Oxidative stress responses in *Campylobacter jejuni*: the role of the Peroxide Regulator PerR

A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

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Abstract

Campylobacter jejuni is a microaerophilic foodborne pathogen capable of surviving the stressful, oxygen-exposed route from an avian host and entering into the human food chain.

The ability of *Campylobacter* to survive oxidative stress is thought to contribute to the annual ~500,000 UK cases of campylobacteriosis. One of the main regulatory proteins involved in the protective response of *C. jejuni* to oxidative stress is the regulatory protein PerR, which regulates gene expression in a metal-dependent manner, controlling the transcription of a set of peroxidases.

In this study the *perR* gene was inactivated and characterised using phenotypic tests and transcriptomic investigations. We investigated the role of PerR in the regulation of oxidative stress defences in *C. jejuni* and demonstrated that a *perR* mutant has increased aerotolerance and survival against exposure to oxidative stress. A *C. jejuni perR* mutant also demonstrated no defect in growth, motility or virulence in the *Galleria mellonella* insect model.

Analysis of microarray data using *perR*, *fur* and *fur perR* mutants allowed the identification of PerR-repressed genes (e.g. *ahpC*, *katA*, *trxB*). Differential RNA sequencing was used to identify target promoters for PerR. Proteomics (2D gel electrophoresis) and gel shift assays were also used to confirm direct regulation by PerR. The combination of these technologies allowed us to focus and hone in on the core members of the PerR regulon.

The mechanism by which PerR senses oxidative stress is still unknown, although iron has been suggested to function as co-factor. To investigate this, we expressed and purified *C. jejuni* PerR in *E. coli*. Pure PerR was used in biochemical and genetic characterisation experiments including protein crystallography trials and absorption spectrum analysis.

Further investigations are required into why the *perR* gene is evolutionary maintained in *C. jejuni* despite the beneficial nature of its absence for oxidative stress survival.

Declaration

I hereby declare that this thesis and the work described herein are original, except where indicated by reference or otherwise, and has not previously been submitted for any degree at this or any other university.

Signed: ……..

R A Handley

Date: ……..

Parts of the research described in this thesis have appeared in the scientific literature as follows:

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Collaborative Work

Throughout

- Media was kindly prepared by Maddy Houchen and glassware provided by Valerie Russell.

Chapter 3: Characterisation of the role of *perR* in *C. jejuni*

- Electron Microscopy was performed with technical and practical support from Dr Louise Salt.

Chapter 4: Characterisation of PerR and fur regulated genes in *C. jejuni*

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Chapter 5: Cloning, Characterisation and Comparison of the *C. jejuni* Peroxide Regulator (PerR)

- Mass spectrometry protein identification was performed by Dr Francis Mulholland
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1 : Chapter One

Introduction

1.1 History of *Campylobacter*

The first known description of *Campylobacter* was made by paediatric physician Theodor Escherich. In 1886 Escherich published an article in the German journal 'Münchener Medizinische Wochenshrift', noting the presence of 'spiral shaped' bacteria in the colons of infants who had died of what he described as '*Cholera infantum*'. He also confirmed the frequent presence of similar non-culturable, spiral shaped bacteria in stool samples from infants suffering from enteric disease (Escherich, 1886). Unfortunately, Escherich was unable to culture the spiral bacteria on solid medium, he also believed there was no aetiological role of the bacteria in enteric disease and did not pursue his research any further. Escherich's observations remained largely unrecognised until 1985 when his findings were rediscovered by Kist in 1985 (Escherich, 1886; Kist, 1985).

In the years preceding Escherich's article there were several reports of similar, non-culturable, spiral-shaped bacteria but the first isolation of any member of the *Campylobacter* species did not occur until 1906, 40 years later. In 1906 John McFadyean and Stewart Stockman isolated a *Vibrio* from the uterine mucus layer of a pregnant ewe, taken from a flock of sheep that had been experiencing high rates of epizootic abortion (McFadyean & Stockman, 1913). In 1919 Smith and Taylor identified a 'spirillum' whilst investigating bovine abortion and linked this back to the earlier work conducted by McFadyean and Stockman. Smith and Taylor proposed the bacteria be named *Vibrio fetus* (now *Campylobacter fetus*) (Smith & Taylor, 1919).

Prior to 1963, bacterial classifications were based on growth requirements, cell morphology and biochemical and immunological testing. The genus *Vibrio* was comprised of bacteria that resembled the causative agent of cholera (*V. cholerae*) and consequently contained species of bacteria that had curved or spiral shaped cell morphology. In 1963 Sebald and Véron proposed the creation of a new bacterial genus, '*Campylobacter*' (Kampylos – Greek meaning 'curved'). This new genus was posed to accommodate the microaerophilic *Vibrio* with low GC contents and distinguish them from the more classical cholera and halophilic *Vibrio* groups (On, 2005; Sebald & Veron, 1963; Skirrow, 1977).

It was not until 1968 that any *Campylobacter* species were linked to enteric disease in humans. Almost 90 years after Escherich's first recordings, a 'related *Vibrio*' (*C. jejuni*) was isolated from the faeces and blood of a 20-year old woman hospitalised with severe diarrhoea and fever. Previous isolations of '*Vibrio*' from human faeces had been hampered by technical difficulties (Dekeyser *et al.*, 1972). Enteric pathogens were difficult to isolate from faecal matter due to the vigorous overgrowth of common faecal bacterial flora (Butzler *et al.*, 1973; Skirrow, 1977). A breakthrough was achieved by Butzler and Dekeyser, who used differential filtration techniques on faecal suspensions in combination with selective media to successfully culture these '*Vibrios*' (*C. jejuni*) from stool samples. As *C. jejuni* was the only enteric pathogen cultured from the stool, Butzler and Dekeyser were able to link the intestinal bacterial infection as the cause of the patients symptoms and bacteraemia (Dekeyser *et al.*, 1972).

In succeeding years, the selective media and filtration methods developed by Butzler and Dekeyser aided the discovery of more '*Vibrio*-like' bacterial species from a range of sources with varying clinical symptoms. It was not until more recent years where whole genome studies of bacterial species has presented an opportunity to produce more robust phylogenetic trees.

1.2 Taxonomy

The *Campylobacter* genus is located in the order *Campylobacterales* in the Epsilon Proteobacteria class of the bacterial phylogenetic tree. The C*ampylobacterales* (Figure 1.1), although mainly comprised of the *Campylobacter* species, incorporates several other species of bacteria including *Arcobacter, Wolinella* and *Helicobacter.* The *Campylobacterales* also consists *Bacteroides ureolyticus* and the genus *Sulfurosprillum* (Debruyne *et al.*, 2008).

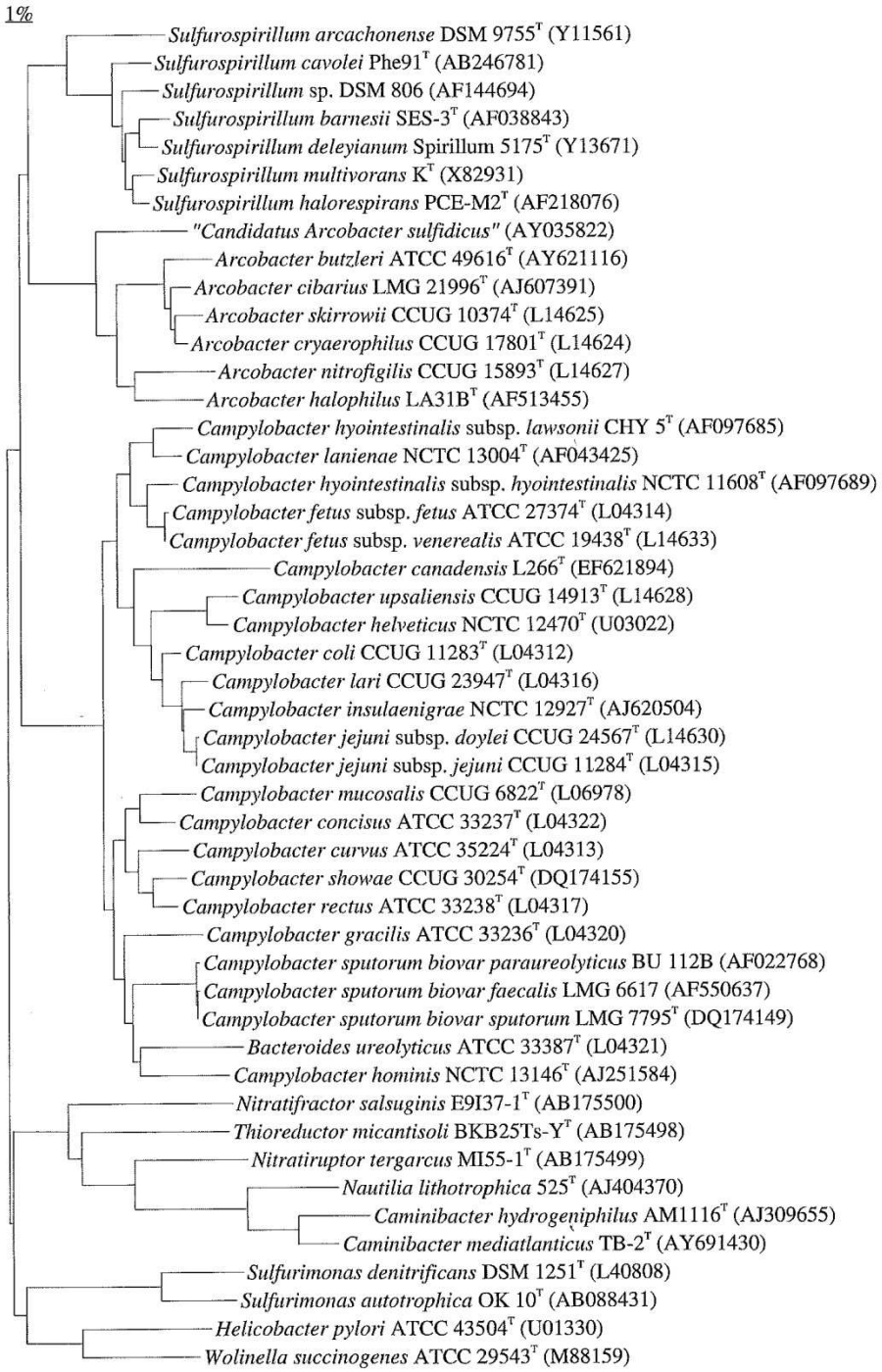

Figure 1.1: Phylogenetic tree of the family *Campylobacteraceae* and related bacteria, based on percentage 16S rRNA gene sequence similarity (Debruyne *et al.*, 2008).

As previously defined by Sebald and Véron in 1963, members of the *Campylobacteraceae* are Gram negative bacteria, with low GC content, are commonly asaccharolytic and require a microaerobic (or anaerobic)

atmosphere for growth. In general the *Campylobacteraceae* family members are curved, S-shaped or spiral rods and exist as free living organisms or live as commensals or parasites on humans and domesticated animals (Debruyne *et al.*, 2008).

The *Campylobacter* genus currently contains 17 species and six sub species, with *C. fetus* as the type strain. The *Campylobacter* genus has significant clinical and economic importance. The thermophilic *Campylobacter* species *C. jejuni* and *C. coli* are now firmly established as the most common causal agents of acute bacterial enteritis worldwide, affecting both humans and animals (Tauxe, 1992).

1.3 Campylobacter jejuni

Campylobacter jejuni is comprised of two recognised sub-species, *C. jejuni subsp. jejuni* and *C. jejuni subsp. doylei*. Compared to *C. jejuni subsp. jejuni*, relatively little is known about *C. jejuni subsp. doylei* (Parker et al., 2007). The two strains vary biochemically, *C. jejuni subsp. doylei* does not reduce nitrate, is cephalothin sensitive and has weak catalase activity (Allos, 2001; Debruyne *et al.*, 2000). The pathogenic role of *C. jejuni subsp. doylei* is also poorly understood, although it is frequently isolated in human infants with bacteraemia (Debruyne *et al.*, 2000). For clarity, throughout this thesis '*C. jejuni*' will refer solely to *C. jejuni subsp. jejuni.*

1.3.1 Morphology

C. jejuni are small (between 0.2 - 0.8 µm wide and 0.5 - 5.0 µm long), Gram negative bacteria with a spiral, S-shaped rod cell morphology, as is typical for members of the *Campylobacter* species. Their spiral shape and unsheathed, bipolar flagella make them highly motile. They move in a characteristic corkscrew-like motion making them well adapted to movement through the thick, viscous mucus layers of the gastrointestinal tract (Ferrero & Lee, 1988; Fields & Swerdlow, 1999; Griffiths & Park, 1990). They are also non spore forming and catalase, oxidase and hippurate hydrolysis positive (Griffiths & Park, 1990).

1.3.2 Growth Requirements

C. jejuni is frequently described as a fastidious organism, largely due to past difficulties in growing the bacterium in a laboratory setting. As a microaerobic and capnophilic organism, *C. jejuni* grows optimally in a range of 3-15% O_2 and 3-10% CO_2. Remarkably *C. jejuni* shows great resilience in sub-optimal growth conditions. During its natural lifecycle and infection cycle, *C. jejuni* is frequently challenged by sub-optimal atmospheric growth conditions, which can vary from aerobic to anaerobic (Svensson *et al.*, 2008). *C. jejuni* is a thermophilic organism that grows in a range of temperatures between 30 °C to 44 °C, which is thought to be an adaptation for growth in the avian cecum, its usual niche (Krause *et al.*, 2002; van Vliet *et al.*, 2002).

C. jejuni is able to grow in a range of pH conditions between 4.9 and 9.0, but grows optimally between pH 6.5 - 7.5. *C. jejuni* will not survive below pH 3.5, it is also sensitive to salinity, desiccation and freezing (Altekruse *et al.*, 1999; Le *et al.*, 2012).

1.3.3 Pathogenesis

C. jejuni is a successful pathogen, as evidenced by the high incidence of disease. This success is the result of an arsenal of virulence factors including chemotaxis, motility, secretion of cell adhesion and invasion proteins, haemolysin secretion, toxin production, biofilm formation, capsular antigens and lipooligosaccharides (Grant *et al.*, 1997; Karlyshev *et al.*, 2000; Szymanski *et al.*, 1995; Wassenaar, 1997; Wooldridge & Ketley, 1997).

The only toxin expressed by *C. jejuni* is the cytolethal distending toxin (CDT), which plays a role in cell cycle control, particularly induction of host cell apoptosis (Parkhill *et al.*, 2000; Pickett & Whitehouse, 1999).The motile nature of *C. jejuni* is an important factor in pathogenesis and non-motile strains have defects in host invasion and colonisation (Newell *et al.*, 1985; Yao *et al.*, 1994). The flagella of *C. jejuni* not only function as a mode of transport allowing the bacterial cells to reach attachment sites on host intestinal epithelial cells, the flagella themselves also secrete *Campylobacter* invasive antigens (cia) (Dasti *et al.*, 2010).

In addition to the virulence factors encoded in the *C. jejuni* genome, an estimated 17% of *C. jejuni* clinical isolates also contain a plasmid, *pVir* (Tracz *et al.*, 2005). *pVir* was initially identified in *C. jejuni* strain 81-176. It is

approximately 37.5 Kb in size and encodes a type 4 secretion system – an important virulence factor for many major bacterial pathogens (Bacon *et al.*, 2000; Bleumink-Pluym *et al.*, 2013; Christie, 2001).

1.3.4 Genomics

C. jejuni has a genome between 1.6 -1.8 Mbp, which encodes approximately 1600 genes and 54 stable RNA species, this is a relatively small number of genes compared to over 5000 genes expressed in the *Salmonella* sp (FSA, 2005). Data from the Sanger Institute indicates that *C. jejuni* has an AT rich genome and approximately 94.3% of the genome contains coding genes, giving *C. jejuni* a very compact genome structure (Parkhill *et al.*, 2000). The specific, fastidious growth requirements of *C. jejuni* are often attributed to the small genome size of *C. jejuni*. In 2007 the *C. jejuni* NCTC 11168 genome was re-annotated, making it the most comprehensively studied *Campylobacter sp* genome (Gundogdu *et al.*, 2007).

1.4 Epidemiology of *C. jejuni*

C. jejuni is well known for its ability to induce gastroenteritis in humans. However, in addition to this it is also able to cause a variety of other diseases, such as meningitis, septicaemia, proctitis, abortion and an array of auto immune diseases (Reiters arthritis, Miller-Fischer syndrome and Guillian Barre syndrome (GBS)). Gastroenteritis is by far the most frequent manifestation of *C. jejuni* infection, the symptoms of which will be discussed below in the context of a human infection (Young & Mansfield, 2005).

1.4.1 Symptoms of human *C. jejuni* infection

In the developed world a *C. jejuni* infection presents itself as an acute, self-limiting gastrointestinal illness. Early symptoms are non-specific and typically begin 1 to 3 days after ingestion of *C. jejuni*, however in the case of a low infection dose an incubation period of up to 1 week is not uncommon (Skirrow & Blaser, 1995). Symptoms commonly begin with headaches, aching limbs (myalgia), chills and fever (~ 40 ^{o}C). This is followed by the major clinical manifestation of *C. jejuni*, the onset of profuse, bloody

diarrhoea lasting for a period of 1 to 4 days. Other commonly reported symptoms include severe central or upper abdominal pain and nausea, although vomiting is rare (Butzler & Skirrow, 1979; Butzler, 2004; Skirrow, 1977). The nature of the disease is self-limiting, the disease peak may last 24 to 48 hours before gradually resolving itself, however if left untreated, 20% of affected persons will experience a relapse (Skirrow & Blaser, 1995).

In the developed world, the clinical manifestation of a *C. jejuni* infection varies greatly. Despite being more prolific, symptomatic *C. jejuni* infections primarily present in young children, causing substantial levels of morbidity (Calva *et al.*, 1988). The number of cases and duration of *C. jejuni* illness reduce with age, for example an infection may result in watery, non-inflammatory diarrhoea and many adult human hosts are asymptomatic (Friedman *et al.*, 2000; Oberhelman & Taylor, 2000; Taylor *et al.*, 1993). The symptomatic differences between developed and developing countries are attributed to natural variances in *C. jejuni* strains and the immune status of the host. In developing countries humans are exposed to high levels of *C. jejuni* in early life and appear to develop a gradual protective immunity (Blaser, 1997; Skirrow & Blaser, 1995).

1.4.2 Complications arising from *C. jejuni* infection

Campylobacteriosis sufferers have a chance of developing a severe post-infection complication, which may manifest itself up to one year after the infection with *C. jejuni*. The occurrence of post-infection complications is rare and often only associated with specific serotypes of *C. jejuni*. Complications may manifest in a variety of ways. Local complications can occur due to the direct spread of *C. jejuni* from the gastrointestinal tract, these include gastrointestinal haemorrhaging, pancreatitis, peritonitis and cholecystitis (Acheson & Allos, 2001). Manifestations of *C. jejuni* outside of the intestine are rare but may include meningitis, endocarditis, septic arthritis, osteomyelitis and neonatal sepsis (Acheson & Allos, 2001; Skirrow *et al.*, 1993).

The most clinically significant and the most widely studied complication of a *C. jejuni* infection is Guillian-Barre syndrome (GBS). GBS is an acute, demyelinating neurological disease of the peripheral nervous

system. *C. jejuni* infections are a common trigger of GBS, responsible for approximately 30% of cases, other bacterial and viral infections are also associated with causing GBS (Allos, 2001). GBS contracted post-*C.jejuni* infection is often more severe than when acquired from other sources. *C. jejuni* GBS patients experience a more severe disease with extensive axonal injury, increased chances of irreversible neurological damage and a greater likelihood of requiring mechanical ventilation. Typically symptoms of GBS will occur 1 to 3 weeks after the onset of diarrhoea. The molecular mimicry of *C. jejuni* lipooligosaccharides to the peripheral nerve gangliosides results in the production of auto-immune antibodies, which induce inflammation and damage host nervous tissue (Komagamine & Yuki, 2006).

The incidence of developing GBS post *C. jejuni* infection is approximately less than 1 per 1000 cases, but it may also occur in asymptomatic hosts of *C. jejuni* (Allos, 1997; Allos, 2001; Kuroki *et al.*, 1993).

Other important post infection complications include Miller-Fischer syndrome, Crohns disease, reactive arthritis and ulcerous colits (Hannu *et al.*, 2002; Nachamkin, 2002; Palyada *et al.*, 2009) Bacteraemia is seen in less than 1% of patients, typically those with underdeveloped or compromised immune systems (Skirrow *et al.*, 1993).

1.4.3 Incidence of infection

In the past 30 years, research has raised *Campylobacteriosis* from relative obscurity into a position where isolation rates surpass those of other common, more widely known enteric pathogens such as the *Salmonella* spp. and *Shigella* spp. (CDSC, 2001; Moore, 2001).

Campylobacteriosis refers to infections caused by either *C. jejuni* or *C. coli*. However 93% of laboratory confirmed cases of C*ampylobacteriosis* in England and Wales are due to *C. jejuni* (Gillespie et al., 2002). *Campylobacteriosis* is a global issue and *Campylobacter* are the most commonly isolated cause of bacterial gastroenteritis in both developed and developing countries (Allos, 2001).

The accurate assessment of the true number of *C. jejuni* cases that occur each year is marred by poor reporting. Due to the self-limiting nature of the illness, many cases go unreported, are not laboratory confirmed and as a

result the true population burden is far greater than indicated by national surveillance. It is estimated that the ratio of infections to reported infections for the *Campylobacter sp.* is 10 to 1 (Tam *et al.*, 2012; Wheeler *et al.*, 1999). Annually in England and Wales there are between 40,000 and 60,000 laboratory confirmed cases, but due to poor reporting this is estimated to represent an actual annual figure of over 300,000 infections, which is similar to that of other industrialised countries (McCarthy *et al.*, 2012; PHE, 2000-2011; Wheeler *et al.*, 1999).

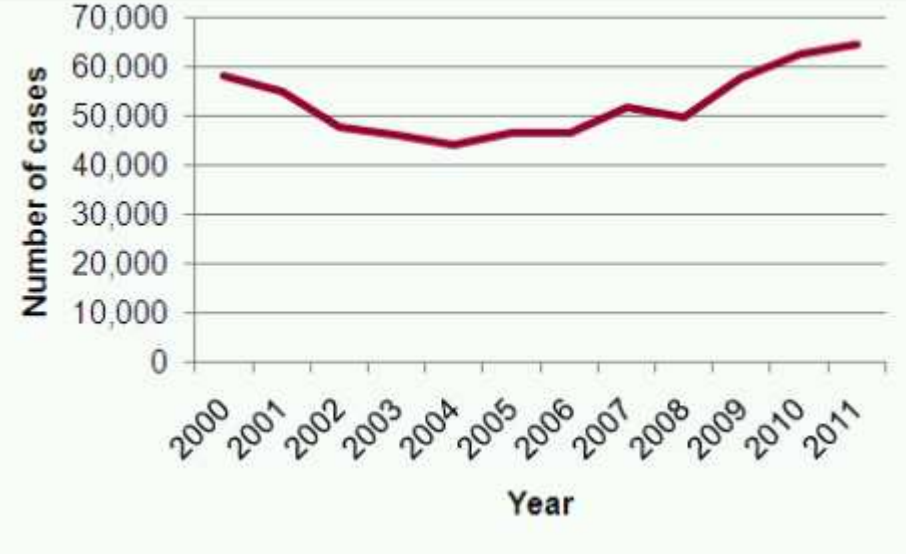

Figure 1.2: Laboratory reports of *Campylobacter* sp. infections by year in England and Wales, 2000-2011 (Public Health England data) shows a recent upward trend in the incidence of *Campylobacter* related food poisoning.

The general incidence of *C. jejuni* enteritis maintained a sustained upward trend throughout 1980 to 1990, with the reported number of *C. jejuni* infections peaking in 1998 with over 58,000 laboratory confirmed cases. Since the 1990s the incidence of *C. jejuni* infections fell, when in 2004 just over 44,544 cases were reported in the UK and Wales (PHE, 2000-2011) The number of laboratory confirmed cases of *Campylobacteriosis* detailed in Figure 1.2, shows the initial reduction in incidence lasting from 2000 to 2004 but unfortunately from 2004 to 2011, incidence of *Campylobacter* infections has increased. This increase in incidence shows a contrast to other common

food borne pathogens such as *Salmonella,* where the incidence of infection has been in decline since 1997 (FSA, 2005).

The economic burden of such a high number of infections is largely due to the loss of productive output and days taken as sick leave (Moore, 2001). In the UK in 2009 there were approximately 371,000 cases of *C. jejuni* food poisoning, resulting in more than 17,500 hospitalisations and 88 deaths. This corresponds to an economic burden of approximately £583 million pounds in England and Wales (FSA, 2005). In 1987 it was estimated that a single case of *Campylobacter* enteritis had a tangible cost of £587 per patient in the UK, however this is likely to have increased significantly in today's economy (Sockett & Pearson, 1987). In the United States the economic burden of *Campylobacter enteritis* is just as large and is estimated to cost $4.3 billion dollars annually, without considering extra costs associated with long term sequelae (Buzby & Roberts, 1997).

Incidence rates of *C. jejuni* infection vary amongst the human demographic. In the developed world the highest associated risk of infection is seen in infants, typically between 0 to 5 years of age. However it is likely that that the high risk level associated with infants is an artefact, as the tendency to retrieve samples from young children is higher and therefore isolation rates are also higher. In addition to this risk group, secondary and tertiary peaks in infection risk are seen in young adults and 50-54 year old respectively (PHE, 2000-2011; Roberts *et al.*, 2003; Tam, 2001). Higher incidences of infection are also seen in males compared to females (Skirrow, 1987).

One striking feature of *C. jejuni* enteritis in humans is the pronounced and consistent seasonal pattern of incidence throughout the year seen in temperate climates. Temperate climates show a late spring/early summer peak in *C. jejuni* infections, where the average number of cases reported may double for the same few weeks each year (Kovats *et al.*, 2005; Nylen *et al.*, 2002). Such seasonal increases in infection numbers are also seem in colder climates, although typically later on in the year (Nylen *et al.*, 2002).

The cause of *C. jejuni* seasonality is unknown, although several theories have been postulated. Increase in infections may be due to increased exposure to differing sources of *Campylobacter*, often commonly

described as ‘Barbeque season’, or alternatively the warmer climate may increase the dose of *C. jejuni* present in sources of infection (McCarthy *et al.*, 2012; Wilson *et al.*, 2008). Other more circumstantial causes have also been suggested, including the introduction of puppies into the home and birds contaminating milk supplies (Evans, 1993; Southern *et al.*, 1990).

1.5 Transmission

The successful movement from one environment to another is essential for the survival and spread of *C. jejuni*. The bacteria must be able to survive stress experienced during transmission in conditions that are growth limiting. In order to spread from one niche to another, *C. jejuni* must be able to adequately detect and respond to a host of environmental stresses. *C. jejuni* has a fastidious nature, which would be expected to limit the bacterium’s ability to survive outside normal growth conditions. However, *C. jejuni* possesses an arsenal of defences making it remarkably resilient and able to survive transmission through a variety of mediums and colonise a number of animal hosts (Purdy *et al.*, 1999).

1.5.1 Niches of *Campylobacter*

The association of poultry (chickens, turkeys, ducks and geese) and *Campylobacter* has long been recognised (Winkenwerder, 1967). Poultry is now considered to be the primary source of *C. jejuni* infections and is responsible for up to 70% of *Campylobacteriosis* infections in humans (Harris et al., 1986; Stern, 1992). *C. jejuni* is commonly regarded as commensal organisms in poultry, forming part of the natural gut flora of chickens and inducing little or no immune reaction (Stern, 1992).

C. jejuni colonises the intestinal tract of chickens, particularly the mucosal layer overlying the epithelial cells of the small intestine and ceca (Newell & Fearnley, 2003). The immune systems of chickens provides little resistance to colonisation by *Campylobacter*, which is illustrated by the absence of clinical illness in most poultry and the relatively low infection dose required for colonisation (40 CFU) (Cawthraw *et al.*, 1996; Newell & Fearnley, 2003). *C. jejuni* must pass through the avian digestive tract prior to colonisation, but it has sufficient acid resistance mechanisms to survive

exposure to the acidic proventriculus (glandular stomach) in chickens (Avila et al., 1986; Le et al., 2012). Studies indicate that young chicks become colonised with *C. jejuni* between 2 – 4 weeks of age, not from birth, suggesting that transmission occurs via an environmental source (Berndtson *et al.*, 1996; Evans & Sayers, 2000; Jacobs-Reitsma *et al.*, 1995).

The initial source of *Campylobacter* is likely to vary from flock to flock, but most studies agree that once an initial bird is infected, transmission throughout the rest of the flock occurs rapidly (Evans & Sayers, 2000; Jacobs-Reitsma *et al.*, 1995). *C. jejuni* are shed from colonised birds in high numbers and bird to bird contamination occurs via the faecal-oral route (Wesley et al., 2000). Typically the levels of *Campylobacter* on a colonised chicken ranges between 5 to 9 log CFU per gram in the small intestine and ceca reducing to between 2 to 6 log CFU per gram on a chicken carcass after slaughter and processing (Rosenquist et al., 2006). It is likely that oxidative stress during freezing accounts for some reduction in *C. jejuni* numbers of chicken carcasses.

In addition to poultry, *C. jejuni* also frequently infects other avian species and several groupings of wild birds are considered to be reservoirs of *Campylobacter*, including waterfowl, shorebirds, gulls and corvids (Waldenstrom *et al.*, 2002). After birds, there are several other major reservoirs of *C. jejuni*, these include livestock (beef, pork, lamb), dairy products, surface water and fresh produce (fruits, vegetables), all of which are important risk factors when considering the high incidence of *C. jejuni* in humans (Miller & Mandrell, 2005). In the developed world, animals for consumption are considered the primary source of *Campylobacter* infections (Adak et al., 1995; Harris et al., 1986).

Infection can easily be spread to other sources, the use of animal manure as a fertilizer promotes the further spread of *C. jejuni*, while many vegetables are not cooked or heated and may be consumed unwittingly by humans who are unaware they may carry *C. jejuni* on their surface. Surface water run-off from animal farms can contaminate ground water supplies (such as ponds and streams) with *C. jejuni*. Growing research provides evidence for the survival of the *Campylobacter* species in water, whether via interaction with protozoa or the formation of biofilms. The most common

sources of *C. jejuni* infection are undercooked meat products and unpasteurised dairy, respectively. Contaminated meat may be a source of further outbreaks through the spread *C. jejuni* to other foods in food preparation areas when food hygiene standards are not adhered to (Young *et al.*, 2007).

1.5.2 Survival of *C. jejuni* during transmission

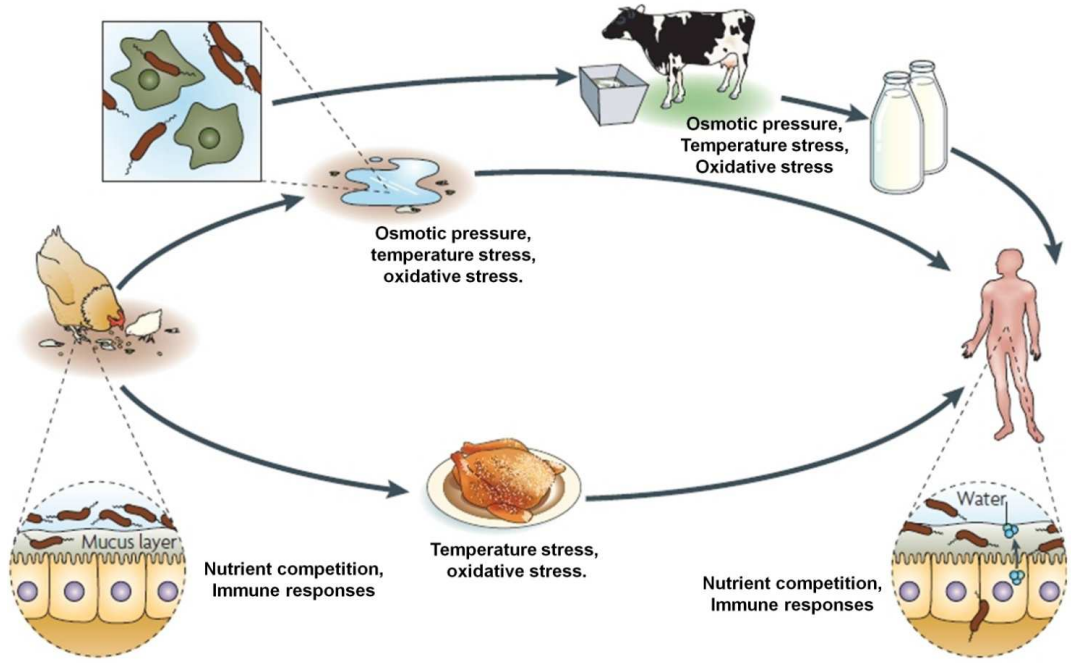

Figure 1.3: The transmission routes of *C. jejuni* leading to human infection. *C. jejuni* must survive various stresses encountered during transmission, some of which have been labelled [From (Young *et al.*, 2007)]

The spread and transmission of *C. jejuni* throughout the food chain involves many challenging conditions for the survival of a thermophilic, microaerobic organism (Figure 1.3). The route of *C. jejuni* infection from contaminated food to the gastrointestinal tract means that bacteria must survive several stressful encounters before reaching the gut (Crushell *et al.*, 2004). These conditions include the low pH of the stomach, high osmolarity, reactive oxygen and nitrogen species, low iron concentrations and various changes in nutrient concentrations (Guiney, 1997). Further determinants in

the success of colonisation include motility, invasion route, and adherence to host cells (Crushell *et al.*, 2004).

C. jejuni demonstrates a strong ability to survive exposure to these exogenous stresses, particularly oxidative and aerobic stress. As of yet, no dedicated pathway for aerobic survival or aerotolerance has been identified in *Campylobacter,* although many different genes have been implicated in the aerotolerance of *C. jejuni*, with mutants typically showing a reduced ability to survive aerobic exposure (See Section 3.3.5.4).

The production of biofilms have been linked to the increased survival of *C. jejuni* in aerobic environments. Biofilms are protective extracellular matrices secreted by C. jejuni and other bacteria, that act as a defensive, protective barrier to harsh environmental conditions (Reeser *et al.*, 2007). The ability of *C. jejuni* to form biofilms to thought to enhance the bacteria's ability to survive exogenous stress during transmission from one niche to another, by physically sheltering the bacteria from conditions unsuitable for growth. Within a biofilm bacteria are capable of generating a microenvironment within the biofilm that is more favourable for survival. *C. jejuni* biofilms may be produced on food preparation surfaces, in slaughter houses and have also been implicated in the increased planktonic survival of *C. jejuni* (Reeser et al., 2007). The effect of mutation on *C. jejuni* biofilm formation is investigated further in Chapter 3.

1.6 Oxidative stress and the reactive oxygen species (ROS)

Aerobic metabolism generates reactive oxygen species (ROS) within the bacterial cell. One of the ways ROS are generated is during the incomplete reduction of oxygen to water or step by step reduction of molecular oxygen to more reactive compounds (Figure 1.5) (Atack & Kelly, 2009a; van Vliet *et al.*, 1999).

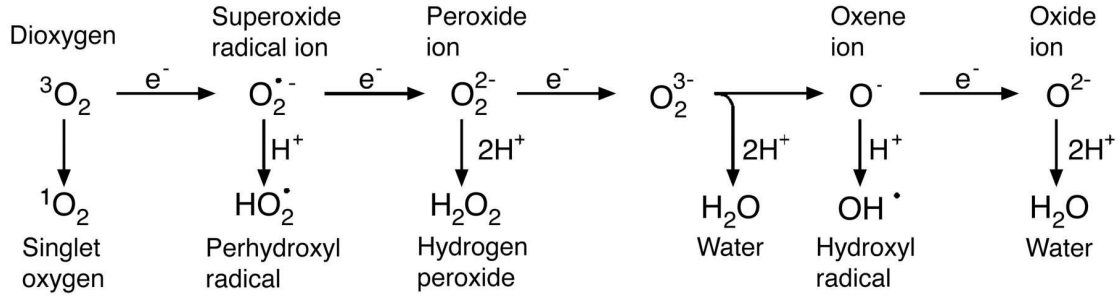

Figure 1.4: The generation of reaction oxygen species by energy transfer or reduction of oxygen [From (Apel & Hirt, 2004)].

These reactive compounds include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\cdot}$) (Atack & Kelly, 2009a; Imlay, 2003). *C. jejuni* will also encounter ROS as part of host defences. ROS are produced both inside and outside *C. jejuni* animal hosts and are a component of the lysosome, which *Campylobacter* will be exposed to during phagocytosis (Svensson *et al.*, 2008). H_2O_2 and O_2^- are also formed by the auto-oxidation of respiratory dehydrogenases (Imlay, 2008).

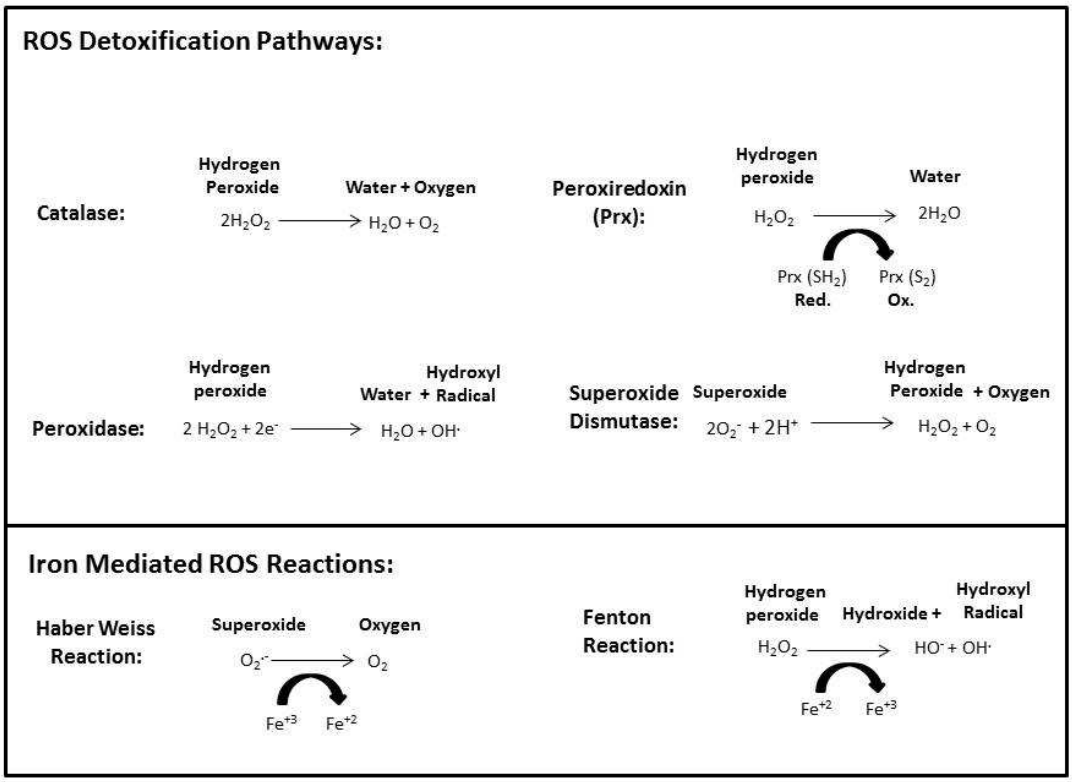

Figure 1.5: Major pathways for the generation and break down of the reactive oxygen species in *C. jejuni*.

ROS may also be formed by reaction of oxygen with cellular iron, this will be discussed later (Figure 1.5). ROS produced from a variety of sources still have the same detrimental effects on bacterial cells. They can damage DNA and proteins via oxidation, and also induce peroxidation of lipids causing damage to cell membranes (Svensson *et al.*, 2008). Methionine residues oxidised by ROS are a major source of cell damage, the formation of methionine sulfoxide can produce conformational changes in proteins (Skaar et al., 2002; Weissbach et al., 2002).

1.7 Metal Ions

Metal ions are required as cofactors for the activity of approximately one third of all proteins (Holm *et al.*, 1996). To acquire and use metal ions, bacteria have evolved a variety of high affinity transport systems that enable them to acquire them from the environment. The expression of bacterial metal acquisition systems is typically either induced or de-repressed when metal ions become a limiting growth factor, however when metal ions are in

excess the cell responds by inducing the expression of efflux pumps to mitigate toxic effects of an overload of metal ions. It is this balance that requires tight control, usually at the transcription level (Helmann *et al.*, 2007). Many genes that are involved in the uptake of metal ions are directly repressed by a protein that binds the cognate metal ion as a co-repressor (Fur/ DtxR/ NikR families).

Maintaining transition metal homeostasis requires both a sensor system to monitor cytoplasmic concentrations and a regulator system to control the balance if it shifts. These two systems are often combined in the context of available binding sites in metalloproteins (Maret, 2010).

The nutritionally essential metal ions from the first transition series are required for metalloprotein function. These ions can be ordered into the affinity at which the divalent metal ions interact with ligands, known as the Irving-Williams series. This abides to the following order of affinity (Irving & Williams, 1948):

$$\mathbf{Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}}$$

Of these metal ions, manganese (Mn), iron (Fe), and copper (Cu) are redox active, and their affinities for ligands can be altered by either oxidation (Fe and Mn) or reduction (Cu). Cobalt is also redox active, as part of cobalamin (Vitamin B12) (Silverman & Dolphin, 1973). Zn is considered redox inert in biological systems (Maret, 2010).

The affinity of a protein for a metal ion is usually high if the ion is required for the protein to fulfil its biological function (Maret, 2010). In catalytic and structural metal ions, the equilibrium between free and protein bound metal is far to the right. Free metal concentrations under equilibrium can also be predicted using the Irving Williams series order of metal affinities. Free metal ion concentrations are important to biological functions as they determine boundaries for regulatory function and adverse effects on biological processes. Based on equilibrium considerations, metal ions must be buffered in narrow ranges within a cell to prevent interference with functions of other metal ions (Li & Maret, 2009).

The best studied and most biologically widespread metal responsive regulators are members of the ferric uptake regulator (Fur) family of proteins (Ahn *et al.*, 2006). Fur orthologues act as global regulators to maintain metal

ion homeostasis, by specifically binding Fur operators in promoters and controlling their own homeostasis (Patzer & Hantke, 1998). In addition to roles in homeostasis, Fur family proteins are also associated with roles in anti-oxidative responses and virulence factors (Hantke, 2001; Ratledge & Dover, 2000).

1.7.1 Iron and oxidative stress

Iron is an essential micronutrient for almost all living organisms and its acquisition from surrounding environments is especially important to bacteria (Wooldridge & Williams, 1993). As a transition metal, iron participates in electron transfer reactions, has key roles as an enzyme cofactor and it participates in redox reactions (van Vliet *et al.*, 2002). Iron has many other significant biological roles in proteins. Proteins containing iron in the form of heme or iron-porphyrin compounds are associated with roles in basic cell metabolism, such as roles in bacterial respiration, electron transport and the reduction of peroxide. Other iron containing proteins include iron-sulphur proteins, which have roles in anaerobic respiration, energy and amino acid metabolism and electron transport reactions. Also other non-heme, non-iron sulphur proteins are used during DNA synthesis, superoxide removal and during protein and amino acid biosynthesis (Earhart, 1996; van Vliet *et al.*, 2002).

Iron is a versatile cation often existing in ferrous (Fe^{2+}), ferric (Fe^{3+}) and ferryl (Fe^{4+}) forms, producing a redox potential spanning from -500 mV to +300 mV versus normal hydrogen electrode (NHE) (Andrews *et al.*, 2003). However, in solution the presence of oxygen and the physiological pH produce an oxidising environment that favours the formation of ferric (Fe^{3+}) iron, which forms an insoluble hydroxide (Holmes *et al.*, 2005).

Iron homeostasis is carefully monitored on many levels, including uptake, utilization, and storage (van Vliet *et al.*, 2002). This is due to the risks associated with excess ferrous iron and the production of harmful hydroxyl radicals via the Fenton and Haber Weiss reactions, as shown in Figure 1.5 (Palyada *et al.*, 2009).

In Fenton-Haber Weiss reactions, ferrous iron aids the formation of the hydroxyl radical via the breakdown of hydrogen peroxide. It is for this

reason that iron homeostasis is often paired with oxidative stress responses within the cell and oxidative stress responsive proteins use iron cofactored prosthetic groups. Excess iron may also lead to iron toxicity within the cell (Storz & Imlay, 1999; Touati, 2000; van Vliet *et al.*, 2002). However reduced iron availability is as equally detrimental to bacteria as an excess of iron. Low iron conditions may lead to diminished bacterial growth. It is for this reason that low iron concentrations can serve as an environmental signals for bacterial gene expression, typically those involved in iron uptake or bacterial virulence (Litwin & Calderwood, 1993).

Many pathogenic enteric bacteria are known to possess multiple iron uptake systems. Siderophores are low molecular weight iron chelating compounds produced by bacteria, expression of siderophores is often coupled with expression of high molecular weight outer membrane proteins that act as iron-siderophore complex receptors (Field *et al.*, 1986). The *Campylobacter* species do not produce their own siderophores, they are able to survive and successfully compete for iron in the gastrointestinal tract by parasitizing the iron-chelating compounds produced by other indigenous bacterial species in the gut (Field *et al.*, 1986; Parkhill *et al.*, 2000).

