. Data presented are the means of triplicate sampling from two independent experiments with the standard error. N.B. Data illustrated in this figure are replicates of the RR2 growth assay data in Figure 4.13 and presented with a smaller Y-axis scale to aid visualisation.
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The effects of mutational changes and iron on the whole cell fumarase activities were assessed for NCTC 11168 wild-type and RR2 using the method described by Hassett \textit{et al.} (1997). AB3 (\textit{racR} mutant) and AV17 (\textit{furCj} mutant) were also included in order to correlate the changing in fumarase activities with the transcriptional regulation of \textit{fumC} by RacR and Fur\textit{Cj}. The wild-type and mutant cells were cultured in the same growth and iron conditions as used for total RNA purification (2.3.1) with the addition of 4 mM malate. Whole cell extracts were prepared for each sample by sonication and the concentration of each sample was measured and standardised (2.4.12) prior to measuring the conversion of malate to fumarate at 25 °C.

As shown in Figure 4.15, fumarase activity was not detected for RR2 in both iron conditions which strongly indicated that \textit{fumC} is the only fumarase coding gene in the \textit{C. jejuni} genome and therefore plays a critical role in maintaining a functional TCA cycle. Fumarase activities of various levels in response to iron concentrations were detected for the wild-type AB3 and AV17 which illustrated the involvement of both Fur\textit{Cj} and RacR in the iron responsive regulation of \textit{fumC}. The patterns of FumC expression (measured in the form of fumarase activity) did not match the \textit{fumC} transcriptional profiles demonstrated by northern blot analysis which suggested the involvement of post-transcriptional or translational regulation of \textit{fumC}. However as malate was demonstrated to improve cell growth when supplemented in MH broth, the possibility of malate-induced alterations in gene expression could not be overlooked.
Figure 4.15: Fumarase activity assays of the wild-type NCTC 11168, RR2, AV17 and AB3 under different high iron (40 μM FeSO₄) and low iron (20 μM Desferal) conditions along with BSA controls. N.B. Data presented in this figure are from a single preliminary experiment.
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4.4 Discussion

The expression of three *E. coli* fumarase genes is controlled in a hierarchical manner by several global regulators that allow *E. coli* to adapt to various environmental conditions by maintaining the TCA cycle flux (Park and Gunsalus, 1995; Tseng *et al.*, 2001). Although *fumC* has been proposed to be the only fumarase coding gene in *C. jejuni* and oxygen-dependent variation of fumarase activity were not observed (Smith *et al.*, 1999), iron and oxygen responsive regulation of *fumC* have been demonstrated recently by microarray analysis where the expression of *fumC* was mediated by the global iron regulator FurCj and the RacR-RacS TCS (Holmes *et al.*, 2005; van Mourik *et al.*, 2009). By a combination of mutational analysis and northern blot analysis, the functional importance and transcriptional regulations of *C. jejuni fumC* were revealed in this study. Both NCTC 11168 RacR and RacS-HK were also purified and the interaction of RacR with RacS-HK and RacR regulated promoters were characterised *in vitro*.

4.4.1 NCTC 11168 fumC and its functional importance

To determine the functional importance of *C. jejuni fumC* in active cell growth and the role of fumarase in the TCA cycle, a NCTC 11168 *fumC* mutant was constructed and the changes in growth rate and total fumarase activity were assessed in response to iron. The growth of the mutant strain was severely hindered when compared with the wild-type in standard MH broth media under both iron conditions and the mutant failed to grow on standard MH agar plates. This observation clearly demonstrated the impact of the *fumC* gene in maintaining the active cell growth and a functional TCA cycle.
When supplemented with malate, which was determined to enhance cell growth (Hinton, 2006), the growth rate of the mutant strain was expected to restore back to the wild-type level. Although enhanced cell growth was observed for the wild-type and mutant strains under both iron conditions, the growth level for the mutant was still low compared to the wild-type (Figures 4.13 and 4.14) and the growth rate for the fumC mutant was not further enhanced with increased malate concentrations.

This growth discrepancy might be caused by the polar effect of the acid membrane antigen gene amaA located downstream from the fumC gene that is transcribed in the opposite direction. As the ahpA-3 gene used to construct the fumC mutant does not have its own terminator, the expression of the ahpA-3 might lead to the transcription of the amaA from the non-coding strand if the fumC terminator is weak. However as the mutant growth rate was influenced by the presence of malate, the growth discrepancy between fumC mutant supplement with malate and the wild-type strain is more likely caused by the deletion of the fumC gene leading to a disrupted TCA cycle. In addition, as the growth level of the mutant was also increased on MH agar plates supplemented with horse blood, it is likely that other supplements present in the blood are needed to return the mutant growth rate back to the wild-type level. Other possible explanation for this growth difference is the build up of fumarate that negatively affects the cell growth or has a knockback effect on the TCA cycle. As the complemented strain was not constructed due to time limitation, the impact of fumC on the cell growth could not be fully evaluated.
When the total fumarase activity was tested for the \textit{fumC} mutant, no fumarase activity was detected (Figure 4.15) which experimentally proved that \textit{fumC} encodes the only fumarase in \textit{C. jejuni} NCTC 11168. The absence of \textit{fumA} and \textit{fumB} in \textit{C. jejuni} is likely reflected by its strictly microaerophilic growth requirement and the abandonment of both genes through evolution allows \textit{C. jejuni} to maintain a small genome while sustaining a functional TCA cycle. By contrast, FumB is essential for \textit{E. coli} during anaerobic respiration and a relative high level of FumA activity has also been detected in \textit{E. coli} under anaerobic conditions despite the expression of \textit{fumA} is down regulated (Tseng et al., 2001).