Iron acquisition is an important determinant in the success of a pathogen, particularly evident during growth and host colonisation. *C. jejuni* has several systems aiding iron acquisition including a siderophore transport system (enterochelin) (CeuBCDE) (Richardson & Park, 1995), a ferric enterobactin uptake receptor (CfrA) (Guerry *et al.*, 1997), a haemin/haemoglobin-uptake system (ChuA) (Ridley *et al.*, 2006) and a high affinity iron transporter (called P19) (Chan *et al.*, 2010; van Vliet *et al.*, 2002), all of which are regulated by the *C. jejuni* ferric uptake regulator (Fur).

1.7.2 Metalloregulators

Metalloregulation refers to the regulation of gene expression in response to metal ion availability (O'Halloran, 1993). A metalloregulatory protein can sense levels of specific metal ions, and respond via mediating a transcriptional or translational response (Helmann *et al.*, 2007). The most widely known metalloregulatory protein is the ferric uptake repressor (Fur) in *E. coli*, which has often been used as a model for other regulators within the

same family. Fur is known to regulate iron dependent repression of iron uptake pathways (Gaballa & Helmann, 1998).

1.8 Fur Family Metalloproteins

The Fur family metalloregulators are capable of sensing iron (Fur), zinc (Zur), manganese (Mur), Nickel (Nur) and oxidative stress (PerR). It is not uncommon for multiple Fur homologues to be present in a single genome, as seen in *B. subtilis* which has three (Bsat *et al.*, 1998; Gaballa & Helmann, 1998). Some of the bacterial species where homologues of the Fur family have been identified are detailed in Table 1.1 along with their putative roles.

The proteins within the Fur family are likely derived from a common ancestor and have high sequence identity, yet respond to different metals. It is difficult to explain how subtle sequence changes contributes to alternate metal selection and alternate protein functions (Helmann *et al.*, 2007).

Table 1.1: Representatives of the Fur family metalloproteins (Lee & Helmann, 2007). Question marks indicate gaps in knowledge.

Protein subfamily	Organism	Structural/ Regulatory metal ion	Function(s)	Reference(s)
Fur	*E. coli*	Zn^{2+}/Fe^{2+} (Mn^{2+})	Iron uptake Iron sparing	(Mills & Marletta, 2005) (Masse & Gottesman, 2002)
	B. subtilis	Zn^{2+}/Fe^{2+}	Iron uptake	(Bsat & Helmann, 1999)

	C. jejuni	Zn^{2+} /?	Iron uptake	(Butcher *et al.*, 2012)
	P. aeruginosa	Zn^{2+}?/Fe^{2+}	Iron uptake Iron sparing	(Lewin *et al.*, 2002; Pohl *et al.*, 2003) (Wilderman *et al.*, 2004)
	N. meningitidis	?/Fe^{2+}	Iron uptake Respiration	(Delany *et al.*, 2004) (Delany *et al.*, 2004)
	H. pylori	?/Fe^{2+}	Iron uptake Iron storage	(Delany *et al.*, 2001) (Delany *et al.*, 2001)
	B. japonicum	-/Fe^{2+}	Iron uptake	(Friedman & O'Brian, 2004)
Zur	*E. coli*	Zn^{2+}/Zn^{2+}	Zn^{2+} uptake	(Outten & O'Halloran, 2001)
	B. subtilis	Zn^{2+}/Zn^{2+}	Zn^{2+} uptake Zn^{2+} mobilization	(Gaballa & Helmann, 1998) (Akanuma *et al.*, 2006)
Mur	*R. leguminosarum*	-/Mn^{2+} (Fe^{2+})	Mn^{2+} uptake	(Diaz-Mireles *et al.*, 2004)
Nur	*S. coelicolor*	?/Ni^{2+}	Ni^{2+} uptake	(Ahn *et al.*, 2006)

PerR	*B. subtilis*	Zn^{2+}/Fe^{2+} (Mn^{2+})	Oxidative stress *srf* operon	(Lee & Helmann, 2006b) (Hayashi *et al.*, 2005)
	S. aureus	Mn^{2+} (Fe^{2+}?)	Oxidative stress	(Horsburgh *et al.*, 2001)
	S. pyogenes	$Zn^{2+}/Fe^{2+}/Ni^{2+}$	Oxidative Stress	(Makthal *et al.*, 2013)
	C. jejuni	See Chapter Five	Oxidative stress	(Palyada *et al.*, 2009; van Vliet *et al.*, 1999)
Irr	*B. japonicum*	-/Fe-heme	Iron uptake Oxidative stress	(Hamza *et al.*, 1998; Yang *et al.*, 2006) (Rudolph *et al.*, 2006a; Rudolph *et al.*, 2006b)
	R. leguminosarum	?/Fe^{2+}	Iron	(Singleton *et al.*, 2010; White *et al.*, 2011)

The Fur family are likely to maintain a conserved structure, with an amino-terminal DNA binding domain and carboxyl-terminal metal binding domain (Coy & Neilands, 1991; Stojiljkovic & Hantke, 1995). This conservation of structure is likely to include the presence of two metal-binding sites per monomer, comprised of two conserved cysteines and at least one histidine residue forming a tetrahedral zinc site, as well as a second metal binding site that can sense variable divalent cation levels (Gaballa & Helmann, 1998; Jacquamet *et al.*, 1998). The specificity of Fur divalent cation sensing is probably decided by specific, precise, spatial

arrangements of potential metal ligands surrounding the regulatory site. This recognises the relaxation of specification in some Fur family repressors, where some proteins are capable of binding an assortment of metal ions. For example, the peroxide stress response in *B. subtilis* can be elicited by manganese, iron or other weakly bound metal ions; however the repression of iron uptake by Fur is only iron responsive (Bsat *et al.*, 1998; Chen *et al.*, 1993; Chen *et al.*, 1995).

C. jejuni encodes two members of the Fur metalloprotein family, Fur and PerR, the peroxide stress regulator (Chan *et al.*, 1995; van Vliet *et al.*, 1999), the regulatory role of these proteins in *C. jejuni* is explored in Chapter Four.

1.8.1 The Ferric Uptake Regulator (Fur)

Fur was one of the first metalloregulatory proteins to be genetically and biochemically classified (Bagg & Neilands, 1987). Early research showed that *E. coli* Fur mutants were unable to utilise succinate as a carbon source and they also constitutively expressed siderophores and siderophore uptake systems. These Fur mutants were also more resistant to excess manganese (Mn^{2+}). Demonstrating that Mn^{2+} can bind the Fur repressor in the place of iron, potentially preventing cells from obtaining sufficient iron for growth (Hantke, 1987). Work on Fur in *E. coli* provided a genetic selection basis, allowing for identification of *fur* genes in other Gram negative bacteria (Funahashi *et al.*, 2000; Prince *et al.*, 1993; Thomas & Sparling, 1996). The Fur system was also confirmed in Gram positive bacteria when the genome of *B. subtilis* was sequenced in 1997 and three Fur homologues were identified (Kunst *et al.*, 1997).

1.8.1.1 Mode of Gene Regulation

Fur is an iron responsive repressor protein that down regulates expression of iron transport systems in response to high levels of intracellular iron (Hantke, 1981). Repression is achieved when Fe^{2+} levels reach a sufficient cellular concentration to allow one Fe^{2+} to co-ordinate with a Fur dimer (Escolar *et al.*, 1999; Stojiljkovic & Hantke, 1995). This 2Fur- Fe^{2+} dimerization complex then binds a specific, conserved sequence motif

upstream of the target gene; this conserved sequence is known as a Fur box and is located within the promoter region of the Fur regulated gene (Stojiljkovic *et al.*, 1994). The affinity of Fe^{2+} for the Fur protein is sufficiently low to allow cellular iron levels to accumulate enough to activate essential metalloenzymes (for heme and iron sulphur cluster synthesis) prior to the iron transport machinery being suspended (Lee & Helmann, 2007).

Fur is capable of binding to other metals as well as iron, but with varying dissociation constants; these metals includes Mn^{2+}, Co^{2+} and Cu^{2+}, all of which can activate Fur (Hamed, 1993). It is likely that Fur has adapted to react with many other metal ions as well as iron, but with the exception of Mn^{2+} other common metal ions rarely reach high enough concentrations to activate Fur. For example, Copper binds Fur with greater affinity than iron, but there is no free copper in the cytosol of bacteria, so binding rarely occurs (Changela *et al.*, 2003).

Genes typically under the regulation of Fur include siderophore uptake systems, which are directly involved in iron uptake. Hence when iron reaches a sufficient intracellular concentration transcription of genes that are involved in iron uptake are repressed (Coy & Neilands, 1991). Drops in iron concentration lead to the competitive removal of iron from the Fur complex, causing Fur to disassociate from target gene promoters, allowing transcription to occur (Palyada *et al.*, 2004). Fur is a repressor, but equally has been shown to have roles as an activator, via repressing genes that inhibit the repression of other nearby sequences.

Fur regulation is usually via direct repression of gene expression in response to iron, but additional more complex regulation is also evident. Transcriptomic experiments indicate genes may be expressed at higher levels in iron replete conditions, indicating repression of gene expression by apo-Fur (Delany *et al.*, 2001; Delany *et al.*, 2004). Fur regulation in other systems has been demonstrated to be mediated indirectly via small RNAs, best characterised is RyhB of *E. coli* (Masse & Arguin, 2005); RyhB orthologues have also been described in *Shigella* and *Vibrio* (Mey *et al.*, 2005; Payne *et al.*, 2006).

1.8.1.2 Fur Boxes

Fur has been demonstrated to regulate genes by the recognition of a unique sequence upstream from the target gene, known as a 'Fur Box' (See Figure 1.7 for *C. jejuni* Fur box). Fur boxes are predicted to be 19-nucleotide long sequences found preceding putative iron uptake genes in a wide range of bacteria (Panina *et al.*, 2001; Rodionov *et al.*, 2004).

Early theories predicted that Fur binding occurred by the recognition of tandem arrays of at least three hexamers (GATAAT) (Escolar *et al.*, 1999). Revised models of Fur binding, based on *B. subtilis,* identified a shorter putative Fur Box consisting of 15 bp (Baichoo & Helmann, 2002). A sequence alignment of approximately twenty Fur regulated genes in *B. subtilis* revealed a conserved binding motif of 15 base pairs comprised of a 7-1-7 base pair, inverted repeat (Baichoo *et al.*, 2002). Two copies of this motif overlap to form the typical 19 nucleotide Fur box, which corresponds to the binding of two Fur dimmers to the DNA (Lavrrar & McIntosh, 2003). There is some flexibility in this binding site, most Fur proteins conform to the larger 19 nucleotide Fur box sequence, but at some promoters, Fur boxes match the 7-1-7 consensus.

In addition to Fur, the other two Fur homologues of *B. subtilis* bind consensus Fur Box-like DNA sequences that only differ from the Fur box by one or two base pairs per half-site (Fuangthong & Helmann, 2003).

1.8.1.3 The Structure of Fur

The structure of Fur has been solved from a number of bacterial species. It is believed a non-classical helix-turn-helix motif in the N-terminus of the Fur protein is responsible for DNA binding and the C-terminus contains the dimerization and Fe^{2+} domains (Gonzalez de Peredo *et al.*, 2001; Stojiljkovic & Hantke, 1995).

The *E. coli* Fur protein contains two metal binding sites; a structural, tightly bound zinc site involving Cys-92, Cys-95 and one histidine (Althaus *et al.*, 1999; Coy *et al.*, 1994; Jacquamet *et al.*, 1998). The other metal binding site is found within a hexacoordinated histidine and carboxylate ligand environment and binds exchangeable Fe^{2+} (Helmann *et al.*, 2007).

The crystal structure of the Fur protein from *P. aeruginosa* showed an N-terminal binding domain and a C-terminal dimerization domain. Two metal

binding sites per monomer were observed, and one of these sites connects the two domains; via the co-ordination of side chain residues (Pohl *et al.*, 2003). The iron binding site is within the dimerization domain of the protein, and is co-ordinated by His86, Asp88, Glu107, and His 124 residues (Helmann *et al.*, 2007).

There is evidence that the structure of the Fur metal binding sites is variable. The Fur of *P. aeriginosa* has only one cysteine, this is analogous to Cys-92 of *E. coli* Fur, however it is not involved in metal binding. In *P. aeriginosa*, Fur metal binding sites are highly conserved compared to Fur homologues in other species, but in *Bradyrhizobium japonicum* multiple mutations can be made in the protein sequence of the Fur metal binding sites and the Fur protein will still react to Fe^{2+} (Friedman & O'Brian, 2004).

1.8.1.4 *C. jejuni* Fur

In the genome of *C. jejuni*, *cj0400* encodes a Fur homolog with 40% identity to the *E. coli* Fur protein, with the majority of the identity residing in the C-terminus containing the iron binding sites (Chan *et al.*, 1995; Parkhill *et al.*, 2000; Schaffer *et al.*, 1985). The *C. jejuni* Fur protein was functionally identified via reporter gene analysis, mutant complementation and also independently via upstream sequencing of the *lysS* gene (Chan *et al.*, 1995; Wooldridge *et al.*, 1994).

When compared to the Fur from other bacteria, *C. jejuni* Fur has many different features (Butcher *et al.*, 2012; van Vliet *et al.*, 2000). The *C. jejuni fur* gene is located in an operon along with housekeeping genes, *lysS* and *glyA*. A formation usually found only in *Campylobacter upsaliensis*, a member of the *Campylobacter* family that is rarely clinically isolated (Bourke *et al.*, 1998; Chan *et al.*, 1995; van Vliet *et al.*, 2000). Fur is not auto-regulated in *C. jejuni* and unlike several other well studied Gram-negative bacteria, it has been possible to experimentally inactivate *C. jejuni* Fur via insertional mutagenesis, allowing for more in depth research into the role of *fur* (van Vliet *et al.*, 2000).

Fur mutants in *C. jejuni* show decreased growth compared to wild-type strains (although this may possibly be an artefact produced by downstream effects) and decreased resistance to hydrogen peroxide and

cumene hydroperoxide (van Vliet *et al.*, 1998; van Vliet *et al.*, 1999) (See Chapter Three). Transcriptomic analysis of *C. jejuni* Fur mutants reveal a large number of differentially expressed genes, many of which are typically associated with Fur regulation including as major iron transport systems for haemin, ferric iron and enterochelin, as well as many iron transport genes (*p19, cj1658, cj0177, cj0178,* cj1663 and *cfrA*) (Butcher *et al.*, 2012; Holmes *et al.*, 2005; Palyada *et al.*, 2004; van Vliet *et al.*, 1998). In addition to the genes classically regulated by Fur, *C. jejuni* Fur also regulates several oxidative stress responsive proteins (PerR, KatA, AhpC, Tpx and SodB) and other proteins thought to be physiologically relevant under iron limited conditions (FldA, TrxA, TrxB) (Palyada *et al.*, 2004). The regulon of *C. jejuni* Fur is further explored in Chapter Four of this thesis.

In *C. jejuni* no uniform Fur box has been identified for all transcriptional targets of Fur. Instead *C. jejuni* Fur recognises multiple consensus sequences depending on regulation. Holo-Fur recognises different sequences for activated and repressed genes. The holo-Fur repressed consensus sequence has a similar 7-1-7 palindromic structure seen in other Fur boxes. However the holo-Fur activated sequence is markedly different from other known fur boxes and is non-palindromic and comprised of contains two direct 5 bp repeat sequences (Butcher *et al.*, 2012). No consensus Fur box has yet been identified for apo-Fur in *C. jejuni*.

Structurally *C. jejuni* Fur is distinct from other characterised Fur homologues in *H. pylori*, *Vibrio cholerae* and *Pseudomonas aeruginosa* (Figure 1.6)(Dian *et al.*, 2011; Lewin *et al.*, 2002; Sheikh & Taylor, 2009). *C. jejuni* apo-Fur protein has the canonical V-shaped dimer of previously characterised holo-Fur proteins, where apo-Fur in *B. subtilis* loses its V-shape (Butcher *et al.*, 2012).

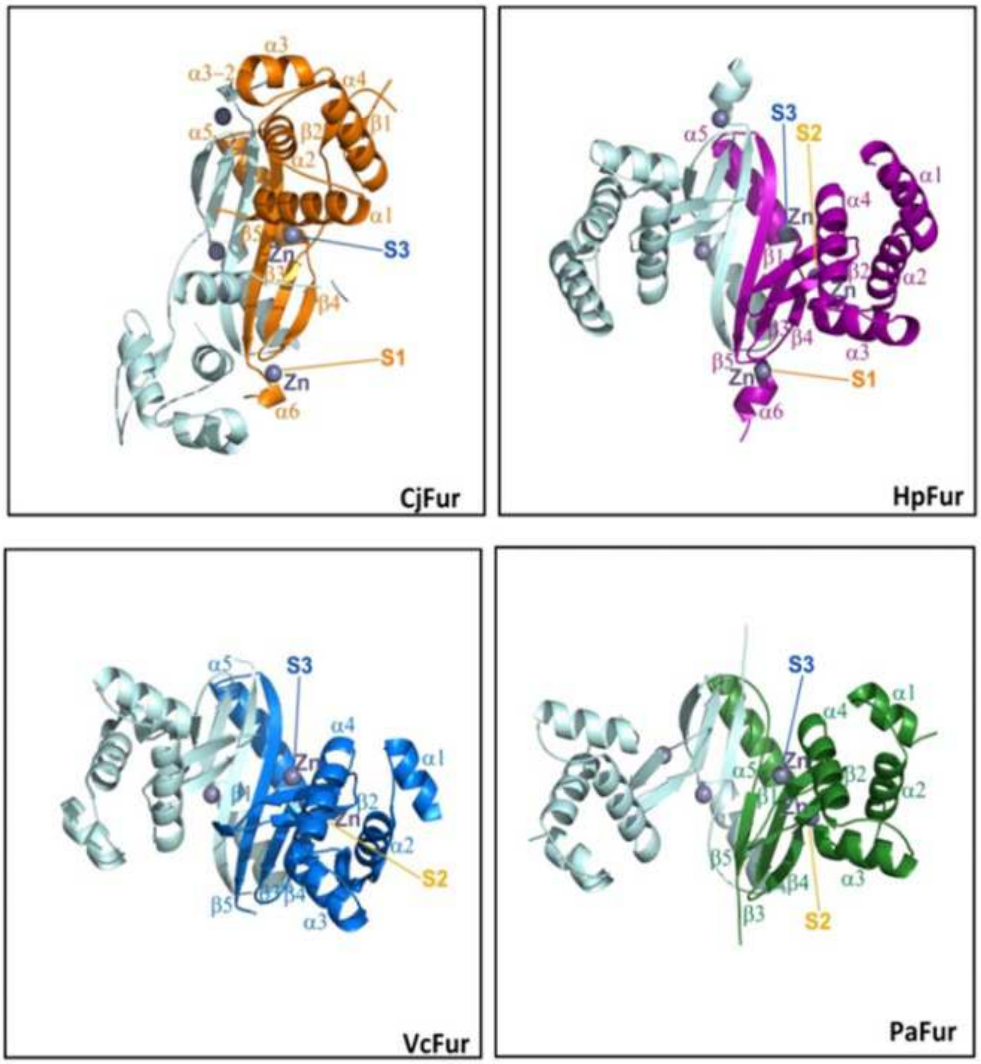

Figure 1.6: An overview of Fur protein structures (Protomer A) from *C. jejuni* (orange), *H. pylori* (purple), *V. cholerae* (blue) and *P. aeruginosa* (green). Beta sheets, alpha helices and metal binding sites of each Protomer A are labelled. The dimerization domain of all structures are shown in the same orientation [Figure adapted from (Butcher *et al.*, 2012) supplementary material].

The *C. jejuni* Fur V-shaped dimer is comprised of a structurally unique hinge domain that modulates the position of the Fur DNA binding domain upon binding of iron. This hinge domain has a 180 degree rotation compared to other Fur structures, putting the DNA binding domain of *C. jejuni* apo-Fur on the exterior of the protein structure. The rotation of the DNA binding domain is due to the unique elongation of the hinge region in *C. jejuni* Fur,

showing that the hinge region has an important role in modulating DNA binding in *C. jejuni* Fur. The movement of the DNA binding domain likely explains the ability of *C. jejuni* apo-Fur to recognise different DNA binding sequences for gene repression and activation. Holo-Fur DNA activation and repression was also observed yet no distinct consensus DNA binding sequences could be identified (Butcher *et al.*, 2012).

Fur purified from *C. jejuni* with two zinc ions (one coordinated by four cysteines), it is regarded as apo-Fur as two binding sites (including the regulatory binding site) were empty. These two sites were non-permissive to metal ion binding due to the rotation of the DNA binding domain. (Butcher *et al.*, 2012).

1.8.2 The Peroxide Repressor (PerR)

PerR (the peroxide repressor) is the central regulator of oxidative stress detoxification pathways in *B. subtilis*, amongst other species. It provides an inducible response to hydrogen peroxide where gene regulation is mediated by metal availability and the presence of reactive oxygen species. The PerR system is functionally analogous to the *E. coli* OxyR and SoxRS systems, these DNA regulators control the transcription of a set of oxidative stress detoxification pathways (Aslund *et al.*, 1999; Herbig & Helmann, 2001; Zheng *et al.*, 1998).

1.8.2.1 PerR Gene Regulation

Members of the *B. subtilis* PerR regulon include several adaptive responses to hydrogen peroxide such as KatA (a vegetative catalase), AhpCF (alkyl hydroperoxide reductase), MrgA (Dps-like binding protein), ZosA (a Zn^{2+} uptake P-type ATPase), HemAXCDBL (a haem biosynthesis operon), Fur and PerR itself (Antelmann *et al.*, 1996; Bsat *et al.*, 1996; Chen & Helmann, 1995; Chen *et al.*, 1995; Fuangthong *et al.*, 2002; Hartford & Dowds, 1994).

PerR acts as a repressor of gene function in a similar manner to Fur, by recognising specific binding sites (PerR Boxes) upstream of regulated genes and blocking access of the transcription machinery (Herbig & Helmann, 2001).

Exposure to oxidative stress alleviates PerR repression of DNA, although the mode of PerR activation is still debated.

One hypothesis for the mode of action of PerR is based on structural characteristics of the PerR protein. It has been proposed that PerR senses oxidative stress via metal-catalysed oxidation of a histidine residue (Lee & Helmann, 2006b). Histidine oxidation in PerR occurs when bound iron (Fe^{2+}) incorporates an oxygen atom into His-37 or His-91. Oxidation leads to iron release and dissociation of the PerR-DNA adduct (See Figure 1.7).

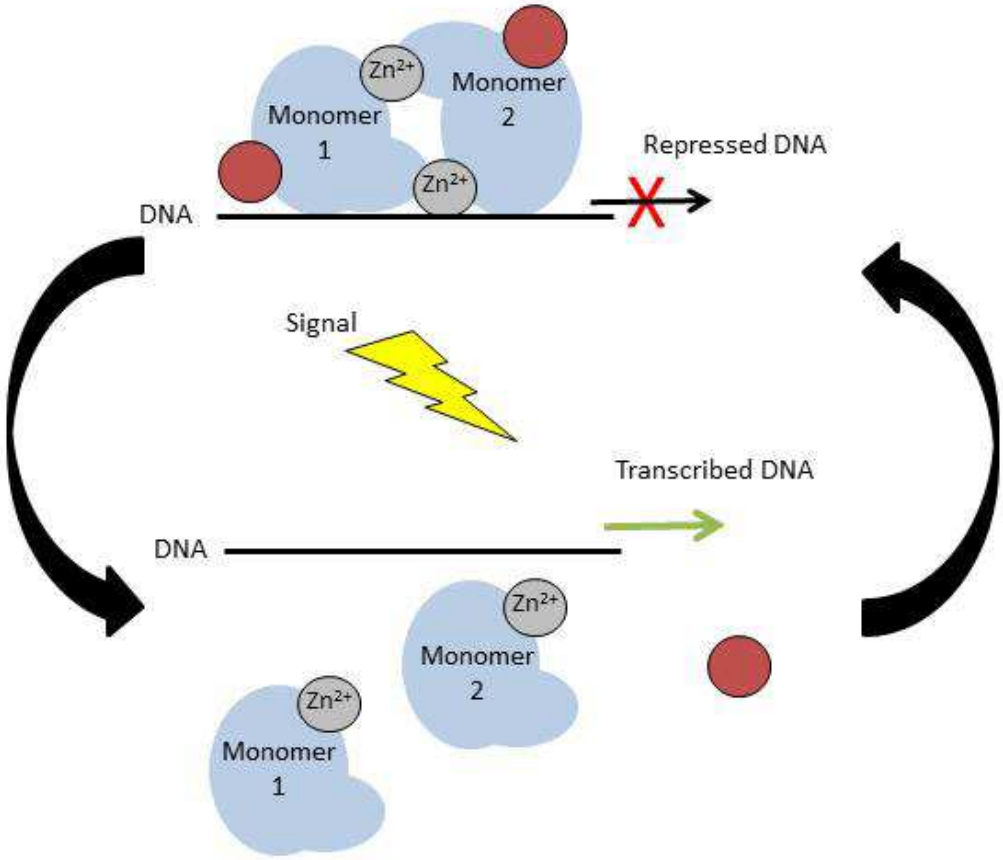

Figure 1.7: The mode of action of PerR mediated DNA transcription. In *B. subtilis* PerR represses DNA by binding DNA and blocking access of the transcription machinery to the DNA. PerR binds DNA as a dimer, the formation of which is stabilised by the binding of one zinc ion per monomer. Each PerR also binds a secondary metal ion, typically either iron of manganese. In response to a signal, such as oxidative stress the PerR dimer disassociates from the DNA, allowing transcription of the DNA to occur.

This mechanism relies on the reduction of ROS by iron and the subsequent production of a reactive hydroxyl radical. The hydroxyl radical produced may then react with histidine residues generating the 2-oxo-histidinyl radical intermediate and finally producing 2-oxo-histidine (Schoneich, 2000; Uchida & Kawakishi, 1993).

In vivo PerR can exist in two different metallated forms: PerR-Zn-Fe or Per-Zn-Mn, whether Fe^{2+} or Mn^{2+} is bound, PerR acts as a repressor of gene function. However in both *B. subtilis* and *Streptococcus pyogenes*, only iron co-factored PerR is sensitive to peroxide stress - iron is required for the generation of the hydroxyl radical needed for histidine oxidation (Lee & Helmann, 2006b; Makthal *et al.*, 2013). Hydrogen peroxide (H_2O_2) causes PerR to dissociate from DNA, however when Mn^{2+} is present PerR-peroxide induction does not occur and PerR remains bound to the DNA. In contrast, if iron is bound peroxide induction is maximal and produces expression of the *perR* regulon comparable with the *perR* null mutant (Fuangthong *et al.*, 2002; Herbig & Helmann, 2001).

1.8.2.2 Structure of PerR

Bacillus subtilis is the first organism whose PerR crystal structure was solved and characterised and as such has been extensively characterised (Lee & Helmann, 2006a; Traore *et al.*, 2006; Traore *et al.*, 2009).

The B. subtilis PerR crystal structure revealed two metal binding sites per monomer (Lee & Helmann, 2006a; Traore *et al.*, 2006; Traore *et al.*, 2009). Metal ions have been shown to play an important part in the role of *B. subtilis* PerR. The two metal binding sites of *B. subtilis* PerR have very distinct functions, with one assigned a structural role and the other as a regulatory site (Herbig & Helmann, 2001).

The zinc (Zn^{2+}) binding site is comprised of all four cysteine residues in the *B. subtilis* PerR amino acid sequence, Cys-96, 99,136 and 139. These are arranged in two Cys-$(X)_2$-Cys motifs, a strictly conserved characteristic of PerR-like proteins. Zinc is co-ordinately tetrahedrally amongst the cysteines with bond lengths ranging from 2.32-2.36 Å, which is suggestive of co-ordinated by four thiolates (2.31 is the expected distance for a thiolate-Zn^{2+}). The tertiary fold of PerR is locked in place by the binding of a metal ion and

is an essential part of the PerR dimerization domain, explaining why metal binding plays an important role in maintaining the structural stability of *B. subtilis* PerR (Traore *et al.*, 2006).

Evidence suggests that five residues, His-37, His-91, His-93, Asp-85 and Asp-104 are likely to form the second metal binding site in *B. subtilis* PerR. All of these five residues are conserved amongst the protein sequences of PerR-like family members. Several metal ions have been demonstrated to bind this site, but it is unlikely that many of these have physiological significance, except for manganese (Mn^{2+}) and iron (Fe^{2+}), which have been shown to effect PerR transcription. In *B. subtilis*, expression of PerR can be blocked by divalent metal ions Mn^{2+} and Fe^{2+} and growth of *B. subtilis* in Mn^{2+} rich media prevents high level resistance of *B subtilis* to hydrogen peroxide (Bsat *et al.*, 1996; Chen *et al.*, 1995).

Together these five residues (His-37, His-91, His-93, Asp-85 and Asp-104) have a strong affinity for binding iron (Fe^{2+}) and all of these residues have been shown to be essential for the metal binding and repressor function of PerR (Lee & Helmann, 2006a). Of the residues forming the second regulatory metal binding site, one (His-37) is located in the N-terminal domain, whereas the other four are in the C-terminal. The binding of His-37 to a metal ion in this site draws the N and C-terminal domains close together allowing the formation of a DNA binding domain in the tertiary fold. Oxidation of His-37 may prevent iron binding and therefore interfere with the DNA binding activity of PerR (Lee & Helmann, 2006a; Traore *et al.*, 2006).

The structure of PerR has also been solved in *S. pyogenes*, a member of the Group A *Streptococcus.* Structural analysis of *S. pyogenes* PerR indicates a different mechanism of PerR metal binding and peroxide sensing than that described in *B. subtilis*. The PerR of *S. pyogenes* has a unique, 11-amino acid, N-terminal arm, which has not been observed in any other members of the Fur family metalloproteins, yet it is conserved across PerR in the *Streptococcus* genus. Like *B. subtilis* PerR, *S. pyogenes* PerR has two metal binding sites per monomer. The four cysteine, structural zinc site is highly similar to that seen in *B. subtilis* and other Fur family proteins, however the second regulatory metal binding site in *S. pyogenes* is comprised of residues located on the unique, N-terminal arm. Unlike *B.*

subtilis, the respective His-37 residue of *S. pyogenes* does not participate in metal binding and is involved in inter-domain interactions meaning *S. pyogenes* PerR likely has a different mechanism of oxidative stress sensing compared to *B. subtilis (Makthal et al., 2013)*.

1.8.2.3 *C. jejuni* PerR

In *C. jejuni*, *perR* is located approximately 70kb from *fur* on the genome (van Vliet *et al.*, 1999). Until recently it was believed that in *C. jejuni* PerR was unlikely to be a global regulator as only two proteins had been shown to be under its regulation (catalase and alkyl hydroperoxide reductase), and the growth of these mutant strains mimics that of the parental strain (van Vliet *et al.*, 1999). However new transcriptomic studies have indicated that PerR may have much broader control over gene expression. A genome wide transcriptional profile of the PerR regulon revealed a total of 143 genes that are differentially regulated by PerR with at least 104 genes belonging to the PerR regulon (Palyada *et al.*, 2009). Interestingly this study also revealed a subset of genes that did not respond to the oxidant stimulus hinting at alternative regulatory roles for PerR.

Data from Palyada *et al* showed PerR to be a major regulator of oxidative stress responses in *C. jejuni*, with greater control over oxidative stress responses compared to Fur, despite the two regulons having functional overlap (approximately 50 co-regulated genes in *C. jejuni*) (Palyada *et al.*, 2009; van Vliet *et al.*, 1999). Microarray data showed PerR regulates genes in response to oxidative stress-inducing chemical treatments. PerR controlled 74% of genes responding to stimulation by cumene hydroperoxide, 73% of genes in response to stimulation by menadione, and 85% of genes regulated in response to hydrogen peroxide (with or without the presence of iron) (Palyada *et al.*, 2009). Of the genes regulated in response to the oxidant stimulus, many were unique to the stimulus, with 11, 12 and 8 genes being uniquely up-regulated in response to cumene hydroperoxide, menadione and hydrogen peroxide respectively, indicating the existence of oxidant-specific transcriptome changes (Palyada *et al.*, 2009). However it is likely that regulation in *C. jejuni* has higher levels of complexity, as genes that have previously been shown to be involved in

oxidative stress responses, such as *katA, ahpC* and ferritin (*cft*) were repressed in low iron conditions in a wild-type strain. This is despite the need for iron for PerR to function as a repressor (Palyada *et al.*, 2009). PerR regulation in *C. jejuni* is discussed in further detail in Chapter 4.

A putative consensus sequence for PerR binding has been described (Kim *et al.*, 2011) (Figure 1.8), however due to the AT rich nature of the *C. jejuni* genome it is difficult to identify PerR regulatory targets based on this sequence alone.

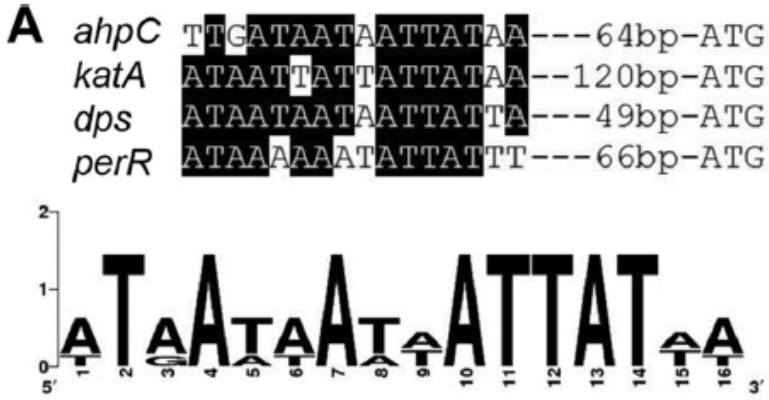

Figure 1.8: The putative *C. jejuni* PerR binding sequence. A multiple alignment of PerR regulated genes with putative PerR binding sequences highlighted in black. A sequence logo of the putative PerR box generated using WebLogo 2.8.2 [From (Kim *et al.*, 2011)]

It is believed that whilst PerR only recognises PerR boxes, Fur may be able to recognise both Fur and PerR boxes in DNA sequences (Baillon *et al.*, 1999; van Vliet *et al.*, 2002).

1.8.2.4 PerR Mutant Characterisation in *C. jejuni*

A *C. jejuni* strain with a 69% deletion of the *perR* gene showed similar growth properties to the wild-type strain (Palyada *et al.*, 2009). To assess the role of *perR* in response to oxidative stress the same mutant was exposed to a range of oxidants: cumene hydroperoxide, menadione and hydrogen peroxide (Palyada *et al.*, 2009). The mutant showed increased resistance to both cumene hydroperoxide and hydrogen peroxide compared to the wild-type as a result of *perR* being a repressor of *katA* and *ahpC* expression

(Palyada *et al.*, 2009; van Vliet *et al.*, 1999). The *perR* mutant showed increased sensitivity to menadione, supporting the notion of PerR also being an activator of genes, possibly involved in menadione resistance (Palyada *et al.*, 2009). PerR mutants are reported to demonstrate a significantly altered ability to colonise in chick animal models. Compared to the wild-type levels of $2x10^7$ CFU per gram of caecal content, a PerR mutant exhibited a decrease to $2x10^4$ CFU per gram. However PerR mutants are also reported to have decreased motility compared to wild-type strains which may allow for easier immune clearance (Crushell *et al.*, 2004; Palyada *et al.*, 2009). A double mutant of *fur* and *perR* in *C. jejuni* is still viable, however colonisation is greatly reduced to below 100 CFU per gram of caecal content (Palyada *et al.*, 2009; van Vliet *et al.*, 1999), showing that iron regulation and oxidative stress responses are vital for gut colonisation.

The phenotype of a *C. jejuni* PerR mutant is further explored and characterised in Chapter Three of this thesis.

1.9 Campylobacter Oxidative Stress Defences

To actively resist or avoid the damage caused by ROS, protective proteins are constantly expressed in low levels by bacterial cells to protect against, and repair any cellular damage (Traore *et al.*, 2006). Characteristically, these proteins are negatively controlled by regulators creating an inducible, flexible response system (Mongkolsuk & Helmann, 2002). Some of the major defensive proteins and their regulators are detailed in Figure 1.9.

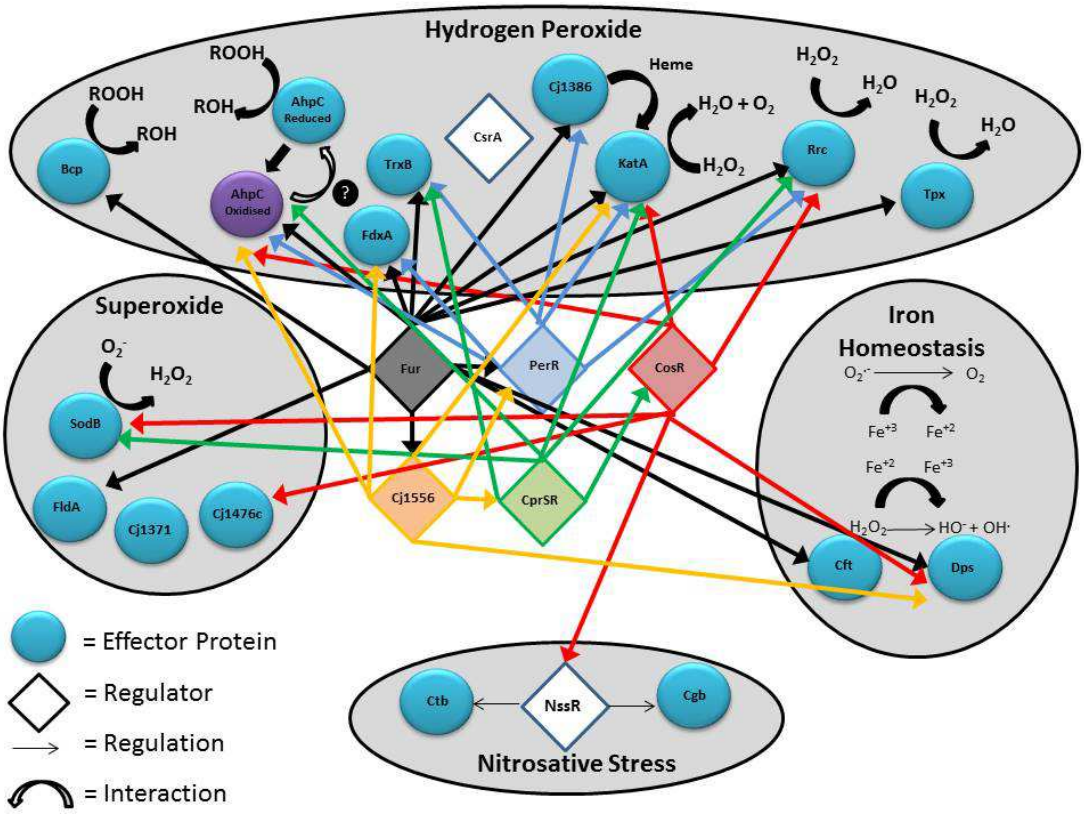

Figure 1.9: Major oxidative stress effector proteins in *C. jejuni* and their regulators.

1.9.1 Peroxide Resistance Effector Proteins

Peroxides are generated at various stages throughout *C. jejuni* transmission and can damage *C. jejuni* both intra- and extracellularly. Peroxides form a major part of the ROS stress burden on *C. jejuni,* and as a result the bacterium has evolved a number of different detoxification pathways. *C. jejuni* contains multiple detoxification pathways for combating general hydroperoxides and more specifically hydrogen peroxide, with systems proposed to function in different cell locations, such as the periplasm or cytoplasm. Some of these major peroxide detoxification systems, as shown in Figure 1.9 will be discussed here.

1.9.1.1 Alkyl hydroperoxide reductase, Ferredoxin and Thioredoxin Reductase

In *C. jejuni* an alkyl hydroperoxide reductase (AhpC, Cj0334) has been linked to increased aerotolerance (Baillon *et al.*, 1999). AhpC proteins

are also referred to as thiol-specific antioxidants (TSA) or peroxiredoxins (See Figure 1.5). When reduced, they degrade alkyl hydroperoxides to the corresponding alcohols whilst becoming oxidised in the process (ROOH + $AhpC^{red}$ → ROH + $AhpC^{ox}$). Oxidised AhpC proteins can be recycled back to the reduced protein by a recycling partner, similar to the AhpF protein in *E. coli*. AhpF re-reduces AhpC by transferring electrons from NAD(P)H to the oxidised AhpC (Poole, 1996; Poole & Ellis, 1996). However, in *C. jejuni* and the closely related pathogen *Helicobacter pylori*, no AhpF homologs have been found – indicating an alternative method of AhpC recycling (Tomb *et al.*, 1997). Proteins such as ferredoxin (FdxA) or thioredoxin reductase (TrxB) have been suggested as potential recycling partners in *C. jejuni*; their roles as potential recycling partners for AhpC will be discussed below.

The role of AhpC in *C. jejuni* aerotolerance was noted by Baillon *et al*, (1999) who showed that in stationary phase cells, an *ahpC* mutant had a significantly reduced ability to survive in an aerobic environment (aerotolerance) compared to the wild-type strain. Alkyl hydroperoxide reductases are able to detoxify peroxidated molecules and destroy hydroperoxide intermediates generated by reactive oxygen species, which typically challenge *C. jejuni* during aerobic exposure (Baillon *et al.*, 1999). Ferredoxins (*fdxA*) are reducing agents (the *C. jejuni fdxA* gene encodes a 4Fe-4S ferredoxin) and *fdxA* is divergently located from *ahpC* in the *C. jejuni* genome, which is why it is a tempting candidate as an AhpC recycling partner (van Vliet *et al.*, 2001b) However, both *ahpC* and *fdxA* have differential regulation under iron depletion. Whereas *ahpC* expression is induced in low iron conditions under the control of PerR and the *Campylobacter* Oxidative Stress Regulator, CosR (discussed in Section 1.10.3), *fdxA* is repressed and regulated by Fur (Baillon *et al.*, 1999; Holmes *et al.*, 2005; Hwang *et al.*, 2011; Palyada *et al.*, 2009; van Vliet *et al.*, 2001b). In *C. jejuni* strain 81116, an *fdxA* mutant showed reduced aerotolerance and increased sensitivity to hydrogen peroxide and cumene hydroperoxide, indicating a potential role during *C. jejuni* survival on food surfaces and in the environment (van Vliet *et al.*, 2001a; van Vliet & Ketley, 2001).

Thioredoxin reductase (TrxB) is a flavoprotein that reduces disulphide-containing substrates via electron transfer from pyrimidine nucleotides by a

flavin carrier. In the *C. jejuni* genome, it is located adjacent to *trxA*, which encodes a thioredoxin that undergoes reversible oxidation and reduction mediated by TrxB and NADPH (Arner & Holmgren, 2000; Cohen *et al.*, 1993). Under iron limitation, both TrxA and TrxB have increased expression, owing to TrxB's strong reductase activity and similar regulation to AhpC, it makes a plausible candidate as a recycling partner (Holmes *et al.*, 2005). However, both TrxB and FdxA are regulated by Fur, whereas AhpC is not, indicating further levels of complexity in the search for a recycling partner for AhpC.

1.9.1.2 Bcp (Cj0271)

Bacterioferritin Comigratory protein (Bcp) is a thiol peroxidase with a broad substrate range in *C. jejuni*, although in *E. coli* and *H. pylori* Bcp uses linoleic acid hydroperoxide as a preferred substrate. In *C. jejuni* Bcp provides important protection against protein oxidation, aerobic exposure and lipid peroxidation. Lipid peroxidation is particularly important when host inflammatory responses are triggered during infection where membrane lipids will be oxidised (Jeong *et al.*, 2000; Wang *et al.*, 2005)

1.9.1.3 Catalase and Cj1386

Catalase enzymes are ubiquitous in bacteria and several different types of catalase enzymes exist, these show intricate patterns of regulation and substrate specificity. The catalase enzyme rapidly dismutates hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2), removing the hydrogen peroxide generated by the dismutation of superoxide by SodB (Grant & Park, 1995; van Vliet *et al.*, 2001a). *C. jejuni* contains a single heme-cofactored catalase that is important for *C. jejuni* survival against aerobic and oxidative stresses in the environment and against macrophage killing by oxidative bursts. Therefore it may also contribute to cell survival during infection (Grant & Park, 1995). However, in *C. coli*, *katA* mutants did not show a reduced ability to survive aerobically on chicken skin and skimmed milk (Purdy *et al.*, 1999; Stead & Park, 2000). This hints at the presence of alternative hydrogen peroxide detoxification pathways, for example AhpC (see above) or Rrc encoded by the *Cj0012c* gene, a recently characterised unique

desulforubrerythrin, which possesses a significant hydrogen peroxide reductase activity (Pinto *et al.*, 2011; Yamasaki *et al.*, 2004).

In the *C. jejuni* genome, directly downstream from catalase is (*cj1386*) and despite not being co-transcribed with catalase, it is believed to function within the same hydrogen peroxide detoxification pathway. KapA may function in heme trafficking to catalase (Flint *et al.*, 2012), heme is required for catalase to breakdown hydrogen peroxide.