4.4.2 The iron and oxygen responsive regulation of \textit{fumC} and \textit{racR}

4.4.2.1 Factors affect \textit{fumC} expression

As the only fumarase coding gene in the \textit{C. jejuni} genome, regulation of \textit{fumC} expression was predicted to involve a complicated regulatory network in order to compensate for the absence of \textit{fumA} and \textit{fumB} and to respond to suboptimal growth conditions such as iron limitation or oxidative stress. \textit{fumC} was initially demonstrated to be induced by iron when total RNA used for northern blot analysis were purified from cell grown in conditions routinely used for \(\beta\)-galactosidase assays. The exact mechanism of this positive regulation was unclear as the \textit{fumC} transcriptional profiles were not altered in the \textit{furCj} mutant (Figure 3.15) indicating the iron-responsive regulation of \textit{fumC} is mediated by another regulator.
When growth conditions mimicking the conditions used by Holmes et al. (2005) were analysed, a potential negative regulation of *fumC* by iron was observed (Figure 3.17) though the involvement of Fur$_{Cj}$ and PerR$_{Cj}$ remained inconclusive. One of the major differences between the two culture conditions was the growth phase variation although no changes in *fumC* regulation between the two phases were detected using northern blot and β-galactosidase assays and this observation was consistence with Smith et al.’s observation that fumarase activity is not affected by the age of cultures (Smith et al., 1999). As the levels of internal cellular iron at the start of liquid culture inoculation were also different between the two culturing methods, the variation of *fumC* expression and regulation observed by northern blot analysis is therefore most likely to be caused by the difference in growth rate arising from internal iron availability. In another word, prior to inoculating into MHB supplemented with 20 μM Desferal, the initial intercellular iron concentration is much higher for *C. jejuni* grown on MHA plates using the Holmes’s method (Holmes et al., 2005) than using the β-galactosidase assay method, and this key variation could greatly affect the regulation of *fumC*.

Interestingly the iron responsive regulation of *fumC* was not detected in a separate study of the *C. jejuni* Fur$_{Cj}$ regulon (Palyada et al., 2004) where cells used for RNA purification were grown at 37 °C rather than 42 °C. The different outcomes between these two studies were likely to be caused by this temperature variation that might have had a direct effect on iron responsive regulation of *fumC* or indirect effects on the
growth rate. In fact, a recent comparison of the *C. jejuni* proteome between cells cultured at these two temperatures demonstrated a 2-fold increase in the level of FumC at 37 °C, thus potentially indicating the expression of *fumC* is temperature-dependent. In addition, the minimal essential medium MEM α used by Palyada *et al.* (as supposed to MH used by Holmes *et al.* ) may also have direct or indirect effects on the expression of *fumC* (Palyada *et al.*, 2004; Holmes *et al.*, 2005).

The temperature-dependent cell growth of *C. jejuni* has been previously investigated by Brás *et al.* (1999) where the RacR-RacS TCS was characterised. RacR has been recently demonstrated to alter the expression of several metabolism genes including *fumC* although the regulation of *fumC* was mediated by oxygen limitation rather than temperature (van Mourik *et al.*, 2009). The regulation of *fumC* and *racR* in response to iron and oxygen availability and the involvement of FurCJ, PerRcJ and RacR were comprehensively analysed by northern blot assays in this study and is summarised in Figure 4.16.

### 4.4.2.2 Regulation of *fumC* expression under high iron conditions

Using the culturing conditions described by Holmes *et al.* (2009), *fumC* is transcribed in both iron conditions and it is repressed by RacR and FurCJ. The level of *fumC* expression is also marginally decreased in high iron conditions, though the physiological relevance of this transcriptional variation (if any) is unknown and quantitative measurement by densitometry is required to validate this observation. RacR
Figure 4.16: Transcriptional regulation of \textit{fumC} by several regulators in response to oxygen and potentially iron availability. Arrow heads represent gene induction whereas gene repression is indicated by flat heads. The effects of oxygen on PerR\textsubscript{Cj}, apo-Fur\textsubscript{Cj} and IR2 are uncertain and are indicated by dash arrows. The level of \textit{fumC} expression is represented by the size of black arrows.
is the dominant repressor of \textit{fumC} under low oxygen conditions (3\%) where \textit{racR} itself is highly induced by iron and an unknown regulator. Fur\textsubscript{Cj} plays a less significant role in the repression of \textit{fumC} under this condition and as iron containing Fur\textsubscript{Cj} does not contact the \textit{fumC} promoter directly, the exact mechanism of \textit{fumC} repression by Fur\textsubscript{Cj} is unclear. One possible explanation is the repression of \textit{fumC} by Fur\textsubscript{Cj} using other metal ions such as manganese as the co-factor. Alternatively, \textit{fumC} may be indirectly repressed by Fur\textsubscript{Cj} through an unknown intermediate regulator (IR1) which itself is negatively regulated by Fur\textsubscript{Cj} and iron. As \textit{C. jejuni fumC} shares a high degree of identity (76.9\%) with the \textit{H. pylori fumC} (Smith \textit{et al.}, 1999), it’s highly possible that like in \textit{H. pylori} 26695 (Xiao \textit{et al.}, 2009) the identity of IR1 is a sRNA located on the opposite strand as the \textit{fumC} gene. Sequence alignment of \textit{C. jejuni NCTC 11168 fumC} and \textit{H. pylori 26695 fumC} (2.5.1) indicated that the two genes shares 68.5\% identity and the region that encodes the sRNA in \textit{H. pylori} 26695 shares 64.3\% identity with the corresponding region in \textit{C. jejuni NCTC 11168 fumC} (data not shown). Therefore further characterisation of this region by northern blot and real time RT-PCR is required to confirm the identity of IR1.

Once the oxygen concentration reaches an optimal level (7\%), the expression level of \textit{racR} is reduced which allows Fur\textsubscript{Cj} to play more significant roles in the repression of \textit{fumC} under high iron conditions. When the oxygen level increases to 11\%, the iron-induced activation of \textit{racR} becomes dependent on PerR\textsubscript{Cj} despite that \textit{perR\textsubscript{Cj}} itself is normally expressed under iron-limited conditions. However the up regulation of \textit{perR\textsubscript{Cj}} expression has been previously observed from microarray data where the level of
perR_Cj mRNA is elevated in the presence of iron and hydrogen peroxide (Palyada et al., 2009). The induction of racR by PerR_Cj under oxidative stress conditions presumably allows RacR to resume the dominant role as the fumC repressor in order to eliminate the possible derepression of fumC caused by the interaction of reactive oxygen species and the iron co-factor of the Fur_Cj protein.

The interplay of both RacR and Fur_Cj in the regulation of fumC is also reflected by the two fumC transcripts determined by northern blot analysis (3.16) and during RACE (3.18). One of these transcripts is a longer but weaker transcript detected in a racR mutant background under high iron conditions and as racR is induced by iron, it’s most likely that this weaker (secondary) transcript is repressed solely by RacR. The stronger (primary) fumC transcript on the other hand is presumably repressed by RacR when racR is high expressed and indirectly by Fur_Cj when RacR becomes less dominant such as in 7% oxygen.

4.4.2.3 Regulation of fumC expression under low iron conditions

Compared to the repression of fumC by RacR and Fur_Cj under high iron conditions determined by northern blot analysis, the positive influences of fumC expression by PerR_Cj and Fur_Cj under iron-limited conditions are less clear (Figure 4.16). At 3% oxygen, the expression of fumC is derepressed in the absence of RacR and as the oxygen concentration increases to 7%, Fur_Cj and PerR_Cj also positively influence the expression of fumC. The activation of fumC by Fur_Cj under iron-limited conditions is
particularly puzzling as FurCj normally does not function under this condition due to the loss of its iron co-factor. The only possibility is the involvement of apo-FurCj as a repressor which represses an intermediate repressor (IR2) and in turn allows the expression of fumC. Although apo-FurHp repression has been exclusively studied in H. pylori (Delany et al., 2001; Ernst et al., 2005), a recent investigation of the C. jejuni dsbA2 promoter indicated a direct repression of dsbA2 by apo-FurCj in vitro and in vivo (Grabowska et al., 2011). As this is the only example of apo-FurCj repression in C. jejuni to date, further characterisations of apo-FurCj repression and its role in the indirect positive regulation of fumC are required. In contrast to furCj, perRcj is induced under low iron conditions therefore whether PerRcj has a direct positive influence on fumC expression or through the repression of IR2 or yet another intermediate is unclear.

As the oxygen level reaches 11%, the positive influence of fumC expression by FurCj and PerRcj increases as demonstrated by the strong repression of fumC under iron limited conditions in AV17 and AV63. As a peroxide sensing regulator, the expression perRcj is induced in the presence of hydrogen peroxide. However as the repressive effect of PerRcj on the oxidative stress response genes is also released in the presence of hydrogen peroxide, the involvement of oxygen in the positive regulation of fumC by PerRcj cannot be easily determined without the expression profiles of perRcj gene itself and the identity of IR2.

Despite the changing of oxygen concentrations, the overall level and pattern of
iron-responsive regulation of \textit{fumC} is unaffected and this finding is in full agreement with the previous observation that the \textit{C. jejuni} fumarase activity remains constant under different oxygen tensions (Smith \textit{et al.}, 1999). This unaltered expression of \textit{fumC} is expected considering the importance of FumC in maintaining the active cell growth and a functional TCA cycle, however regulation of \textit{fumC} by several global regulators in a complicated overlapping network has therefore evolved in order the compensate for the lack of FumA and FumB and further experiments are required to fully reveal the identity and mechanisms of all the regulators involved in this network. 

Although the expression of \textit{fur}_{Cj} and \textit{perR}_{Cj} has been comprehensively characterised (van Vliet \textit{et al.}, 2000; Palyada \textit{et al.}, 2009), different culturing conditions used in these studies and in this current study such as temperature, agitation and oxygen levels making the expression of \textit{fur}_{Cj} and \textit{perR}_{Cj} difficult to predicate and to compare with previous studies. Therefore one critical experiment to further characterise the regulation of \textit{fumC} as well as \textit{racR} by Fur\textsubscript{Cj} and Per\textsubscript{Cj} is to repeat the northern blot assays with the addition expression profiles of \textit{fur}_{Cj} and \textit{perR}_{Cj}. The addition of a \textit{fur}_{Cj} and \textit{perR}_{Cj} double mutant (AV67) is also important to understand the co-involvement of Fur\textsubscript{Cj} and Per\textsubscript{Cj} in the regulation of \textit{fumC}, especially under iron-limited conditions. Due to the inability to obtain sufficient amount of AV67 for RNA purification and the inaccuracy of northern blot analysis, real time RT-PCR is more preferable to accurately determine the transcriptional profiles of \textit{fumC} and each of the regulator coding genes.
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4.4.2.4 Possible post-transcriptional and translational regulation of \textit{fumC}

The identity of IR2 is difficult to determine which requires the characterisation of other oxidative stress response regulators and one of the possible candidates is the carbon starvation regulator CsrA. CsrA has been identified in \textit{E. coli} as a posttranscriptional regulator (Romeo, 1996) and it plays important roles in \textit{H. pylori} in the regulation of motility, oxidative stress resistance and mouse colonisation (Barnard \textit{et al.}, 2004). CsrA also has significant regulatory roles in \textit{C. jejuni} pathogenesis as it contributes to oxidative stress survival, biofilm formation and host cell invasion (Fields and Thompson, 2008). The expression of over 100 proteins have been demonstrated by proteomic analysis to alter in the \textit{C. jejuni} 81-176 \textit{csrA} mutant (Thompson \textit{et al.}, 2009) and FumC is potentially one of these putative CsrA targets (S. Thompson, personal communication).