1.9.1.4 Tpx (Cj0779)

The *C. jejuni* thiol peroxidase (Tpx) belongs to a group of peroxiredoxins that are commonly found throughout bacterial species (Cha *et al.*, 2004; Rho *et al.*, 2006; Wang *et al.*, 2005). In other bacteria, Tpx is capable of breaking down a variety of peroxides, similar to Bcp (Section 1.9.1.2). However, in *C. jejuni* Tpx is a dedicated hydrogen peroxide reductase and shows no activity against other peroxides. A *C. jejuni tpx* null mutant shows poor growth capabilities in aerobic conditions and *tpx* also has increased expression when *C. jejuni* cells are in a biofilm (Atack *et al.*, 2008; Kalmokoff *et al.*, 2006). Both of these findings are indicative of a role for Tpx in the survival of *C. jejuni* during transmission throughout aerobic environments.

1.9.1.5 Rrc (Cj0012c)

The **R**ubredoxin oxidoreductase-**R**ubreythrin like protein of ***C****. jejuni* (Rrc), also termed Desulforubreythin (DRbr) is a non-heme, iron- containing protein. It was first recognised in *C. jejuni* for its apparent degradation after *C. jejuni* cells were exposed to hydrogen peroxide (Yamasaki *et al.*, 2004). Rrc is a novel multidomain metalloprotein and the first characterized example of a protein containing an N-terminal desulforedoxin like domain, a four helix bundle domain and a C-terminal rubredoxin domain. Rrc is conserved in all sequenced *Campylobacter* species and has a significant NADH-linked hydrogen peroxide reductase activity (Pinto *et al.*, 2011). Rrc is also unique in that currently it is the only oxidative stress effector protein to be jointly regulated by all four major oxidative stress regulation systems in *C. jejuni*, incorporating PerR, Fur, CosR and CprSR regulation (Holmes *et al.*, 2005;

Hwang *et al.*, 2011; Palyada *et al.*, 2009; Svensson *et al.*, 2009). This tight regulation hints that Rrc has a significant role in oxidative stress detoxification, one that has not yet been fully recognised. The role of Rrc in oxidative stress defences in *C. jejuni* is further investigated in Chapter Six of this thesis.

1.9.2 Superoxide Effector Proteins

Superoxide is a harmful member of the ROS, and the sole *C. jejuni* superoxide dismutase (*cj0169*) was the first ROS-responsive gene identified in *C. jejuni*. It is responsible for catalysing the dismutation of superoxide to H_2O_2 and dioxygen (Pesci *et al.*, 1994; Purdy & Park, 1994; Svensson *et al.*, 2008). SOD genes are classified by their metal co-factors, commonly copper-zinc, nickel, manganese or iron. However, unlike *E. coli,* which expresses three SOD proteins (Fe, Mn and CuZn-cofactored), *sodB* encodes the sole SOD present in *C. jejuni* and requires iron as a cofactor (Lynch & Kuramitsu, 2000; Pesci *et al.*, 1994; Purdy & Park, 1994). SodB and its role during *C. jejuni* transmission are discussed here, along with other proteins that have been shown to function in response to paraquat, a superoxide generating compound.

1.9.2.1 Superoxide Dismutase

The superoxide dismutase enzyme is ubiquitous in all kingdoms of life, and catalyses the dismutation of superoxide into hydrogen peroxide and oxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$). The hydrogen peroxide generated during this process is then further detoxified by catalase (KatA). *Campylobacter* species have a single SOD enzyme (Cj0169), which contains iron as co-factor (Pesci *et al.*, 1994; Purdy & Park, 1994); this plays important roles in the protection of *C. jejuni* and *C. coli* against superoxides and other oxidative stresses. In *C. coli*, *sodB* null mutants showed reduced aerotolerance in stationary phase cells, had a reduced ability to survive freeze-thawing, and were less able to survive on foods such as chicken skin and skimmed milk (Purdy *et al.*, 1999; Stead & Park, 2000).

1.9.2.2 Other Superoxide Effector Proteins: Flavodoxin (FldA), Cj1371, and Cj1476c

Garenaux *et al* (2008) looked at the responses of *C. jejuni* to paraquat, a superoxide anion generating compound. Using global proteomic profiling they observed differential regulation of a number of *C. jejuni* genes in response to paraquat exposure, some of these were known oxidative stress response effector proteins, whereas other proteins identified were less well characterised. These proteins include FldA, Cj1476c, Cj1371 and the orphan response regulator CosR (Garenaux *et al.*, 2008a).

Flavodoxin and Cj1476c, a pyruvate-flavodoxin oxidoreductase were over expressed in response to paraquat, both suggesting roles in oxidative or redox stress responses. *C. jejuni* flavodoxin is an electron acceptor for the pyruvate oxidoreductase complex (POR) responsible for catalysing NADPH production by oxidative decarboxylation. Flavodoxin is iron repressed and located in an operon (*Cj1384c-Cj1383c-fldA*) divergently transcribed from catalase (*katA).* In comparison, little is known about Cj1371, it is co-ordinately expressed with flagellar proteins although not directly involved in motility (Garenaux *et al.*, 2008a; Palyada *et al.*, 2004). In other bacteria such as *Pseudomonas putida,* homologues of Cj1371 have been shown to function in response to other sources of oxidative stress, namely phenol induced oxidative stress (Santos *et al.*, 2004).

1.9.3 Nitrosative Stress Effector Proteins

Nitric oxide and its redox intermediates are intricately linked with the ROS. Nitrosative stress forms part of the protective response of humans, which *C. jejuni* encounters during infection and invasion. The stomach is a source of nitrosative stress, generating nitric oxide during the digestion of food. Nitrosating compounds also form part of the protective inducible defence systems of intestinal epithelial cells, which are encountered by *C. jejuni* during cell invasion (Dykhuizen *et al.*, 1996; Elvers *et al.*, 2004).

1.9.3.1 Haemoglobins (Ctb, Cgb, NssR)

C. jejuni expresses two putative haemoglobins: a single domain globin, known as Cgb (*Campylobacter* Globin, Cj1586) and a truncated

globin Ctb (Cj0465c). Cgb has been implicated in a protective response to nitrosative stress, being up-regulated when *C. jejuni* is challenged with nitrosative stress. Mutants lacking Cgb are hypersensitive to nitrosating agents, which may indirectly link with oxygen-related stresses. *cgb* mutants also show a reduced ability to survive exposure to aerobic environments (Elvers *et al.*, 2004; Wainwright *et al.*, 2005). The *C. jejuni* truncated haemoglobin Ctb is also thought to function in the nitrosative stress response regulon, which is controlled by the regulatory protein NssR (Cj0466), although it may also play a role in moderating O_2 flux in *C. jejuni* (Elvers *et al.*, 2005; Wainwright *et al.*, 2005).

1.9.4 Iron Homeostasis Effector Proteins

Iron homeostasis is carefully monitored on many levels including uptake, utilisation, and storage. To date, nine iron uptake and transport systems have been identified in *C. jejuni* (van Vliet *et al.*, 2002). When excess iron and ROS are both present there is a risk of cell damage (Palyada *et al.*, 2009) from Haber-Weiss reaction and Fenton chemistry (Figure 1.5).

Iron homeostasis proteins that are also known to function during aerobic stress are illustrated in Figure 1.5; these include *Campylobacter* ferritin (Cft), a Dps homologue (Dps) and the ferric uptake regulator (Fur).

1.9.5 Ferritin (Cft) and Dps

Campylobacter ferritin (Cft) has a significant role in *C. jejuni*, functioning in both iron storage and intracellular iron homeostasis. Iron homeostasis has a dual role, by limiting intracellular free iron concentrations, ferritin also protects *C. jejuni* from iron mediated oxidative stress, such as the Fenton and Haber Weiss reactions (Garenaux *et al.*, 2008a). In other species, over-expression of ferritin has been shown to increase aerotolerance by removing free iron from the cell. This prevents the Fenton reaction from occurring and allows bacteria to survive in an aerobic environment for much longer (Touati *et al.*, 1995; Wai *et al.*, 1996).

1.9.5.1 Dps

The Dps protein is encoded by the c*j1534c* gene. It derives its name from the *E. coli* counterpart (DNA binding protein from starved cells) and is important for oxidative stress resistance. It may play a role similar to bacterioferritin by sequestering iron, thus effectively reducing the opportunity of free metal ions to produce harmful Fenton Chemistry intermediates (Ishikawa *et al.*, 2003).

The *C. jejuni* Dps protein shows high affinity DNA binding in the presence of Fe^{2+} or hydrogen peroxide under acidic conditions, as shown with both electrophoretic mobility shift assays and surface plasmon resonance (Huergo *et al.*, 2013). Moreover, Dps-DNA complexes are protected from DNase I degradation and damage from hydroxyl radicals, suggesting a direct role for *C. jejuni* Dps in protecting DNA from oxidative damage. Two studies have shown that inactivating the *dps* gene in *C. jejuni* results in increased sensitivity to hydrogen peroxide (Huergo *et al.*, 2013; Ishikawa *et al.*, 2003).

1.10 Regulation of Aerobic and Oxidative Stress Responses

Gene regulation in *C. jejuni* is complex and control of oxidative stress effector proteins is no exception. PerR - the peroxide regulator, and Fur - the ferric uptake regulator, are two major transcriptional regulators in *C. jejuni* however several other regulators have been recognised as controlling regulation of oxidative stress effector proteins, these are summarised below.

1.10.1 CprSR

The *Campylobacter* planktonic growth regulation system *cprSR* (*cj1227-1226*) encodes a two-component regulatory system, which consists of a membrane-associated signal-transducing histidine kinase (CprS) and a cytoplasmic response regulator (CprR). *C. jejuni* cells lacking CprS show colonisation defects in chickens, but enhanced motility and biofilm formation (Svensson *et al.*, 2009). The CprSR system also influences transcription levels of genes encoding different proteins involved in oxidative stress responses, such as *katA*, *trxB* and *ahpC* (repressed) and *sodB* and *rrc* (induced).

1.10.2 Cj1556

The *C. jejuni* genome encodes several putative regulatory proteins for which no specific function can be predicted. One of these genes (*cj1556*) encodes a MarA family transcriptional regulator, when inactivated results in a reduced ability to form biofilms, decreased infection of the *Galleria mellonella* invertebrate virulence model and increased sensitivity to aerobic and oxidative stress (Gundogdu *et al.*, 2011).

1.10.3 CosR

The *Campylobacter* oxidative stress regulator is an essential OmpR type response regulator in *C. jejuni* that controls expression of approximately 93 proteins, including some of the major oxidative stress effector proteins (SodB, Rrc, Dps, AhpC and KatA) (Hwang *et al.*, 2011; Hwang *et al.*, 2012). The *cosR* gene in *C. jejuni* is essential and cannot be inactivated. In order to study genes under its regulation, expression of *cosR* was suppressed using antisense peptide nucleic acids (PNA).

CosR differentially regulates expression of a number of genes important to the *C. jejuni* protective response to oxidative stress, including *dps*, *rrc, ahpC*, *sodB, luxS* and *katA.* LuxS itself has been shown to regulate *ahpC* and *tpx* in the presence of hydrogen peroxide in *C. jejuni* (He et al., 2008). CosR acts a repressor of *sodB*, *dps*, *rrc* and *luxS*, and an activator of *katA* and *ahpC* (Hwang *et al.*, 2012). Expression of *cosR* in *C. jejuni* can be manipulated by the presence of oxidative stress. Expression was reduced in response to challenging *C. jejuni* with paraquat, a super-oxide generating compound, which is in keeping with its role in repressing oxidative stress responses (Garenaux *et al.*, 2008a). However expression was not altered by the addition of hydrogen peroxide, indicating CosR has more functions in response to superoxide stress, which is implied by its regulation of superoxide dismutase (Hwang *et al.*, 2011).

1.10.4 CsrA

CsrA is a homolog of the *E. coli* carbon storage regulator and a putative transcriptional regulator in *C. jejuni*. In *E. coli* CsrA is an RNA-binding global regulator that mediates gene expression by post transcriptionally regulating translation, decaying specific mRNA targets (Liu &

Romeo, 1997; Romeo, 1998). More specifically, CsrA inhibits the ribosome from binding the ribosome binding site by itself binding the 5' untranslated region of target mRNAs. This prevents the initiation of translation and may increase or decrease the half-life of the mRNA (Baker *et al.*, 2002; Romeo, 1998). CsrA also potentially has many roles in *C. jejuni*; it has been identified as having regulatory roles in motility, adherence and oxidative stress responses. In *C. jejuni* strain 81-176, a *csrA* mutant had a reduced ability to survive oxidative stress, including hydrogen peroxide stress and reduced ability to survive aerobic exposure compared to the wild-type. A *csrA* mutant also had reduced motility and biofilm formation, indicating an activating role for CsrA in these processes. Interestingly, the *csrA* mutant had an increased ability to invade human cells; this suggests that CsrA may have important functions during *C. jejuni* pathogenesis. At present little is known about *C. jejuni* CsrA, such as details of its mRNA targets and during what stages of the *C. jejuni* growth phase it is active. Further work is required to understand this regulator, however it is clear it has important functions in *C. jejuni* survival after exposure to stress (Fields & Thompson, 2008).

Thesis Aims

C. jejuni is frequently described as being a fastidious organism due to its microaerophilic, capnophilic and thermophilic nature (Atack & Kelly, 2009b). These conditions mimic those of the avian jejunum, the natural niche and large reservoir of *C. jejuni* (Atack, 2010; Jacobs-Rietsma *et al.*, 2000). However, oxygen is present throughout the *C. jejuni* infection cycle (Figure 1.3) and the oxidative stress burden upon this microaerophilic organism whilst exposed to aerobic conditions will be substantially greater than that of its normal environment in the avian cecum. So how does a microaerophilic organism such as *C. jejuni* survive in an aerobic environment? Many of the oxidative stress detoxification proteins of *C. jejuni* are regulated by PerR (See Figure 1.9), and therefore PerR may have a significant role in the survival of *C. jejuni* within the human food chain. The number of cases of Campylobacteriosis seen annually are increasing and greater understanding of the survival mechanisms of *C. jejuni* are required if rates of infection are to

be reduced. Whilst transcriptomic studies of *C. jejuni* have been performed, little is known about the mechanism by which PerR functions and it is not clear how PerR protein is activated and by what signal (Palyada, 2009). Therefore the aims of this thesis are:

- To characterise the regulatory role of PerR in *Campylobacter jejuni* oxidative stress defence.
- To investigate the biochemical properties of *C. jejuni* PerR protein.

2 : Chapter Two

Materials and Methods

2.1 General Lab Methods

2.1.1 Chemicals

All chemicals and synthesized oligonucleotides used in this investigation were purchased from Sigma-Aldrich, unless otherwise stated.

2.1.2 Autoclaving and Sterilisation Techniques

All microbiological work was carried out according to standard laboratory aseptic techniques. When possible work was carried out within a Class II Microbiology Safety Cabinet (Walker BS 5726).

All solutions, pipette tips and glassware were sterilised by autoclaving at 69 KPa for 20 minutes in a B&T Autoclave 225 5H. When autoclaving was unsuitable, solutions were filter-sterilised using 0.22-0.4 μM Ministart filter units from Sartorius Stedim Biotech.

A MilliQ Ultrapure water system (Barnstead) was used to provide microbiology grade water throughout this investigation.

2.2 Bacterial Growth

2.2.1 Bacterial Strains Used

Table 2.1: Bacterial strains used in this investigation.

Strain	Genotype	Source
Escherichia coli BL21 (DE3)	F $^-$ ompT hsdSB ($r_B^- m_B^-$) gal dcm	Novagen
Escherichia coli Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen

Campylobacter jejuni NCTC 11168	Wild-type	(Parkhill *et al*., 2000)
Campylobacter jejuni 11168 Δ*flaAB*	NCTC 11168 (Δ*cj1338, Δcj1339c*)::kan^{R}	(Reuter & van Vliet, 2010)
Campylobacter jejuni 81116 (NCTC 11828)	Wild-type	(Pearson *et al.*, 2007)
Campylobacter jejuni 81-176	Wild-type	(Hofreuter *et al.*, 2006)
Campylobacter jejuni 11168 Δ*fur*Δ*perR*AS	NCTC 11168 (Δcj0400, Δcj0322^{-}) ::kan^{R},cm^{R}	(Palyada *et al*., 2009)

2.2.2 Growth Media

2.2.2.1 Luria Broth (LB) Medium

- For the standard growth of *E. coli* strains

10 g Bacto^{TM} tryptone (Difco Laboratories)
5 g Bacto^{TM} yeast extract (Difco Laboratories)
10 g NaCl
pH to 7.0 with NaOH

2.2.2.2 Super Optimised Broth with Catabolite Repression (SOC)

- Nutrient rich media for the growth of *E. coli* during transformation.

2 % (w/v) Tryptone
0.5 % (w/v) Yeast extract
8.56 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose
H_2O to 1000 ml
pH to 7.0 with NaOH

2.2.2.3 Brucella Medium

- For the standard growth of *C. jejuni.*

10 g Bacto™ Pancreatic digest of casein (Difco Laboratories)
10 g Bacto™ Peptic digest of Animal Tissue (Difco Laboratories)
1 g Dextrose
5 g Yeast extract
5 g NaCl
0.1 g Sodium bisulfite

2.2.2.4 Skirrow Medium (recipe from Blood agar base No.2, Oxoid Ltd.)

- Selective media for the growth of *C. jejuni* only.

15 g Proteose peptone
2.5 g Liver Digest
5.0 g Yeast Extract
5.0 g NaCl
pH 7.4
Campylobacter selective supplement (Skirrow, SR0069E, Oxoid Ltd.) Final conc. 0.1 mg.ml-1 vancomycin, 50 µg.ml-1 Trimethoprim, 2500IU.L-1 Polmyxin B) was added after auto-claving.

2.2.2.5 Horse Blood Agar (BAB, 5%HB, 1% Yeast Extract)

- Nutrient rich media for the growth of *C. jejuni* during transformation.

40 g Blood Agar Base No. 2 (Oxoid)
5 % Horse Blood, oxalated (Oxoid)
10 g Yeast Extract

2.2.2.6 Advanced D-Modified Eagle Flex Media

Table 2.2: Advanced D-Modified Eagle Media (DMEM)/F-12-Flex Media Formulation (Pre-made) (Sigma).

Component	Concentration (mM)
Glycine	0.25

L-Alanine	0.05
L-Arginine hydrochloride	0.699
L-Asparagine-H2O	0.05
L-Aspartic acid	0.05
L-Cysteine hydrochloride-H2O	0.0998
L-Cysteine 2HCl	0.1
L-Glutamic Acid	0.05
L-Histidine hydrochloride-H2O	0.15
L-Isoleucine	0.416
L-Leucine	0.451
L-Lysine hydrochloride	0.499
L-Methionine	0.116
L-Phenylalanine	0.215
L-Proline	0.15
L-Serine	0.25
L-Threonine	0.449
L-Tryptophan	0.0442
L-Tyrosine disodium salt dehydrate	0.214
L-Valine	0.452
Ascorbic Acid phosphate	0.00863
Biotin	0.0000143
Choline chloride	0.0641
D-Calcium pantothenate	0.0047
Folic Acid	0.00601
Niacinamide	0.0166
Pyridoxine hydrochloride	0.00971
Riboflavin	0.000582
Thiamine hydrochloride	0.00644
Vitamin B12	0.000502
i-Inositol	0.07
Calcium Chloride (CaCl2) (anhyd.)	1.05
Cupric sulfate (CuSO4-5H2O)	0.0000052
Ferric Nitrate (Fe(NO3)3"9H2O)	0.000124

Ferric sulfate (FeSO4-7H2O)	0.0015
Magnesium Chloride (anhydrous)	0.301
Magnesium Sulfate (MgSO4) (anhyd.)	0.407
Potassium Chloride (KCl)	4.16
Sodium Bicarbonate (NaHCO3)	29.02
Sodium Chloride (NaCl)	120.61
Sodium Phosphate dibasic (Na2HPO4) anhydrous	0.5
Sodium Phosphate monobasic (NaH2PO4-H2O)	0.453
Zinc sulfate (ZnSO4-7H2O)	0.003
AlbuMAX® II	∞
Human Transferrin (Holo)	∞
Insulin Recombinant Full Chain	∞
Glutathione, monosodium	0.00326
Ammonium Metavanadate	0.0000026
Manganous Chloride	0.0000003
Sodium Selenite	0.0000289
D-Glucose (Dextrose)	17.51
Ethanolamine	0.0195
Hypoxanthine Na	0.015
Linoleic Acid	0.00015
Lipoic Acid	0.00051
Phenol Red	0.0215
Putrescine 2HCl	0.000503
Sodium Pyruvate	1
Thymidine	0.00151

Plus 20 mM Lysine and/or Arginine.

2.2.2.7 Minimal Essential Media (MEM)-alpha with Arginine

Table 2.3: d-MEM-alpha Media Formulation (Pre-made)

Component	Concentration (mM)

Glycine	0.667
L-Alanine	0.281
L-Arginine	0.498
L-Asparagine-H_2O	0.333
L-Aspartic acid	0.226
L-Cysteine hydrochloride-H_2O	0.568
L-Cystine 2HCl	0.099
L-Glutamic Acid	0.51
L-Glutamine	2
L-Histidine	0.2
L-Isoleucine	0.4
L-Leucine	0.397
L-Lysine	0.399
L-Methionine	0.101
L-Phenylalanine	0.194
L-Proline	0.348
L-Serine	0.238
L-Threonine	0.403
L-Tryptophan	0.049
L-Tyrosine disodium salt	0.198
L-Valine	0.393
Ascorbic Acid	0.284
Biotin	0.00041
Choline chloride	0.00714
D-Calcium pantothenate	0.0021
Folic Acid	0.00227
Niacinamide	0.0082
Pyridoxal hydrochloride	0.0049
Riboflavin	0.000266
Thiamine hydrochloride	0.00297
Vitamin B12	0.001
i-Inositol	0.0111
Calcium Chloride (CaCl2) (anhyd.)	1.8

Magnesium Sulfate (MgSO4) (anhyd.)	0.814
Potassium Chloride (KCl)	5.33
Sodium Bicarbonate (NaHCO3)	26.19
Sodium Chloride (NaCl)	117.24
Sodium Phosphate monobasic (NaH2PO4-H2O)	1.01
D-Glucose (Dextrose)	5.56
Lipoic Acid	0.000971
Phenol Red	0.0266
Sodium Pyruvate	1

Plus 20 mM L-Arginine

2.2.3 Media Additives

2.2.3.1 Antibiotics

Antibiotics were made to concentrated solutions in water or ethanol and filter sterilised (0.2 µM Sartorius Stedim Biotech) then added aseptically to the medium after it had been autoclaved.

2.2.3.1.1 Ampicillin and Carbenicillin

In this investigation ampicillin and carbenicillin were used interchangeably. Carbenicillin was typically used in cultures grown for longer duration, due to its increased stability at lower pH levels.

Ampicillin and carbenicillin were both used at a final working concentration of 100 µg/ml, stock solution of 100 mg/ml were stored at -20 °C.

2.2.3.1.2 Chloramphenicol

Chloramphenicol was prepared as a 30 mg/ml stock in 96% ethanol and stored at 4 °C. Final working concentrations of 15 µg/ml were used for *C. jejuni* and 30 µg/ml for *E. coli*.

2.2.3.1.3 Kanamycin

Kanamycin was prepared as a 100 mg/ml stock solution in MilliQ water and stored at -4 °C. Kanamycin was used at a working concentration of 100 µg/ml.

2.2.3.2 X-gal

X-gal (Bromo-chloro-indolyl-galactopyranoside) was made into solution with dimethyl formamide and added aseptically to the medium after autoclaving. X-gal was stored in the dark at -20 °C when not in use.

2.2.3.3 IPTG (Isopropyl β-D-1-thiogalactopyranoside

IPTG (Isopropyl β-D-1-thiogalactopyranoside) was dissolved in MilliQ water, filter sterilised (0.22 µM) and added aseptically to the medium after autoclaving. IPTG was made fresh, prior to use. Typically IPTG was used at a final concentration of 0.4 mM in the bacterial culture.

2.2.3.4 Metal Ions

Where additional metal ions were added to growth media, this was to a final concentration of 100 µM. Stock solutions of metal ions (100 mM) were made by dissolving either metal chloride or sulphate (Sigma) in MilliQ water. All solutions were filter sterilised prior to use in a 0.22 µM filter.

2.2.4 Growth of Bacteria

2.2.4.1 Escherichia coli

All strains of *Escherichia coli* were grown aerobically in LB broth at 37 ^{o}C with 200 rpm agitation in an Innova 4230 incubator (New Brunswick Scientific). The growth of bacterial cultures was routinely monitored by optical density (OD) readings at 600 nm. Cells were harvested after several hours of growth, or upon reaching a set optical density, depending on the experiment. *E. coli* strains were also grown on LB agar in Petri dishes at 37 ^{o}C, LB media was supplemented with appropriate additives (e.g. antibiotics, X-GAL).

2.2.4.2 Campylobacter jejuni

All strains of *Campylobacter jejuni* were grown in Brucella broth at 37 or 42^{o}C in a microaerobic cabinet (MACS-MG-100 Don Whitley Scientific) under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) with 200 rpm agitation. Cells were harvested after 16 hours growth or when an appropriate, according to the experiment.

Alternatively *C. jejuni* growth was monitored in a FluoroStar Omega controlled atmosphere plate reader. Small volume (200 µl) *C. jejuni* cultures were grown in clear, flat-bottomed, 96-well plates under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) at 37-42 °C, shaking at 600 rpm (double orbital). Additionally *C. jejuni* growth was monitored by 10-fold, serial dilution of broth cultures, 5 µl of which was plated onto Brucella broth, grown for 48 hours and used to assess the log number of viable cells present.

2.2.5 Glycerol stocks

2.2.5.1 Escherichia coli

E. coli strains were grown overnight at 37 °C in LB broth (10 ml). To make glycerol stocks, 200 µl of sterile 100 % glycerol was added to 1 ml of bacterial culture in a 2 ml screw cap Eppendorf tube. The tube was mixed by vortexing and frozen at -80 °C for storage.

2.2.5.2 Campylobacter jejuni

C. jejuni strains were grown overnight on Brucella agar. Bacteria were harvested from the plate into 2 ml of Brucella broth using an inoculating loop to gently resuspend the bacteria. Resuspended bacteria were mixed with 200 µl sterile 100% glycerol in a 2 ml screw cap Eppendorf and stored at -80 °C as a frozen stock.

Frozen stocks (50 µl) were grown overnight on Brucella agar and this process was repeated. The resuspended bacteria plus glycerol was then divided into 50 µl aliquots to serve as single use bacteria aliquots for plate inoculation.

2.3 Molecular Biology

2.3.1 Analytical Biology Kits

All kits were used as per manufacturer's instructions. A GenElute™ Plasmid Midiprep Kit (Sigma Aldrich) was used to isolate plasmid DNA from *E. coli* cell pellets, and a DNeasy Blood & Tissue Kit (Qiagen) was used to isolate genomic DNA from *C. jejuni*. A QIAquick PCR Purification Kit (Qiagen) and QIAquick Gel Extraction Kit (Qiagen) were used to isolate and purify DNA amplified by PCR. A 2D Quant kit (GE Healthcare) was used to quantify protein concentrations in soluble cell protein extracts.

2.3.2 dNTPs

A 50 mM working stock of dNTPs was used in PCR reactions. For each base (A, T, G and C) 100 µl of 100 mM dNTP's (New England Biolabs) were mixed together in a 1.5 ml Eppendorf, 100 µl of this was added to 100 µl MilliQ water to create a 50mM working stock used in PCR reactions, this was stored at -20 °C.

2.3.3 Polymerase Chain Reaction (PCR)

Amplification of target DNA was performed in a Multigene Optimax Thermal Cycler (LabNet). Typically, PCR reactions were analysed on a 0.8 % agarose gel. Successful amplifications were purified using a QIAgen PCR Purification kit (as per manufacturer's instructions) and concentrated, as required, in a Speedy Vac concentrator (Savant). In general HotStarTaq master mix was used for routine PCR reactions and Phusion Taq (New England Biolabs) was used when high fidelity PCR was required (e.g. Gene amplification).

2.3.3.1 HotStarTaq Master Mix (Qiagen)

DNA oligonucleotides (10 µM, 2.5 µl each) were added to a reaction mixture of 25 µl HotStarTaq Master Mix, 19 µl MilliQ water and 1 µl genomic DNA. A single cycle activation stage of 95 °C for 15 minutes was used, followed by 30 cycles of 94 °C for 45 seconds, 50-60 °C (varies depending of the melting temperature of the oligonucleotides used) for 45 seconds and 72°C

for 45 seconds (30 seconds per 1 kb). A final single finishing step of 72 °C for 10 minutes and then the reaction was cooled to 4 °C prior to analysis. Colony PCR were also performed using HotStarTaq, instead of a genomic DNA template a small volume of *E. coli* cells from a bacterial colony were used.

2.3.3.2 Phusion Taq (New England Biolabs)

DNA oligonucleotides (10 µM, 5 µl each) were added to a reaction mixture of 10 µl 5x High Fidelity Buffer, 0.5 µl 50 mM dNTP's, 0.5 µl Phusion DNA Polymerase, 28 µl MilliQ water, 1µl genomic DNA.
A single cycle denaturing stage of 98 °C for 2 minutes was used, followed by 30 cycles of 98 °C for 30 seconds, 50-60 °C (varies depending of the melting temperature of the oligonucleotides used) for 30 seconds and 72°C for 15 seconds (30 seconds per 1 kb). A final single finishing step of 72 °C for 15 minutes and the reaction was cooled to 4 °C prior to analysis.

2.3.4 Restriction Enzymes

Restriction enzymes used in this thesis were purchased from New England Biolabs or Promega. Digests were performed as per the manufacturer's instructions. Where instructed, incubations were carried out in a heated water bath for up to 3 hours. Once complete, the DNA was purified in a QIAgen PCR Purification Kit.

2.3.5 Agarose Gel Electrophoresis

Agarose gels were prepared for the routine size analysis of DNA using 0.8 – 1.0 % (w/v) electrophoresis grade agarose in 0.5 x TBE buffer containing 1 % (v/v) SafeView staining dye (NBS Biological). DNA samples were mixed with 6x DNA loading buffer (New England Biolabs) prior to gel loading. A 1 kb DNA ladder (New England Biolabs) was loaded on all gels, alternatively a low molecular weight DNA (New England Biolabs) ladder was used where appropriate (Figure 2.1). Once all samples were loaded, agarose gels were run at 100 volts for 30 minutes using a BioRad PowerPack 200 power supply. Gels were visualised and photographed under ultra-violet radiation (300 nm) in a U:Genius gel dock system (Syngene).

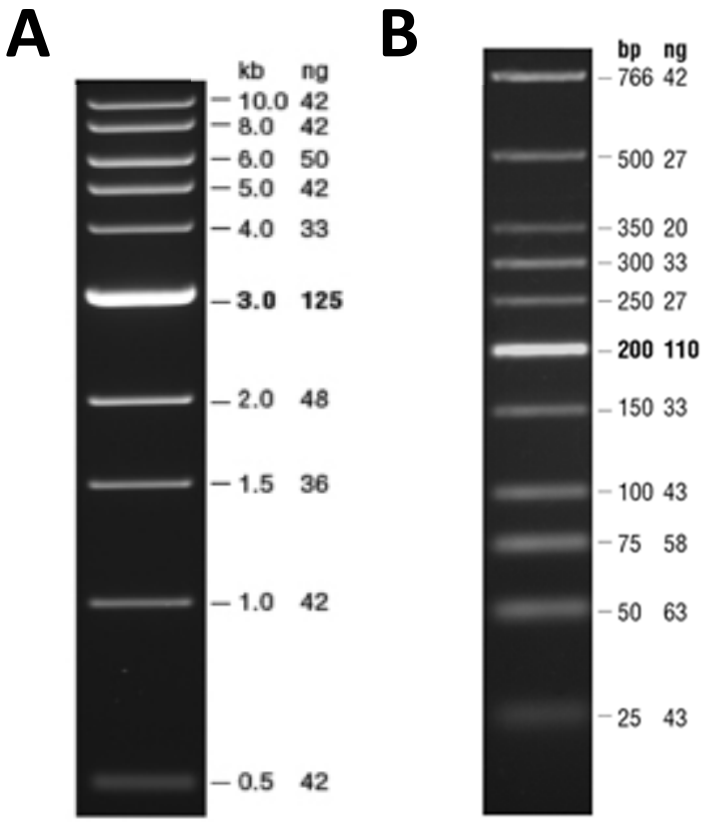

Figure 2.1: DNA molecular weight ladders from New England Biolabs. [A] A 1 kb DNA ladder and [B] a low molecular weight ladder.

2.3.6 DNA Ligation

The ligation of DNA fragments into vectors was performed using T4 DNA Ligase (New England Biolabs). Typically a 10 µl reaction volume was used, the DNA insert was added at a 3 : 1 ratio to the vector (~5 µl) with 1 µl 10x Ligase Buffer, 1 µl T4 DNA Ligase, up to 10 µl with DNase/RNase free water. The reaction was incubated at 4 °C overnight.

2.3.7 Transformation

2.3.7.1 Escherichia coli

One-Shot® Top10 Chemically competent *E. coli* (Invitrogen) were transformed with 1 µl vector (5 µl if a ligation) by heat shock (30 minutes on ice, 1 minute 42°C, 5 minutes on ice). Cells were then incubated in 1 ml SOC media at 37 °C (200 rpm shaking), then plated onto the appropriate selective media and grown at 37 °C overnight.

2.3.7.2 Campylobacter jejuni

C. jejuni (50 µl from glycerol) was grown on Skirrow agar for 48 hours in microaerobic conditions. Cells were then harvested by gentle agitation into 2 ml Brucella broth and the cells were collected in a pellet by centrifugation (22,000 x *g*, 5 minutes). The supernatant was discarded and the cell pellet was washed twice in 1 ml *Campylobacter* Competent Cell Buffer (272 mM sucrose, 15 % (v/v) glycerol in 100 ml water (filter sterilized) and finally resuspended in 500 µl *Campylobacter* Competent Cell Buffer. For each transformation, 100 µl of cell suspension was mixed with 5 µl plasmid DNA and placed in an electroporation cuvette. Samples were electroporated at 2.5 kV, a load resistance of 200 ohms and 25 µF capacitance. Cells were removed from the electroporation cuvette in 1 ml Brucella broth and plated onto pre-warmed Blood Agar Base (BAB) plates*. C. jejuni* was grown on BAB plates for 5 hours, resuspended by agitation into 1 ml Brucella broth and plated onto Brucella agar containing an appropriate antibiotic. Brucella plates were grown microaerobically for 48 hours, or until colonies were large enough to pick.

2.3.8 Isolation of Plasmid DNA from *Escherichia coli*

Plasmid DNA was extracted from *E. coli* cell pellets grown overnight in selective LB broth cultures (ranging from 10-50 ml). Plasmid DNA was extracted using a Sigma Midi Prep Kit.

Plasmids were identified by restriction digestion or by plasmid DNA sequencing.

2.3.9 Isolation of genomic DNA from *C. jejuni*

C. jejuni was grown overnight on Skirrow agar and cells were harvested in 2 ml Brucella broth. The cells were centrifuged at 4,500 x *g* for 10 minutes and then frozen at -80 °C, to aid cell lysis. Prior to genomic DNA isolation using the QIAgen Blood and Tissue Kit, cell pellets were thawed on ice.
Cell pellets were resuspended in 180 µl ATL buffer, 20 µl proteinase K (600 mAU/ml) was then added and the cell suspension was vortexed for 30 seconds and incubated at 56 °C for 20 minutes. Then 20 µl of 20 mg/ml RNAse A was added, mixed and incubated at room temperature for two minutes. Once the incubation was completed 200 µl AL buffer and 200 µl 96-100% ethanol were added, mixed and then the solution was centrifuged through a DNeasy Mini Spin column (6,500 x *g*, 1 minute). The flow through was discarded and the column was washed with 500 µl AW1 buffer (6,000 x *g,* 1 minute), 500 µl AW2 buffer (20,000 x *g*, 3 minutes) and the sample eluted in 200 µl AE buffer (1 minute incubation, 6,000 x *g*, 1 minute).

Purified DNA (1 µl) was run on an agarose gel (as previously described) to ascertain the quality of the purified DNA. The concentration of DNA was determined using a NanoDrop 2000 (ThermoScientific) as per the manufacturer's instructions.

2.3.10 DNA Sequencing Analysis

All sequencing reactions were performed by The Genome Analysis Centre (TGAC) (Norwich, UK) using the AbiPrism 3730 high throughput capillary sequencer. Sequence data was interpreted using Vector NTI analysis software (Invitrogen) by comparison to published sequences.

2.3.11 Buffers

-Phosphate Buffer Saline (PBS)
-1M Tris Borate (TB) Buffer (pH 7)
-1M Tris-EDTA (TE) Buffer (pH 8)

2.3.12 Construction of *C. jejuni* knockout mutations

2.3.12.1 ∆*perR* (*cj0322*)

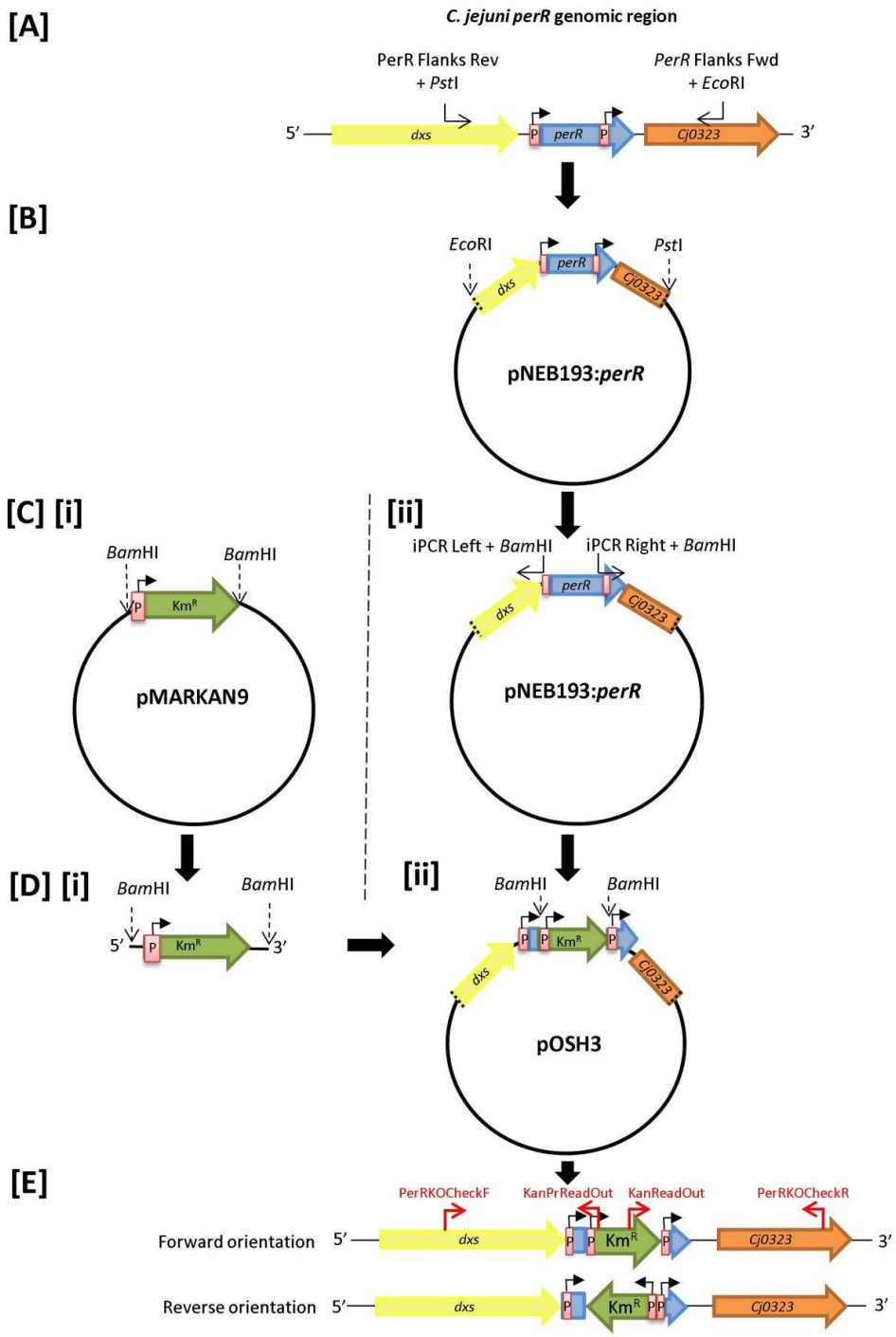

Figure 2.2: Illustration detailing the process of vector construction and *perR* mutagenesis in *C. jejuni.* [A] The *perR* loci of the *C. jejuni* NCTC *11168* genome. PCR amplification of *perR* and 500 bp flanking regions, adding 5' and 3' restriction sites. [B] Double restriction digest of the *perR* loci PCR fragment and pNEB193 with *Eco*RI and *Pst*I, and subsequent ligation of the DNA fragment into cut pNEB193 [C] [i]

Restriction digestion of pMARKAN9 with *Bam*HI to excise the Kanamycin resistance cassette. [ii] Inverse PCR (iPCR) amplification of pNEB193 to amplify *perR* flanking regions and add 5' and 3' *Bam*HI restriction enzyme sites. [D] [i] The Kanamycin resistance cassette with *Bam*HI sticky ends. [ii] Restriction digest of the pNEB193:*perR* inverse PCR product with *Bam*HI, then ligation of the kanamycin resistance cassette to the inverse PCR product, forming a new vector 'pOSH3'. Two orientations of the kanamycin cassette are possible. [E] Transformation of pOSH3 into the *C. jejuni* NCTC 11168 wild-type genome. The two possible orientations of the kanamycin cassette are shown. 'P' (pink box) indicates a gene promoter. Primers are shown in red.

The region containing the *cj0322* gene and approximately 500 nucleotides of flanking sequence on each side was PCR amplified by Phusion DNA Polymerase (New England Biolabs) using oligonucleotide primers PerRFlanksFwd and PerRFlanksRev, which added an *Eco*RI and *P*stI restriction site to either end of the gene fragment (Figure 2.2A). This amplified fragment was purified using a QIAgen PCR Purification Kit, digested with *Eco*RI (New England Biolabs) and *Pst*I (Promega) and then ligated into pNEB193 (New England Biolabs) to form plasmid pOSH2 (pNEB193:*perR*) (Figure 2.2B). The ligation mixture was transformed into chemically competent *E. coli* strain Top10 (Invitrogen). Plasmid-containing cells were selected on LB agar plates containing 100 µg ml^{-1} ampicillin and 20 µg ml^{-1} X-GAL.

To make the Δ*perR* (*cj0322*) insertional inactivation construct, pOSH2 was used as a template for inverse PCR using the oligonucleotide primers PerRInverseRight and PerRInverseLeft (Figure 2.2Ci). The kanamycin cassette from pMARKAN9 (Figure 2.2Di) and the inverse PCR product from pOSH2 were digested with *Bam*HI (New England Biolabs) and ligated to form plasmid pOSH3 (Figure 2.2 Dii). Newly formed ligated pOSH3 plasmids were transformed into *E. coli* strain Top10 and positive, kanamycin resistant transformants were selected for by plating on LB agar supplemented with 30 µg ml^{-1} kanamycin. Plasmids were recovered from *E. coli* using a Sigma Midi

Prep kit as previously described, all constructs and insert orientations were confirmed by restriction digestion analysis and sequencing (TGAC, Norwich, UK) prior to transformation into *C. jejuni*.

Wildtype *C. jejuni* NCTC11168 was transformed (See Section 2.3.7.2) with plasmid pOSH3 (Figure 2.2E). *C. jejuni perR* mutants were isolated after transformation and subsequent selection on kanamycin-containing agar. Colonies were screened by PCR using oligonucleotides that anneal outside of the cloned flanking regions (PerRKOCheck FWD and PerRKOCheck REV, see Table 2.4 for all oligonucleotides) in combination with antibiotic cassette specific primers (KmPrReadOut and KmReadOut) (Figure 2.2E).

2.3.12.2 ∆*fur* (*cj0400*) and ∆*fur*∆*perR*

Mutation of the *cj0400* gene (*fur*) in *C. jejuni* NCTC 11168 was performed essentially as described above for *perR (cj0322*), with a few changes. Oligonucleotide primers FurFlanksFwd and FurFlanksRev (Table 2.4) were used to amplify the gene fragment, once inserted into pNEB193 the vector was designated pRAH2. The vector pRAH2 was amplified via inverse PCR using primers FurInverseLeft and FurInverseRight (See Table 2.4), which added *Bgl*II end restriction sites. The inverse PCR product was digested with *Bgl*II and vector pAV35 was digested with *Bam*HI to release a chloramphenicol resistance cassette, these were then ligated as previously described to form vector pRAH3. *C. jejuni fur* mutants were created by transforming the *C. jejuni* NCTC 11168 wildtype strain with pRAH3 and correct mutants were screened for using primers FurKOCheck Fwd, FurKOCheck Rev, CmPrReadOut and CmReadOut (See Table 2.4).

A *C. jejuni* ∆*fur*∆*perR* double mutant was generated by the transformation of *C. jejuni* NCTC 11168 ∆*perR* with vector pRAH3. Successful transformants were screen for as previously described.