The genomic DNA of \textit{C. jejuni} 81-176 \textit{csrA} mutant was obtained, though further investigations were not performed due to time limitations. Further characterisation of \textit{fumC} regulation by CsrA requires the initial construction of NCTC 11168 \textit{csrA} mutant from the 81-176 mutant and subsequently analyse the expression profiles of \textit{fumC} as well as \textit{csrA} in response to iron and oxygen and the possible regulation of \textit{csrA} by Fur\textsubscript{Cj} and PerR\textsubscript{Cj}. As CsrA acts as a posttranscriptional regulator, the exact mechanism of \textit{fumC} regulation by CsrA is difficult to determine by northern blot assays alone. EMSA analysis of purified CsrA and \textit{fumC} mRNA is one alternative method to study the interaction between CsrA and the 5’ UTR of \textit{fumC} as direct binding of 81-176 CsrA and
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*C. jejuni* RNA have been demonstrated previously (Thompson et al., 2009).

The expression of FumC may also be affected on the translational level as the transcriptional profiles of *fumC* did not match the results of fumarase activity assays. This result discrepancy is potentially caused by the temperature sensitive nature of the fumarase activity assay and a temperature controlled automated spectrometer is more preferable in order to obtain more accurate results. Alternatively, western blot assays can be used to determine the FumC expression profiles although anti-FumC antibodies are required for this method. Regardless of the chosen method, the effects of malate supplementation on the expression of *fumC* and other genes cannot be overlooked and therefore the *fumC* mutant strain (that requires malate supplementation) needs to be excluded from the fumarase activity assays in order to determine the translational regulation of *fumC*.

4.4.3 *The interaction of RacR with RacS-HK and RacR regulated promoters*

The His6-tagged NCTC 11168 RacR and the histidine kinase domain of RacS were purified and the *in vitro* transfer of phosphate between the two proteins was observed which demonstrated that RacR and RacS form a TCS. High affinity binding of RacR and the *aspA* promoter was determined in the presence of RacS-HK and ATP and interestingly RacR also interacts with the *aspA* promoter without the presence of ATP and this interaction was partially inhibited by RacS-HK. This observation suggested that RacS plays important roles in preventing interactions between unphosphorylated RacR
and RacR regulated genes and the mechanism involved in this process does not require the transfer of phosphate. As this unphosphorylated RacR-DNA interaction was only observed for the _aspA_ prompter, and too faint to be observed for _fumC_, EMSA assays with other RacR regulated genes are required to fully understand this process.

A relatively weak RacR-DNA interaction was determined for the _fumC_ promoter and this finding was in agreement with the partial repression of _fumC_ by RacR revealed by northern blot analysis. DNase I footprinting assays performed to determine the RacR binding site were unsuccessful despite the strong protein-DNA interactions observed in EMSAs. The result discrepancy between EMSA and DNase I footprinting assays could be explained by a “caging” effect where in EMSA, the protein-DNA complex was stabilised by the non-denaturing polyacrylamide gel, whereas in DNase I footprinting experiments, protein-DNA interaction was weak in solution and the complex could be easily dissociated by DNase I (Fried and Liu, 1994). However due to the high affinity of RacR binding to the _aspA_ promoter even without the presence of RacS and ATP, the negative DNase I footprinting results were likely to be caused by incompatibility between the buffers used for phosphorylation, DNA-protein interaction and DNase I footprinting assays. As RacR alone interacts with the _aspA_ promoter, a DNase I footprinting assay without the presence of RacS and ATP will be able to eliminate the buffer incompatibilities and further buffer optimisations are required in order to determine the RacR binding site on the _fumC_ promoter.
4.4.4 The regulation of chuA by PerR<sub>Cj</sub> and RacR

When the expression profiles of chuA were analysed as a positive control using northern blot assays, a transcript of approximately 1.6 Kb was detected (Figures 4.2-4.4). Although the actually size could not be accurately determined, it nevertheless suggests that chuA is predominantly transcribed as a monocistronic mRNA and the downstream chuBCD genes may have their own promoter(s).

Additionally, the transcription of chuA was also found to be indirectly repressed under high iron conditions and 11% oxygen by PerR<sub>Cj</sub> and this repression was mediated by RacR (Figure 4.4). This repression of chuA by PerR<sub>Cj</sub> under elevated oxygen levels eliminates the possible derepression of chuA by Fur<sub>Cj</sub> due to reactive oxygen species and prevents further uptake of iron under oxidative stress conditions. chuA has been previously determined to be repressed by PerR<sub>Cj</sub> though this repression was mediated by hydrogen peroxide in the absence of iron (Palyada et al., 2009). The mechanism of this repression was speculated to be caused by the release of iron from iron-sulphur in the presence of hydrogen peroxide and subsequently led to the repression of chuA by Fur<sub>Cj</sub> (Palyada et al., 2009). The expression of chuA was also found to be up regulated in the presence of both oxygen and iron and this activation was thought to enhance hydrogen peroxide detoxification by producing haem that is essential for KatA’s catalytic activity (Palyada et al., 2009). The culturing temperature and oxygen levels used in this current investigation may explain the results discrepancy between these two studies and the expression of chuA as well as racR in response to hydrogen peroxide requires further
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investigation. Both studies however demonstrated the interconnected link between oxidative stress and iron metabolism and the involvement of oxygen- and iron-responsive regulators in this regulatory network.
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Regulation of gene expression in response to environmental or intracellular stimuli is an essential process for the survival of every single living organism and this is particularly so for pathogenic microorganisms. Effectively regulated global and specific regulatory networks allow pathogenic bacteria to survive in the natural environment during transmission, to adapt to the host environment and avoid the host immune response, and to cause disease (Snyder and Champness, 2003). For the foodborne enteric pathogen C. jejuni, the co-regulation of iron homeostasis and oxidative stress responses by FurCj and its homologue PerRj is such an example of global gene regulation. Both transcriptional regulators are vital for C. jejuni in in vivo survival and colonisation (Palyada et al., 2004; 2009).

Understanding the interaction between FurCj and its operator sequence, the FurCj box, is essential for revealing the mechanisms of FurCj regulation and for identifying FurCj regulated genes. The aim of this research was to investigate the base-specific interaction of FurCj and the FurCj box by a mutagenesis approach using the FurCj box of the classically repressed chuA promoter and a FurCj box-like sequence of the non-classically FurCj regulated fumC promoter. Additionally, the transcriptional regulation of fumC was further investigated to determine the regulatory role of FurCj and the interplay between FurCj and other regulators in the regulation of fumC expression in C. jejuni.

5.1 FurCj-FurCj box interaction
By using a combination of EMSA, β-galactosidase and DNase I footprinting assays, it was demonstrated that in the functional state, two Fur$_{Cj}$ dimers cooperatively recognise and bind to the Fur$_{Cj}$ box. Binding of the two Fur$_{Cj}$ dimers is likely to occur at the opposite face of the Fur$_{Cj}$ box and Fur$_{Cj}$ polymerisation was not observed indicating that the binding of two Fur$_{Cj}$ dimers is sufficient, at least for chuA, to mediate transcriptional repression. Although this mechanism of Fur$_{Cj}$-Fur$_{Cj}$ box interaction is well conserved among many well characterised Fur orthologues, including Fur$_{Ec}$ (Lavras et al., 2002), Fur$_{Bs}$ (Baichoo and Helmann, 2002) and Fur$_{Pa}$ (Pohl et al., 2003), the putative Fur$_{Cj}$ box sequence proposed by van Vliet et al. (2002) and later by Palyada et al. (2004) matched poorly with the 19 bp Fur$_{Ec}$ box consensus sequence (de Lorenzo et al., 1987). The putative Fur$_{Cj}$ box sequence proposed by Palyada et al. (2004) was experimentally verified in this study as this sequence was enclosed in the 31 bp Fur$_{Cj}$ protected region of the chuA promoter and it was able to promote Fur$_{Cj}$ binding when introduced into a functionally unrelated fumC promoter (Table 5.1). This consensus sequence however was only found in 11 out of the 53 Fur$_{Cj}$ regulated genes indentified by Palyada et al. (2004) thus further in vitro binding studies of other Fur$_{Cj}$ repressed promoters are required to experimentally refine or possibly redefine a consensus Fur$_{Cj}$ box sequence. The experimental design used in this study should also be applied to the newly purified PerR$_{Cj}$ protein (Handley et al., 2010) in order to facilitate the understanding of the Fur$_{Cj}$ regulator family as a whole. However future mutational analyses of the Fur$_{Cj}$ or PerR$_{Cj}$ binding site should always be accompanied by detailed promoter structural studies to avoid any undesired disruption of the functional promoter.
Table 5.1: Illustration of the Fur<sub>Cj</sub> protected regions observed for the wild-type chuA and the fumC10/13/19 promoters. The chuA Fur<sub>Cj</sub> box is highlighted in yellow and the mutated Fur<sub>Cj</sub> box-like sequence for fumC10/13/19 is highlighted in red. The base positions of the 19 bp proposed Fur<sub>Cj</sub> box are numbered and the four bases identified to play essential roles in Fur<sub>Cj</sub>-Fur<sub>Cj</sub> box interaction are coloured in green.
A 7 bp Fur$_{Cj}$ protected region was observed for chuA10/19 which was likely to be caused by the binding of a single Fur$_{Cj}$ dimer although only one of the Fur$_{Cj}$ monomers was tightly bound, presumably due to the mutations toward the centre and the 3’ end of the Fur$_{Cj}$ box. This observation potentially suggests that the Fur$_{Cj}$ box can be interpreted by the (7-1-7)$_2$ model proposed by Baichoo and Helmann (2002). However no symmetric elements can be observed in the 19 bp consensus sequence indicating that unlike most bacteria, the exact sequence of the Fur$_{Cj}$ box may not play a vital role in Fur$_{Cj}$-Fur$_{Cj}$ box interaction. Additionally, although mutations of the 1$^{\text{st}}$, 7$^{\text{th}}$, 13$^{\text{th}}$ and 19$^{\text{th}}$ bases of the chuA Fur$_{Cj}$ box introduced in this study as well as mutations of several bases of the p19 operon promoter introduced in previous work (Breg, 2007) have negative effects on the affinity of Fur$_{Cj}$ binding, the result obtained by these two studies did not correlate well with each other. This result discrepancy also suggests that the overall architecture of a Fur$_{Cj}$ regulated promoter plays a more significant role in Fur$_{Cj}$ box recognition and Fur$_{Cj}$ binding rather than the sequence of the Fur$_{Cj}$ box per se. This conclusion is in full agreement with the latest view on the H. pylori Fur$_{Hp}$ box (Carpenter et al., 2009). Fur$_{Hp}$ shares 32.6% identity and 52.2% similarity with Fur$_{Cj}$ (Miles et al., 2010a), and as in C. jejuni, no strong consensus Fur$_{Hp}$ box sequence is present in the promoter regions of Fur$_{Hp}$ regulated genes (Merrell et al., 2003). Interestingly, although the Fur$_{Cj}$ box does not share any sequence homology with the Fur$_{Hp}$ box, fur$_{Cj}$ when expressed in trans was able to complement Fur$_{Hp}$ in the repression of the amiE promoter (Miles et al., 2010a), which further indicated that the AT-rich characteristic of both C. jejuni and H. pylori promoters is a key feature in the
Fur-mediated iron-responsive repression for both bacteria.