2.3.12.3 ∆*rrc* (*cj0012c)*

Mutation of the *cj0012c* gene (*rrc*) in *C. jejuni* NCTC 11168 was performed essentially as described above for *perR*, with the use of different oligonucleotides. Oligonucleotide primers RrcFlanksFwd and RrcFlanksRev

were used to amplify the gene fragment, once inserted into pNEB193 the vector was designated pINK2. The vector pINK2 was amplified via inverse PCR using primers RrcInverseLeft and RrcInverseRight (Table 2.4), which added *Bam*HI end restriction sites. The inverse PCR product and vector pMARKan9 were digested with *Bam*HI and these were then ligated to form vector pINK3, which was transformed into the *C. jejuni* wildtype strain. *C. jejuni rrc* mutants were screened for using primers RrcKOCheck Fwd, RrcKOCheck Rev, KanPrReadOut and KanReadOut (Table 2.4).

2.3.13 Construction of *perR* complementation constructs

To ensure that phenotypic changes observed could be restored, *perR* was complemented *in trans* in all three *C. jejuni perR* mutant backgrounds. *C. jejuni perR* mutants were complemented by inserting the *perR* gene with its own promoter into a *C. jejuni* pseudogene (*cj0046*), as is described in Figure 2.3 (Reuter & van Vliet, 2013; Thomas *et al.*, 2011).

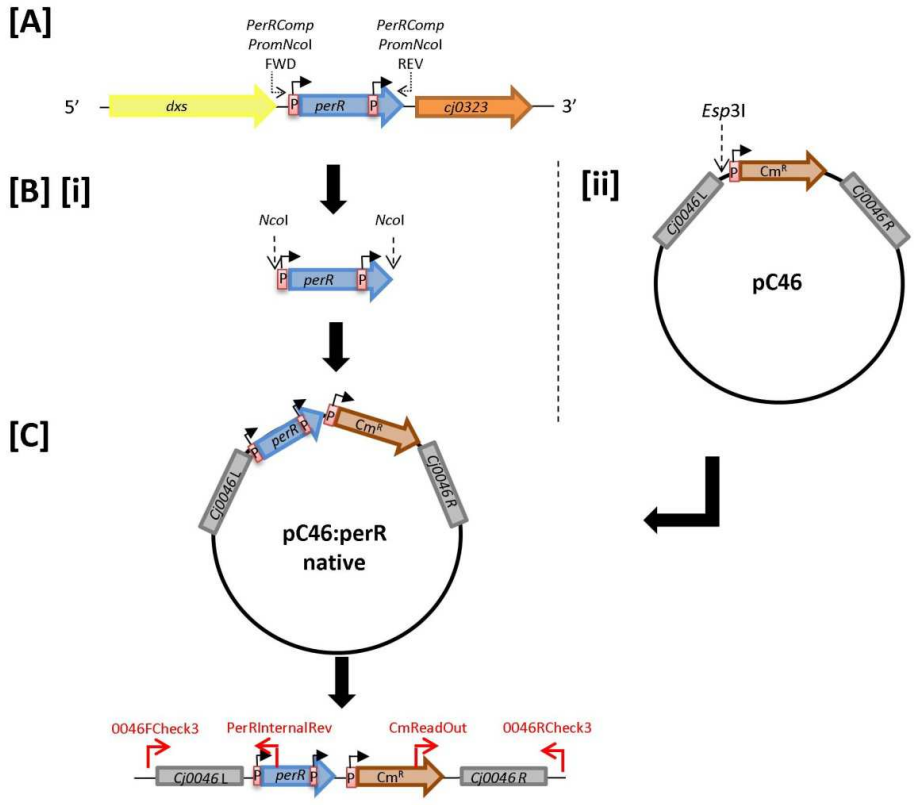

Figure 2.3: Illustration detailing the process of vector construction and transformation required for *perR* complementation in *C. jejuni*. [A] PCR

amplification of *perR* plus and its native promoter using primers that add 5' and 3' *Nco*I restriction digest sites. [B] [i] Restriction digestion of the *perR* PCR amplified fragment with *Nco*I. [ii] *Esp3*I (*Bsm*BI) restriction digestion of vector pC46, [C] subsequent ligation of *perR* (plus promoter) into pC46 forming pC46:*perR* and the *cj0046* region after homologous recombination with pC46:*perR*. Primers are shown in red.

Restriction digestion of DNA with *Esp*3I or *Nco*I restriction enzymes leads to the formation of DNA with a single stranded DNA overhang. The overhangs produced by these two enzymes are complementary to each other aiding the ligation of *perR* and pC46. The ligation of *perR* into pC46 may occur in two orientations either the forward or the reverse orientation. The construction and orientation of pC46:*perR* was confirmed by restriction digestion and plasmid DNA sequencing.

Once confirmed, the plasmid pC46:*perR* was transformed into the isogenic *C. jejuni perR* mutants by electroporation. Successful transformants were selected for by screening for chloramphenicol resistance. Correct integration of the *perR* complementation construct into the *C. jejuni* genome was validated by PCR amplification. As previous, PCR primers were designed to anneal outside of the homologous recombination zone to ensure a complete double crossover of genetic material onto the *C. jejuni* genome from vector pC46:*perR*. The primers used include 0046FCheck3 versus PerRInternalRev and CmReadOut versus 0046FCheck3 (See Table 2.5). Complementation of *perR* (under control by its native promoter) by insertion into the *C. jejuni* genome was performed as gene expression levels are more likely to mimic wildtype expression than if replacement copies of the deleted gene were provided on a vector.

Complemented Δ*perR* strains are referred to throughout as ' Δ*perR*::*perR*$^{+}$' (See Table 2.1).

2.3.1 The generation of a *fur* mutant and a *fur:perR* double mutant in *C. jejuni* NCTC 11168

The mutation of *fur* was performed essentially as described above for *perR.* A vector was constructed containing an antibiotic resistance cassette, flanked by 500 bp of the upstream and downstream regions of the *fur* gene as found in the *C. jejuni* genome.

Oligonucleotide primers FurFlanksFwd and FurFlanksRev were used to amplify the *fur* gene fragment, once inserted into pNEB193 the vector was designated pRAH2 (See Table 2.5 and Table 2.6). The vector pRAH2 was amplified via inverse PCR using primers FurInverseLeft and FurInverseRight, which added *Bgl*II end restriction sites. The inverse PCR product was digested with *Bgl*II and vector pAV35 was digested with *Bam*HI to release a chloramphenicol resistance cassette, these were then ligated as previously described to form vector pRAH3. *C. jejuni fur* mutants were created by transforming the *C. jejuni* NCTC 11168 wildtype strain with pRAH3 and correct mutants were screened for using primers FurKOCheck Fwd, FurKOCheck Rev, CmPrReadOut and CmReadOut.

A *C. jejuni* Δ*fur*Δ*perR* double mutant was generated by the transformation of *C. jejuni* NCTC 11168 Δ*perR* with vector pRAH3. Successful transformants were screened for as previously described for both *fur* and *perR* gene deletions.

Table 2.4: The mutants and complement strains generated in this chapter

Name	Inactivated Gene(s)	Gene Annotation	*C. jejuni* Strain	Genetic complementation
11168 Δ*perR*, kan^R	Cj0322	Peroxide Stress DNA regulator	NCTC 11168	-
81176 ΔperR, kan^R	Cj0322	Peroxide Stress DNA regulator	81-176	-
81116 Δ*perR*, kan^R	Cj0322	Peroxide Stress DNA	81116	-

		regulator		
11168 Δ*perR::perR*$^{+}$, kan^{R}, chlor^{R}	Cj0322	Peroxide Stress DNA regulator	NCTC 11168	NCTC 11168 Cj0332 under native promoter
81176 Δ*perR::perR*$^{+}$, kan^{R}, chlor^{R}	Cj0322	Peroxide Stress DNA regulator	81-176	NCTC 11168 Cj0332 under native promoter
81116 Δ*perR::perR*$^{+}$, kan^{R}, chlor^{R}	Cj0322	Peroxide Stress DNA regulator	81116	NCTC 11168 Cj0332 under native promoter
11168 Δ*flaAB*, kan^{R}, chlor^{R}	Cj1339, Cj1338c	Structural flagellin proteins FlaA and FlaB. Used as a non-motile control.	NCTC 11168	-
11168 Δ*fur,* chlor^{R}	Cj0400	Ferric uptake regulator	NCTC 11168	-
11168 Δ*fur* Δ*perR*, kan^{R}, chlor^{R}	Cj0400, Cj0322	Peroxide Stress DNA regulator, Ferric uptake regulator	NCTC 11168	-

2.4 Phenotypic Analyses in *C. jejuni*

2.4.1 Motility

The A_{600} of an overnight *C. jejuni* culture was adjusted to 0.4 using sterile PBS. Bacterial motility was assessed by spotting 10 µl of this culture onto the centre of a 0.4 % agar Brucella plate. Plates were photographed at 24, 48, and 72 hours and the diameter of the halo was measured using ImageJ software (version 1.41; National Institute of Health [http://rsbweb.nih.gov/ij/]). A *C. jejuni* NCTC 11168 Δ*flaAB* non-motile mutant was included in all experiments as a control.

2.4.2 Aerotolerance Assay

Aerotolerance assays were adapted from (Baillon *et al.*, 1999) with some alterations. Cultures (20 ml) were grown overnight in Brucella broth then adjusted to an A_{600} of 0.4 using PBS. For each strain, cultures were split into two 10 ml aliquots in separate flasks, with one grown microaerobically (85% N_2, 5%O_2, 10% CO_2) and the other aerobically at 37^{o}C shaking at 200 rpm. Samples of each culture were taken at three hour time intervals. Serial 10-fold dilutions were used to assess cell survival; 5 µl of each dilution was spotted onto Brucella agar plates and incubated under microaerobic conditions for 2 days at 37°C.

2.4.3 Disc Inhibition Assay

Resistance to oxidative stress was determined using disc inhibition assays. *C. jejuni* were grown overnight on Skirrow plates at 37 ^{o}C. Cells were harvested into 2 ml of Brucella broth. *C. jejuni* was then added to 3 ml 0.4 % Brucella agar to a final A_{600} of 1.0 and poured onto a Brucella plate. Samples (10 µl) of hydrogen peroxide (0-30% v/v in water), gentamicin (0-30 % in water) or cumene hydroperoxide (0-6 % v/v in dimethyl sulfoxide) were applied to 6 mm diameter sterile 3M Whatmann paper discs which had been placed on the soft agar surface using flame sterilized tweezers.

Plates were incubated overnight in microaerobic conditions at 37 °C and the zone of inhibition (no growth) was measured using ImageJ image analysis software (National Institute of Health).

2.4.4 Oxidative Stress in Broth Culture

Fresh cultures of 11168 *C. jejuni* wildtype, Δ*perR* and Δ*perR*::*perR*+ were grown overnight on Skirrow plates. Each strain was inoculated into 50ml Brucella broth to a final A_{600} of 0.05 and grown overnight to stationary phase. The A_{600} of all three strains was fixed to 0.4 using sterile PBS buffer. A 20µl sample of each culture was taken and diluted into 180µl PBS buffer to sample cell viability, this was then diluted to 10^{-8} by serial dilution and 5µl of each dilution was plated onto Brucella agar and grown for two days. Hydrogen peroxide solution (30% v/v) was then added to each culture to a final concentration of 3% hydrogen peroxide (1M). Samples of each culture were taken over the course of two hours to assess cell survival. Time points were taken at 0, 2, 5, 10, 15, 30, 60, 120 and 180 minutes.

2.4.5 Wax Moth Larvae (*Galleria mellonella*) Infection Model

The *Galleria mellonella* infection model was used to compare bacterial pathogenicity and has been adapted from (Gundogdu *et al.*, 2011) and (Champion *et al.*, 2010). *Galleria mellonella* larvae were obtained from Livefoods.co.uk (United Kingdom). Larvae were inoculated in the upper right pro-leg by microinjection (Hamilton, Switzerland) with 10 µl overnight *C. jejuni* culture, which had been adjusted to an A_{600} of 1.0 (an infectious dose of approximately 10^7 CFU). Mock infection and injection with 10 µl of PBS were also performed in each experiment. The larvae were incubated at 37 °C, with percentage survival scored at 24 hour intervals. For each experiment, ten *G. mellonella* larvae were infected and a total of five independent experiments were performed.

2.4.6 Filter Scanning Electron Microscopy (performed by Dr Louise Salt)

C. jejuni was grown overnight in a 50 ml Brucella and cells were pelleted by centrifugation (4,500 x *g*), then resuspended in 500 µl PBS. Approximately 200µL of sample was pipetted drop by drop onto an Isopore membrane polycarbonate filter (HTTP01300, Millipore, UK) which had been trimmed with a razor blade so that the inoculated surface could be identified. The cells were left to adhere to the surface for 5 minutes after which the filters were placed in 2.5% glutaraldehyde in 0.1 M PIPES buffer (pH 7.2) and fixed for 1

hour. After washing with 0.1M PIPES buffer, each sample was carefully inserted into a critical point drying capsule and dehydrated in a series of ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, 3x 100%) and 3x 100% ethanol.

Samples were critical point dried in a Polaron E3000 critical point drier using liquid carbon dioxide as the transition fluid. The filters were carefully mounted onto SEM stubs using sticky tabs, ensuring that the inoculated surface was facing upwards. The samples were coated with gold in an Agar high resolution sputter-coater apparatus. Scanning electron microscopy was carried out using a Zeiss Supra 55 VP FEG SEM, operating at 3 kV.

2.4.7 Electrophoretic Mobility Gel Shift Analysis

DNA fragments (180-200 bp) located upstream of target genes translational start sites were amplified by PCR using 5' DIG labelled PCR primers (See Table 2.5) and quantified by Nanodrop. Recombinant PerR protein (0 - 5 nM) was incubated with 0.5 nM of DIG labelled DNA in EMSA binding buffer (50 µM $MnCl_2$, 20 mM Tris-Borate pH7.4, 50 mM KCl, 3 mM $MgCl_2$, 5 % glycerol, 0.1 % Triton X-100), in a total volume of 20 µl. The reaction was left for 60 minutes at room temperature. Reactions were assayed for protein-DNA binding by native PAGE, on a 12% acrylamide gel at 150 V for 50 minutes. Gels were electroblotted onto a positively charged nylon membrane (Hybond N+, Amersham Biotech) at 130 mA for 1 hour. DNA was cross-linked to the membrane using UV radiation (1200 kJ cm^2) and incubated with Anti-Digoxigenin-AP fab fragments (Roche). CDP Star ® (SIGMA) was used to detect DIG-labelled fragments on the nylon membranes, as per the manufacturer's instructions.

2.4.8 RNA-seq analysis (Performed by Dr Arnoud van Vliet and Dr Mark Reuter)

2.4.8.1 Isolation of RNA from *C. jejuni*

RNA for RNA-seq analyses was extracted from *C. jejuni* NCTC 11168 wildtype and Δ*perR*Δ*fur* mutants. A *C. jejuni* Δ*perR*Δ*fur* double mutant was

used in place of single Fur and PerR mutants due to the cost of the RNA sequencing. The sequencing of RNA is expensive, and a double mutant was used to ensure the maximum number of results would be returned for the price of the experiment. Experimentally, genes could be identified as either Fur or PerR regulated by comparison to microarray data.

Cultures (40ml) were grown in MEM-*a* (plus 20 mM pyruvate) to mid log phase (A_{600} 0.2). Cells were added to falcon tubes containing 1/10 (v/v) of Cold Stop Solution (10% phenol in ethanol) and harvested at 6,500 x *g* for 20 minutes. The supernatant was discarded and the cell pellet resuspended in 800 µl TE buffer and 80 µl 10 % SDS solution, then incubated at 64 °C for 6 minutes, inverting periodically. After incubation, samples were cooled on ice and transferred to a 2 ml Eppendorf tube. Then 88 µl sodium acetate, pH 5.2 and 1 ml of buffer saturated phenol was added to the cell mixture, and then centrifuged at 20,000 x *g* for 10 minutes at 4 °C. After centrifugation, only the aqueous layer was removed and added to a fresh Eppendorf tube containing 1 ml chloroform, which was mixed by inversion and centrifuged at 20,000 x *g* for 10 minutes at 4 °C. Again, the aqueous layer was removed and placed in a clean Eppendorf tube, 10 % (v/v) 3M sodium acetate and 1 mM EDTA (pH8) were added. Then 2.5 volumes of cold ethanol were added to the mixture and incubated at -80 °C overnight.

Samples were removed from incubation and spun at 20,000 x g for 30 minutes, at 4 °C. The ethanol was carefully removed in order to not disturb the RNA pellet. Cell pellets were washed three times with 1 ml cold ethanol, with centrifugation steps in between until the final stage when the ethanol was removed. Residual ethanol was removed by 25 minutes air drying, on ice. The dry pellet was resuspended in 50 µl RNase-DNase free water and analysed on an agarose gel.

Total RNA was purified omitting size selection, to avoid the loss of small RNA molecules. The exclusion of rRNA and tRNA was also omitted, to avoid the potential loss of other RNA species. RNA was isolated using hot phenol (Mattatall & Sanderson, 1996) to ensure that small RNAs would not be removed by the extraction procedure.

2.4.9 Sequencing of the RNA

The RNA was treated with DNase I to remove residual genomic DNA, followed by optional treatment with Terminator Exonuclease (TEX, Peicentre Biotechnology) for enrichment of primary RNAs (Dugar *et al.*, 2013; Sharma *et al.*, 2010), and treatment with Tobacco Acid Phosphatase (TAP, Cambio, UK) to generate 5'-P ends for downstream ligation of 454 adapters (Sharma *et al.*, 2010). After ligation of an RNA oligonucleotide (AUAUGCGCGAAUUCCUGUAGAACGAACACUAGAAGAAA) to the phosphorylated 5'-ends of RNA, and polyadenylation of RNA, first strand cDNA was generated using an oligo-dT containing 454-B primer. The cDNA fragments were bar-coded and amplified, and used for generation of cDNA libraries for the 454 FLX system at Vertis Biotech, Germany.

2.4.10 Analysis of RNA Sequencing Data

Libraries were analysed using a Roche Titanium sequencer. The same RNA-samples were also used to generate strand-unspecific RNA-seq libraries for Illumina sequencing, using instructions from the manufacturer (Illumina), and further analysed using an Illumina HiSeq2000 sequencer. Sequencing reads were grouped based on the barcode tag, the 5' adapter was clipped, and reads of >70% A were removed. The remaining reads were aligned against the *C. jejuni* genome NCTC 11168 genome sequence using Segemehl version 0.0.9.3 (Hoffmann *et al.*, 2009), and converted into number of reads per nucleotide position. Graphs representing the number of mapped reads per nucleotide were visualized using the Integrated Genome Browser software from Affymetrix (See Figure 4.8) (Nicol *et al.*, 2009) and analysed as described previously (Porcelli *et al.*, 2013; Sharma *et al.*, 2010). Transcript levels of individual genes were expressed as Reads Per Kilobase per Million mapped reads (RPKM) values (See Figure 4.7), calculated after mapping of reads using CLC Genomics Workbench v5 (CLC Bio).

2.5 Protein Purification

2.5.1 Generation of Native Over-Expression Constructs

C. jejuni PerR was expressed recombinantly in *E. coli* strain BL21 (DE3) (Novagen) using the *perR* over-expression vector pOSH1. Vector pOSH1 was generated by amplifying the *perR* gene using Phusion DNA Polymerase (New England Biolabs) with '*perR*pET21a' primers *perR*pET21aF and *perR*pET21aR. The amplified fragment was digested with restriction enzymes *Bam*HI and *Nde*I (New England Biolabs) and ligated into pET21a, which had been digested by the same enzymes. Ligations were transformed into chemically competent *E. coli* Top10 cells (Invitrogen). Transformed *E. coli* were grown on selective LB media plus ampicillin and grown overnight at 37 °C. Successful transformants were confirmed via colony PCR using primers T7Fwd and T7Rev. Cultures (10 ml) of successful *E. coli* transformants were grown overnight in LB media plus ampicillin and cells were harvested by centrifugation (18,000 x *g*, 10 minutes). Plasmid DNA was extracted from the *E. coli* cell pellets using a QIAgen Plasmid Purification kit. Successful constructs, named pOSH1, were confirmed by restriction digestion and plasmid DNA sequencing (TGAC, Norwich, UK).

2.5.2 Generation of His-Tag Over-Expression Constructs

The *perR* gene was amplified and digested as previously described for insertion into pET21a. The *rrc* gene was amplified as described for *perR* except using primers *rrc*pTY28aF and *rrc*pET28aR. Vector pET28a was digested with *Nde*I and *Bam*HI and ligated with the *perR* or *rrc* digested fragment. Chemically competent *E. coli* Top10 cells (Invitrogen) were transformed with the vectors constructed by the ligation reactions. The transformed *E. coli* was grown on selective LB media plus kanamycin and grown overnight at 37 °C. Successful transformants were confirmed via colony PCR using primers T7Fwd and T7Rev. Cultures (10 ml) of successful *E. coli* transformants were grown overnight in LB media plus kanamycin and cells were harvested by centrifugation. Plasmid DNA was extracted from the *E. coli* cell pellets using a QIAgen Plasmid Purification kit. The successful construction of plasmids, named pET28a:*perR*, or pINK1 (*rrc*) were confirmed by restriction digestion.

2.5.3 IPTG Induction of Protein Expression in *E. coli*

2.5.3.1 Native Protein Purification

The *E. coli* strain Bl21 (DE3) was transformed with the vector pOSH1, used to recombinantly over express *C. jejuni* PerR protein. *E. coli* BL21 (DE3) plus pOSH1 were grown in 10 ml LB broth with an ampicillin or carbenicillin supplement overnight at 37 °C. The 10 ml overnight culture was used to inoculate 500 ml LB broth plus carbenicillin to a starting $A_{600\ nm}$ of 0.05. Cultures were then grown at 37 °C, shaking at 180 rpm to an $A_{600\ nm}$ of 1.0. The expression of recombinant PerR was induced by addition of 1.0 mM IPTG (isopropyl-β-$_D$-thiogalactopyranoside) for 4 hours at 30 °C. Bacterial cells were harvested by centrifugation (18,000 x *g* for 15 minutes at 4 °C), washed twice in Buffer A [20 mM Tris-HCl [pH8.0], 50 mM NaCl, 10 mM EDTA] and cell pellets were frozen at -80 °C.

2.5.3.2 His-Tagged

E. coli Bl21 (DE3) were transformed with plasmids pET28a:*perR or pINK1*, bacteria were then grown at a range of temperatures from 20 °C to 37 °C to assess the effect on solubility of the protein. Cells were grown at 37 °C until induction with 1 mM IPTG when an A_{600} of 0.4 had been reached. Then growth temperatures were reduced to 20-30 °C for 4 hours. Cells were harvested as described previously for the native protein over expression.

2.5.4 Cell Lysis

E. coli cell pellets were defrosted and resuspended in 40 ml of either 20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM EDTA (Native) or 20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM Imidazole (His-Tagged). Cells were lysed on ice by sonication for 3 minutes, in six passes of 30 seconds. Soluble cell extract was isolated by centrifugation at 23,000 x *g* $_f$or 30 minutes at 4 °C. The soluble cell extract was filtered to remove large particulates and was then ready to be used in affinity chromatography.

2.5.5 Affinity Chromatography

2.5.5.1 Native Protein Purification

A HiTrap™ Heparin affinity column was equilibrated in five column volumes of Buffer A [20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM EDTA].The soluble cell fraction was loaded onto a HiTrap™ Heparin affinity column attached to an AKTA FPLC at a flow rate of 1 ml/min. Either 1 ml or 5 ml HiTrap™ Heparin affinity columns were used depending on the volume of cell lysate. Once loaded onto the column, the column was washed with five column volumes in Buffer A. The flow through and column wash were collected for SDS-PAGE analysis. *C. jejuni* PerR was eluted from the heparin column across a salt gradient with high salt Buffer B [20 mM Tris-HCl [pH8 .0], 1 M NaCl, 10 mM EDTA]. Fractions of eluted protein were collected in 1 ml aliquots in glass test tubes. The purity of eluted protein was assessed by SDS-PAGE and further refined by gel filtration if required.

2.5.5.2 His-Tag Protein Purification

The soluble cell extract containing His-tagged protein was loaded onto a 5 ml Nickel NTA linked sepharose column. Cell contents passed through the column under gravity and the column was washed for ten column volumes in 20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM Imidazole. Once washed, the column was capped and 100 units of thrombin in 2 ml buffer were added, and left to incubate overnight. The column was uncapped and eluted protein was collected in 1 ml fractions. Finally the column was washed in 3 ml buffer. Eluates were analysed by SDS-PAGE.

2.5.6 Buffer Exchange

Fractions of pure protein were exchanged by dialysis into Buffer C [20 mM Tris, pH 8.0, 300 mM NaCl, 10 mM EDTA] after affinity chromatography. Dialysis was performed in Slide-A-Lyzer dialysis cassettes (Pierce), 3,500 (MWCO) overnight at 4 °C.

2.5.7 Sample concentration

Protein samples were concentration to desired volumes or desired concentrations using VivaSpin Centrifugal Concentrators (GE Healthcare) 3,500 MWCO. Concentrators were pre rinsed with ultra-pure water then

loaded with the protein sample and centrifuged at 6,000 x *g* for 15 minute intervals at 4 °C.

2.5.8 Gel Filtration

Gel filtration was used to further purify protein samples after affinity chromatography. A Sephacryl-200 High Resolution column was equilibrated with Buffer C [20 mM Tris, pH 8.0, 300 mM NaCl, 10 mM EDTA] at 0.2 ml/min overnight. The gel filtration column was calibrated with proteins/molecules of known molecular weight; 3 mg/ml Aprotinin (6,500 Da), 2 mg/ml Cytochrome C (12,400 Da), 2 mg/ml Carbonic Anhydrase (29,000 Da), 5 mg/ml Bovine Serum Albumin (66,000 Da) and 1 mg/ml Blue Dextran (2,000,000 Da). All samples were loaded onto the column using a 2 ml sample volume. The volume that each calibrant eluted from the column was recorded (V_e), the elution volume of Blue Dextran was taken as the column's void volume (V_o). A standard curve was constructed plotting the V_e/V_o ratio of each protein versus its molecular weight.

Semi pure PerR protein was loaded onto the equilibrated Sephacryl-200 High Resolution for further purification. The volume that proteins are eluted from the column is approximately proportional to the molecular weight of the protein. Therefore PerR protein could be separated from many of the high molecular weight contaminant proteins present in the sample. As the column had been calibrated the eluted volume of PerR can also be used to estimate molecular weight.

2.5.9 Protein Quantification

The Bradford method was used to determine protein concentration. Bradford Reagent (BioRad) was diluted (1 volume Reagent in 5 volumes of water) and added to a series of known concentrations of bovine serum albumin (BSA) (0.2-1.0 mg/ml^{-1}). BSA was incubated in Bradford reagent for 10 minutes at room temperature, then the absorbance of these solution was read at 595 nm. Absorbance at 595 nm directly correlates to the protein concentration. Readings from the known concentrations of BSA were taken in triplicate and used to establish a standard curve from which the concentration of protein of

an unknown sample could be determined, based on its absorbance at 595 nm.

Where low sample volume was a concern, a NanoDrop 2000 (Thermo Scientific) was used to measure protein concentration by reading the absorbance of a 1 µl protein sample at 280 nm. This value could then be used to calculate protein concentration (M) by division by the extinction co efficient (12295 M^{-1} cm^{-1}), and then multiplication by the dilution factor.

2.5.10 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Samples for ICP-MS analysis were concentrated to 100 µM using Vivaspin centrifugal concentrators (as previously described). Sample concentration was determined by NanoDrop analysis (Thermo Scientific) and 500 µl of each sample was submitted to North Western University for ICP-MS analysis.

2.5.11 Protein Crystallography

Pure native *C. jejuni* PerR protein was concentrated to approximately 20 mg/ml and then diluted to final concentrations of 5 and 10 mg/ml in Buffer C [20 mM Tris, pH 8.0, 300 mM NaCl, 10 mM EDTA]. PerR at 5 and 10 mg/ml was used in crystallography trial screens MD1-01 Structure Screen 1 and MD1-02 Structure Screen 2 and MD1-37 JCSG+ (Molecular Dimensions). Vapour diffusion reservoirs were established in 96-well plate format, with two protein concentrations per well. A 50 µl reservoir volume was used and 2 µl of reservoir buffer was mixed with 2 µl of each protein concentration. All plates were sealed and stored at 4 °C and 16 °C and monitored for one week.

2.5.12 Proteome Analyses

2.5.12.1 Cell Growth and Protein Isolation

C. jejuni cells were harvested from broth culture (50 ml) by centrifugation at 4,000 × *g*, 10 minutes at room temperature. Cell pellets were resuspended in 500 µl lysis buffer (50 mM Tris (pH 7.5), 0.3% sodium dodecyl sulphate

(SDS), 0.2 M dithiothreitol, 3.3 mM $MgCl_2$, 16.7 µg ml^{-1} of RNase, and 1.67 U ml^{-1} of DNase) and lysed (Soniprep 150 MSE; Sanyo) on ice until clear. The samples were then centrifuged (20,000 × *g*, 20 min, 4°C) to remove any unlyzed cells. Total cell protein was quantified using a 2D Quant kit (GE Healthcare) as per the manufacturer's instructions.

2.5.12.2 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using pre-cast 12% RunBlue SDS-PAGE gels (Expedeon). Typically samples were loaded onto SDS-PAGE gels after treatment with 6x SDS PAGE loading buffer (Invitrogen) in total volumes of 5 -20 µl. A molecular weight marker (5 µl) was typically used to indicate protein size, either InstantBlue Prestained Molecular Weight Marker (Expedeon) or Precision Plus All-Blue Prestained Standards (BioRad). Gels were run using an XCell SureLock Mini-Cell system (Invitrogen) in 1 x Nu PAGE MOPS SDS Running Buffer for Bis-Tris Gels (Invitrogen) and run at 180 V for 50 minutes using a High Current PowerPac (Biorad). Gels were stained by covering in Coomassie Blue stain (0.5 % Coomassie Blue in 50% methanol, 10% acetic acid)and heating in a microwave at 600 watts for 30 seconds. Coomassie stain was removed and gels were left to de-stain overnight in 50 ml SDS-PAGE Destain Buffer (20 % Methanol (v/v)l, 10 % Acetic Acid (v/v), 70 %l water (v/v)).

2.5.12.3 2D Gel Electrophoresis

2.5.12.3.1 1st Dimension Electrophoresis

C. jejuni protein (100 µg) in 400 µl of IPG strip rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 0.1% Bromophenol Blue, 28 mg.ml-1 DTT) was loaded onto an Immobiline DryStrip gel (IPG strip) (GE Healthcare) (pH 3-11 NL 24-cm) in a strip re-swelling tray. All air bubbles were carefully removed and strips were covered in a 2.5 ml mineral oil. The gel strips were left at 20 °C overnight to rehydrate.

Once rehydrated the IPG gel strips were placed in IPGphor (GE Healthcare) for isoelectrical focussing at 44.7 kV per hour at 20°C for 8

hours. All focused IPG strips were then conditioned for use in the second dimension or stored at – 80 °C.

To condition focussed IPG strips for the second dimension, strips were incubated in filtered (0.45 µm, Sartorius Stedim Biotech) equilibration buffer (5% SDS and 0.01% bromophenol blue in 0.122 M Tris/acetate, Genomic Solutions) for 30 minutes with gentle shaking (Rotatest shaker, R100/TW, Luckham). The strips were then incubated in 8 mg/ml^{-1} DTT in equilibration buffer (9 ml) for 30 minutes to reduce and alkylate cysteines. Finally the strips were transferred into 25 mg/ml iodoacetamide in equilibration buffer (9 ml) for 30 minutes with gentle rotation.

2.5.12.3.2 2D Gel Electrophoresis: 2nd Dimension Electrophoresis

The second dimension was performed to separate proteins according to the molecular weight (kDa). Gels were run in an Investigator 2nd Dimension Running System (Genomic Solutions) was used using a maximum power of 20 W per gel. The running system was filled with cathode buffer (200 mM Tris base, 200 mM Tricine, 14 mM SDS,) and anode buffer (25 mM Tris-acetate buffer, pH 8.3) prior to the running of the gels. Gels were stained with Sypro Ruby (Sigma) overnight and imaged on a Pharos FX+ molecular imager using Quantity One imaging software (v4.6.1; Bio-Rad). A 532 nm excitation laser with a 605 nm band-pass emission filter was used to scan gels at 100 µm resolution, producing a 16 bit image. Laser strength was adjusted for each image to optimise the maximum signal without saturation of gel image pixels. Gel images were compared by producing overlay images using Proteom Weaver analysis software (v3.0.1; Definiens).

2.5.12.4 Protein identification using LC-MS/MS

Proteins were removed from SDS PAGE or 2D Gels using a ProPick excision robot (Genomic Solutions) and were trypsin digested in-gel using a ProGest protein digester (Genomic Solutions). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using a LTQ-Orbitrap mass spectrometer (Thermo Electron) and a Nanoflow-HPLC system (nanoACQUITY; Waters).

Peptides were trapped on line to a Symmetry C_{18} trap (5 µm, 180 µm by 20 mm) which was then switched in-line to a UPLC BEH C_{18} column (1.7 µm, 75 µm by 250 mm) held at 45 °C. Peptides were eluted on a gradient of 0 to 80% acetonitrile in 0.1 % formic acid over 50 min at a flow rate of 250 nl min^{-1}. The mass spectrometer was operated in positive-ion mode with a nanospray source at a capillary temperature of 200 °C. The Orbitrap was run at a resolution of 60,000 over the mass range *m/z* 300 to 2,000, with an MS target of 10^6 and a 1-s maximum scan time. The MS/MS was triggered by a minimal signal of 2,000 with an automatic gain control target of 30,000 ions and maximum scan time of 150 ms. For MS/MS events, the selection of 2+ and 3+ charge states was used. Dynamic exclusion was set to 1 count and a 30-s exclusion time with an exclusion mass window of ±20 ppm. Proteins were identified by searching the Thermo RAW files converted to Mascot generic format by DTA supercharger (**http://www.msquant.sourceforge.net**) against *C. jejuni* protein sequences in a monthly updated copy of the SPtrEMBL database, using an in-house version (v2.2) of the Mascot search tool (Matrix Science, Inc.).

Table 2.5: A list of oligonucleotides used in this thesis

Primer Name	Oligonucleotide Sequence (5'-3')
PerRFlanksF	GGACCTATTGAATTCCGTTATCCTAG
PerRFlanksR	GGAAACTGCAGGCTAAAATTCTG
PerRInverseRight	GATC-GGATCC-GCTAAGCTTTATGAATATCAAGAGC
PerRInverseLeft	GATC-GGATCC-CACATAGTCTTTGCGGAGTAGC
PerRKOCheck FWD	GCCCCAAGAGATGAACAAATGATGC
PerRKOCheck REV	GCTTATCTTTTCTCTCTAAAGATAAAGTCC
KmPrReadOut	GCGATATCTTCTATATAAGCGTACCG
KmReadOut	CGGGGAAGAACAGTATGTCGAGC
PerRcomppromFWD	GAAAAAGATGTCCATGGTTTGTTGACG
PerRcompREV	CCCTTAAAATCCATGGGTTATTTTAACC
RrcPlusFlankRight	GCAGTTTGGAATTCAGATTAAAGC
RrcPlusFlankLeft	CGCTTAGTTTTTTAGGATCCCACAACC
Rrc Pet21a FWD	GGAGTCCATATGAGACAATATGAAACC
Rrc Pet21a REV	GGATAAGGATCCCTTAACTTAACCC
Rrc Inverse Left	GATC-AGATCT-CCAAAAGAGTATTTTAAACGCGAATTTTTGG
Rrc Inverse Right	GATC-AGATCT-CCTACATTTTGAACTTCTACTTCATTGCC
Rrc KO check FWD	CCCTTAAAGCTCCCATTAACATACCAGG
Rrc KO check REV	GCGTCGATTTGTTCTGAAGAAAAAGC
Fur plus flanks left	GGCTTAAGGGAATTCTCAATGG
Fur plus flanks right	GGATTAAACTCACCACTGCAGAACGC
Fur Inverse Left	GATC-AGATCT-GCTTAGTATATTTAAGTCCGCC
Fur Inverse Right	GATC-AGATCT-GCAGCTTTATGGTGTTTGTGG
Fur KO Check Left	GGTTTGATGCTTTTATTTTAGG
Fur KO Check Right	GGAGTTTCTACTTCTAAAAAGCC

PerRCompNativeFwd NcoI	GGAAAACCATGGAGAAAAAGATG
PerRCompNativeRev NcoI	GGGTTATTTTAACCATGGTTTGC
0046FCheck3	GCAGAGCACTTGATTTTAGTGTGTGC
0046RCheck3	CCTGGAGAAGTATTAGATAGTAGCGG
PerRInternalRev	GCACAAACAGACAGATGATTGACG
CmPrReadOut	GGTCGAAATACTCTTTTCGTGTCC
CmReadOut	CGTTTGTGACGGCTTTCATGTTTGC
T7Fwd	TAATACGACTCACTATAGGG
T7Rev	GCTAGTTATTGCTCAGCGG
PerRpET21aF	GGAATAAATCATATGGAATTACTACAAATG
PerRpET21aR	CCCTTAAAATATATGGATCCTTTTAACC
KatA Prom Fwd	5’-[DIG]-CCAATAACCAAAACTGACATAAATTCTCC-3’
KatA Prom Rev	5’-CGTTAGTCAATTTTTTCATTGTTTTCTCC-3’
DnaE Prom Fwd	5’-[DIG]-GCAATTTTTACTTTCATCATTTCATCC-3’
DnaE Prom Rev	5’-GCAAGTGTGTAAATTGACTCATTTTCTTCC-3’
AhpC Prom Fwd	5’-[DIG]-GCAATACATATATCTGTAATTTTTACAGC-3’
AhpC Prom Rev	5’- GCTTTTTTAGTAACTATCATATTTTCTCC-3’
PerR Prom Fwd	5’-[DIG]-GGAAAAACAAGTGAGGTGG-3’
PerR Prom Rev	5’- GCTTTTAACTCATGTTTTTTAAGC-3’
TrxB Prom Fwd	5’-[DIG]-GGTGAAGTTGTAGATCAACTCGTTGGTGC-3’
TrxB Prom Rev	5’-CCAGCAGGACCTCCACCTATGATTGC-3’
Rrc Prom Fwd	5’-[DIG]-GCTTAATACATGTGAAATTTAT-3’
Rrc Prom Rev	5’-[CCTACATTTTGAACTTCTACTTCATTGCC-3’

Table 2.6: A list of vectors used and generated during this thesis

Vector	Description	Source
pNEB193	General sub-cloning vector	New England Biolabs
pET21a	T7 inducible over expression vector, Amp^r	Novagen
pET28a	Vector for the recombinant expression of proteins with an N-terminal, cleavable, His-tag under the control of the T7 promoter, Kan^r	Novagen
pET28a:*perR*	For the expression of *C. jejuni* PerR in *E. coli* with a thrombin cleavable, 6 His tag.	This study
pOSH1	For the recombinant expression of native *C. jejuni* PerR in *E. coli*	This study
pOSH2	*C. jejuni* 11168 *perR (cj0332)* plus 500 bp flanking sequences ligated into pNEB193.	This study
pOSH3	For replacement of *perR (cj0332*) with a kanamycin resistance cassette	This study
pMARKan9	Vector containing a *Bam*HI digestible kanamycin resistance cassette.	(Reuter & van Vliet, 2013)
pAV35	Vector containing a digestible chloramphenicol resistance cassette.	(van Vliet *et al.*, 1998)
pRAH2	pNEB192 plus *fur* (*cj0400*) and 500 bp flanking regions ligated into pNEB193.	This study

pRAH3	For replacement of *fur (cj0400*) with a chloramphenicol resistance cassette.	This study
pINK1	For the expression of *C. jejuni rrc* in *E. coli* with a cleavable 6 His tag.	This study
pINK2	*C. jejuni* 11168 *rrc (cj0012c)* plus 500 bp flanking sequences ligated into pNEB193.	This study
pINK3	For replacement of *rrc (cj0012c*) with a kanamycin resistance cassette	This study
pC46	For gene complementation into the Cj0046 pseudogene region	(Reuter & van Vliet, 2013)
pC46::perR	For complementation of PerR with native promoter into the Cj0046 pseudogene region.	This study

2.6 Bioinformatics

Several data analysis packages were used throughout this document for statistical evaluation or for the presentation of data. These include:

Artemis Genome Comparison Tool (Wellcome Trust Sanger Institute) was used for the viewing genomes and the alignment of sequences to the *C. jejuni* genome (www.artemis.co.uk).

BLAST (Basic Local Alignment Search Tool) (NCBI), was used to find proteins homologous to PerR and identify species of bacteria containing members of the fur family metalloproteins (http://blast.ncbi.nlm.nih.gov)

Graph Pad Prism (GraphPad Software) statistics package was used to perform the majority of statistical analyses on the data sets obtained during

this thesis, including statistical tests such as the determination of the standard error of the mean, T-tests and Two-Way ANOVA's. Alternatively, **OMEGA Mars Data Analysis software** (BMG LabTech) was used to calculate the mean and standard error of the mean when working with growth data obtained from OMEGA machinery.

StringDB (www.string-db.org) functional protein association networks were used to identify species of bacteria containing homologues of both PerR and Rrc in Chapter One (Franceschini *et al.*, 2013).

Cytoscape Network Analysis (www.cytoscape.org) used in Chapter Four is an open source platform for the complex analysis of gene expression profiles and was used to integrate multiple microarray data sets and illustrate interactions between them.

IrfanView Image Analysis Software (www.irfanview.com) was used to scale and measure images of disk assays in Chapter Three.

Swiss Protein Data Bank (PDB) DeepViewer (ExPASy) was used to model the sequence of *C. jejuni* PerR onto the structure of *B. subtilis* PerR, as seen in Chapter Five (
Figure 5.12) (http://spdbv.vital-it.ch/).

3 : Chapter Three

The Response of *Campylobacter jejuni* to Oxidative Stress

3.1 Background

The lifestyle of the food-borne, bacterial pathogen *Campylobacter jejuni* presents an interesting paradox. *C. jejuni* requires microaerobic conditions (3-15% O_2 and 3-10% CO_2) for growth in laboratory conditions (low levels of oxygen are essential for metabolism) however *C. jejuni* has a strong sensitivity to oxidative stress and the damage caused by reactive oxygen species (Hazeleger *et al.*, 1998).

C. jejuni are unable to proliferate in the non-permissive aerobic atmospheric conditions that are encountered during transmission and infection, however *C. jejuni* are able to survive these conditions for extended periods. Under these conditions, *C. jejuni* will be under oxidative stress and hence its ability to deal with such stresses is thought to contribute significantly to its success as bacterial pathogen (Davis & DiRita, 2008; Garenaux *et al.*, 2008b; Garenaux *et al.*, 2009; Svensson *et al.*, 2009).

Comparative analyses of genome sequences have shown that *C. jejuni* lacks many of the oxidative stress resistance systems characterised in other bacterial pathogens, such as the SoxRS and OxyR regulons from the Enterobacteriaceae (Parkhill *et al.*, 2000; van Vliet *et al.*, 2002). In contrast, *C. jejuni* utilises members of the Fur family of metalloregulatory proteins to control oxidative stress responses, as both the Ferric Uptake Regulator (Fur) and the Peroxide Stress Regulator (PerR) have been shown to control expression of oxidative stress defence systems such as peroxidases and superoxide dismutase (Holmes *et al.*, 2005; Palyada *et al.*, 2009; van Vliet *et al.*, 1999).

In *C. jejuni*, PerR was shown to mediate iron-dependent regulation of catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (van Vliet *et al.*, 1999). Subsequent transcriptional profiling of the PerR regulon in *C. jejuni* revealed that a total of 143 genes are differentially expressed in a *perR* mutant, with at least 104 genes belonging to the *perR* regulon (Palyada *et al.*, 2009).

This chapter focuses on the role of two global DNA regulators in *C. jejuni*, the ferric uptake regulator (Fur) and the Peroxide Regulator (PerR). In order to understand how PerR and Fur mediate oxidative stress responses in *C. jejuni*, the phenotypes of *C. jejuni perR* and *fur* mutants in response to different sources of oxidative stress were characterised. The phenotypes of *C. jejuni fur perR* double mutants were also characterised in an attempt to unravel the interactions and functional overlap of these two DNA regulators during oxidative stress responses. Previously reported phenotypes of *C. jejuni* Δ*perR* have varied, so to confirm that any phenotypes observed in the *perR* mutant was due to the disruption of the *perR* gene, the same gene was inactivated in two additional *C. jejuni* reference strains: 81-176, and 81116.

3.2 Objectives

- To phenotypically characterise the responses of *C. jejuni* to a variety of sources of oxidative stress.

- To investigate the role of *perR* (*cj0322*) and *fur* (*cj0400*) in oxidative stress resistance

3.3 Results

3.3.1 The generation of Δ*perR* mutants in *C. jejuni* strains NCTC 11168, 81116 and 81-176

During the generation of *C. jejuni* PerR mutants (described in detail in Materials and Methods) approximately 100 bp of the 3' end of the *perR* gene region was not removed, this was to avoid disruption of the promoter of the downstream gene (*cj0323*), which overlaps with the 3' end of the *perR* gene sequence (See Figure 3.1).