In addition to iron-dependent repression, \textit{apo-Fur}_{Cj} has been recently demonstrated to repress the \textit{dsbA2} promoter both \textit{in vitro} and \textit{in vivo} (Grabowska \textit{et al.}, 2011). Although several instances of \textit{apo-Fur}_{Hp} regulation have been investigated in \textit{H. pylori} (Delany \textit{et al.}, 2001; 2002; 2003; Ernst \textit{et al.}, 2005), whether \textit{apo-Fur}_{Hp} functions as a monomer or a dimer is uncertain. The recently resolved \textit{Fur}_{Hp} crystal structure reveals the presence of an N-terminal extension that functions in stabilising the \textit{Fur}_{Hp} structure in the absence of a metal co-factor (Dian \textit{et al.}, 2011). This extension is also present in \textit{Fur}_{Cj} (Miles \textit{et al.}, 2010a) suggesting that both proteins function as dimers in their \textit{apo}-form. EMSAs of the \textit{dsbA2} promoter with \textit{apo-Fur}_{Cj} revealed one shift species whereas multiple species were observed for the \textit{chuZ-chuA} intergenic region with \textit{Fur}_{Cj} (Grabowska \textit{et al.}, 2011), which potentially suggested that a single dimer is the functional state of \textit{apo-Fur}_{Cj} repression. Although the \textit{apo-Fur}_{Cj} operator sequence has not been determined by DNase I footprinting assays, \textit{fur}_{Cj} when expressed \textit{in trans} was not able to complement \textit{apo-Fur}_{Hp} in the repression of the \textit{pfr} promoter, indicating that unlike Fur, \textit{apo-Fur} of \textit{C. jejuni} and \textit{H. pylori} recognise their own unique operator sequence.

Although the DNase I footprint assay and EMSA were successfully used in this study to demonstrate the interaction between \textit{Fur}_{Cj} and the \textit{chuA} promoter \textit{in vitro}, those techniques are only suitable for studying individual \textit{Fur}_{Cj}-regulated promoters. In order
to further reveal the global regulatory role of Fur$_{CJ}$ in both the iron-bound and apo-form, a comprehensive investigation of Fur$_{CJ}$-DNA interactions on a genome-wide basis is ultimately required. Such investigation could be achieved by the ChIP-on-chip or the ChIP-Seq techniques, which combine chromatin immunoprecipitation with microarray technology or high-throughput DNA sequencing respectively to allow the study of \textit{in vivo} interactions between proteins and DNA (Ren \textit{et al.}, 2000; Johnson \textit{et al}, 2007). The outcome of such investigations would allow the identification of genes that are directly regulated by Fur$_{CJ}$ or apo-Fur$_{CJ}$ and an alignment of these Fur$_{CJ}$ regulated promoter regions would confirm the presence or the absence of a Fur$_{CJ}$ box and an apo-Fur$_{CJ}$ box consensus sequence. The ChIP-on-chip technique could also be used in a Fur$_{CJ}$ mutant background that has been complemented \textit{in trans} by a fur orthologue. Such a study would reveal the key differences in the mechanism of DNA recognition and DNA-binding between Fur$_{CJ}$ and other Fur orthologues at a genome-wide level.

Prior to the submission of this dissertation, a ChIP-chip characterisation of Fur$_{CJ}$ was presented in the CHRO 2011 meeting (Butcher and Stintzi, 2011). In this study, approximately 90 genes involved in DNA replication, flagella and surface structure composition, iron acquisition and energy metabolism were indentified to be directly Fur$_{CJ}$ regulated (Butcher and Stintzi, 2011). Such observations further illustrated the global regulatory role of Fur$_{CJ}$ and the knowledge acquired from this study would provide a definitive determination of the Fur$_{CJ}$ box and a better understanding of the Fur$_{CJ}$-Fur$_{CJ}$ box interaction.
5.2 Regulation of *chuA* expression

Although the haem OM receptor *chuA* is perhaps one of the best characterised Fur$_{Cj}$ repressed iron acquisition genes in *C. jejuni* (van Vliet *et al.*, 1998; Ridley *et al.*, 2008), in this study the analysis of *chuA* promoter structure, transcriptional regulation, and interaction with Fur$_{Cj}$ revealed new insights into the regulation of *chuA* expression (Figure 5.1). Under iron-rich conditions in optimal or low oxygen concentrations, Fur$_{Cj}$ binds to the Fur$_{Cj}$-box in the 5’ UTR of *chuA* and prevents the transcription of the downstream coding region by RNA pol. When the extracellular oxygen concentration increases, *chuA* is co-repressed by Fur$_{Cj}$ and RacR. Although EMSA was not carried out for the *chuA* promoter with RacR due to time limitations, northern blot analysis demonstrated that the deletion of either *racR* or *fur$_{Cj}$* led to a derepression of *chuA* under high iron conditions. RacR presumably binds to the *chuA* promoter region close to the *chuA* Fur$_{Cj}$ binding site as the expression of the upstream *chuZ* promoter was not affected by RacR. Although *chuA* has been considered to form an operon with the downstream *chuBCD* genes as no intergenic regions are present (Miller *et al.*, 2009), northern blot analysis of *chuA* expression indicates that *chuA* is primarily transcribed as a monocistronic mRNA. As *chuBCD* are also regulated by Fur$_{Cj}$ in response to iron availability (Palyada *et al.*, 2004; Holmes *et al.*, 2005), it is likely that a Fur$_{Cj}$-regulated promoter is present in the *chuA* coding region that functions in controlling the downstream *chuBCD* genes and this promoter is also potentially regulated by RacR under high oxygen concentrations. To test this hypothesis, northern blot analyses using
Figure 5.1: Iron-responsive repression of *chuA* at different oxygen concentrations by *Fur*<sub>Cj</sub> and *RacR*. In 3-7% oxygen (a), two *Fur*<sub>Cj</sub> dimers bind to the 5' UTR of *chuA* and repress gene expression by preventing RNA pol from transcribing the DNA template. In 11% oxygen (b), *PerR*<sub>Cj</sub> activates the expression of *racR*, and then *RacR* and *Fur*<sub>Cj</sub> co-repress the expression of *chuA*. Genes and promoter regions are not drawn in proportion to their sizes and the exact mechanisms of *PerR*<sub>Cj</sub> and *RacR* regulation are unknown.
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*chuBCD*-specific probes would confirm the co-expression of these three genes and their regulation by RacR, and *lacZ* reporter assays using the *chuA* coding region would confirm the presence of a promoter for *chuBCD*.