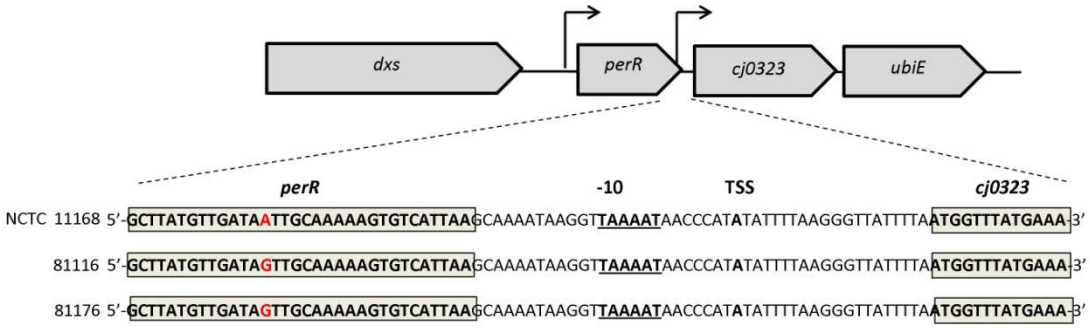

Figure 3.1:The *perR* genomic region is conserved across *C. jejuni* reference strains NCTC 11168, 81116 and 81-176, including a promoter downstream of *perR* driving transcription of *cj0323* (Dugar *et al.*, 2013). The final 54 nucleotides of the 3' end of *perR* were not deleted in the Δ*perR* mutants, to avoid disruption of the *cj0323* promoter. Promoters are indicated by arrows, with -10 sequences, transcription start sites and the respective genes highlighted.

The *perR* loci are conserved, the 3' end of the *perR* gene's sequence identity is highly similar across all three *C. jejuni* reference strains used (See Figure 3.1). There is only one base pair difference between the three strains in the region shown, a nucleotide transition (labelled in red in Figure 3.1 of a G to A in the *C. jejuni* NCTC 11168 *perR* sequence compared to the *perR* sequences from 81116 and 81176, which codes for an asparagine in place of a serine. Equally, the sequence shown in Figure 3.1, plus an additional 71 bp upstream region were not removed from the *C. jejuni* genome during *perR*

mutagenesis in order to prevent disruption of the transcription of the downstream gene, *cj0323*. The promoter sequence of *cj0323* is located in the intergenic region between *perR* and *cj0323*, and deletion of the 3' end of *perR* may interfere with the transcription of *cj0323*. The function of *cj0323* is unknown and therefore it was important to not disrupt transcription, as this may have led to polar effects and *perR* independent phenotypes in the ∆*perR* mutant

The mutagenesis of *perR* in *C. jejuni* was achieved by the insertational inactivation of *perR* with a kanamycin resistance cassette, as described in the Materials and Methods section. For a full list of vectors and strains used in this chapter, also refer to Chapter 2: Materials and Methods.

3.3.2 Physiological characterisation of *C. jejuni* ∆*perR* and Δ*fur* mutants

To investigate the physiological and regulatory role of PerR (*cj0322*) in *C. jejuni* the isogenic Δ*perR* mutants in all three *C. jejuni* strains (11168, 81-176 and 81116) were phenotypically analysed using a range of analyses covering growth, motility and survival. Additionally, as PerR is a Fur homologue and interplay between PerR and Fur in *C. jejuni* has been reported (van Vliet *et al.*, 1998; van Vliet *et al.*, 1999), a *C. jejuni* NCTC 11168 *fur* single mutant was also constructed and characterised (as previously described in Materials and Methods), as well as a *C. jejuni* NCTC 11168 *ΔfurΔperR* double mutant. This is to allow distinction between PerR and Fur functions in *C. jejuni*.

The phenotypes of all mutant strains were compared to the wildtype and complemented strains.

3.3.2.1 Growth of *C. jejuni* strains at 37 °C and 42 °C

To determine whether mutant strains had any defects in growth that may affect the results in other phenotypical analyses, the growth of *C. jejuni* was assessed by measuring the optical density (absorbance) of *C. jejuni* bacterial cultures periodically over the course of ten hours. All *C. jejuni* strains were at grown in a FLUOstar Omega incubator at either 37 °C or

42°C, under microaerobic conditions, shaking at 600 rpm in a double orbital configuration. All cultures were grown in Brucella broth at an approximate starting A_{600} of 0.05 in a final culture volume of 280 µl.

3.3.2.1.1 NCTC 11168

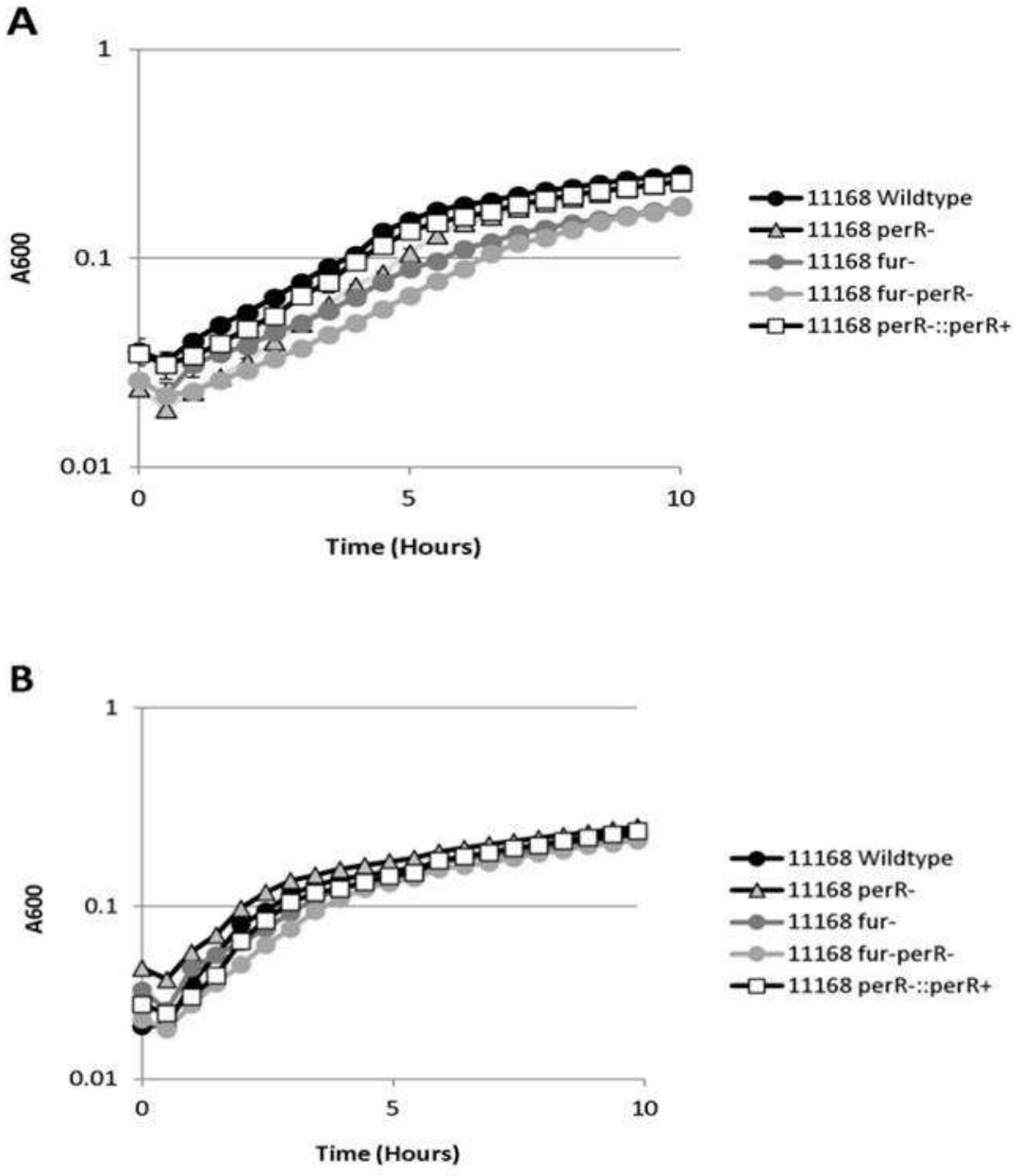

Figure 3.2: *C. jejuni* NCTC 11168 mutants and complemented strains grown at the same rate as the wildtype strain at 37 °C (A) and 42 °C (B). Experiments were performed in triplicate, error bars show standard

error of the mean as calculated by Mars Data Analysis Software (BMG LabTech).

When grown microaerobically in Brucella broth at either 42 °C or 37 °C, there are no significant growth differences between a *C. jejuni* Δ*perR* mutant in 11168 and the wild-type species.

In *C. jejuni* NCTC 11168, the Δ*fur* and Δ*fur*Δ*perR* mutants also have wildtype growth at 42 °C, but appear to have a reduced exponential growth rate at 37 °C in Brucella broth. This has been shown previously, a *C. jejuni* NCTC 11168 Δ*fur* mutant had a reduced growth rate compared to the wild-type strain in iron rich conditions at 37 °C but still reached a similar final A_{600} (van Vliet *et al.*, 1998).

3.3.2.1.2 Other *C. jejuni* strains: 81116 and 81-176

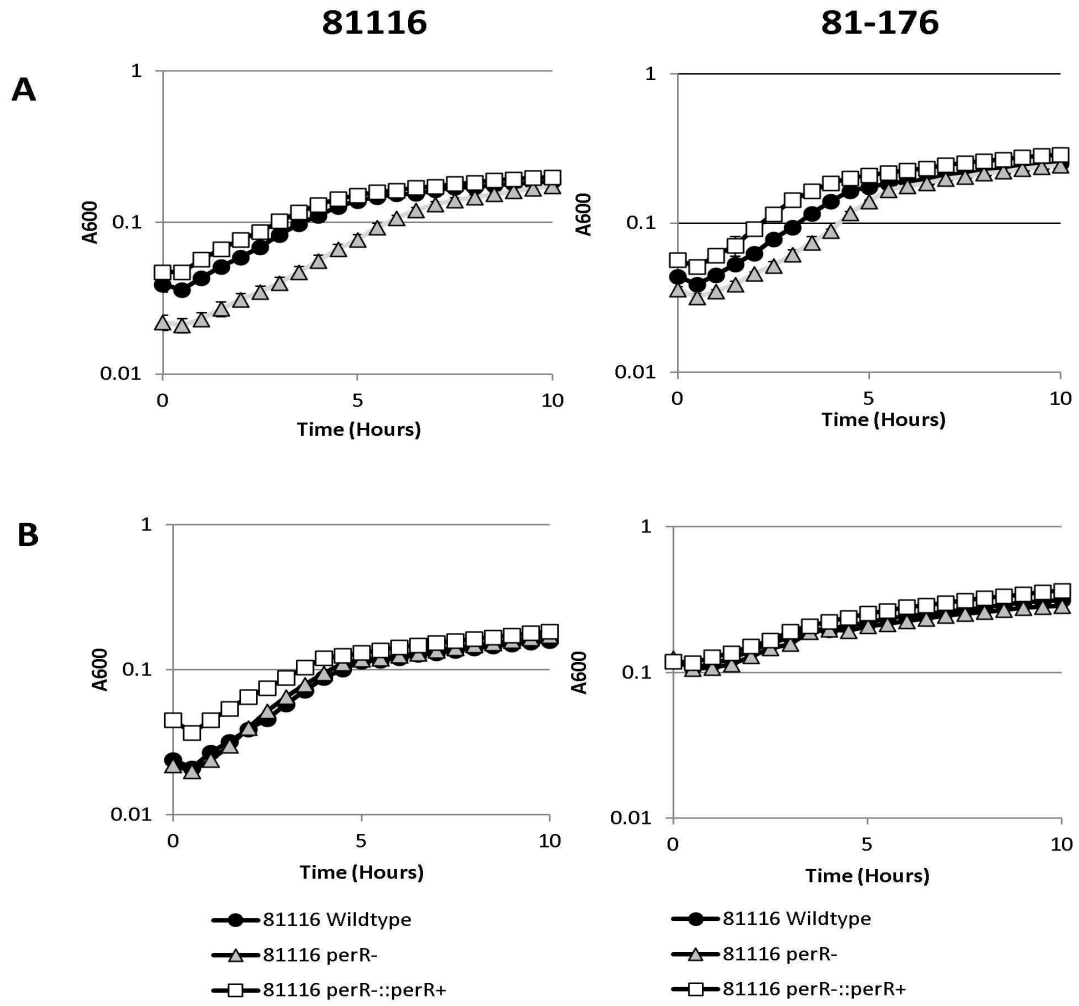

Figure 3.3: *C. jejuni* 81-176 and 81116 *perR* mutants and complemented strains grown at 37 °C (A) and 42 °C (B). Experiments were performed in triplicate, error bars show standard error of the mean as calculated by Mars Data Analysis Software (BMG LabTech).

The *C. jejuni* 81116 Δ*perR* mutant appears to have consistently lower A_{600} reading compared to the 81116 wildtype and complemented strains, however this is probably a reflection of the lower starting A_{600}, as the same pattern is not seen at 42 °C. Otherwise, all Δ*perR* mutants show a similar phenotype to *C. jejuni* NCTC 11168, having wildtype levels of growth at both 37 and 42 °C.

3.3.2.2 Motility

Motility is a key virulence factor for many bacteria, previous studies have implicated both Fur and PerR as having roles in the regulation of motility in *C. jejuni* NCTC 11168 (Palyada *et al.*, 2009).

C. jejuni strains were cultured in Brucella broth for 16 hours, then 5 µl of culture from each strain was placed onto the surface of a soft agar plate (0.4 % agar). Motility is measured as a function of bacterial movement across the surface of the soft agar. Motile bacteria strains spread outwards across the surface of the semi-solid agar, whilst non-motile strains do not. For comparison the movement of *C. jejuni* strains across the semi-solid (0.4%) agar plates were compared to a non-motile control, 11168 Δ*flaAB* (Figure 3.4F), a strain where the structural gene for the flagella are disrupted by a kanamycin resistance cassette (Reuter & van Vliet, 2013).

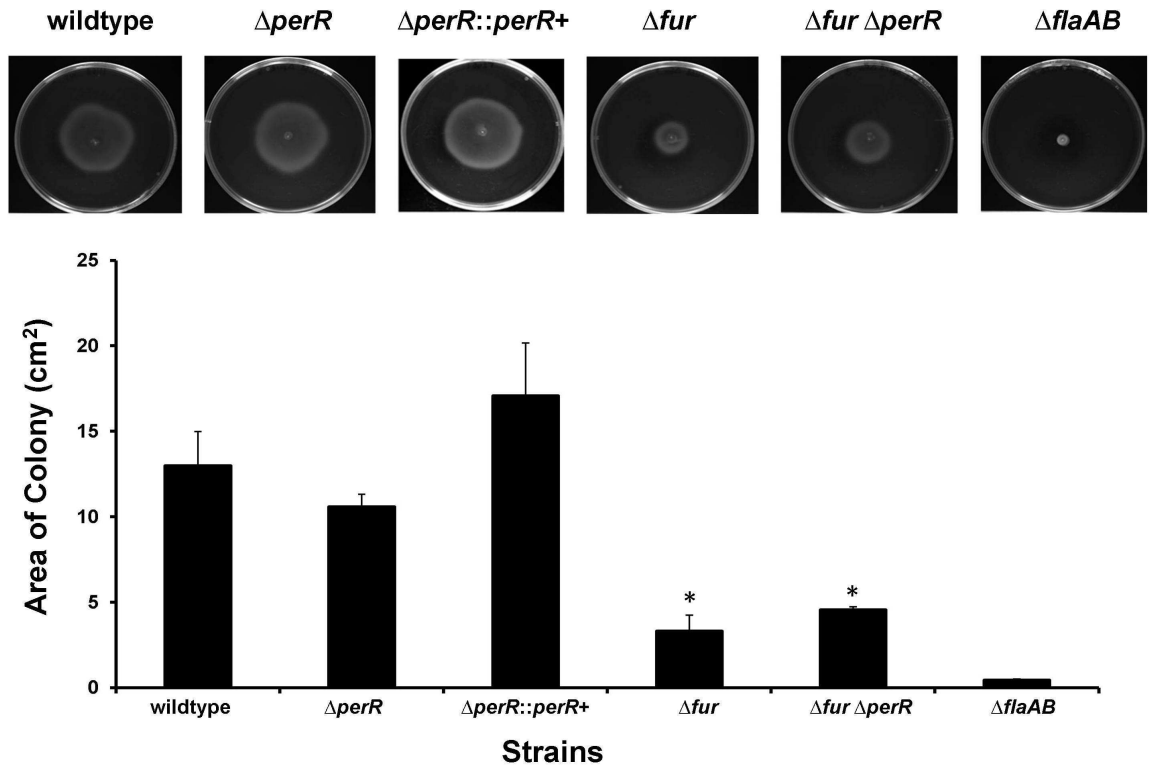

Figure 3.4: The motility of *C. jejuni* NCTC 11168 mutant and complement strains. The inset shows images of *C. jejuni* strains motility on soft agar after three days growth, from a single experiment.

The graph shows the average area of each bacterial halo after three days as determined by ImageJ image analysis software. Experiments were performed in triplicate, error bars show standard error of the mean as calculated GraphPad Prism. * indicates result is statically significant compared to the wildtype (Two-Way ANOVA, P-Value = 0.37 (Δ*fur*) and 0.012 (Δ*fur*Δ*perR*)).

The *C. jejuni* NCTC 11168 wild type strain shared similar motility profiles with the Δ*perR* mutant and complementation. The Δ*fur* and Δ*fur*Δ*perR* mutants both had greatly reduced motility compared to the wildtype strain, however neither was completely non-motile. A Two-Way ANOVA indicates results for both Δ*fur* and Δ*fur*Δ*perR* are statistically different from the wildtype strain phenotype.

In order to confirm and further characterise the role of PerR in *C. jejuni* motility, motility assays were additionally performed in *perR* mutants in 81116 and 81-176.

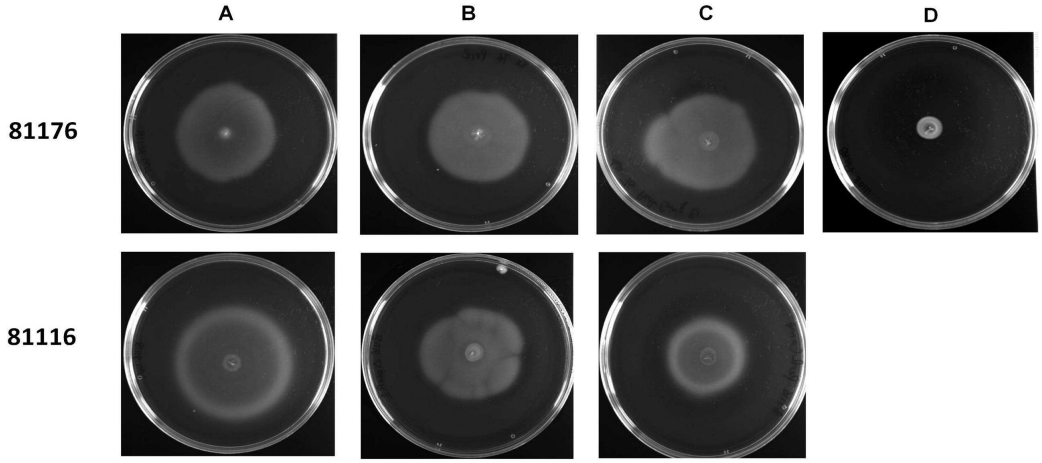

Figure 3.5: Motility of *C. jejuni* 81-176 and 81116 is unaffected by the mutation of *perR*. Representative data from the wildtype strain (A), the Δ*perR* mutant (B) and the complemented Δ*perR*::*perR*$^{+}$ mutant (C) is shown in comparison to a non-motile control (D) (11168 Δ*flaAB*).

PerR mutants in *C. jejuni* strains 81116 and 81-176 have wildtype motility phenotypes and no defect or reduction in motility was seen in any of

the strains tested (Figure 3.5). This corresponds to the motility phenotype seen in the *C. jejuni* NCTC 11168 Δ*perR* mutant (Figure 3.4).

3.3.2.3 Biofilm Formation

C. jejuni employs a variety of strategies to survive environmental stress and one such feature of *C. jejuni* is the ability to form biofilms. Biofilms are matrix enclosed populations of bacteria that can adhere to surfaces. Biofilms confer conditions suitable for bacterial survival and act as a buffer zone from environmental stresses such as desiccation and high oxygen atmospheric content. The ability to form biofilms is thought it be a key feature in the aerobic survival of *C. jejuni* (Buswell *et al.*, 1998; Costerton *et al.*, 1995; Reuter *et al.*, 2010).

We assessed the ability of *C. jejuni* mutants to form biofilms by leaving static cultures of *C. jejuni* in test tubes in both aerobic and microaerobic conditions (37 °C). The biofilm formed by each culture was quantified after two days by removing planktonic cells and growth media from the test tube, then using crystal violet to stain any remaining adhered biofilm cells left attached to the glass test tube.

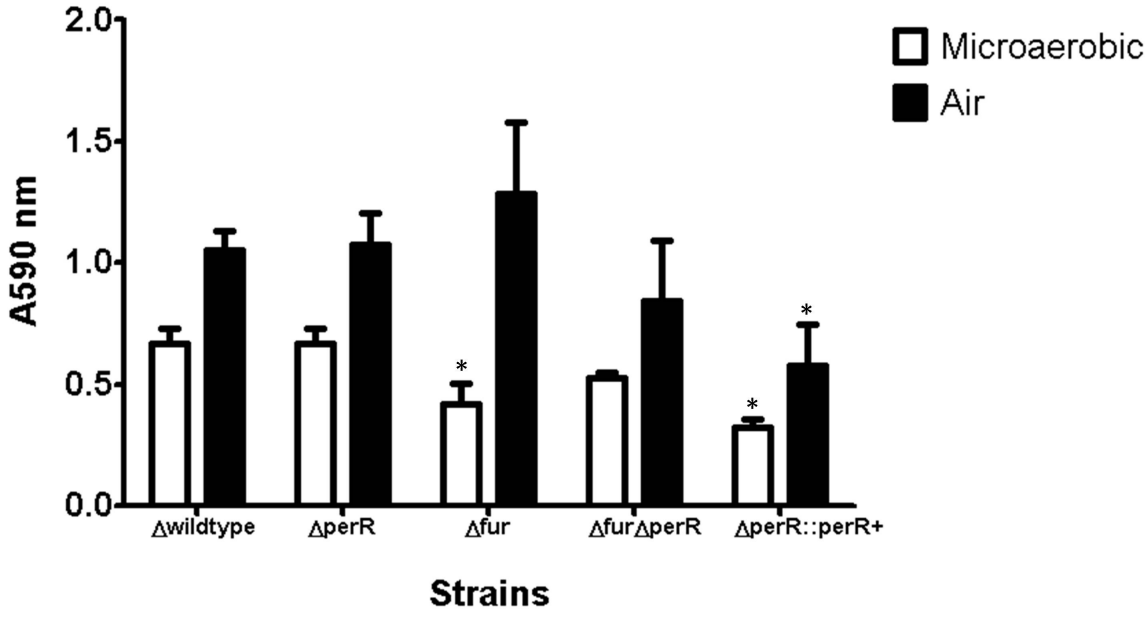

Figure 3.6: The formation of biofilms by *C. jejuni* NCTC 11168 mutant strains when grown either microaerobically or in air at 37 °C in Brucella

media. N=5 * indicates result is statistically significant (P= <0.05) from the wildtype as determined by T-test (GraphPad Prism).

Biofilm experiments were repeated five times, with three technical replicates per strain in each experiment. All strains follow the wildtype trend of biofilm formation in aerobic conditions, aerobic culture results in more biofilm compared to microaerobic conditions, as has been previously reported (Reuter *et al.*, 2010).

The Δ*perR* mutant shows wildtype biofilm production, however the Δ*fur* mutant has reduced biofilm production under microaerobic growth conditions compared to the wildtype strain, as determined by an unpaired T test (P-value = 0.04). The reduction in biofilm formation in a *fur* mutant is perhaps a reflection of the reduced growth (Figure 3.2) and the reduced motility of this strain (Figure 3.4), as flagella are key instruments in adhesion and biofilm formation.

The complemented Δ*perR* (Δ*perR::perR*+) mutant has significantly reduced biofilm production under both aerobic and microaerobic conditions. This is likely an artefact of *C. jejuni* genetic manipulation and not a true phenotype, as similar results are not seen in the wildtype or Δ*perR* mutant.

3.3.2.4 Analysis of *C. jejuni* NCTC 11168 wildtype, Δ*perR* and Δ*perR:perR*+ by Electron Microscopy

Filter electron microscopy was used to view *C. jejuni* NCTC 11168 strains at high magnification. This was to determine whether mutation of *fur* or *perR* had any visible effect on cell morphology or flagella formation.

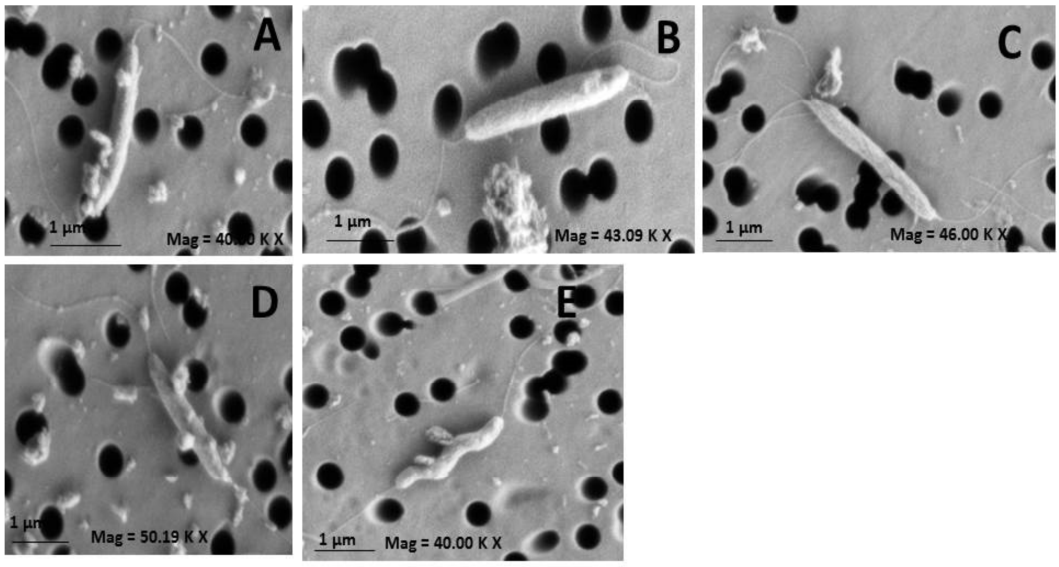

Figure 3.7: Filter Electron microscopy analysis of *C. jejuni* NCTC 11168 cell morphology, including the wildtype (A), Δ*perR* (B), Δ*perR::perR+* (C), Δ*fur* (D) and Δ*fur*Δ*perR* (E).

Filter EM analysis reveals that all strains have bipolar flagella including the Δ*fur* mutant, which showed a motility defect. Unfortunately, the filter EM sample preparation process is abrasive, therefore flagella length could not quantified as many are broken such as Figure 3.7C. Cells from all strains except Δ*fur*Δ*perR* (E) maintain the wildtype rod shaped morphology. The Δ*fur*Δ*perR* mutant has reverted to a twisted rod morphology, as is commonly seen in environmental *C. jejuni* isolates. All *C. jejuni* strains were confirmed by strain specific PCR, to ensure that all isolates were correct.

The individual length of *C. jejuni* cells from filter electron microscopy images were measured using ImageJ photo analysis software.

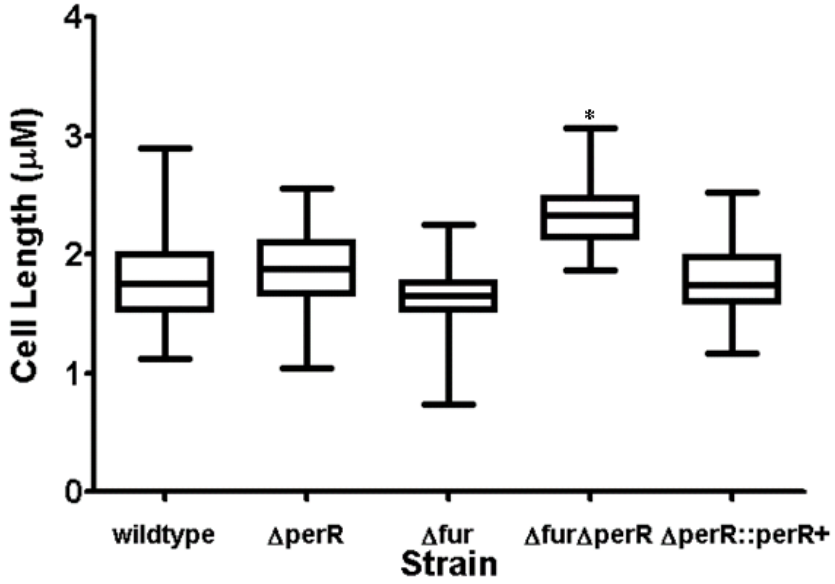

Figure 3.8: Filter Electron Microscopy analysis of *C. jejuni* NCTC 11168 mutant strains cell length. * indicates statistically different from wildtype (P= <0.05) as determined by a One-Way ANOVA test (GraphPad Prism) (N = 25).

Analysis of cell length in *C. jejuni* NCTC 11168 strains shows no significant differences between the average lengths of the wildtype, Δ*perR* mutant, Δ*perR* complemented strain and the Δ*fur* mutant, as determined by a 1-way ANOVA analysis. In contrast, the Δ*fur*Δ*perR* double mutant has increased cell length, perhaps indicating excess elongation, an indication of cell stress, however the spiral morphology made measurement of cell length difficult.

3.3.2.5 Determining virulence of a Δ*perR* mutant using an invertebrate virulence model

The *G. mellonella* wax moth larvae infection model was employed to assess virulence of *C. jejuni* NCTC 11168 and the isogenic mutant strains (Δ*perR* and Δ*perR::perR*+). Bacteria were grown on Brucella agar overnight to an A_{600} of 1.0 (an infection dose of approximately 10^6 cfu) and 10 µl cell suspension was injected into the uppermost right proleg of each larvae.

Mock injection and injection with 10 µl PBS were included in each assay as controls. Survival of the larvae was assessed 24 and 48 hours post injection.

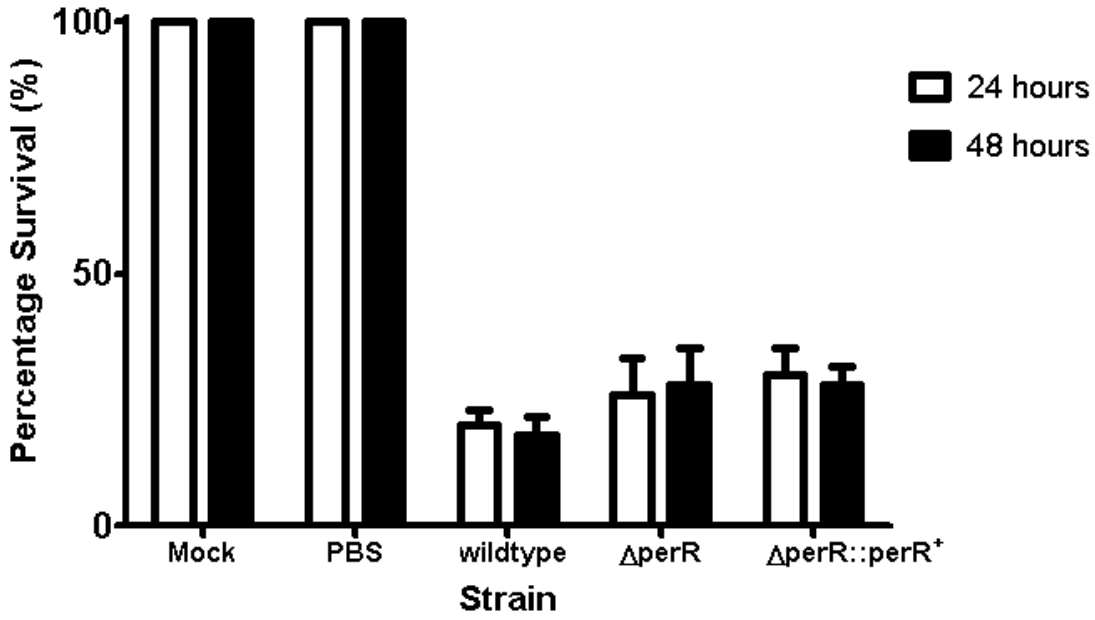

Figure 3.9: The *G. mellonella* infection model shows no significant variation in bacterial pathogenicity between the *C. jejuni* NCTC 11168 wildtype, Δ*perR* and Δ*perR::perR+*. Experiments were repeated five times, error bars show standard error of the mean (GraphPad Prism).

Infection with *C. jejuni* Δ*perR* and Δ*perR::perR*+ resulted in no significant variation in *G. mellonella* survival compared to the *C. jejuni* NCTC 11168 wildtype strain at 24 or 48 hours after infection (Figure 3.9), although a decrease in survival was observed between all strains and the control experiments. The survival of *G. mellonella* is assessed on the ability of the larvae to rectify itself if turned. Survival increased between 24 and 48 hours in some cases, as some larvae appeared to recover over time.

3.3.3 Response of *C. jejuni* to Oxidative Stress

Although PerR is a 'Peroxide Regulator' many bacterial stress responsive proteins have multiple functions, it was therefore important to characterise the responses of *C. jejuni* Δ*perR* to a wide range of oxidative stress sources.

3.3.3.1 Hydrogen Peroxide

As PerR is primarily associated with peroxide resistance, *C. jejuni* Δ*perR* strains were challenged with hydrogen peroxide in both liquid and solid mediums.

C. jejuni NCTC 11168 strains were exposed to hydrogen peroxide on solid media by means of a disc inhibition assay. Filter paper discs were placed onto the surface of a plate of bacteria, and spotted with 10 µl of varying concentrations of hydrogen peroxide. Sensitivity of *C. jejuni* to hydrogen peroxide is indicated by a zone in which no bacterial growth is seen around the filter paper disk.

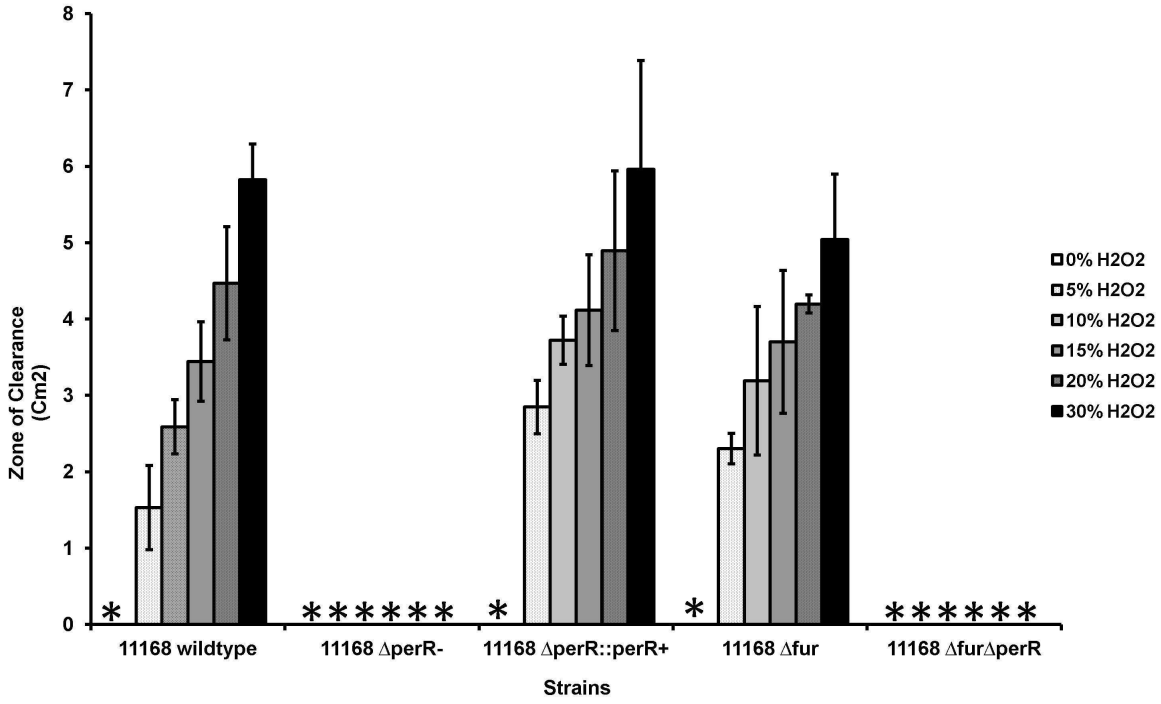

Figure 3.10: *C. jejuni* Δ*perR* and Δ*fur*Δ*perR* mutants is resistant to 30% hydrogen peroxide (~ 9M) in disc inhibition assays, whereas growth of the other strains is inhibited. Error bars indicate standard error of the mean calculated over three replicates (GraphPad Prism). *; Indicates data point is equal to zero.

C. jejuni NCTC 11168 wild type shows a positive correlation between increasing concentrations of hydrogen peroxide and the area of inhibited growth, the same pattern of sensitivity is seen in the Δ*perR* complemented strain and the Δ*fur* mutant. However, the Δ*perR* and Δ*fur*Δ*perR* mutants are resistant to hydrogen peroxide, even at the highest concentration of 30%.

The same assay was performed in *C. jejuni* backgrounds 81116 and 81-176 to confirm the resistance of *C. jejuni* Δ*perR* to hydrogen peroxide.

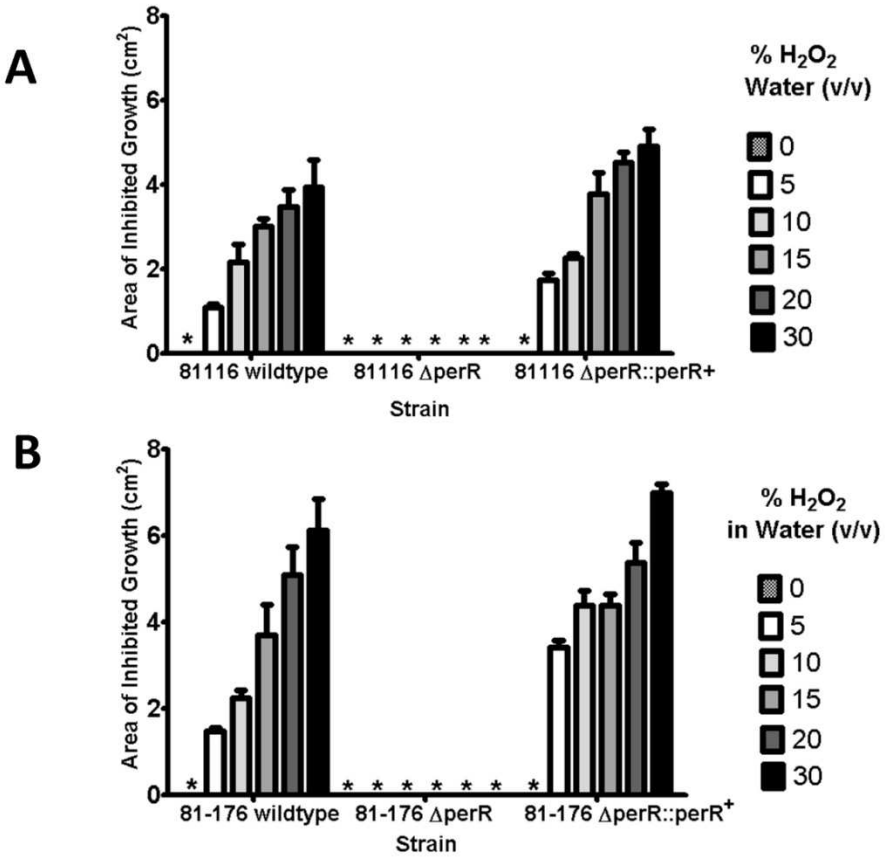

Figure 3.11: PerR mutants in *C. jejuni* 81116 [A] and 81-176 [B] are resistant to 30% hydrogen peroxide. Error bars indicate standard error of the mean calculated over three replicates (GraphPad Prism). *; Indicates data point is equal to zero.

As with *C. jejuni* NCTC 11168, Δ*perR* mutants in strains 81116 and 81-176 were highly resistant to hydrogen peroxide, a phenotype restored in the complemented mutant strain.

Figure 3.10 and Figure 3.11 illustrate the exposure of *C. jejuni* to increasing concentrations of hydrogen peroxide (0-30% v/v) and the areas of growth inhibition correspond to the relative resistance of each strain to the source of oxidant.

The *perR* mutants in *C. jejuni* strains NCTC 11168, 81116 and 81-76 and the Δ*fur*Δ*perR* double mutant in *C. jejuni* NCTC 11168 had no zone of inhibited growth for any concentration of hydrogen peroxide used.

C. jejuni Δ*perR* strains were also challenged to hydrogen peroxide in liquid media. *C. jejuni* strains were grown in Brucella broth overnight and then adjusted to an A_{600} of 0.4. Hydrogen peroxide was directly added to *C. jejuni* cultures at a final concentration of 0, 3 or 6% hydrogen peroxide (v/v). Cultures exposed to hydrogen peroxide were left shaking, at 37 °C in microaerobic conditions for three hours. Survival of cultures was assessed periodically by monitoring the cell viability of each strain after the addition of hydrogen peroxide. Cell viability was measured by serial dilution and spot plating to count the number of viable cells, to the nearest log value, remaining in each flask. Survival of *C. jejuni* Δ*perR* was compared to the corresponding wild type and complemented mutant.

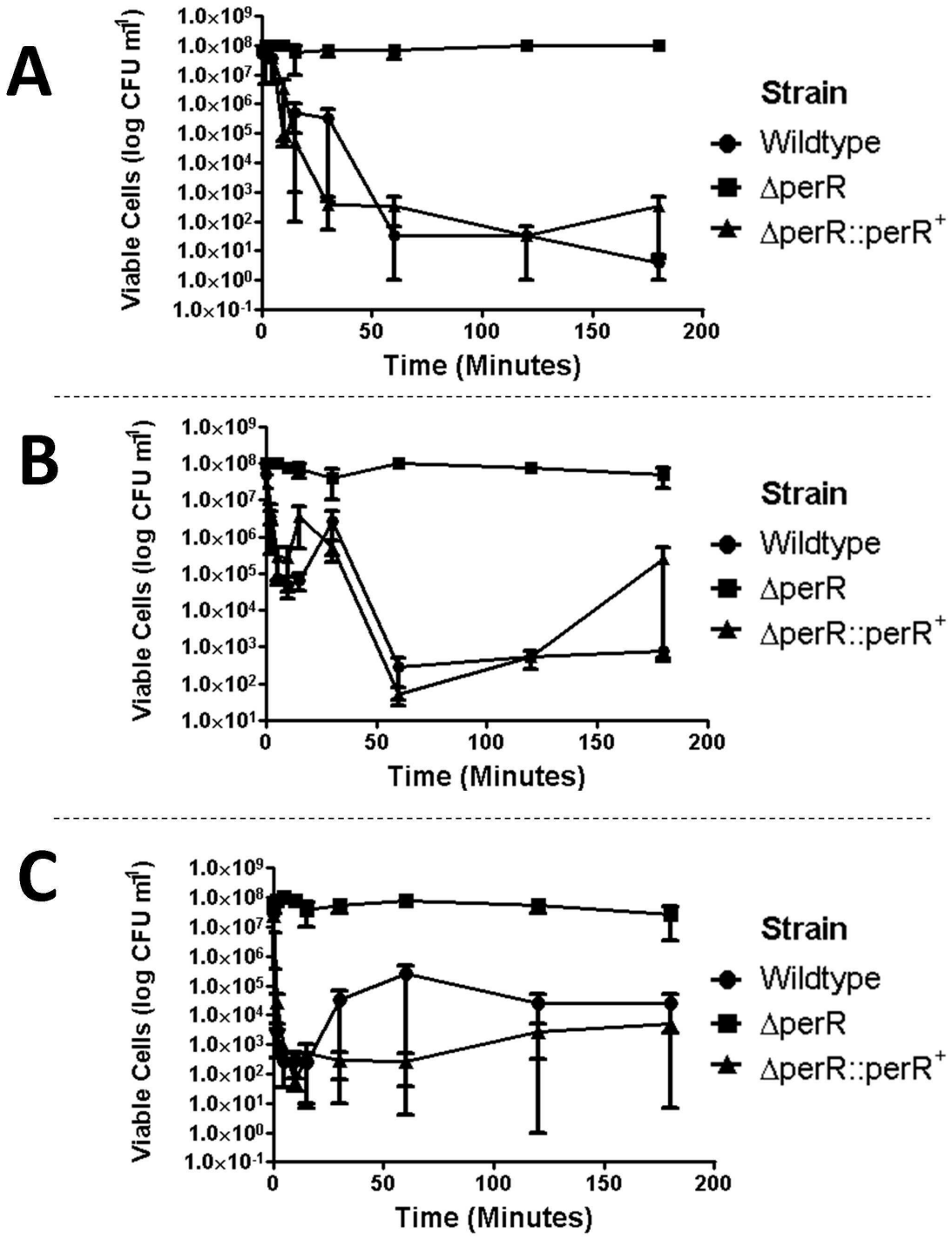

Figure 3.12: *C. jejuni* Δ*perR* mutants in strains NCTC 11168 [A], 81116 [B] and 81-176 [C] are resistant to hydrogen peroxide in broth culture. Experiments were repeated in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

The Δ*perR* mutant has the same phenotype in all three *C. jejuni* background strains. Upon addition of hydrogen peroxide to a broth culture the number of viable cells of the wildtype and Δ*perR* complemented strain (Δ*perR::perR*+) begin to decrease. However the Δ*perR* mutant did not show a decrease in viable cells over the course of the assay.