Interestingly, the regulation of *chuA* expression by RacR is modulated by PerR<sub>Cj</sub> (Figure 5.1) and although the mechanism of *racR* activation by PerR<sub>Cj</sub> is unknown, this observation is in agreement of with the recent characterisation of the PerR<sub>Cj</sub> regulon, which demonstrates that the expression of *chuA* (as well as *chuBCD*) is regulated by PerR<sub>Cj</sub> and Fur<sub>Cj</sub> in response to iron and exposure to hydrogen peroxide (Palyada et al., 2009). The outcome of the current study and Palyada et al.’s investigation (2009) collectively illustrate an intricate regulatory network of *chuA* expression involving three transcriptional regulators. Under high-rich conditions, Fur<sub>Cj</sub> represses the expression of *chuA* to prevent further iron uptake. When the extracellular oxygen concentration increases, PerR<sub>Cj</sub> activates the expression of *racR* which with Fur<sub>Cj</sub> in turn strengthens the repression of *chuA* to minimise iron-mediated oxidative stress. However, when the cell is exposed to hydrogen peroxide, both PerR<sub>Cj</sub> and Fur<sub>Cj</sub> are presumably inactivated due to the oxidation of their ferrous iron co-factor, and this leads to a down regulation of *racR* and subsequent derepression of *chuA*. The expression of *chuA* allows further haem uptake into the cell to enhance hydrogen peroxide detoxification as haem has been shown to exhibit antioxidant properties (Nagababu and Rifkind, 2004) and it is also required for the catalytic activity of the catalase, KatA (Palyada et al., 2009). This derepression of *chuA* to enhance haem uptake, but not for the actual iron content is
further supported in this study and Palyada et al.’s investigation (2009) in that the expression of chuZ is not affected by the increasing oxidative stress. Additionally, although chuA is not required for C. jejuni intestinal colonisation (Haigh et al., 2010), the tight regulation of chuA by three regulators suggests that it is likely to play other essential roles in pathogenesis such as deep tissue survival (R. Haigh, personal communication). Furthermore, although the expression of other C. jejuni iron uptake systems has also been shown to be regulated by FurCj and PerRCj (Palyada et al., 2004; 2009), the exact mechanism of PerRCj regulation and the possible involvement of RacR in these regulatory networks are currently unknown. Therefore, further transcriptional characterisation of other iron uptake systems in response to iron and oxygen is required to reveal the involvement of any multilevel regulation as seen with chuA.

5.3 Regulation of fumC expression
The co-involvement of FurCj, PerRCj and RacR in oxygen- and potentially iron-responsive gene regulation has been further demonstrated for the regulation of fumC. Encoding the only functional fumarase in C. jejuni, fumC is essential for supporting active cell growth and maintaining a functional TCA cycle. As illustrated in Figure 4.16, the expression of fumC is primarily repressed by RacR, but the transcription level is also potentially regulated by FurCj, apo-FurCj and PerRCj through two hypothetical intermediate regulators IR1 and IR2. As well as iron and oxygen levels, the expression of fumC is also likely to be dependent on growth rate and growth temperature and evidence of post-transcriptional and translational regulation of fumC.
have been demonstrated both in a previous study of the CsrA regulon (S. Thompson, personal communication) and using fumarase activity assays in this study. Further transcriptional studies of \textit{fumC} as well as \textit{fur}_{Cj} and \textit{perR}_{Cj} are required to fully appreciate the extent of this complicated regulatory network, but due to technical impracticability and inaccuracy of northern bolt analysis, a quantitative method such as reporter assays or real-time PCR would be more appropriate. Although further characterisation of \textit{fumC} expression is required, \textit{fumC} regulation nevertheless serves as a good example to illustrate the necessity for \textit{C. jejuni} to cooperatively regulate essential gene expression by several regulators in response to different environments. This allows \textit{C. jejuni} to efficiently use its rather limited set of regulators to effectively control the expression of this essential TCA cycle enzyme under the sub-optimal conditions usually encountered during transmission and colonisation.