3.3.3.2 Resistance of *C. jejuni* to cumene hydroperoxide (CHP)

Plate inhibition assays (as previously described for hydrogen peroxide) were used to assess the resistance of *C. jejuni* Δ*perR* strains to cumene hydroperoxide (CHP). CHP is an organic hydroperoxidase and an alternate source of oxidative stress for bacteria.

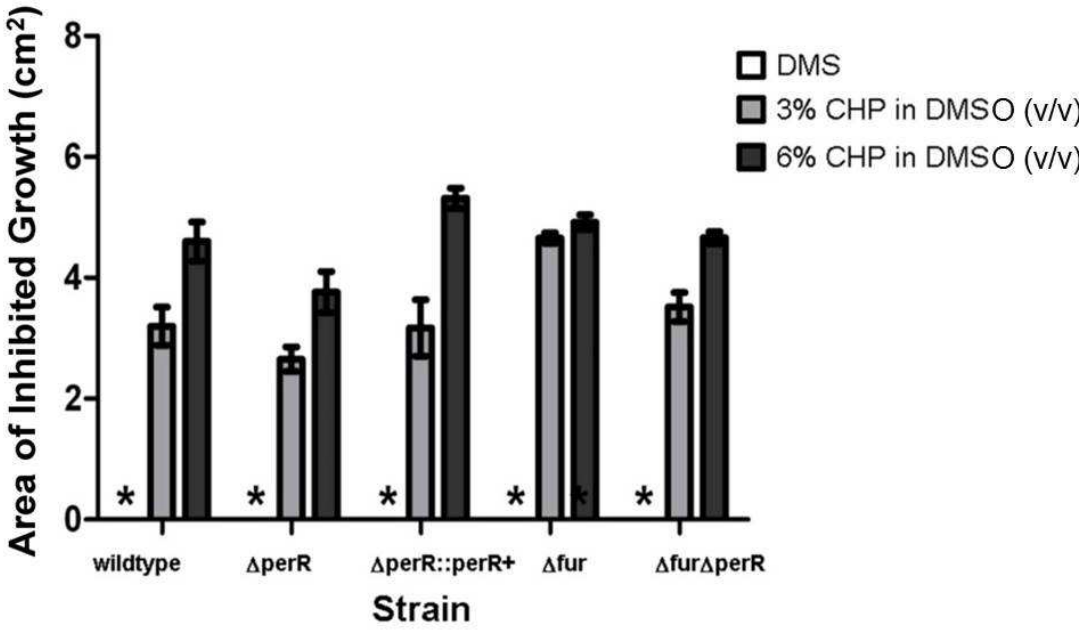

Figure 3.13: *C. jejuni* NCTC 11168 strains are sensitive to cumene hydroperoxide (CHP) in dimethyl sulfoxide (DMS). Error bars indicate standard error of the mean calculated over three replicates. *; Indicates data point is equal to zero.

C. jejuni NCTC 11168 demonstrates sensitivity to both 3 and 6 % cumene hydroperoxide, indicated by the zone of inhibited growth observed during the disk assay. When grown in the presence of 3-6% cumene hydroperoxide (CHP) (v/v in dimethyl sulfoxide (DMSO) all strains showed a reduction in growth as the concentration of CHP increases. This phenotype is slightly reduced in the 11168 Δ*perR* indicating greater resistance and

ability to survive CHP. Wildtype sensitivity to CHP is observed in both the Δ*perR::perR*+ and Δ*fur*Δ*perR* strains, however the Δ*fur* mutant has increased sensitivity to 3% CHP compared to the wildtype strain. This is most likely due to increased iron availability in the Δ*fur* mutant (due to disruption of iron homeostasis) leading to increased generation of reactive oxygen species through Fenton chemistry.

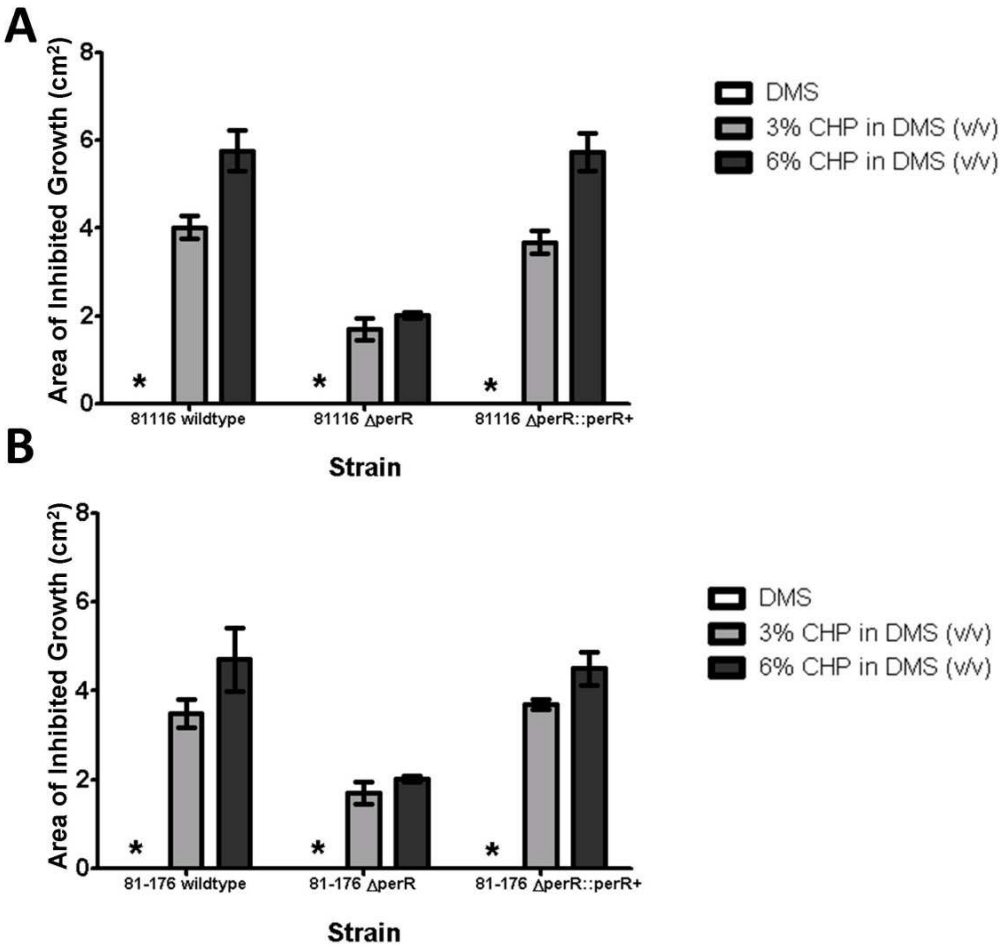

Figure 3.14: A *C. jejuni perR* mutant is more resistant to cumene hydroperoxide (CHP) than the wildtype strain in *C. jejuni* strains 81116 [A] and 81-176 [B]. Error bars indicate standard error of the mean calculated over three replicates (GraphPad Prism). *; Indicates data point is equal to zero.

C. jejuni 81116 and 81-176 Δ*perR* mutants, were able to grow better in the presence of CHP compared to the comparative wildtype strains, as is evident by the reduced zones of no growth. The is the same trend seen in Figure 3.13 for *C. jejuni* strain NCTC 11168, although the increased resistance of the Δ*perR* mutant is less defined in NCTC 11168.

3.3.3.3 The role of PerR in resistance to bactericidal antibiotics

Gentamicin is a bactericidal antibiotic that induces bacterial killing through the production of reactive oxygen species, particularly hydroxyl radicals (Kohanski *et al.*, 2007). Plate inhibition assays were performed to assess whether a *C. jejuni* 11168 Δ*perR* deletion in *C. jejuni* provided broad oxidative stress protection and increased resistance to bactericidal killing by antibiotics.

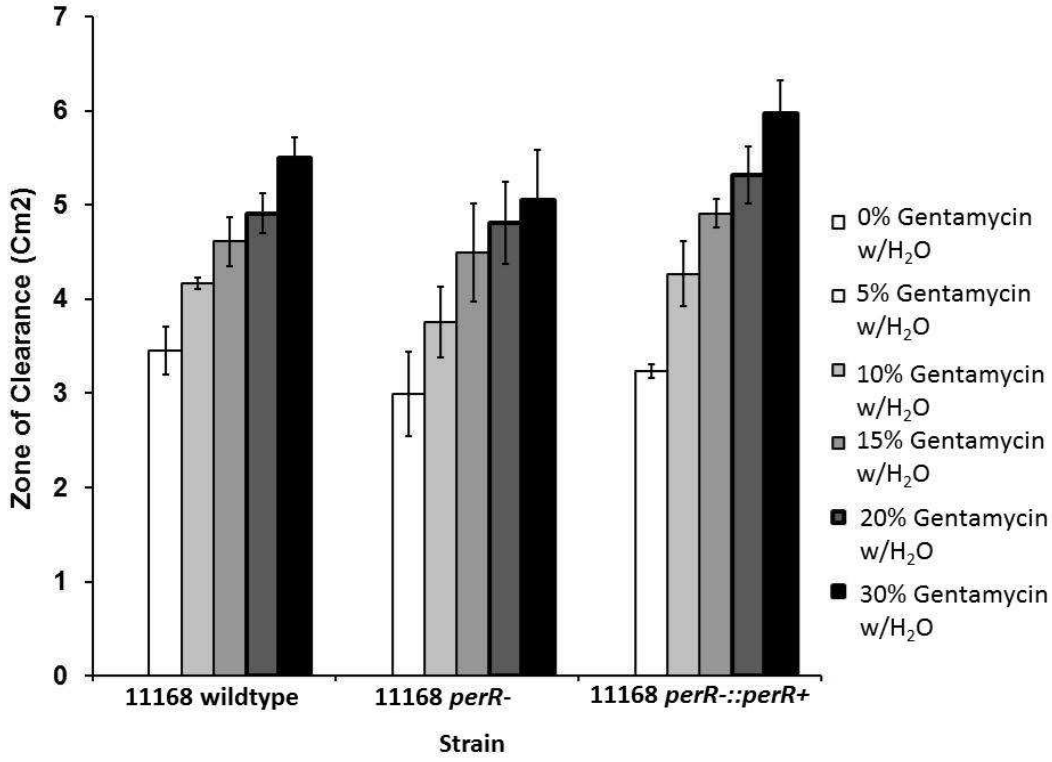

Figure 3.15: A Δ*perR* mutation in *C. jejuni* NCTC 11168 does not provide resistance to killing by gentamicin. Experiments were performed in triplicate, error bars indicate standard error of the mean (GraphPad Prism)

C. jejuni NCTC 11168 Δ*perR* has no reduction in susceptibility to killing by gentamicin exposure. All strains show wildtype sensitivity to killing by gentamicin.

3.3.3.4 Investigating the aerobic tolerance of *C. jejuni*

For a microaerobic organism, aerobic stress is abundant during the transmission of *C. jejuni* to food preparation surfaces. Tolerance of aerobic environments may be an important factor in the spread and survival of *C. jejuni* in the environment. To explore aerobic tolerance, broth cultures of *C. jejuni* were grown microaerobically overnight at 37 °C. The A_{600} of these cultures were adjusted to 0.4 using PBS and then moved into an aerobic 37 °C incubator. The *C. jejuni* cultures were monitored for cell viability by plating serial dilutions of the cultures at 0, 3, 6 and 9 hours aerobic exposure.

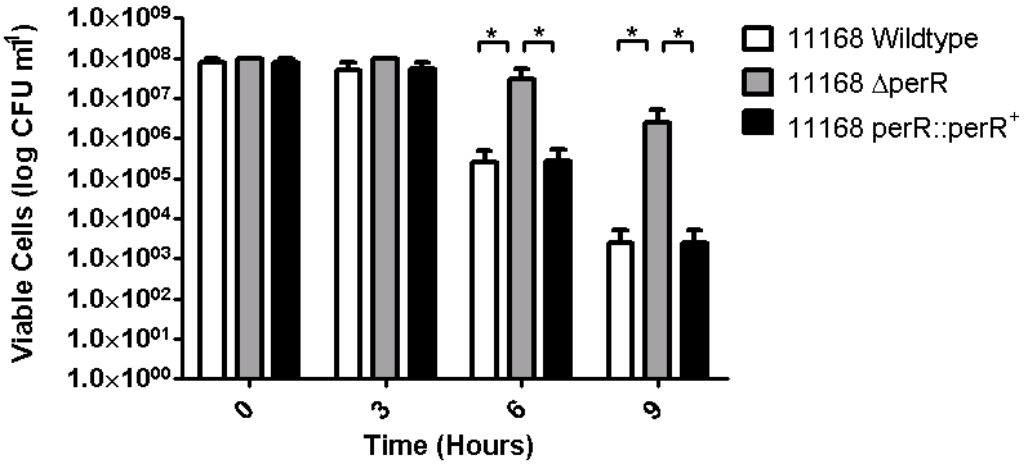

Figure 3.16: Survival of *C. jejuni* NCTC 11168 wildtype, Δ*perR* mutant and Δ*perR::perR*+ stationary-phase cells kept under atmospheric oxygen conditions at 37°C. Aerobic survival is expressed as the number of viable cells remaining in a culture exposed to air, as determined by ten-fold serial dilution of a 20 µl culture aliquot. Statistically significant differences are indicated (*) as determined by an unpaired T test.

After exposure of stationary phase cells to an aerobic environment, the *C. jejuni* NCTC 11168 wild type and Δ*perR::perR*+ strains showed a reduction in cell viability. The number of viable cells for these two strains declined by 2 logs to $1.0x10^6$ after 6 hours in an aerobic environment (Figure 3.16). In contrast, the *C. jejuni* NCTC 11168 Δ*perR* mutant showed a comparatively increased tolerance to an aerobic atmosphere with over $1.0x10^6$ cells surviving after 9 hours exposure to air.

3.4 Discussion

3.4.1 Characterisation of *C. jejuni* NCTC11168 mutant strains : Δ*perR*

The mutation of *fur* and *perR* in the *C. jejuni* genome was successfully performed, as confirmed by DNA sequencing and PCR analysis. The deletion of DNA regulator *perR* conferred few changes to wildtype phenotype in tests analysing growth (Figure 3.2 and Figure 3.3), motility (Figure 3.4 and Figure 3.5), biofilm formation (Figure 3.6), cell length (Figure 3.8) and cell morphology (Figure 3.7). However, some of these results are in contention with previously reported results stating that *C. jejuni* NCTC 11168 *perR* mutants have reduced motility (Palyada *et al.*, 2009). This phenotype was not seen in our NCTC 11168 *perR* mutant, or in the *perR* mutants generated in *C. jejuni* strains 81116 and 81-176.

In league with these results are our findings that *C. jejuni* NCTC 11168 Δ*perR* has wildtype pathogenicity in the *G. mellonella* infection model (Figure 3.9). This is also in contrast to previously published data, where a *C. jejuni* 11168 Δ*perR* mutant showed reduced colonisation of the chick intestine, indicating that a Δ*perR* mutant strain may be less 'fit' than the wildtype strain (Palyada *et al.*, 2009). However when we investigated pathogenicity, we saw no significant difference between the ability of *C. jejuni ΔperR* to kill *G. melonella* compared to the wildtype (indicating no reduced 'fitness' of the strain). However, these are two very different models exploring both the colonisation and pathogenicity of *C. jejuni*, yet it is likely that the reduced motility of the Δ*perR* mutant explains these differences in strain 'fitness'. Motility is an important virulence factor for *C. jejuni* and required for successful colonisation of the chick gastrointestinal tract (Hendrixson & DiRita, 2004).

It has not been possible to determine why the reported motility phenotypes of *C. jejuni* Δ*perR* vary. It is tempting to theorize that motility fluctuations may be mediated by iron availability in the media, as PerR is known to differentially regulate genes in response to iron concentrations, although further investigation is required (Palyada *et al.*, 2009; van Vliet *et*

al., 1999). Unfortunately, it is also possible that the previously reported motility defect in *C. jejuni* Δ*perR* was a non-specific phenotype conferred during the mutation process, rather than a direct or indirect link between PerR and motility.

3.4.2 Δ*fur*

In contrast to *perR*, the inactivation of *fur* from *C. jejuni* had numerous effects on basic cell functions, including slightly reduced growth at 37 °C (Figure 3.2), reduced motility (Figure 3.4), reduced biofilm production in microaerobic conditions (Figure 3.6) and reduced cell length (Figure 3.8). From these basic phenotype analyses, it is evident that *fur* mutation has a detrimental effect on *C. jejuni*.

Previous studies confirm our reports of reduced growth and motility of *C. jejuni* 11168 NCTC Δ*fur* mutants, this was attributed to the down regulation of housekeeping genes *lysS* (cj0401) and *glyA* (Cj0402), both downstream from *fur* (cj0400), which may be a result of downstream effects from Fur disruption (Holmes *et al.*, 2005).

Interestingly our EM analysis reveals that *C. jejuni* Δ*fur* do produce flagella, despite reports of *C. jejuni* Δ*fur* mutants lacking flagellar expression (Holmes *et al.*, 2005) (Figure 3.7). Unfortunately due to the abrasive EM sample preparation, it is not possible to quantify the flagella of *C. jejuni* Δ*fur* mutants, therefore we are unable to conclude whether the reduced motility of C*. jejuni* Δ*fur* is a result of disrupted flagella biosynthesis or the reduced growth rate of the strain.

Motility is a key factor in *C. jejuni* biofilm formation. The flagellar motility complex is absolutely required for the initial attachment of *C. jejuni* during biofilm formation and also aids cell to cell interactions. It is therefore easy to postulate that the reduced motility of the Δ*fur* strain results in the strains reduced ability to attach and form a biofilm under microaerobic conditions.

3.4.3 Δ*fur*Δ*perR*

The *C. jejuni* Δ*fur*Δ*perR* mutant phenotype was essentially similar to the Δ*fur* single mutant, showing slightly reduced growth at 37 °C, reduced motility and reduced biofilm formation. However, EM analysis of the Δ*fur*Δ*perR* cell morphology revealed striking changes to the cell shape (Figure 3.7). It is important to mention that the *C. jejuni* 11168 strain used in these studies is phenotypically different from the sequenced *C. jejuni* NCTC 11168 type strain (Gaynor *et al.*, 2004; Parkhill *et al.*, 2000). Our laboratory wildtype strain is rod shaped, having lost the natural helical cell morphology as seen in other *C. jejuni* strains (81-176), although it retains all other *C. jejuni* 11168 NCTC wildtype phenotypes. Unexpectedly the Δ*fur*Δ*perR* mutant has reverted back to a helical cell morphology hinting at the importance of gene regulation on cell shape. This phenotype is not witnessed in either the Δ*fur* or Δ*perR* single mutants and seems to be a unique characteristic of the Δ*fur*Δ*perR* double mutant, perhaps due to co-regulation of genes by Fur and PerR. It is likely that the altered morphology of *C. jejuni* 11168 Δ*fur*Δ*perR* cells accounts for the increase in cell length seen in Figure 3.8.

3.4.4 Characterisation of the roles of Fur and PerR in oxidative stress resistance in *C. jejuni*

A *C. jejuni* Δ*perR* mutation confers a very high resistance to oxidative stress. The same trends of high oxidative stress resistance can be seen for Δ*perR* mutants in all three *C. jejuni* strains (NCTC 11168, 81116 and 81-176) during exposure to hydrogen peroxide in broth or disc assay format (Figure 3.10-15). As previous phenotypic analyses showed differed from published results the use of multiple *C. jejuni* strains proved to be an advantage for confirming the validity of the phenotype experiments.

Disc inhibition assays showed that a *C. jejuni* NCTC 11168 *perR* mutant was resistant to 30% hydrogen peroxide (~ 9M), a 10-fold greater resistance to hydrogen peroxide than had previously been reported (Palyada *et al.*, 2009)(Figure 3.10). To ensure high hydrogen peroxide resistance was not due to diffusion of the oxidant into the solid media *C. jejuni* was also exposed to hydrogen peroxide in broth culture.

After exposure to 3% hydrogen peroxide (0.88 M) in broth culture, a *perR* mutant maintained its level of viable cells ($1.0x10^8$), whereas the number of viable wildtype and complemented cells began to decline significantly soon after the addition of H_2O_2 (Figure 3.12). Equal levels of hydrogen peroxide resistance were confirmed in *C. jejuni perR* mutants in strains 81116 and 81-176. The *C. jejuni* catalase (KatA) is a long established member of the *C. jejuni* PerR regulon (van Vliet *et al.*, 1999) and highly up regulated in *C. jejuni perR* mutants (Palyada *et al.*, 2009). Catalase enzymes are ubiquitous in bacteria and several different types of catalase enzymes exist showing intricate patterns of regulation and substrate specificity. *C. jejuni* contains a single heme-cofactored catalase that rapidly dismutates hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (Grant & Park, 1995; van Vliet & Ketley, 2001). It is likely that the high resistance of Δ*perR* mutants to hydrogen peroxide is conferred by the deregulation and therefore excess amounts of catalase within the cells. Complementation of the *perR* mutation restored the wild-type phenotype to the bacteria.

Typically KatA has also been designated part of the *C. jejuni* Fur regulon (van Vliet *et al.*, 1999), confirmed by both microarray (Palyada *et al.*, 2004) and ChIP-chip analysis (Butcher *et al.*, 2012). However, no strong resistance to hydrogen peroxide is witnessed in *C. jejuni* 11168 Δ*fur* and instead shows wild type sensitivity to hydrogen peroxide. Although, in a Δ*fur* mutant one would expect cells to contain excess intracellular iron and therefore more readily generate reactive oxygen species via Fenton Chemistry when challenged with hydrogen peroxide. Therefore the absence of abundant sensitivity to hydrogen peroxide in high iron conditions in the Δ*fur* mutant may be evidence of strong expression of oxidative stress responsive pathways

PerR is repressed under high iron conditions (Butcher *et al.*, 2012; Kim *et al.*, 2011), therefore it is possible that the identification of *katA* initially as a member of the Fur regulon is due to the increased iron uptake in a Δ*fur* mutant leading to increased intracellular iron concentrations and repression of *perR* causing deregulation of *katA* as a member of the PerR regulon.

However, in contrast to this theory is the identification of a Fur binding box upstream of the *katA* promoter.

Perhaps surprisingly the hydrogen peroxide resistance of *C. jejuni* NCTC 11168 Δ*fur*Δ*perR* double mutants is on par with the Δ*perR* single mutation, and are able to overcome the detrimental effects of the Fenton Chemistry generated by high intracellular iron concentrations (caused by *fur* mutation) and hydrogen peroxide exposure.

The resistance of *C. jejuni* strains to cumene hydroperoxide (CHP) (Figure 3.13 and Figure 3.14) and gentamicin (Figure 3.15) were also investigated.

C. jejuni Δ*perR* strains demonstrated improved resistance to CHP in 81116, 81-176 and 11168 (although less defined) compared to the isogenic wild type strains. The increased resistance of *C. jejuni* Δ*perR* strains to CHP has been described previously (Palyada *et al.*, 2009). As an organic hydroperoxidase, CHP is typically detoxified in bacteria by alkyl hydroperoxide reductases, and *C. jejuni* alkyl hydroperoxide reductase (*ahpC*) is another established member of the PerR regulon. As with catalase, it is likely that the deletion of PerR leads to the deregulation and therefore increased expression of AhpC. Providing an ability to combat and detoxify greater levels of cumene hydroperoxide than seen in the wildtype strains.

The Δ*fur* strain demonstrates increased sensitivity to 3% CHP compared to the wildtype strain, again this is likely due to increased intracellular iron concentrations readily forming reactive oxygen species. The Δ*fur*Δ*perR* double mutant shows wild type sensitivity to CHP, it is likely that the protective properties of the Δ*perR* deletion outweigh the increased sensitivity conferred by the Δ*fur* deletion in this double mutant strain.

Gentamicin is a member of the aminoglycoside class of antibiotics and is routinely used as a bacteriocide against Gram Negative bacteria. Gentamicin requires the presence of oxygen for oxygen-dependent electron transport across the cell membrane. Once inside the cell its mode of action is the inhibition of ribosomal activity by binding the 30-50S ribosome interface, thereby halting cell growth (Bryan *et al.*, 1979). However the antibiotics bactericidal activity is caused by the oxidation of NADH by the electron

transport chain, which is dependent on the TCA cycle. Hyperactivation of the electron transport chain stimulates the formation of superoxide, which reacts readily with iron sulphur proteins releasing ferrous iron. Ferrous iron is then free to participate in Fenton chemistry and the generation of hydroxyl radicals, causing cell death by the damaging of DNA, proteins and lipids (Kohanski *et al.*, 2007). We investigated whether a *C. jejuni* Δ*perR* mutant was resistant to gentamicin compared to the wildtype strain and saw no increased survival in a disc assay format. However, retrospectively it would be interesting to explore this phenotype further. For wildtype *C. jejuni* killing by gentamicin occurs due to the excess build up and reactive oxygen species and an inability to produce oxidative stress detoxification proteins due to the binding of gentamicin to the ribosome interface. In *C. jejuni* Δ*perR* where oxidative stress detoxification proteins are already in abundance prior to gentamicin exposure, it is tempting to postulate that gentamicin may only have bacteriostatic effects although further research is required. This poses interesting questions about the use and effectiveness of aminoglycoside antibiotics against *C. jejuni*.

3.4.5 A role for PerR in the aerobic tolerance of *C. jejuni* NCTC 11168

Aerobic tolerance assays performed with *C. jejuni* 11168 strains showed that the Δ*perR* mutant had significantly increased survival in an aerobic environment compared to the wild type strain (Figure 3.16). This is the first time, to our knowledge, that PerR has been linked to a role in aerotolerance regulation in *C. jejuni*. The tolerance of aerobic environments is a key phenotype for *C. jejuni*, a microaerobic pathogen that must survive exposure to air in order to spread and proliferate. Aerobic tolerance assays were performed on cells grown overnight to stationary phase, *C. jejuni* cells are likely to be in stationary phase during aerobic food transmission, where growth requirements are typically sub-optimal.

The increased aerotolerance of *C. jejuni* NCTC 11168 Δ*perR* may be linked to the depression of alkyl hydroperoxide reductase (*ahpC*). A decrease in *ahpC* expression in *C. jejuni* has been linked to a reduction in

aerobic survival; therefore it seems reasonable to speculate that an increased expression of *ahpC* may confer increased resistance and survival in aerobic environments although further investigation is required (Baillon *et al.*, 1999).

In *C. jejuni* alkyl hydroperoxide reductase performs the breakdown of organic peroxides to their corresponding alcohols, in which AhpC becomes oxidised in the process. *C. jejuni* lacks a homologue for the known AhpC recycling partner AhpF, which is found in *E. coli* (Poole, 1996; Poole & Ellis, 1996). There have been some speculations over potential candidates for AhpC recycling partners in *C. jejuni*, including ferredoxin which is located divergently on the *C. jejuni* genome (Baillon *et al.*, 1999).

This is the first time the responses of the *C. jejuni* Δ*fur*Δ*perR* double mutant have been characterised against oxidative stress. The deletion of *C. jejuni perR* seems to bring greater stability to the initial *fur* mutation, such as slightly improved motility and greater resistance to oxidative stress.

A *C. jejuni perR* deletion confers high resistance to oxidative and aerobic stress, yet the susceptibility level of *C. jejuni* wildtype strains to oxidative stress is striking. It is perplexing as to why PerR DNA regulation is not switched off in the wildtype allowing expression of oxidative stress defences comparative to those seen in the Δ*perR* mutant. Auto-regulation of PerR has been reported (Kim *et al.*, 2011), it is possible that low levels of PerR are always maintained within *C. jejuni* as a means of energy conservation. Surplus expression of proteins would be taxing on the energy resources within a bacterial cell. It is likely that strict regulation of the PerR regulon in *C. jejuni* confers some benefit to *C. jejuni* bacteria; however this was not within the scope of this study.

To our knowledge, a mutation in PerR is the first such mutation in *C. jejuni* that confers improved phenotypes for survival, compared to the wild type strain. Such high tolerance to oxidative and aerobic stress in *C. jejuni* ΔperR was greatly unanticipated, but hints that *C. jejuni* is a more robust organism than the fastidious organism it is often represented as being, and is capable of surviving and persisting in aerobic environments.

These results may partially explain why *C. jejuni* is prolific in aerobic environments despite being microaerophilic in nature. Although they raise the question as to why *C. jejuni* has such tight PerR- mediated gene regulation if deregulation provides advantageous for survival?

Overall, *fur* mutation in *C. jejuni* was detrimental and increased strain sensitivity to sources oxidative stress. From these results it is safe to conclude that *C. jejuni* PerR is primarily involved in oxidative stress protection, despite some regulatory overlap with Fur. Whereas Fur is primarily associated with factors relating to growth and motility.

4 : Chapter Four
Characterisation of PerR and Fur regulated genes in *C. jejuni*

4.1 Background

C. jejuni contains two Fur family homologues, the Ferric Uptake Regulator (Fur) and the Peroxide Regulator (PerR), as investigated in Chapter Three (Chan *et al.*, 1995; van Vliet *et al.*, 1999). Despite both having different roles in iron homeostasis (Fur) and oxidative stress defence (PerR), there are some functional overlaps between these two Fur family transcriptional regulators.

Several attempts have previously been made to characterise the breadth of genes under the regulatory control of the Fur family metalloregulators in *C. jejuni,* using microarray analysis of gene transcription (Holmes *et al.*, 2005; Palyada *et al.*, 2009). Unfortunately, in previous studies there has been high discordance between the genes identified as being Fur or PerR regulated in *C. jejuni*. The lack of consistency between comparative studies has hampered the effort to identify core regulons for Fur and PerR in *C. jejuni* (Holmes *et al.*, 2005; Palyada *et al.*, 2009).

In this chapter we attempt to further identify and refine a core list of genes under regulatory control by Fur and PerR by combining and comparing current data sets, and using bioinformatics modelling to highlight the most likely members of the Fur and PerR regulons. We have also employed the use of next generation RNA sequencing and DNA binding assays to illustrate direct regulation of *C. jejuni* genes by PerR.

We also aim to characterise the functional overlap between Fur and PerR, by investigating the genes which are co-regulated by both Fur and PerR in *C. jejuni*.

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4.2 Objectives

- To characterise the *C. jejuni* NCTC 11168 PerR and Fur regulons

- To identify the functional regulatory overlap of *C. jejuni* Fur and PerR

- To investigate whether *C. jejuni* Fur has a role in oxidative stress defences

4.3 Results

4.3.1 Analysis of *C. jejuni* soluble protein extract via SDS-PAGE

Analysis of *C. jejuni* Δ*fur,* Δ*perR* and Δ*fur*Δ*perR* mutants revealed different results during phenotypical tests, including deviation from basic cell growth to enhanced oxidative stress responses. To identify if differential expression of proteins has yielded these changes in phenotypes, soluble protein cell fractionations from each strains were analysed by SDS-PAGE.

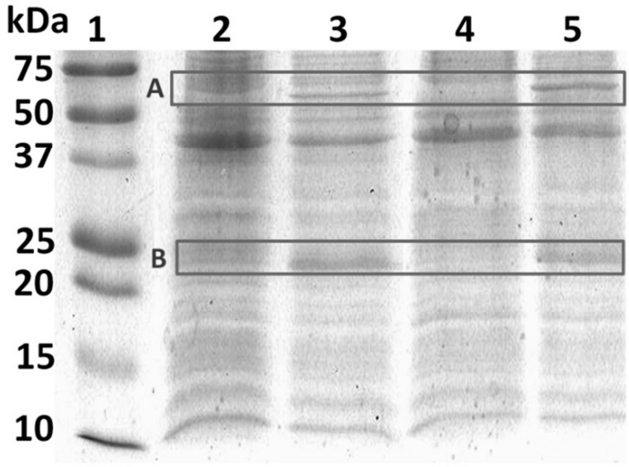


Figure 4.1: SDS-PAGE analysis of the soluble protein fraction of *C. jejuni* NCTC 11168 mutants. 1= Molecular weight ladder, 2 = *C. jejuni* NCTC 11168 wildtype, 3= Δ*perR*, 4=Δ*fur* and 5 = Δ*fur*Δ*perR*. Differentially expressed proteins are highlighted in boxes A and B.

SDS-PAGE analysis shows clear differences in the protein expression profiles of *C. jejuni* Δ*perR* and Δ*fur*Δ*perR* (Lanes 3 and 5) compared to the wildtype strain. The protein bands in boxes A and B have been previously identified as catalase or KatA (A) (55 kDa) and an alkyl hydroperoxide reductase or AhpC (B) (26 kDa) in *C. jejuni* (van Vliet *et al.*, 1999). Both of these genes are known members of the PerR regulon and have functions in the scavenging of internal and external hydrogen peroxide in bacteria (Kim *et al.*, 2011; Seaver & Imlay, 2001; van Vliet *et al.*, 1999).

4.3.2 Bioinformatic analysis of PerR-Fur transcriptomic studies reveals complex co-regulation of several subsets of genes (Cytoscape Web networks)

4.3.2.1 ∆perR

In order to confirm the deregulation of both KatA and AhpC in the ∆*perR* and ∆*fur*∆*perR* mutants and expand upon genes under the regulatory control of PerR, we analysed transcriptomic microarray analyses performed on *C. jejuni* NCTC 11168 ∆*perR* mutants under normal growth conditions. Two different microarray analyses had previously been performed to characterise the *C. jejuni* PerR transcriptome. One performed by Palyada *et al* (Palyada *et al.*, 2009) and the other is unpublished data from this laboratory, Reuter *et al*.

Bioinformatic analyses were used to compare the functional overlap of these two studies, in an attempt to identify core members of the PerR regulon and discriminate between strain variations or non-specific growth effects on transcription. Network maps generated using Cytoscape Web (Lopes *et al.*, 2010) were constructed using the supplementary (Palyada *et al.*, 2009) and raw data available from these studies (Reuter & van Vliet, 2013). Statistical cut-off points were determined by each study to distinguish between significantly up and down regulated genes.

For the Palyada study background subtracted fluorescent intensities for both wave lengths, Channel 1 (indodicarbocynanine [Cy5]) and Channel 2 (indocarbocyanine [Cy3]), were normalised using MIDAS software (from TIGR) and a locally weighted linear regression (Lowess) (Saeed *et al.*, 2003). The ratio of Channel 2 to Channel 1 was calculated, converted into $\log_2$ and statistically analysed using the Bayes method (Long *et al.*, 2001). Genes were considered to be differentially expressed if their *P* value was equal to or below 10^{-6} and their change in transcript abundance was greater than 2-fold (Palyada *et al.*, 2009).

For the Reuter study, genomic controls printed on the array were used to normalize the fluorescence signals for each data set (Cy5 and Cy3) and the Bayesian model was solved using the ArrayLeaRNA algorithm (TGAC).

ArrayLeaRNA was used to statistically analyse raw data and generate ratios comparing the two data sets (Cy5 and Cy3). Only ratios that were 2-fold or greater were considered significant.

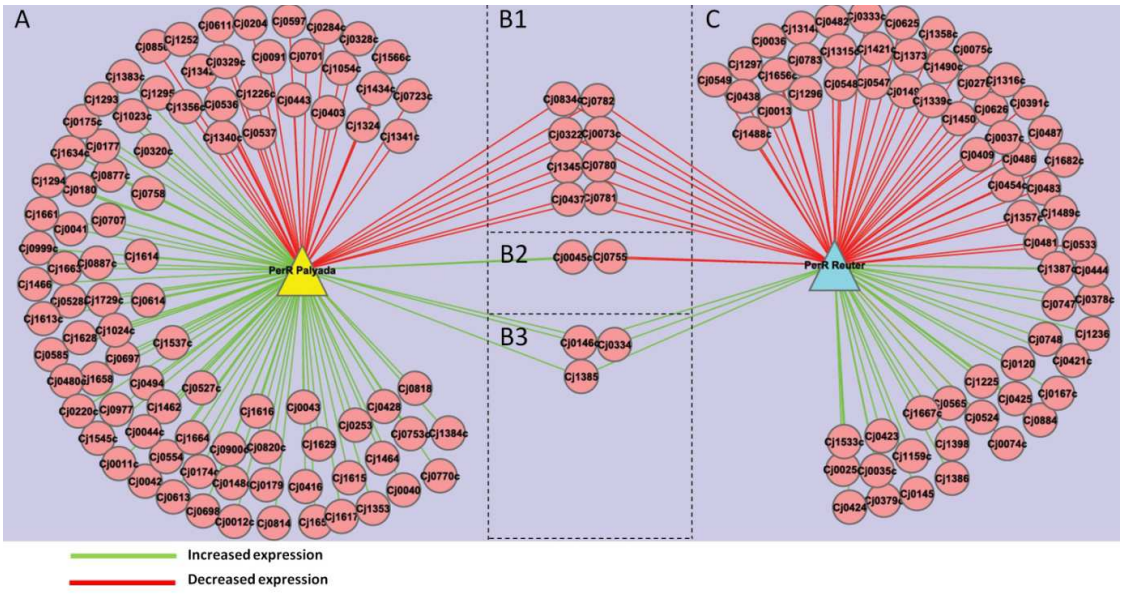


Figure 4.2: A network analysis comparing the genes identified as having altered transcription in two separate microarray analyses of *C. jejuni perR* mutants. Genes identified as being differentially regulated in a *C. jejuni* NCTC 11168 *perR* mutant are segregated based on which study they were identified in. (See Appendix for enlarged version)

Column A contains genes only identified by Palyada *et al* (Yellow), Column B contains genes identified by both studies and Column C contains genes only identified in the Reuter *et al* study (Blue). Boxes B1-B3 are expanded upon in Table 4.1.

In total, 171 genes were shown to be differentially regulated in a *C. jejuni* NCTC 11168 Δ*perR* mutant across both studies. The Reuter study identified 79 differentially regulated genes compared to 105 in the Palyada *et al* study. In total there was an overlap of only 13 genes that were highlighted as being differentially regulated by both studies and are these are listed in Table 4.1 (Figure 4.2:Column B). Of the 13 genes highlighted, 11 were in agreement (Boxes B1 and B3) as having either increased or decreased expression. The remaining 2 genes were identified as members of the PerR

regulon by both studies, however the studies disagree on the regulation of these genes in both studies (Figure 4.2, Box B2).

Many of the genes highlighted as having differential expression in one study but not the other are likely to not be PerR regulated. These variations in gene expression may be due to strain differences or be linked to growth.

Table 4.1: Genes that are differentially regulated in a *C. jejuni perR* mutant across both studies.

Box	Name	Gene	Regulation in Reuter study	Regulation in Palyada study	Function
B1	Cj0073c	Cj0073c	Down	Down	NAD-independent L-Lactate dehydrogenase subunit
B1	PerR	Cj0322	Down	Down	peroxide stress regulator
B1	SdhA	Cj0437	Down	Down	methylmenaquinol fumarate reductase flavoprotein subunit
B1	NapA	Cj0780	Down	Down	periplasmic nitrate reductase
B1	NapG	Cj0781	Down	Down	putative ferredoxin
B1	NapH	Cj0782	Down	Down	putative ferredoxin
B1	Cj0834c	Cj0834c	Down	Down	ankyrin repeat containing protein
B1	Cj1345c	Cj1345c	Down	Down	putative

					periplasmic protein
B2	Cj0045c	Cj0045c	Down	Up	possible iron binding protein
B2	CfrA	Cj0755	Down	Up	probable iron uptake protein
B3	TrxB	Cj0146c	Up	Up	thioredoxin reductase
B3	AhpC	Cj0334	Up	Up	alkyl hydroperoxide reductase
B3	KatA	Cj1385	Up	Up	Catalase

Table 4.1 illustrates that both microarray studies observe PerR repression of oxidative stress responsive proteins (AhpC, KatA and TrxB). However as previously reported, *C. jejuni* PerR seems to have a role in the regulation of genes that are not involved in oxidative stress (Palyada *et al.*, 2009). Both studies agree that several genes that have no links to oxidative stress defence have decreased expression in a *perR* mutant (Table 4.1), these include genes with roles in energy production and metabolism (*oorB, napAGH*) and growth (*cj0073c*) amongst others. However it is unclear whether the decreased expression of these genes in a PerR mutant is due to indirect growth effects caused by PerR mutation or whether expression of these genes is PerR activated.

Interestingly the Palyada study identifies a two iron related genes as being PerR repressed, these are *cfrA* and *cj0045c.* However the Reuter study shows these genes to be down regulated in the *perR* mutant. The use of different growth media, Brucella Broth (Reuter) versus Muller Hinton (Palyada), and therefore different levels of iron availability may account for these variations.

4.3.2.2 Δfur

The same transcriptomic studies have been performed by Palyada *et al* and Reuter *et al* (unpublished data) on *C. jejuni* Fur. Bioinformatic analysis again reveals relatively little consensus between the two data sets for genes differentially regulated in *C. jejuni* NCTC Δ*fur* mutants.

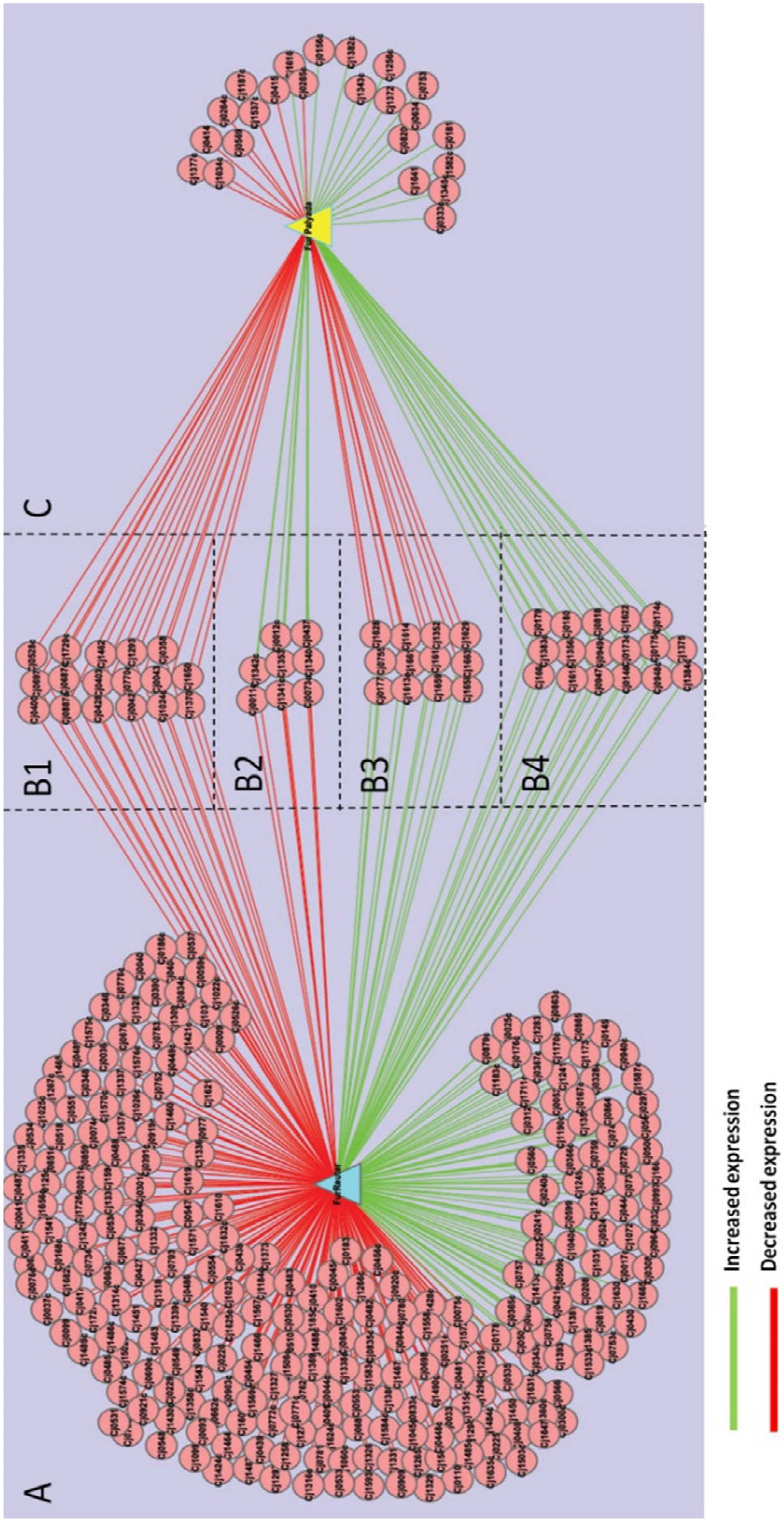

C
B1
B2
B3
B4
A
Increased expression
Decreased expression

Figure 4.3: A network analysis comparing the genes identified as having altered transcription in two separate microarray analyses of *C. jejuni fur* mutants. Genes identified as being differentially regulated in a *C. jejuni* NCTC 11168 *fur* mutant are segregated based on which study they were identified in. (See Appendix for enlarged version)

Column A contains genes only identified by Reuter *et al* (Blue), Column B contains genes identified by both studies and Column C contains genes only identified in the Palyada *et al* study (Yellow). Boxes B1-B4 are expanded upon in Table 4.2.