5.4 The RacR-RacS TCS

The response regulator RacR and its cognate sensor RacS form a TCS and, in NCTC 11168, RacR represses the expression of at least \textit{chuA} and \textit{fumC} in response to iron. Recent characterisation of the 81116 RacR regulon indicates that the expression of many metabolism genes is controlled by RacR in response to low oxygen conditions and direct binding of RacR has been demonstrated \textit{in vitro} for five of these RacR regulated promoters including \textit{aspA} (van Mourik \textit{et al.}, 2009). Further investigation of NCTC 11168 RacR and 81116 \textit{aspA} in this project indicated that when RacS senses a decline in the extracellular oxygen concentration, it phosphorylates RacR and allows it
to repress the expression of *aspA* (Figure 5.2). However without an extracellular signal, unphosphorylated RacS physically interacts with RacR and prevents RacR from forming a non-specific interaction with the *aspA* promoter. This mechanism of RacR repression is also likely to be true for *fumC* although the non-specific interaction was too weak to be observed in this study. Due to technical issues, the RacR binding sites on the *aspA* and *fumC* promoters were unable to be determined by DNase I footprinting assays. However comparative DNase I footprinting analyses of phosphorylated and unphosphorylated RacR with the *aspA* promoter would reveal any physiological role of the non-specific interaction of RacR and RacR-regulated promoters. Additionally, as the expression of *racR* is iron- and PerR<sub>Cj</sub>-induced, the expression of many RacR regulated energy metabolism genes identified by van Mourik *et al.* (2009) are also likely to be indirectly regulated by iron and PerR<sub>Cj</sub>. Characterisation of the expression of these genes under different oxygen levels would allow further demonstration of the global regulatory role of PerR<sub>Cj</sub>.

### 5.5 Final remarks

In conclusion, this study provided a new insight into the Fur<sub>Cj</sub>-promoter interaction and the inter-regulatory effects of Fur<sub>Cj</sub>, PerR<sub>Cj</sub> and RacR in the regulation of *C. jejuni* gene expression in response to iron availability and oxidative stress. This study also provided a novel understanding of the importance of *fumC* in maintaining a functional cellular metabolism and illustrates how *C. jejuni* can effectively utilise its limited set of regulators to control essential gene expression. Ultimately it is hoped that a better
Figure 5.2: Regulation of the aspA promoter by the RacR-RacS TCS in response to low oxygen conditions. When RacR senses a decline in the extracellular oxygen concentration (a), it autophosphorylates and transfers the phosphate group to the response regulator RacR. Phosphorylated RacR binds to the promoter of aspA to repress expression. In the absence of an extracellular signal (b), unphosphorylated RacS interacts with RacR to inhibit the interaction between unphosphorylated RacR and the aspA promoter thus allowing the transcription of aspA by RNA pol.
understanding of the role of iron and oxygen as key environmental signals for controlling of *C. jejuni* pathogenesis will be achieved. The availability of such invaluable information will advance our current knowledge on the physiology and pathogenesis of *C. jejuni* and lead to potential strategies and drugs for detecting and eliminating this harmful human pathogen from infection.
Bibliography


Bagg, A and Neilands, J. B. (1987a) Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* 26:5471-5477


Bsat, N. and Helmann, J. D. (1999) Interaction of *Bacillus subtilis* Fur (Ferric uptake repressor) with the _dhb_ operator *in vitro* and *in vivo*. *J. Bacteriol.* **181:**4299-4307


transferrin utilization and is homologous to TonB-dependent outer membrane receptors. *J. Bacteriol.* **174**:5788-5797


**Cox, C. D.** (1986) Role of pyocyanin in the acquisition of iron from transferrin. *Infect. Immun.* **52**:263-270


**de Lorenzo, V., Giovannini, F., Herrero, M. and Neilands, J. B.** (1988a) Metal ion
regulation of gene expression. Fur repressor-operator interaction at the promoter region of the aerobactin system of pColV-K30. *J. Mol. Biol.* **203:**875-884


Mn(2+)-responsive transcriptional regulator. *Microbiology* **150**:1447-1456


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


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


**Florent, A.** (1953) Isolement d’un vibrion saprophyte du sperme du taureau et du vagin de la vache (*Vibrio bubulus*). *C. R. Soc. Biol.* **147**:2066-2069


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


Corynebacterium diphtheriae dtxR homologue from Streptomyces lividans and S. pilosus encoding a putative iron repressor protein. Gene 166:117-119


Fumarase C activity is elevated in response to iron deprivation and in mucoid, alginate-producing *Pseudomonas aeruginosa*: cloning and characterization of *fumC* and purification of native FumC. *J. Bacteriol.* 179:1442-1451


simulations of the periplasmic ferric-hydroxamate binding protein FhuD. *Biometals* **18**:375-386


Li, Y. (2005) Investigating the role of ferric uptake regulator (Fur) of *Campylobacter jejuni* using the electrophoretic mobility shift assay (EMSA). *M. Sc. thesis*, University of Leicester, Leicester


virulence factor of *Haemophilus influenzae*. *Microbiology* **153**:215-224


**Nowalk, A. J., Tencza, S. B. and Mietzner, T. A.** (1994) Coordination of iron by ferric iron-binding protein of pathogenic *Neisseria* is homologous to the transferrins. *Biochemistry* **33**:12769-12775


Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomic* 10:481


siderophore and exotoxin production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. *J. Bacteriol.* **175**:2589-2598


**Rampersaud, A., Harlocker, S. L. and Inouye, M.** (1994) The OmpR protein of *Escherichia coli* binds to sites in the *ompF* promoter region in a hierarchical manner determined by its degree of phosphorylation. *J. Biol. Chem.* **269**:12559-12566


Bibliography


Smith, T. and Taylor, M. S. (1919) Some morphological and biochemical characters of
the spirilla (*Vibrio fetus* n. sp.) associated with disease of the fetal membranes in cattle. *J. Exp. Med.* **310**:299-312


Thompson, S. A., Li, J. and Fields, J. A. (2009) Post-transcriptional regulation of
Campylobacter jejuni virulence properties. 15th international workshop on campylobacter, helicobacter, and related organisms abstracts. 36


FumC) activity. *J. Bacteriol.* **183**:461-467


Bibliography

Rev. 10: 466-476