Table 4.2: Genes that are differentially regulated in a *C. jejuni fur* mutant across both studies.

	Name	Gene	Regulation in Reuter study	Regulation in Palyada study	Function
B1	FlgD	Cj0042	Down	Down	putative flagellar hook assembly protein
B1	FlgE2	Cj0043	Down	Down	flagellar hook protein FlgE
B1	Cj0358	Cj0358	Down	Down	putative cytochrome C551 peroxidase
B1	Fur	Cj0400	Down	Down	ferric uptake regulator
B1	Cj0403	Cj0403	Down	Down	hypothetical protein Cj0403
B1	Cj0428	Cj0428	Down	Down	Unknown
B1	FlgB	Cj0528c	Down	Down	putative flagellar basal-body rod protein
B1	FlgH	Cj0687c	Down	Down	putative flagellar L-ring protein precursor
B1	FlgG2	Cj0697	Down	Down	putative flagellar basal-body rod protein
B1	Cj0770c	Cj0770c	Down	Down	putative periplasmic protein

B1	FlaD	Cj0887c	Down	Down	putative flagellin
B1	Cj1024c	Cj1024c	Down	Down	signal-transduction regulatory protein
B1	Cj1293	Cj1293	Down	Down	possible sugar nucleotide epimerase/ dehydratase
B1	Cj1370	Cj1370	Down	Down	putative nucleotide phosphoribosyltransferase
B1	FlgI	Cj1462	Down	Down	putative flagellar P-string protein
B1	Cj1650	Cj1650	Down	Down	Unknown
B1	FlgE2	Cj1729c	Down	Down	flagellar hook subunit protein
B2	Cj0011c	Cj0011c	Down	Up	putative non-specific DNA binding protein
B2	Rrc	Cj0012c	Down	Up	non-haem iron protein
B2	Cj0073c	Cj0073c	Down	Up	hypothetical protein Cj0073c
B2	SdhA	Cj0437	Down	Up	succinate dehydrogenase flavoprotein subunit
B2	Cj1340c	Cj1340c	Down	Up	hypothetical protein Cj1340c (1318 family)
B2	Cj1341c	Cj1341c	Down	Up	hypothetical protein Cj1341c (1318 family)
B2	Cj1342c	Cj1342c	Down	Up	hypothetical protein Cj1342c (617 family)
B2	CeuC	Cj1353	Down	Up	enterochelin uptake permease
B3	CfrA	Cj0755	Up	Down	putative iron uptake protein
B3	CeuB	Cj1352	Up	Down	enterochelin uptake permease
B3	Cj1613c	Cj1613c	Up	Down	hypothetical protein

					Cj1613c
B3	ChuA	Cj1614	Up	Down	haemin uptake system outer membrane receptor
B3	ExbB2	Cj1628	Up	Down	putative exbB/tolQ family transport protein
B3	ExbD2	Cj1629	Up	Down	putative exbD/tolR family transport protein
B3	Cj1658	Cj1658	Up	Down	putative integral membrane protein
B3	Cj1661	Cj1661	Up	Down	possible ABC transport system permease protein
B4	Cj0173c	Cj0173c	Up	Up	putative iron-uptake ABC transport system ATP-binding protein
B4	Cj0174c	Cj0174c	Up	Up	putative iron-uptake ABC transport system permease protein
B4	CfbpA	Cj0175c	Up	Up	putative iron-uptake ABC transport system periplasmic iron-binding protein
B4	ExbB1	Cj0179	Up	Up	biopolymer transport protein
B4	ExbD1	Cj0180	Up	Up	biopolymer transport protein
B4	Cj0818	Cj0818	Up	Up	putative lipoprotein
B4	Cj0947c	Cj0947c	Up	Up	putative hydrolase
B4	Cj0948c	Cj0948c	Up	Up	putative transmembrane transport protein
B4	Cj0949c	Cj0949c	Up	Up	hypothetical protein Cj0949c
B4	Cj1356c	Cj1356c	Up	Up	putative integral

					membrane protein
B4	Cj1383c	Cj1383c	Up	Up	hypothetical protein Cj1383c
B4	Cj1384c	Cj1384c	Up	Up	hypothetical protein Cj1384c
B4	ChuB	Cj1615	Up	Up	putative haemin uptake system permease protein
B4	RibD	Cj1622	Up	Up	putative riboflavin-specific deaminase
B4	Cj1663	Cj1663	Up	Up	possible ABC transport system

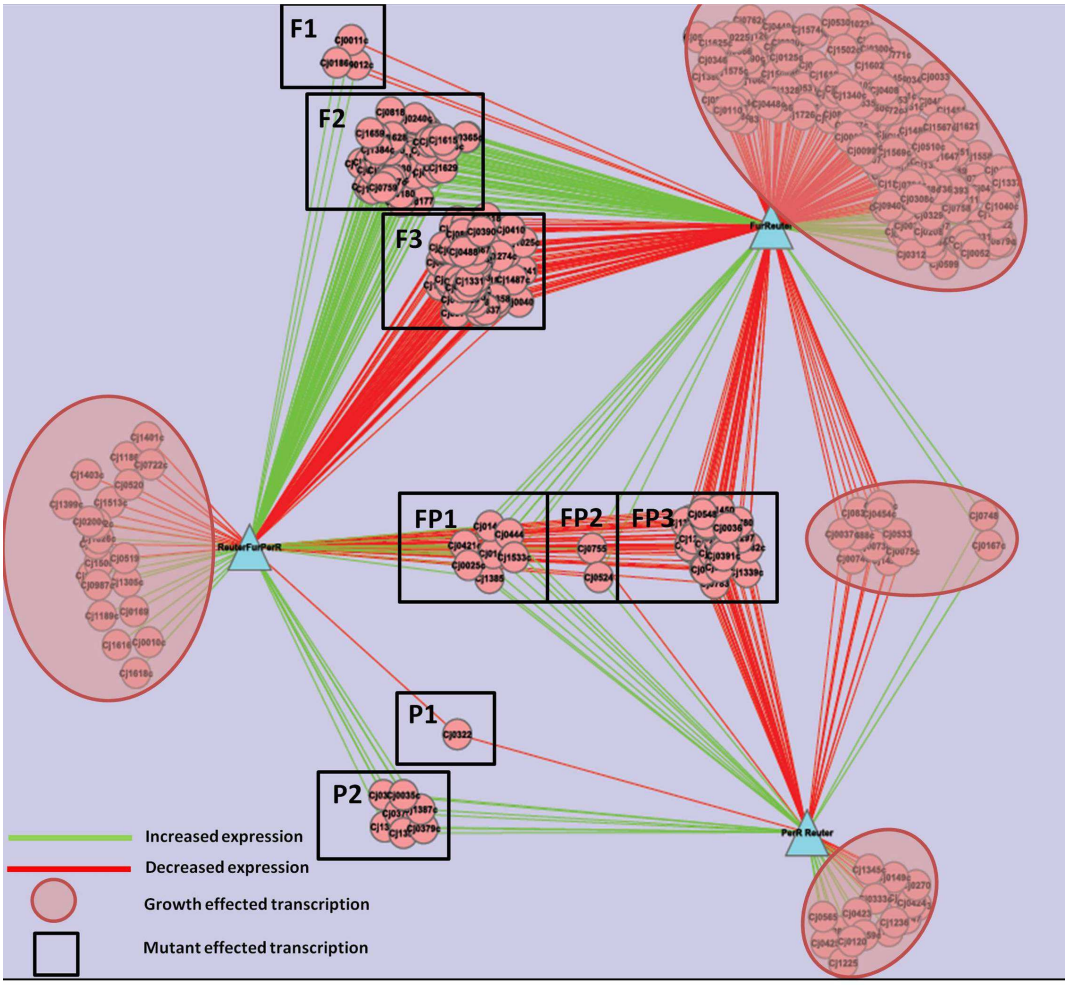
Both microarray studies on *C. jejuni* Δ*fur* identified a total of 347 genes with differential expression, of these genes less than 10% were in agreement in both studies (34 genes). Of the genes identified, 20 had differential regulation (up or down regulated) between the two studies.

The largest subset of genes that both studies agree to be Fur activated are involved in motility and flagella biosynthesis, including *flgB, flgD, flgI, flgE2, flgH, flgG2* and *flaD*. The reduced expression of motility and flagella genes likely accounts for the reduced motility of *C. jejuni* Δ*fur* seen in Chapter 3. Many Fur repressed genes identified in both studies are membrane associated (*cj1373-4c, cj0818, cj0948, cj1356c, exbD1* and *exbB2*) or are related to iron uptake (*chuB, exbD1, exbB1, cfbpA, 0173-4c*). Interestingly the iron related genes identified by the Palyada study as being PerR regulated (*cfrA* and *cj0045c*) are not shown to be Fur repressed by Palyada, whereas in contrast the Reuter study shows *p19, chuA, chuD* and *cfrA* to be Fur repressed. Again these differences could be due to iron availability, or may indicate cross talk between the PerR and Fur regulators.

4.3.2.3 Δfur Δperr

The Reuter study also performed microarrays to investigate the transcriptional expression of a double Δ*fur*Δ*perR* mutant compared to the wildtype NCTC 11168 strain (unfortunately no comparative data set is

available from the Palyada *et al* study). Genes assigned as members of the Fur or PerR regulon according to transcriptional data from the Reuter *et al* single mutants should also show transcriptional changes in the double Δ*fur*Δ*perR* strain. A Cytoscape network map comparing the significantly, differentially-regulated genes in the Reuter *et al* single mutants was compared to the comparative list for the Δ*fur*Δ*perR* . This comparison can be used to validate genes as Fur or PerR regulated by demonstrating differential regulation in two biologically distinct strains, equally it will highlight genes that have been falsely identified due to growth-related transcriptional differences (Figure 4.4).



Box	Regulation
F1	Fur activated, PerR repressed
F2	Fur repressed
F3	Fur activated
FP1	Putative Fur and PerR repressed*
FP2	Differentially regulated *
FP3	Putative Fur and PerR activated*
P1	PerR
P2	PerR repressed

* Indicates putative regulatory targets of Fur or/ PerR. Each genes merit as a regulatory target of Fur and/or PerR was assessed on an individual basis taking into consideration; predicted function, fold expression and a literature search.

Figure 4.4: A Cytoscape network map comparing differentially regulated genes in single Δ*fur* and Δ*perR* mutants to the differentially regulated genes in a Δ*fur*Δ*perR* double mutant from the Reuter *et al* study. (See Appendix for enlarged version)

Genes shown to be differentially regulated by *Reuter et al* in the single mutants that are not recognised as differentially regulated in the double

mutant can be eliminated as members of the Fur or PerR regulon (Figure 4.4, red circles). Gene deletion of the Fur or PerR regulators causes significant metabolic changes inside the *C. jejuni* cell, affecting growth and motility (See Chapter 3). Equally the increased expression of proteins due to de-regulation will be a taxing metabolic load for *C. jejuni* cells (See 2D gel electrophoresis, Figure 4.5). Significant changes in the metabolic activity of *C. jejuni* Δ*fur* and Δ*perR* mutants leads to the differential regulation of many *C. jejuni* housekeeping genes. Although expression of these genes is shown to be altered in a Δ*fur* or Δ*perR* mutant, they are not directly Fur or PerR regulated. As such, genes that fall into this 'Growth Effect' category are indicated in Figure 4.4 (red circles) by the lack of consensus expression in the double Δ*fur*Δ*perR* strain, a detailed list of these genes can be found in the appendix. Many of these genes represent the discord seen between the Reuter and Palyada study in Figure 4.2 and Figure 4.3.

Genes that are highlighted as being differentially regulated in both the single and double mutant (Figure 4.4, Boxes F2, F3 and P2) show validation as members of the Fur and PerR regulons. A total of 112 Fur-only regulated genes (60-Fur activated, 52 Fur-repressed), and 7 PerR-only, repressed genes were identified in the Reuter *et al* study.

In addition to the genes solely PerR or Fur regulated genes, are Boxes FP1-3 in Figure 4.4. These boxes contain 40 genes that were differentially regulated in all three mutant strains analysed and represent the functional overlap or crosstalk between the Fur and PerR regulators in *C. jejuni*. The genes in Boxes FP1-3 may also represent growth mediated gene expression that occurred across all three mutant strains. Therefore all 40 genes were assessed individually based on predicted function and a literature search to distinguish between those that are Fur-PerR regulated or have growth altered transcription, those found to be co-regulated by Δfur and ΔperR are detailed in Table 4.4.

Table 4.3: Cross talk between the Fur and PerR regulators. Genes that have altered transcription in *C. jejuni* NCTC *11168* Δ*fur,* Δ*perR* and

Δ*fur*Δ*perR* mutants compared to the wildtype strain in the *Reuter* et al study.

Gene	Expression	Regulated by	Predicted Function
cj0025c	Up	Fur PerR	Transmembrane symporter
trxB	Up	Fur PerR	Thioredoxin reductase
cj0421c	Up	Fur PerR	Integral membrane protein
cj0444	Up	Fur PerR	TonB dependent outer membrane receptor
katA	Up	Fur PerR	Catalase
cj0045c	Down	Fur PerR	Iron binding protein
cj0409	Down	Fur PerR	Fumarate reductase flavoprotein
sdhA	Down	Fur PerR	Succinate dehydrogenase
sdhB	Down	Fur PerR	Succinate dehrydrogenase iron sulfur protein
uxaA	Down	Fur PerR	Altronate hydrolase N & C terminus
FlaG	Down	Fur PerR	Flagellar protein
fliD	Down	Fur PerR	Flagellar hook associated protein
fliS	Down	Fur PerR	Flagellar protein
napA	Down	Fur PerR	Nitrate reductase
napG	Down	Fur PerR	Ferredoxin
napH	Down	Fur PerR	Ferredoxin
napB	Down	Fur PerR	Cytochrome C type protein
flaA	Down	Fur PerR	Flagellin A
cj1357c	Down	Fur PerR	Periplasmic cytochrome C
cj1373	Down	Fur PerR	Integral membrane protein
cfrA	Down in PerR, Up in Fur and FurPerR	Fur PerR	Iron uptake

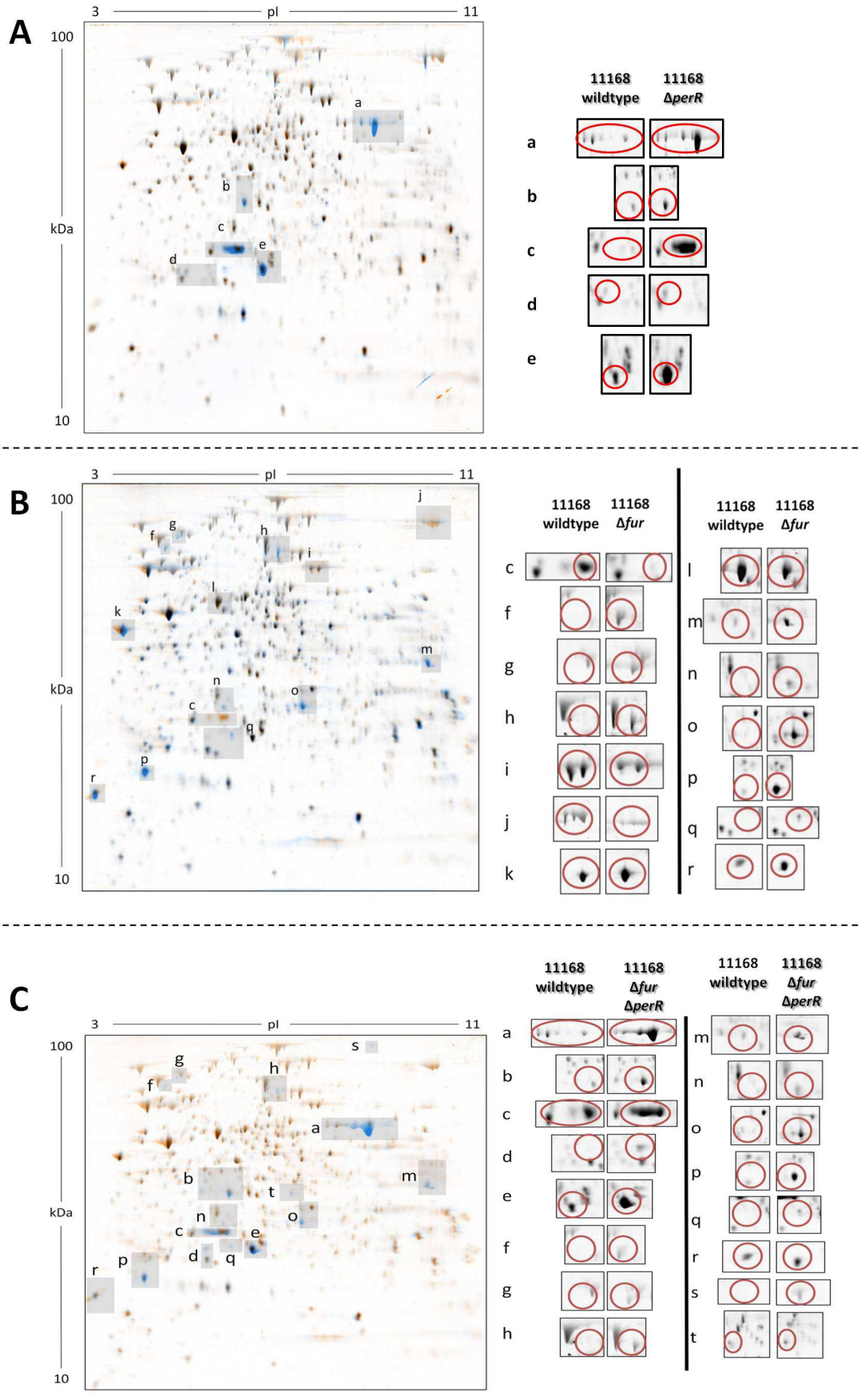
cj0524	Down FurPerR, Up in Fur and PerR	Fur PerR	Pseudogene

In total Boxes FP1-3 contained 22 genes that may be co regulated by Fur and PerR (5 co-repressed and 15 co-activated). The co-repressed genes included notable oxidative stress defence genes, *katA* (catalase) and *trxB* (thioredoxin reductase, whilst many of the co-activated genes had functions involved in motility and membrane transport.

4.3.3 Characterisation of the proteome of *C. jejuni* Δ*fur,* Δ*perR and* Δ*fur*Δ*perR* by two dimensional gel electrophoresis

Due to the labile nature of RNA and relatively poor overlap between the comparative microarray data sets we chose to investigate the proteome of *C. jejuni*.

To identify the proteomic differences between these strains, the proteomic profile of each strain was determined by two dimensional gel electrophoresis, separating proteins firstly by pH and then by molecular weight.


A
3
pI
11
100
kDa
10
a
b
c
d
e
11168 wildtype
11168 ΔperR
B
11168 wildtype
11168 Δfur
C
11168 wildtype
11168 Δfur ΔperR
f
g
h
i
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k
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q
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Figure 4.5: Overlaid 2D gel images depicting the proteome of *C. jejuni* NCTC 11168 wild-type (orange) compared to either a *C. jejuni* Δ*perR* (A) , Δ*fur* (B) or Δ*fur*Δ*perR* mutant (C) (blue). *C. jejuni* were grown in Brucella broth at 37°C under microaerobic conditions (5% O_2, 10% CO_2 with shaking) until late log phase. Differentially expressed proteins are highlighted and described in Table 4.4.

Table 4.4: The identification of proteins that are differentially expressed in *C. jejuni* Δ*perR*, Δ*fur* and Δ*fur*Δ*perR* mutants compared to the wildtype strain as determined by 2D gel electrophoresis.

Box	Name	Gene	Protein
a	KatA	Cj1385	Catalase
b	TrxB	Cj0146c	Thioredoxin reductase
c	Rrc	Cj0012c	Rubrerythin/Rubredoxin like protein of *Campylobacter jejuni*
d	MogA	Cj0725c	Molybdenum cofactor biosynthesis protein
e	AhpC	Cj0334	Alkyl hydroperoxide reductase
f	CfrA	Cj0755	putative iron uptake protein
g	Unknown		
h	ChuA	Cj1614	haemin uptake system outer membrane receptor
i	SdhA	Cj0437	Methylmenaquinol fumarate reductase
j	NapA	Cj0780	Periplasmic nitrate reductase
k	PorA	Cj1259	Major Outer Membrane Protein
l	AspA	Cj0087	Aspartate ammonia-lyase
m	CfbpA	Cj0175c	Iron-uptake ABC transporter, periplasmic iron-binding protein
n	Cj1663	Cj1663	Putative ABC transport system ATP-binding protein

o	Cj1419/20	Cj1419/20	Possible methyltransferase/ hypervariable, Unknown function
p	P19	Cj1659	Periplasmic protein
q	-	-	Chloramphenicol Resistance Cassette
r	FldA	Cj1382c	Flavodoxin
s	KatA	Cj1385	Catalase
t	Cj1429	Cj1429	Hypervariable tract, unknown function.

4.3.3.1 Δ*perR*

The expression levels of several proteins were shown to be significantly de-repressed in the *C. jejuni* mutant strains.

AhpC (*cj0334*), KatA (*cj1385*), TrxB (*cj0146c*),Rrc (*cj0012c*) and MogA (*cj0725c* were are all up regulated in a Δ*perR* mutant and have roles within oxidative and acid stress defence, confirming PerR's role as a repressor of oxidative stress defences in *C. jejuni* (Birk *et al.*, 2012; Palyada *et al.*, 2009).

4.3.3.2 Δfur

There was a notably higher number of proteins differentially expressed in *C. jejuni* Δ*fur* (14 proteins, see Figure 4.5B) compared to *C. jejuni* Δ*perR* (5 proteins). The majority of proteins with differential regulation in a Δ*fur* mutant have functions within iron uptake, transport and homeostasis in *C. jejuni* (Table 4.4), which is in accordance with Fur's role as a regulator of iron uptake (van Vliet *et al.*, 1999). However other proteins with increased expression in *C. jejuni* Δ*fur* have no role in iron homeostasis. Several proteins (PorA, AspA, SdhA and NapA) identified as having lower expression in Δ*fur* compared the wildtype are markers of cell growth, and as such may reflect a slower growth rate of the *C. jejuni* Δ*fur* mutant compared to the wildtype strain. Reduced growth in the *C. jejuni* Δ*fur* mutant was indicated in Chapter 3, and may correspond to some of the disagreement in the Fur transcriptomics.

The remaining proteins that function in neither iron homeostasis or have growth related functions are Rrc and Cj1419/20. Rrc is an iron

containing, oxidative stress responsive protein that is repressed in the absence of the ferric uptake regulator, potentially indicating a role for Rrc in oxidative stress defences during high iron conditions (Pinto *et al.*, 2011; Yamasaki *et al.*, 2004). Finally Cj1419/20, is a hyper variable protein and its altered expression is most likely not related to the absence of Fur (Parkhill *et al.*, 2000).

Δfur ΔperR

The proteomic profile of *C. jejuni* Δ*fur*Δ*perR* largely reflects the combination of the individual Δ*fur* and Δ*perR* regulons, with several notable changes. The expression of KatA is greater in the Δ*fur*Δ*perR* double mutant than the single Δ*perR* mutant, including an additional protein spot (Figure 4.1S) indicating the presence of a degraded form of the protein. There is no increased expression of KatA in a Δ*fur* mutant so the increased levels of protein seen in the double mutant indicate the co-regulation of another protein with transcriptional activity on Catalase by Fur and PerR.

In the Δ*fur* single mutant there is reduced expression of Rrc in *C. jejuni* compared to the wildtype, however in the Δ*fur*Δ*perR* double mutant expression of Rrc is on par with that of the Δ*perR* single mutant.

4.3.4 Electrophoretic mobility shift assays confirm direct regulation of target genes by *C. jejuni* PerR

To investigate whether proteomic changes seen in Figure 4.5 were due to direct regulation by PerR, electrophoretic mobility shift assays were performed. Binding of recombinant PerR protein to approximately 200 bp DNA fragments upstream of major upregulated genes (*katA, rrc, trxB, ahpC*) was observed using chemiluminescence, indicating the formation of a protein-DNA complex and therefore direct regulation. DNA fragments upstream of *perR* and *dnaE* were also facilitated as positive and negative controls, respectively.

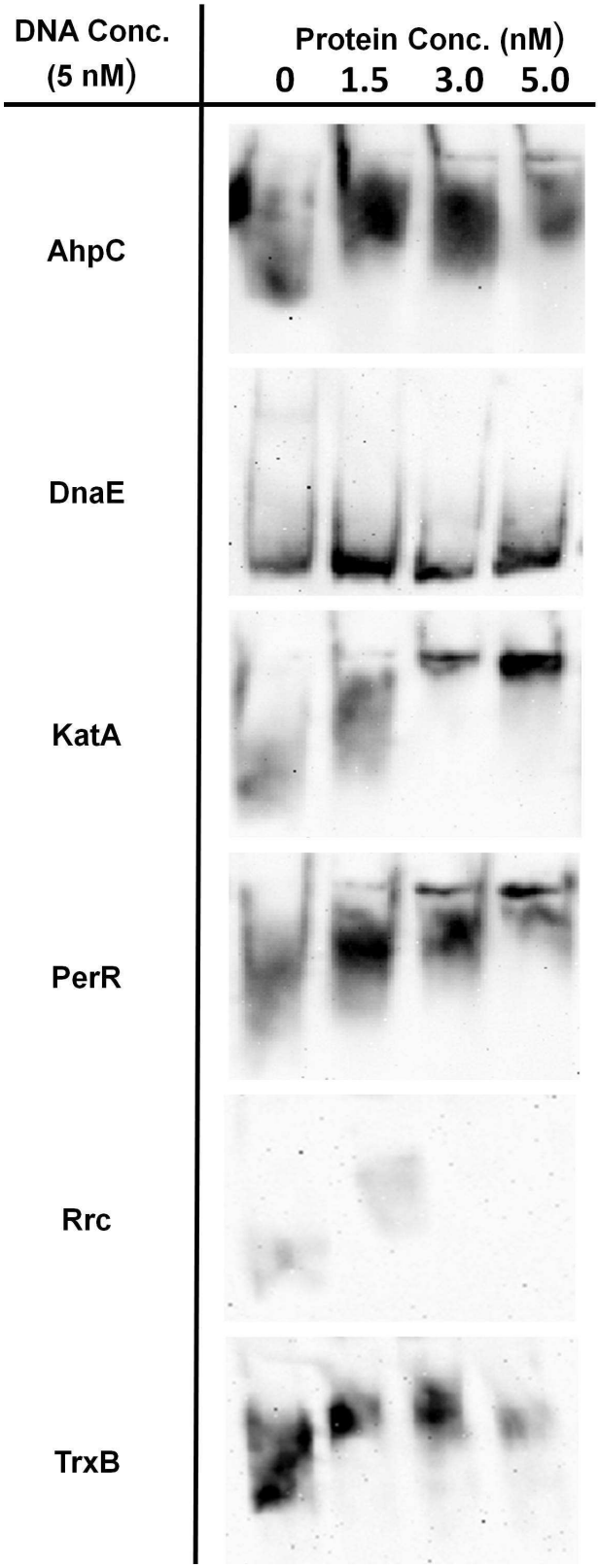


Figure 4.6: Electrophoretic mobility gel shift assays (EMSA's) using purified recombinant PerR protein demonstrate PerR auto-regulation

and direct binding of PerR to promoter regions of genes shown to be differentially expressed in a Δ*perR* mutant by 2D gel electrophoresis.

No protein-DNA complexes were observed for target DNA upstream of *dnaE*, this was included in the experiment as a negative control to rule out unspecific binding of *C. jejuni* PerR protein to *C. jejuni* DNA. PerR auto-regulation has been previously reported, and is confirmed in this study (Kim *et al.*, 2011). Protein-DNA complexes were successfully observed for promoter regions upstream of *ahpC, katA, trxB* and *rrc*, although unfortunately the labelling of *rrc* promoter DNA was not as efficient.

4.3.5 *C. jejuni* PerR controls expression of peroxidases and associated genes at the transcriptional level (RNA-seq)

Differential and normal RNA-seq was performed on a *C. jejuni* NCTC 11168 Δ*fur*Δ*perR* double mutant (Chaudhuri *et al.*, 2011; Porcelli *et al.*, 2013). This allowed the identification of genes under the transcriptional control of Fur or PerR in *C. jejuni* (for comparison to microarray studies), and additionally allowed for the identification of the transcriptional start sites (TSS) for each gene.

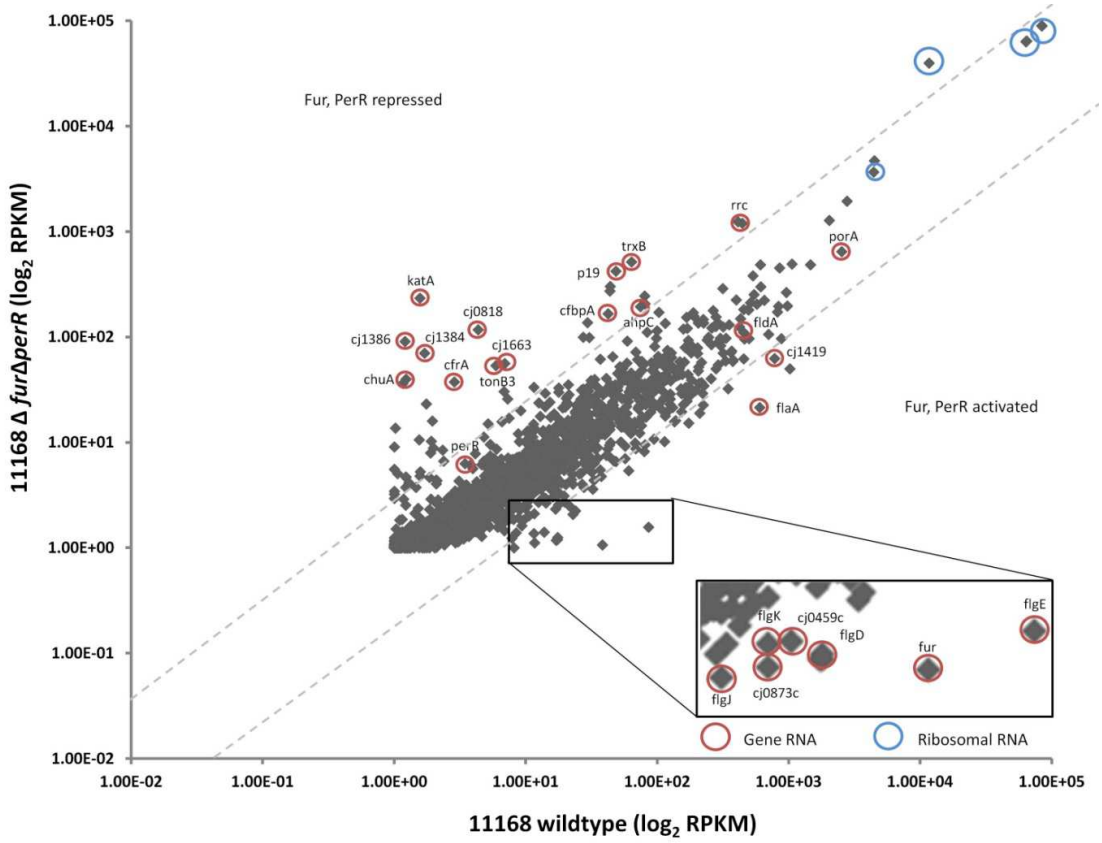


Figure 4.7: Illumina-based RNA sequencing of *C. jejuni* NCTC 11168 wildtype and an isogenic *fur perR* double mutant identifies differentially regulated genes, as shown by RPKM values from Illumina RNA sequencing.

The comparative RPKM values (reads per kilo base of transcript per million mapped reads) for both the *C. jejuni* 11168 wildtype and the Δ*fur*Δ*perR* mutant are plotted in Figure 4.7. Comparison of RPKM values allows the identification of differentially transcribed regions between the two strains, based on the amount of RNA present. We used RNA sequencing data to confirm the transcriptional regulation of genes highlighted during 2D gel electrophoresis (Table 4.4). Many of the genes identified as being Fur-PerR repressed during proteomic analysis of *C. jejuni* NCTC 11168 Δ*fur*Δ*perR* also show increased levels of RNA in an 11168 Δ*fur*Δ*perR* mutant compared to the wildtype strain. Some of these genes include *katA, trxB, rrc, ahpC*, *cfrA, cfbpA* and *p*19. Interestingly flavodoxin (*fldA*) and the molybdenum cofactor biosynthesis protein (*mogA*) do not show higher RNA

reads in the Δ*fur*Δ*perR* mutant compared to the wildtype, increased protein levels may reflect post translational effects or reduced RNA degradation.

Many of the reads that are reduced in the Δ*fur*Δ*perR* mutant compared to the wildtype correspond to genes involved in flagella biosynthesis and motility, such as *flgDEJK* and *flaA*. Although reduced expression of flagella proteins is not reported in Figure 4.5, the reduced motility in Δ*fur* and Δ*fur*Δ*perR* strains compared to the wildtype strain is reported previously in this thesis in Chapter 3.

Differential RNA sequencing also allows the identification of the transcriptional start sites of target genes, typically those that were over expressed in a Δ*fur*Δ*perR* mutant. The identification of transcriptional start sites allows us to determine if gene expression occurs from a single or multiple promoter locations. We analysed the transcriptional start sites of only those genes confirmed as Fur-PerR regulated in both 2D gel electrophoresis and RNA-sequencing (*katA, ahpC, rrc, trxB, cfrA, p19, cfbpA*).

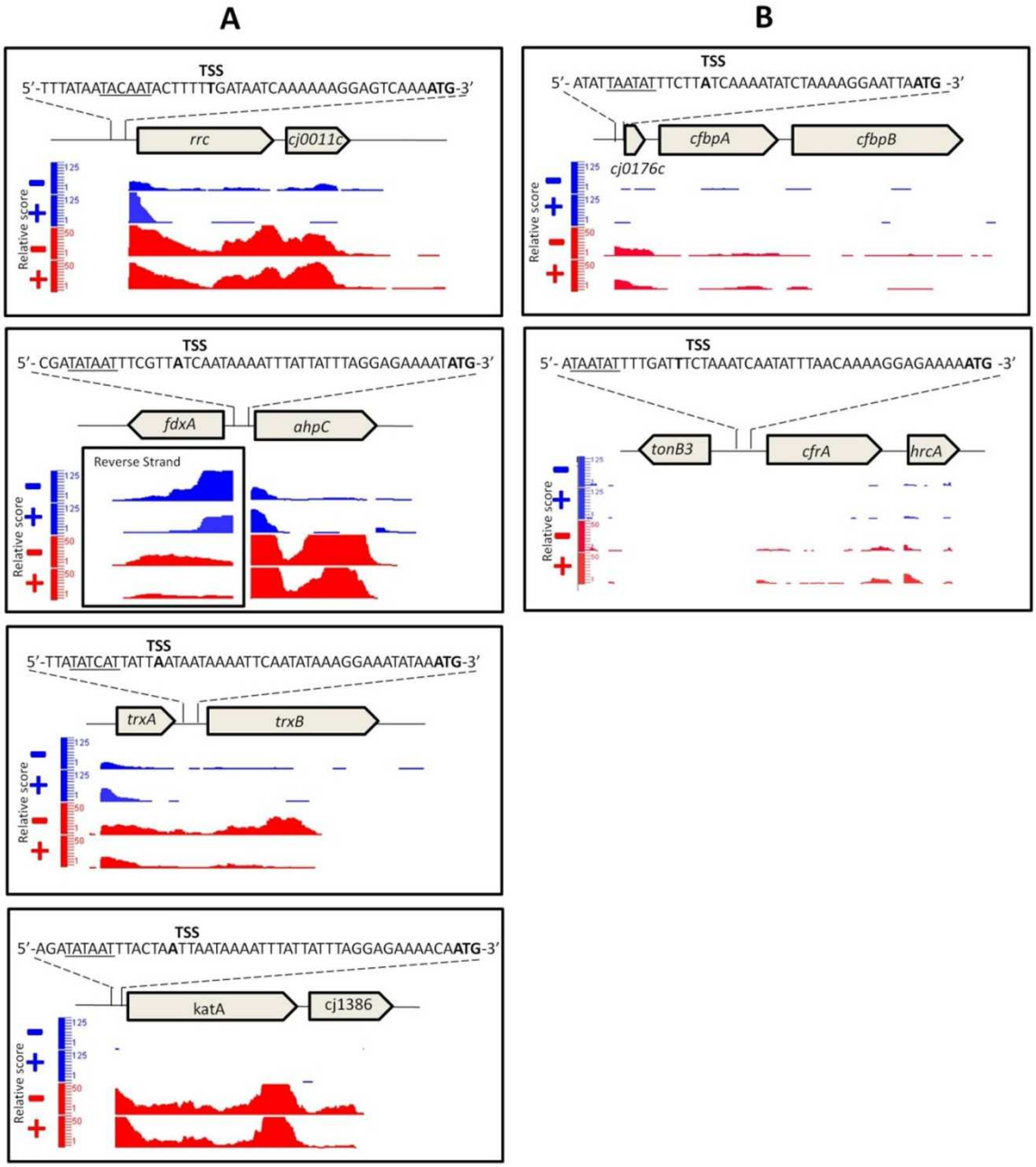


Figure 4.8: Images of normalised histograms showing the transcriptional start sites of A, *rrc, ahpC, trxB* and *katA* and B, *cfbpA, p19* (bacterioferritin-like) and *cfrA*. Histograms in blue indicate wildtype data and red indicates data from the Δ*fur*Δ*perR* mutant. +/-: Refers to 454 differential RNA sequencing performed in the presence of TEX. The -10sequence is underlined (gnTAnaAT).

Single transcriptional start sites were located for the major PerR regulated genes as determined by 2D gel electrophoresis (Figure 4.5). The normalised histograms in Figure 4.8, column A clearly show an increased number of RNA reads for *katA, rrc, trxB* and *ahpC*. Also shown in the genomic region for *ahpC*, is the divergent gene *fdxA*, RNA sequencing clearly shows differential expression of these two genes in a *C. jejuni* Δ*fur*Δ*perR* mutant, demonstrating that these two genes are not co-expressed.

Column B shows Fur regulated genes as determined by 2D gel electrophoresis, although not as distinct as PerR regulated genes, increased numbers of reads can be seen for *cfbpA, tonB* and *p19*. Unfortunately due to the co-expression of upstream and downstream genes, it was not possible to distinguish a transcriptional start site for *p19* in a Δ*fur*Δ*perR* mutant.

4.4 Discussion

4.4.1 Defining the PerR and Fur Regulons in *C. jejuni*

Defining the regulon of a regulatory protein is a difficult task, as is indicated by the discord seen in comparative microarray studies (Figure 4.2 and Figure 4.3). There is little agreement or consensus on which genes are regulated by PerR and Fur in *C. jejuni* based on transcriptomic data alone.

By comparing these studies to proteomic changes (Figure 4.5) and next generation sequencing (Figure 4.7), it is possible to narrow down and identify core members of the Fur and PerR regulons. PerR regulon candidates were then confirmed by direct binding to target DNA upstream of the gene (Figure 4.6). Despite the identification of 13 PerR regulated genes and 34 *Fur* regulated genes by both microarray studies, 2D gel electrophoresis and RNA sequencing were only able to confirm a small fraction of these as being Fur or PerR regulated.

4.4.1.1 The PerR Regulon

Analysis of the comparative microarray studies from Palyada *et al* and Reuter *et al* showed little consensus (Table 4.1). Of the 13 genes identified

as having significantly altered transcription in the microarray studies, only *katA, trxB* and *ahpC* three were confirmed as having differential protein expression using 2D gel electrophoresis. Protein analysis also identified another highly expressed protein that was not identified in the transcriptomic analyses, Rrc (*cj0012c*).

RNA sequencing confirmed increased numbers of reads for *katA, trxB, ahpC* and *rrc* in a Δ*fur*Δ*perR* mutant, and identified the transcriptional start sites (TSS's) for these target genes. Using knowledge of the TSS's, electrophoretic mobility shift assays (EMSA) were then able to confirm binding to promoter regions and therefore direct regulation of *katA, ahpC and trxB* by *C. jejuni* PerR, as well as confirming PerR auto regulation as previously described by Kim et al, 2011 (Kim *et al.*, 2011). Unfortunately, EMSA analysis of PerR's regulation of *rrc* was inconclusive. RNA sequencing also did not identify any alternative or additional TSS for these genes.

4.4.1.2 The Fur Regulon

Similar to PerR, studies aiming to characterise the Fur regulon in *C. jejuni* show little overlap. Several iron related genes were upregulated in the Reuter Δ*fur* microarray study and down regulated in the Palyada data set, such as *chuAB, cfrA*, *exbB2* and *exbD2*. Proteomic data from 2D gel electrophoresis is in agreement with the Reuter study, and shows an increase of ChuA and CfrA expression in a Fur mutant.

A recent study into the *C. jejuni* Fur regulon highlighted the complexity of Fur regulation, showing differential gene regulation and DNA binding in the presence or absence of an iron cofactor (Butcher *et al.*, 2012). It is likely that the regulon of Fur alters with metal ion availability, which may account for the lack of overlap between the comparative transcriptional studies into the Fur regulon.

Comparison of transcriptomic and proteomic approaches was able to confirm *cfrA, chuA* and *sdhA* as being Fur regulated. This study also identified several genes where expression was mediated by both Fur and PerR demonstrating that there is some cross talk between these two

regulators. Typically co-regulated genes had roles in motility, oxidative stress responses and membrane transport.

4.4.2 TrxB is a potential recycling partner for AhpC in *C. jejuni*

C. jejuni AhpC (an alkyl hydroperoxide reductase) has a protective role, and aids the survival of *C. jejuni* in aerobic conditions (Baillon *et al.*, 1999). Typically in other bacteria such as *S. typhimurium.* AhpC is a small protein sub-unit that together with a larger subunit (AhpF) forms an alkyl hydroperoxide reductase capable of scavenging and detoxifying internal and external hydrogen peroxide (Jacobson *et al.*, 1989; Seaver & Imlay, 2001). AhpC contains the catalytic site, mediating the NADPH linked reduction of hydroperoxides to their corresponding alcohols, whilst AhpF acts as an electron donor that reduces and recycles oxidised AhpC (Jacobson *et al.*, 1989; Poole *et al.*, 2000). AhpC readily detoxifies hydroperoxide intermediates and can repair damage caused to membranes by peroxidation. However, unlike *S. typhi*, no homolog of AhpF has been found in *C. jejuni*, and in the absence of AhpF the AhpC subunit is unable to recycle itself (Baillon et al., 1999; Parkhill et al., 2000). It is therefore likely that *C. jejuni* may use another reducing agent for the recycling of oxidised AhpC (Baillon *et al.*, 1999).

Helicobacter pylori, a close relative of *C. jejuni*, also lacks an AhpF homologue and instead facilitates a thioredoxin as an electron acceptor to AhpC (Tomb *et al.*, 1997). It has been postulated that *C. jejuni* may facilitate ferredoxin or thioredoxin to fill this role (Jacobson *et al.*, 1989). Two dimensional gel electrophoresis in Figure 4.5 revealed the over-expression of thioredoxin (TrxB) alongside AhpC at the proteomic level in a *C. jejuni* NCTC 11168 Δ*perR* mutant. RNA sequencing data also confirms the increased transcriptomic expression of *trxB* alongside *ahpC* in a Δ*Fur*Δ*PerR* mutant, whereas ferredoxin (*fdxA*) is regulated oppositely to *ahpC* (Figure 4.8). It is therefore likely that *C. jejuni* uses thioredoxin reductase (TrxB) as a mode of AhpC reduction, similar to that of *H. pylori*, although experimental validation of this is required.

4.4.3 A role for Fur in oxidative stress defence in *C. jejuni*?

Fur has previously been identified as a regulator of a number of oxidative stress defensive proteins in *C. jejuni* Including KatA, Rrc, TrxB, AhpC, Cj0559 (probable oxidoreductase) and KapA (Butcher *et al.*, 2010; Holmes *et al.*, 2005; Palyada *et al.*, 2004). This is in addition to iron containing proteins, such as FdxA and Cft, which may also have roles in oxidative stress.

This investigation failed to confirm the solo role of Fur as a regulator of oxidative stress defences in *C. jejuni*, however did demonstrate Fur-PerR co-regulation of AhpC and TrxB.

KatA (Catalase) is an intriguing regulatory target of Fur. Previous microarray studies on *C. jejuni* Fur have clearly highlighted a role for Fur and iron availability in controlling the transcription of KatA in *C. jejuni*. Yet a single Δ*fur* mutant shows no increased expression of KatA compared to the wildtype, whereas a double Δ*fur*Δ*perR* mutant shows extra KatA expression compared to a single Δ*perR* mutant. This suggests that the regulation of KatA in *C. jejuni* is controlled solely by PerR not Fur, yet iron availability may also have an as yet unexplained role in Catalase expression. To date Fur regulation of PerR has not been demonstrated in *C. jejuni*.

Other regulators have also been shown to effect KatA expression, including CosR (*Campylobacter* oxidative stress regulator), CprSR and Cj1556 (Holmes *et al.*, 2005; Hwang *et al.*, 2011; Palyada *et al.*, 2009; Svensson *et al.*, 2009).

We show no role for Fur in the repression of Rrc, conversely proteomic analysis shows decreased Rrc expression in a Fur mutant. Previous studies confirm decreased *rrc* expression in a Δ*fur* mutant, and go on to show the addition of iron to a Fur mutant activates *rrc* expression (Palyada *et al.*, 2004). The role of Rrc in *C. jejuni* biology has not been fully elucidated, yet its presence during iron abundance and absence during iron limitation may indicate a role for iron-linked, oxidative stress defence, although further investigation is required.

Overall, we show no solo role for Fur in the repression of oxidative stress responsive genes in *C. jejuni*. The only oxidative stress defensive genes regulated by Fur are those co-regulated by PerR. It is likely that the cross talk between Fur and PerR provides a survival advantage, and that gene expression is linked to iron concentrations. Linking oxidative stress defence with iron homeostasis allows *C. jejuni* to increase oxidative stress resistance in high iron conditions to avoid cell damage by Fenton chemistry.

5 : Chapter Five
Cloning, Characterisation and Comparison of the *C. jejuni* Peroxide Regulator, PerR

5.1 Background

Since its first description in *C. jejuni* in 1999 (van Vliet *et al.*, 1999), there have been surprisingly few studies on the PerR regulatory system in the genus *Campylobacter*. In recent years the stimulon and regulon of *C. jejuni* PerR have been characterised, however relatively little is known about the molecular and structural mechanics governing the regulatory activity of PerR (Palyada *et al.*, 2009). The only information on the structure and biochemical activity of *C. jejuni* PerR has been extrapolated from other bacteria, typically *B. subtilis* which is Gram-positive and hence not closely related (See 1.8.2.2) (Bsat *et al.*, 1998; Traore *et al.*, 2006).

The chemistry of PerR is of particular interest as it is known to control part of the oxidative stress defensive pathways in *C. jejuni*. PerR controls transcription of a set of peroxidases (e.g. catalase) aiding the pathogen's survival on food, surfaces and within a host (as discussed in Chapter Four) (Holmes *et al.*, 2005; Palyada *et al.*, 2009). It is therefore crucial to understand the mechanisms behind how the bacterium controls oxidative stress defence systems, before efforts to combat bacterial survival can be truly effective.

In *B. subtilis* PerR is a repressor of gene activity, and a PerR homodimer directly binds DNA upstream of PerR regulated genes and prevents access of the transcription machinery to the target gene (as described in Chapter One 1.8.2.1) . Importantly, one residue of *B. subtilis* PerR (Histidine 37) has been highlighted as a key point of oxidation required for the sensing of oxidative stress by PerR (Lee & Helmann, 2006a).

Purification of *C. jejuni* PerR has been achieved previously, however only in the presence of affinity tags, which may alter the proteins biochemistry and metal binding capabilities (Kim *et al.*, 2011; Palyada *et al.*, 2009). Purified *C. jejuni* PerR protein has only been used for transcriptomic analyses and *C. jejuni* PerR has not thus far been biochemically characterised.

Biochemical characterisation of *C. jejuni* is essential for the advancement of understanding interactions between protein regulators and DNA. PerR belongs to the Fur family metalloregulators and insights into the

C. jejuni PerR mechanism may also provide advantageous when researching other homologues. Therefore in this chapter we aim to investigate several of the key biochemical features governing PerR regulation, such as researching the structure of *C. jejuni* PerR and the role of metal ions in PerR binding.

5.2 Objectives

- To recombinantly express and purify the *Campylobacter jejuni* peroxide regulator (PerR) in an *E. coli* host.

- To biochemically characterise recombinant PerR protein in order to better understand PerR gene regulation.

- To determine the metal binding properties of *C. jejuni* PerR.

5.3 Results

5.3.1 Identification and Comparison of PerR to known orthologues

The *Campylobacter jejuni* gene encoding the PerR orthologue was identified within the published genome sequence of *C. jejuni* NCTC strain 11168 based on sequence similarity to previously characterised *perR* proteins, specifically *Bacillus subtilis* (Figure 5.1A) (Bsat *et al.*, 1998; Parkhill *et al.*, 2000; van Vliet *et al.*, 1999).

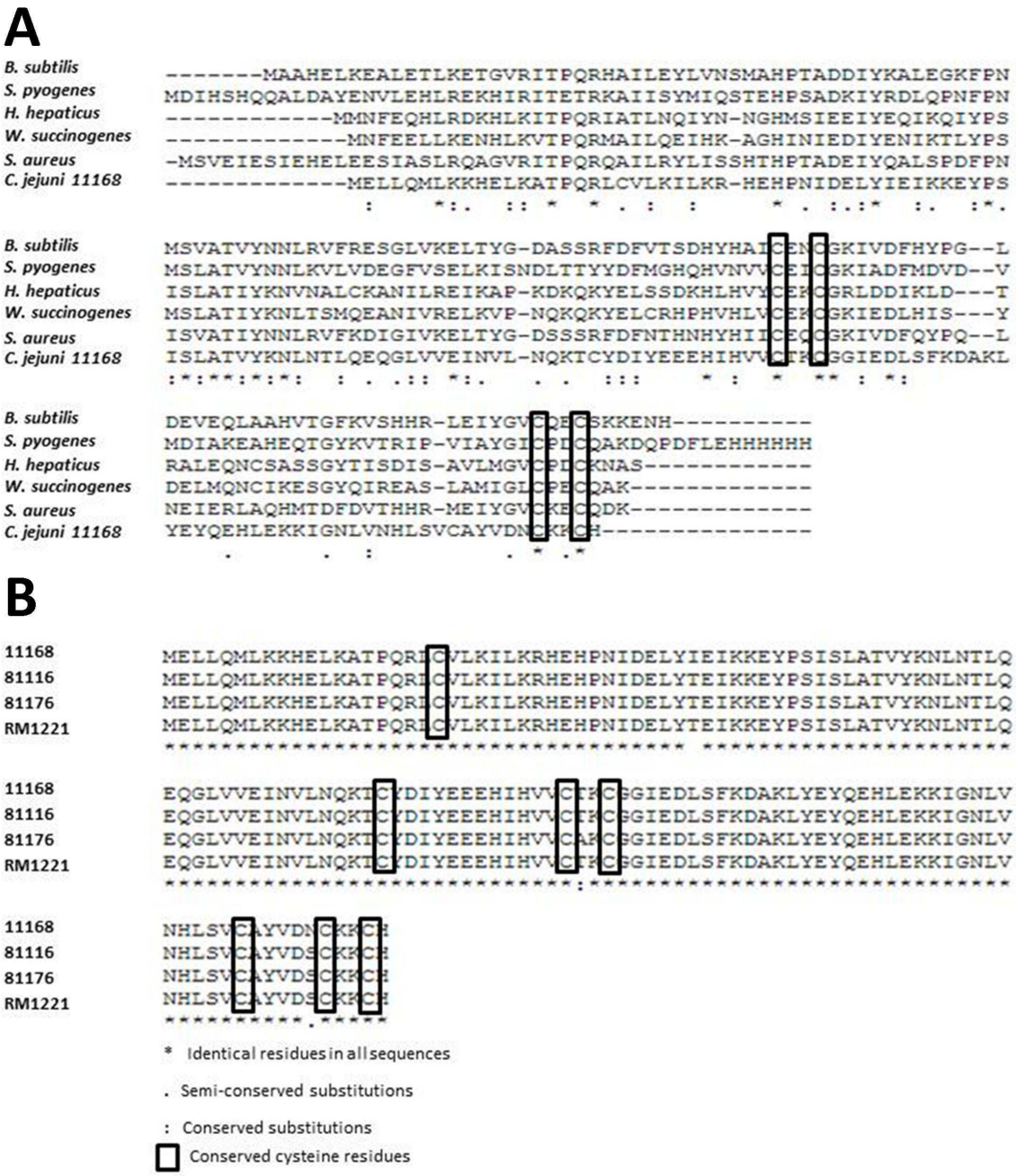


Figure 5.1: Alignment of PerR amino acid sequences using ClustalW (1.83) reveals poor percentage identity across a wide range of known PerR orthologues [A], but strong homology within various *C. jejuni* subspecies [B].

ClustalW sequence alignment reveals the poor conservation of the PerR amino acid sequence across a wide range of bacterial species including *Bacillus subtilis*, *Streptococcus pyogenes*, *Helicobacter hepaticus*, *Wolinella succinogenes*, *C. jejuni* and *Staphylococcus aureus,* these species represent a broad range of bacteria containing oxidative stress responsive, PerR homologues. (Baar *et al.*, 2003; Belzer *et al.*, 2011; Bsat *et al.*, 1998; Horsburgh *et al.*, 2001; King *et al.*, 2000; Parkhill *et al.*, 2000). There is as low as 28.7% identity between the *C. jejuni* and *B. subtilis* PerR amino acid sequences, the latter being the best characterised PerR protein. In total there are 19 conserved amino acid residues across all of the bacterial species in Figure 5.1A. Amongst the conserved residues in Figure 5.1A are the four cysteine residues (Cys-96, 99,136 and 139) that comprise the *B. subtilis* PerR zinc metal binding site, which co-ordinates protein dimerization. Interestingly the residues that co-ordinate the regulatory metal binding site in *B. subtilis* PerR, His-37, His-91, His-93, Asp-85 and Asp-104, are not absolutely conserved. Of these residues, His-37, His- 91 and Asp-104 are conserved, His-93 is conserved in all but *S. pyogenes,* where aspartic acid is replaced by asparagine*.* Asp-85 is replaced by glutamic acid in close relatives of *C. jejuni*, *W. succinogenes* and *H. hepaticus*, yet remains an aspartic acid in *C. jejuni* itself (Parkhill *et al.*, 2000; Traore *et al.*, 2006; Traore *et al.*, 2009).

In contrast, there is very high PerR sequence similarity within the commonly used laboratory strains of *C. jejuni*, shown in Figure 5.1B. All residues identified as having a role in metal binding in *B. subtilis* are conserved amongst the *C. jejuni* strains.

5.3.2 Cloning and Over-Expression of *C. jejuni* PerR in *Escherichia coli*

5.3.2.1 *C. jejuni* PerR-His Over-Expression

The use of selective tags greatly facilitates the ease of purification of recombinantly expressed proteins; therefore we attempted to purify *C. jejuni* PerR with a removable 6-His tag, comprised of six histidine residues added to the C-terminal end of the protein. *C. jejuni perR* was amplified from *C. jejuni* NCTC 11168 genomic DNA and ligated into the over-expression vector pET28a, see Figure 5.2A. The vector pET28a was selected as inserted gene expression is under the tight control of an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 promoter, allowing selective expression of the target gene. Additionally, in pET28a, placed in between the 6-his tag site and the target gene is a thrombin cleavage site, allowing the removal of the 6-his tag after the protein has been expressed.

All vectors were confirmed by DNA sequencing using primers T7 Fwd and Rev (See Table 2.5) to ensure no amino acid substitutions had occurred.

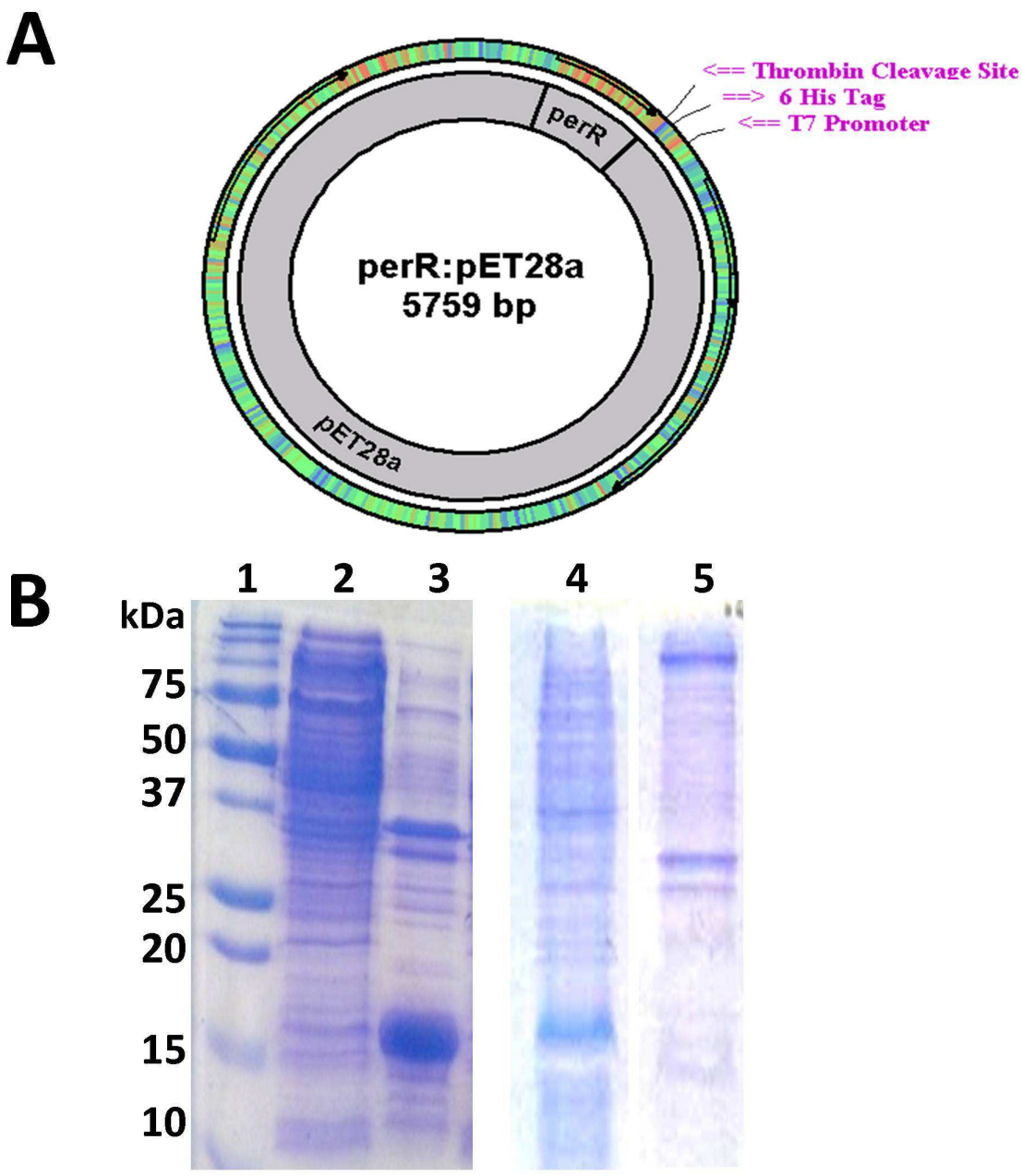


Figure 5.2: Recombinant over-expression of *C. jejuni* PerR-His in *E. coli Bl21* [A] Virtual cloning of *C. jejuni perR* into vector pET28a (pDRAW). [B] SDS-PAGE analysis of the soluble (Lane 2) and insoluble (Lane 3) protein fractions of *E. coli* over-expressing *C. jejuni* PerR-His. Lanes 4 and 5 show soluble and insoluble protein over-expression when *E. coli* were grown at 20 °C after IPTG induction. Lane 1 shows a standard molecular weight ladder.

The successful construction of *perR*:pET28a was confirmed by restriction digest and plasmid DNA sequencing (see Materials and Methods). SDS PAGE analysis revealed the over-expression of a protein approximately 15 kDa in size (Figure 5.2B, Lane 3). However all of the protein was insoluble (Lane 3) and the 15 kDa protein was absent in the soluble fraction (Lane 2).

Reduction of the *E. coli* growth temperature to 20°C after IPTG induction resulted in low levels of protein in the soluble fraction (Figure 5.2B, Lane 4 and 5), but protein yield was low. No manipulation of the IPTG concentration or performing the protein purification at 4°C had any positive effect on protein solubility.

5.3.2.2 Failure of *C. jejuni* PerR-His protein Purification

E. coli cells were lysed by sonication; *C. jejuni* PerR was separated from cell debris by centrifugation at 23,000 x *g* and the pellet was discarded. The soluble fraction was filtered (0.4 µM Mini Start Syringe Filter) and loaded onto a nickel-NTA sepharose column to selectively bind His-tagged proteins. The column was then incubated in thrombin, to cleave the His-Tag from *C. jejuni* PerR-His and release any bound *C. jejuni* PerR from the nickel NTA column. However, no protein was recovered after thrombin cleavage.

The nickel column was stored in 500mM imidazole overnight. Imidazole eluted fractions contained the over-expressed protein, indicating that either *C. jejuni* PerR had bound the Nickel column in the absence of a His-tag, potentially due to its metal ion binding ability or the Thrombin cleavage of the His-tag had failed.

Due to the low levels of soluble protein expression and potential interactions between His-tags and metal binding proteins, use of His-tagged *C. jejuni* PerR was abandoned.

5.3.2.3 Native PerR Over-Expression

The *C. jejuni* PerR orthologue was re-amplified from *C. jejuni* NCTC 11168 genomic DNA and ligated into the over-expression vector pET21a,

also under the control of the T7 promoter. This vector was then transformed into *E. coli* Bl21 (DE3) and protein expression was induced in the presence of IPTG. The predicted molecular weight of PerR protein is 15.9 kDa as calculated based on the *C. jejuni* amino acid sequence. Figure 5.3 shows the successful over-expression of a protein with an approximate molecular weight of 20 kDa, at 1, 3 and 5 hours after IPTG induction.

Native *C. jejuni* PerR was present in both the soluble and insoluble cell fractions (Figure 5.3, Lanes 5 and 6) when induced at 37 °C, unlike the His-tagged version of *C. jejuni* PerR (Figure 5.2B). Attempts were made to improve the ratio of soluble to insoluble PerR. This included altering IPTG concentration (final concentrations of 0.04, 0.1 and 0.4 mM IPTG were tested), altering *E. coli* growth temperatures (30 - 37 °C) and performing protein purifications at room temperature and at 4 °C. After extensive analyses, the highest yield of soluble *C. jejuni* PerR protein was obtained using 0.04 mM IPTG, growing *E. coli* cultures at 37 °C but reducing growth temperatures to 30 °C 1 hour pre-IPTG induction and performing the protein purification at room temperature.

5.3.2.4 Purification of recombinantly expressed native *C. jejuni* PerR protein

E. coli cells pellets containing untagged (native) recombinant PerR protein were lysed by sonication centrifuged to separate the soluble and insoluble fractions and then the insoluble fraction was discarded. The soluble protein fraction (Figure 5.3, Lane 5) was passed through a Hi-Trap ™ heparin column. Bound PerR was eluted from the column across a salt gradient and collected in a series of fractions (Figure 5.4, Lanes 5-10). Typically PerR protein eluted in the presence of 25% Buffer B [20 mM Tris-HCl [pH8.0], 1M NaCl, 10 mM EDTA], which is equivalent to a salt concentration of approximately 265 mM (corresponding to a solution with a specific conductance of 15 mS/cm).

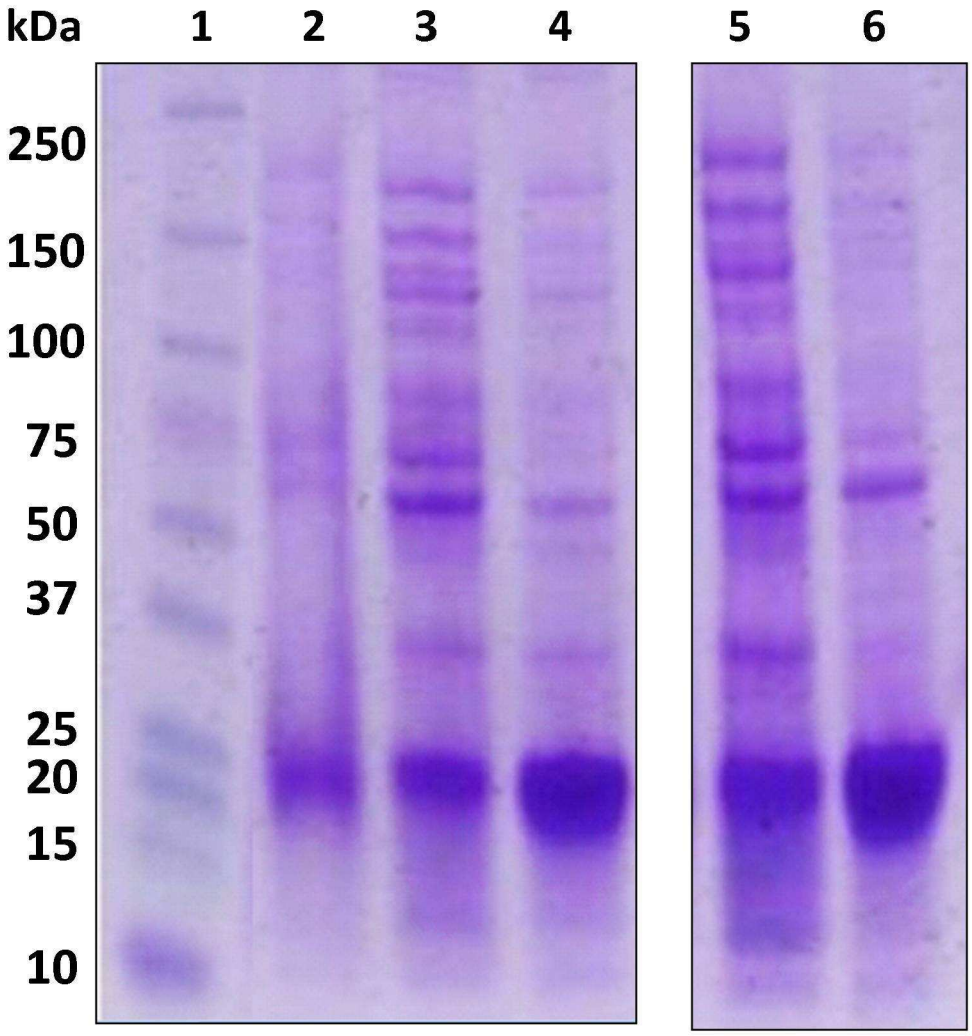


Figure 5.3: SDS-PAGE analysis of whole cell *E. coli* over-expressing *C. jejuni*
PerR at 1, 3 and 5 hours post IPTG induction (Lanes 2-4). Lanes 5 and 6 show the distribution of PerR across the soluble and insoluble cell lysate fractions, respectively. Lane 1 shows a standard molecular weight ladder (Precision Plus (Biorad)), sizes are in kDa.

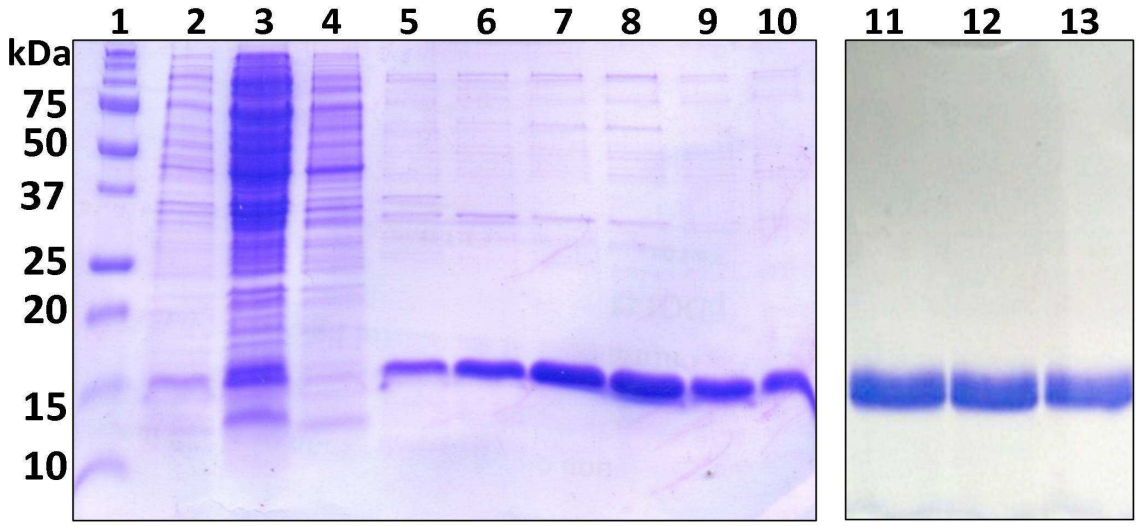


Figure 5.4: SDS-PAGE analysis of *C. jejuni* PerR protein eluted from a Heparin column across a salt gradient. Lane 1 shows a standard molecular weight ladder, sizes are in kDa. Lanes 2-4 show the loaded sample, the column wash and the column flow through, respectively. Lanes 5-10 show PerR protein eluted across fractions 30, 35, 40, 43, 50 and 56, from a Heparin column. Lanes 11-13 show pure PerR protein after gel filtration.

In Figure 5.4, Lane 3 indicates that not all PerR protein bound the Heparin column as PerR protein is present in the column wash (Lane 3, ~15kDa). Once PerR protein was eluted from the heparin column, samples were concentrated and further purified by gel filtration (Figure 5.4, Lanes 11-13) on a Sephacryl-200 High Resolution column.

5.3.3 Confirmation of *C. jejuni* PerR over-expression by mass spectrometry.

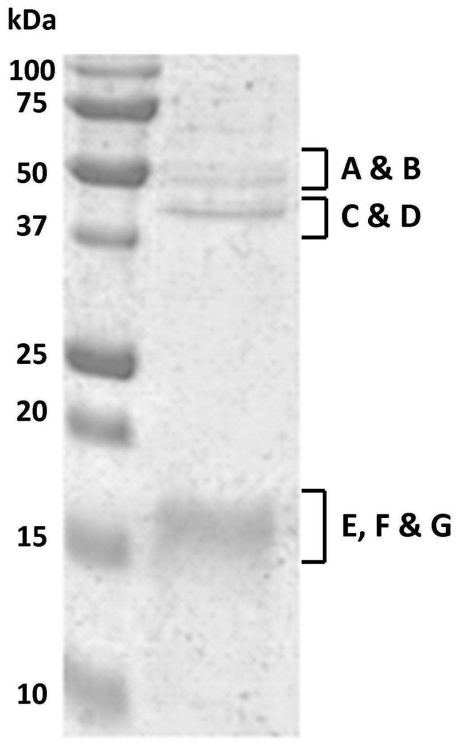
To confirm that the protein over-expressed and purified in Figure 5.3 and Figure 5.4 was *C. jejuni* PerR, a fraction of partially purified PerR protein was run on an SDS-PAGE gel and analysed by mass spectrometry (performed by Dr Francis Mulholland).

Mass spectrometry confirmed that the over-expressed protein induced and purified in Figure 5.3 and Figure 5.4 was *C. jejuni* PerR. Mass

spectrometry also identified several *E. coli* proteins that were co-purified alongside *C. jejuni* PerR, indicating that gel filtration is essential for the isolation of pure protein.

5.3.3.1 Calibrated Gel Filtration to determine the oligomerisation state of recombinantly purified PerR protein

A Sephacryl-200 gel filtration column was calibrated with proteins of known molecular weight (Figure 5.6). The void volume (Vo) of the gel filtration column was determined to be 40 ml, as determined by the elution volume of Blue Dextran through the column. The calibrated gel filtration column was used to investigate the oligomerisation state of purified *C. jejuni* PerR protein. *C. jejuni* PerR passed through the column in an elution volume characteristic of a protein with a molecular weight of 33.9 kDa (See Table 5.1 for working). This corresponds to double the predicted molecular weight of PerR (15.9 kDa), indicating that recombinantly purified *C. jejuni* PerR protein is present in a dimeric state.



	Protein	Mass measured by MS(Da)	Organism
A	6-Phosphogluconate dehydrogenase	51547	*E. coli*
B	Rho	47032	*E. coli*
C	Cysteine desulferase	45232	*E. coli*
D	Phophoglyerate kinase	41264	*E. coli*
E	Catabolite regulation protein (CreA)	17311	*E. coli*
F	Peroxide Stress Regulator	16315	*C. jejuni*
G	50s ribosomal protein L9	15759	*E. coli*

Figure 5.5: Mass spectrometry analysis of a protein fraction eluted from a Heparin column confirms the over expression and purification of *C. jejuni* PerR. The other proteins present in the protein fraction have also been identified.

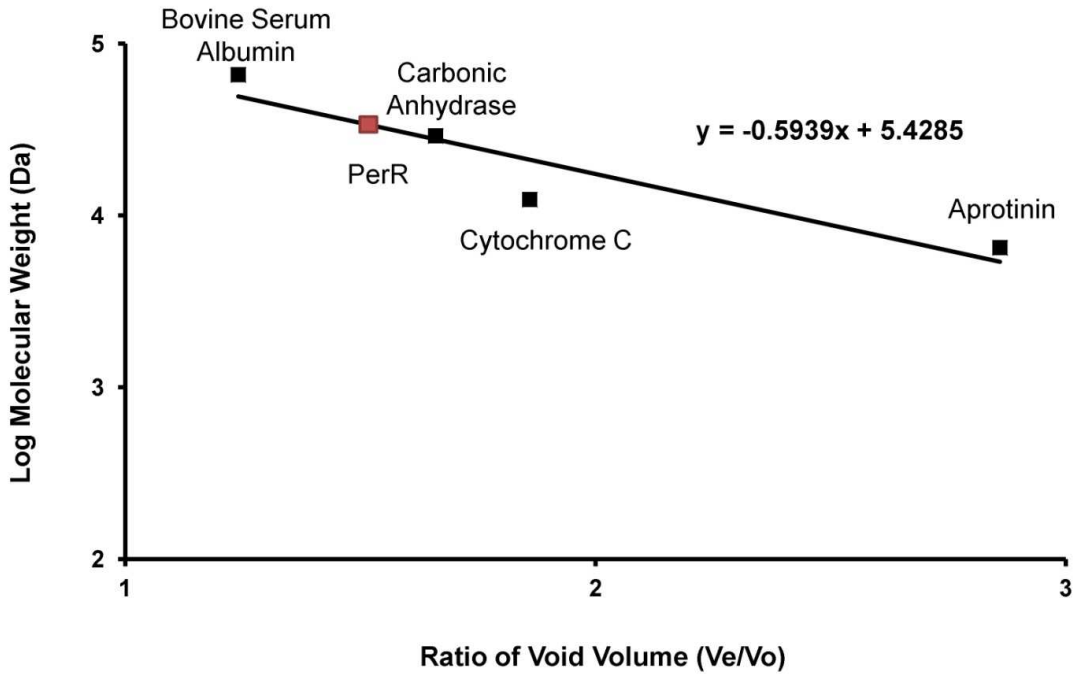


Figure 5.6: Calibration of a Sephacryl-200 column with proteins of known molecular weight.

Once the gel filtration column had been calibrated, semi purified *C. jejuni* PerR protein samples were concentrated to 2 ml and loaded onto the gel filtration column. As the column had been calibrated, it can be used to determine the predicted molecular weight of PerR based on its elution volume from the column (Table 5.1).

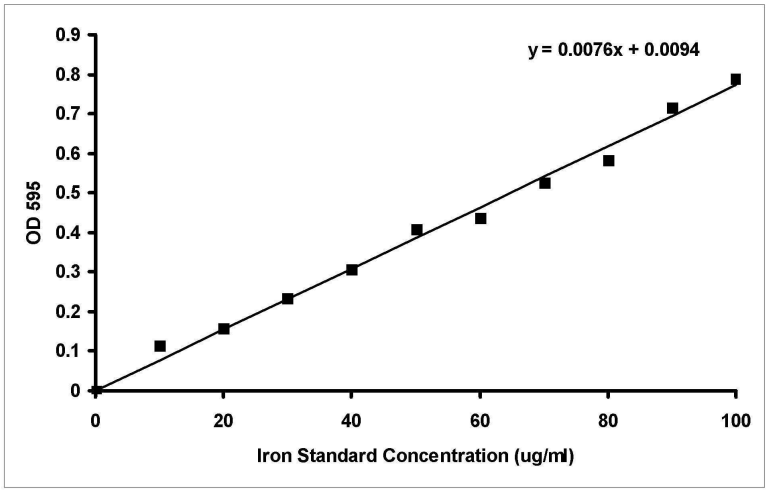
Table 5.1: The calculated molecular weight of purified *C. jejuni* PerR as determined by gel filtration.

Protein	MW (kDa)	Elution Volume [Ve] (ml) [Elution time x flow rate]	Ratio [Ve/Vo]	Calculated Log MW (-0.5939 x [Ve/Vo]) + 5.4	Predicted MW (kDa)
C. jejuni PerR	15.9	60	1.5	4.53	33.9

5.3.4 *C. jejuni* PerR Metal Content Analysis

5.3.4.1 Analysis of the iron content of purified *C. jejuni* PerR protein using a ferene colorimetric iron assay

The compound Ferene (3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid) is a colorimetric indicator of Iron(II) [Fe^{2+}] and was used to analyse the levels of iron present in samples of purified *C. jejuni* PerR protein (Hennessy *et al.*, 1984). PerR protein was denatured and the remaining iron metal ions were quantified. The concentration of the iron present was determined by comparison to a standard curve of known iron concentrations (Figure 5.7).



Dilution Factor (DF)	OD 595	Y= 0.0076x + 0.0094	X Dilution Factor (µg/ml)	Average Concentration (µg/ml)
0	0.0572	7.52	15.07	
2	0.0482	6.34	12.70	**16.22**
10	0.0158	2.08	20.88	

Figure 5.7: Analysis of the iron content of purified *C. jejuni* PerR protein using a ferene colorimetric iron assay indicates that very little iron has been bound by PerR.

The Ferene assay in Figure 5.7 was performed on a 1.75 mg/ml sample of *C. jejuni* PerR. The results indicated a very low level of iron, corresponding to approximately one Fe^{2+} ion per one thousand PerR monomers. This may indicate low levels of iron are present in PerR upon isolation or the sensitivity of this colorimetric assay is too low. Subsequently more robust metal content analyses were subsequently performed.

5.3.4.2 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) of *C. jejuni* PerR

All work completed in this section was performed at the Faculty of Medicine in the University of Ottawa in Canada as part of a research internship funded by the British High Commission in Canada. Bacterial growth and protein purification was performed as a joint collaborative effort with Sabina Sarvan under the supervision of Dr Jean Francois-Couture and Prof. Alain Stintzi. Samples for ICP-MS analysis were sent to North Western University, USA for analysis.

5.3.4.2.1 The addition of metal ions to *E. coli* cultures at the point of induction of PerR over-expression

In an attempt to incorporate metal ions into the metal binding sites of *C. jejuni* PerR, exogenous iron (Fe^{2+}), manganese (Mn^{2+}) and zinc (Zn^{2+}) (100µM) were added to the *E. coli* culture medium at the induction point of protein over-expression. Cobalt and Nickel were also used as media supplements, but these samples were excluded from ICP-MS analysis as the protein isolated from these conditions largely precipitated during protein concentration. The concentration of various metal ions present in a 100 µM sample of pure PerR protein grown in the presence of iron, manganese and zinc is shown was analysed by ICP-MS, as shown in Table 5.2.

Table 5.2: ICP-MS analysis of pure PerR protein over-expressed and grown in the presence of exogenous metal ions. Readings were taken in triplicate and the average value is shown.

Metal Ion	Average concentration of Metal (μM)				
	Fe^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}
PerR + Iron	**2.7690**	0.0681	72.5849	0.0379	2.7313
PerR + Manganese	0.9664	**1.1230**	72.6867	0.0297	1.4964
PerR + Zinc	4.6893	1.2332	**124.5028**	0.0415	1.5636

Table 5.2 indicates that only very low levels of the additional metal ions added were incorporated into *C. jejuni* PerR, suggesting that the exogenous addition of metal ions, apart from the addition of zinc, to the *E. coli* growth media is not an effective method of metal incorporation. The presence of high levels of zinc in all protein samples indicate that this is acquired during growth in *E. coli*. *C. jejuni* PerR purified in the absence of any additional metal ions purifies with one zinc ion per monomer (as determined by Sabina Sarvan, data not shown).

5.3.4.2.2 The addition of metal ions to purified *C. jejuni* PerR protein

As shown previously in Table 5.2, metal ions are not fully incorporated into PerR protein when externally added, only zinc is present in significant concentrations.

ICP-MS was performed on purified PerR protein that had been incubated in a buffer containing a 2:1 (200 μM metal, 100 μM protein) excess of different metal ions (See Table 5.3 and Figure 5.8). To ensure the metal ions had been incorporated into the PerR protein, all samples were gel filtrated before analysis to remove residual metal ions present in the buffer.

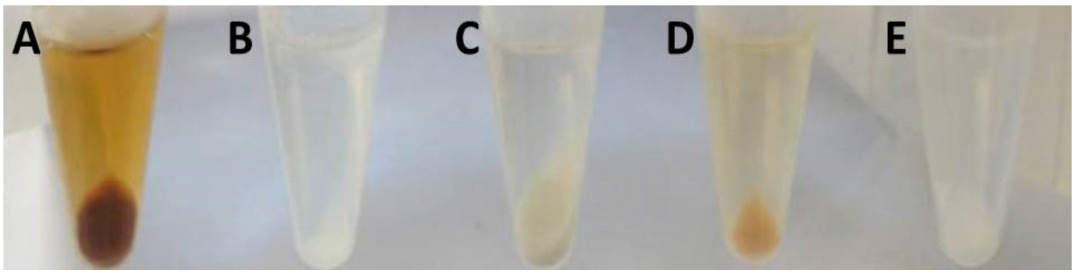


Figure 5.8: The precipitation of *C. jejuni* PerR after the addition of Cobalt (A), Manganese (B), Nickel (C), Iron (D) and Zinc (E) to the protein solution.

Table 5.3: ICP-MS analysis of *C. jejuni* PerR after the addition of metal ions to the purified protein. Readings were taken in triplicate and average results are shown.

Metal Ion	Average Concentration of Metal (μM)				
	Fe^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}
PerR + Iron	**67.93**	2.24	123.21	0.03	1.77
PerR + Manganese	3.4	**93.52**	131.38	0.01	0.85
PerR + Zinc	0.83	1.96	**265.52**	0.03	0.82
PerR + Cobalt	0.48	1.75	119.78	**142.57**	1.03

ICP-MS data from Table 5.3 reveals that all metal ions tested can be incorporated into PerR protein when directly added to a solution of purified PerR protein. The addition of Nickel was also attempted unfortunately no data were obtained as the protein largely precipitated, as shown in Figure 5.8C.

5.3.5 Structural predictions for *C. jejuni* PerR based on observations during protein purification

During purification of *C. jejuni* PerR under normal growth conditions a pigmentation of the protein was seen. When highly concentrated or after the addition of iron (in an aerobic conditions) the protein took on a pale red hue.

Figure 5.9 A-D details observations made during the purification of *C. jejuni* PerR under normal growth *E. coli* conditions in LB media. The pigmentation first became apparent after *E. coli* cell lysis (Figure 5.9A) and persists throughout the purification process until pure PerR protein is achieved (Figure 5.9D). Previously during ICP-MS and the addition of excess metal ions to purified PerR (5.3.4.2.1) it was also noted that after addition of iron the purified protein had a greater red colouration (Figure 5.8D) and ICP-MS results in Tables 5.3. Therefore further purifications were conducted with iron in greater excess. Some images from this purification are shown in Figure 5.10.

When over-expressed in LB plus 100mM iron sulfate the red colouration of *C. jejuni* PerR was increased. The UV-visible absorbance spectrum of the protein was recorded, Figure 5.11.

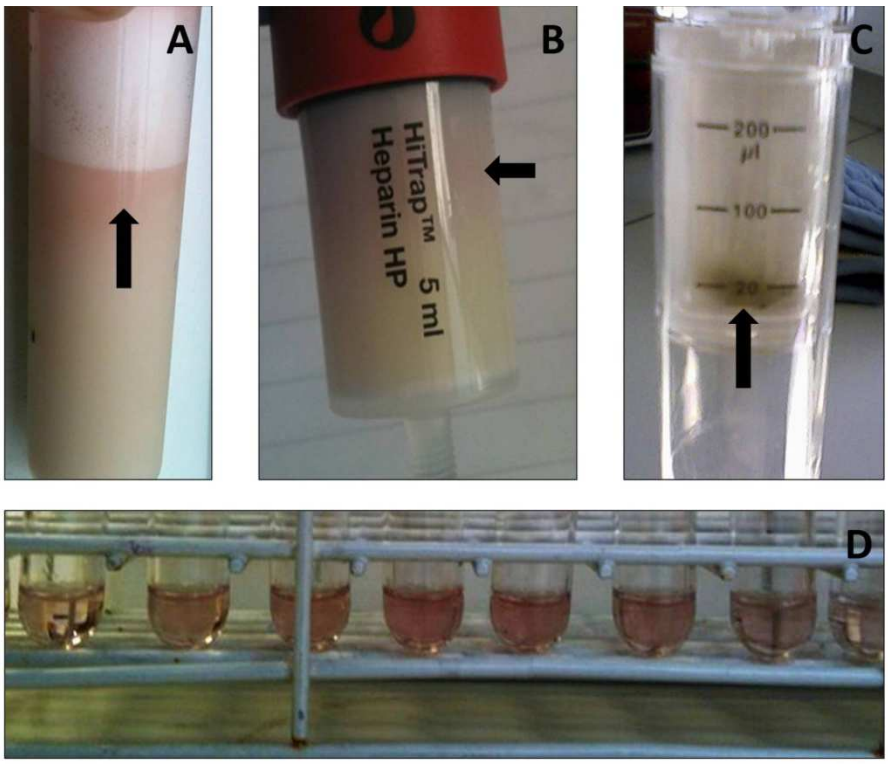


Figure 5.9: Images taken throughout the purification of *C. jejuni* PerR show the protein displaying a red hue (see arrows). [A] Cell lysate in a falcon tube turned red at the air interface after sonication. [B] A heparin column turns red after being loaded with *E. coli* soluble cell lysate containing *C. jejuni* PerR [C] Red-brown pigmentation in a centrifugal concentrator as pure PerR protein is concentrated. [D] A series of fractions containing various concentrations pure *C. jejuni* PerR eluted from a Heparin column.

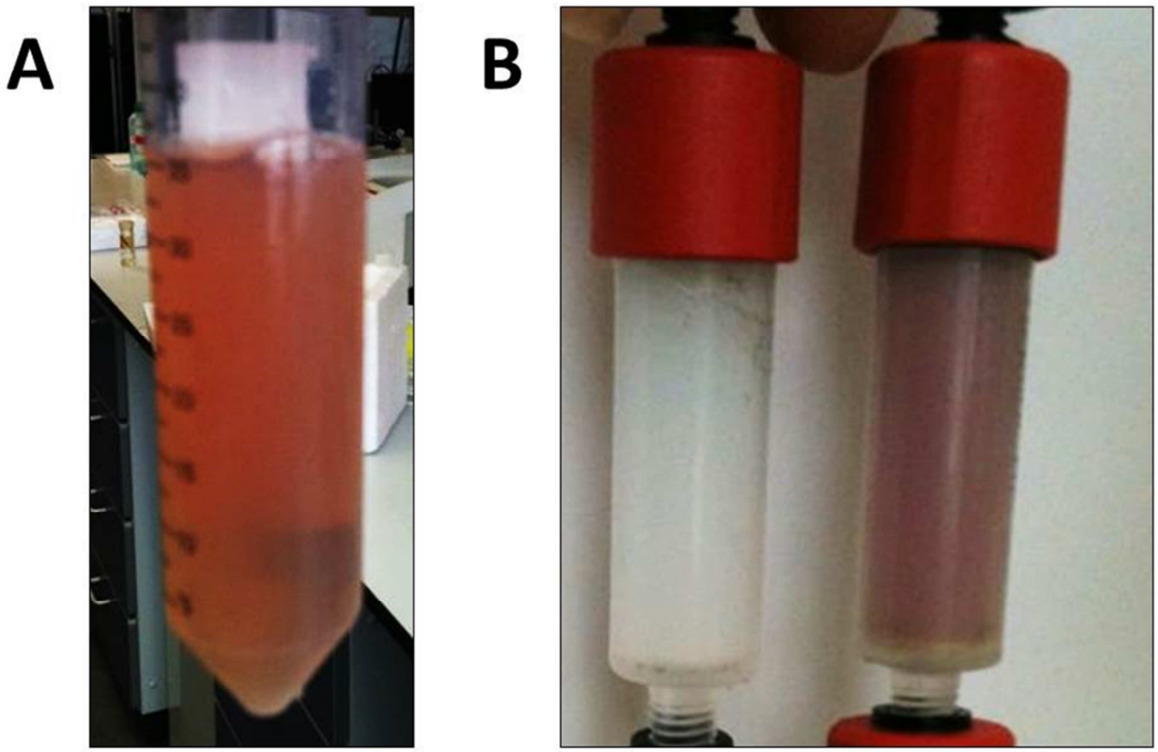


Figure 5.10: *C. jejuni* PerR protein purified from E. coli grown in LB plus 100 mM iron sulphate shows increased red pigmentation. [A] shows the soluble *E. coli* cell lysate and [B] shows this loaded onto a heparin column, next to an unloaded heparin column for comparison.

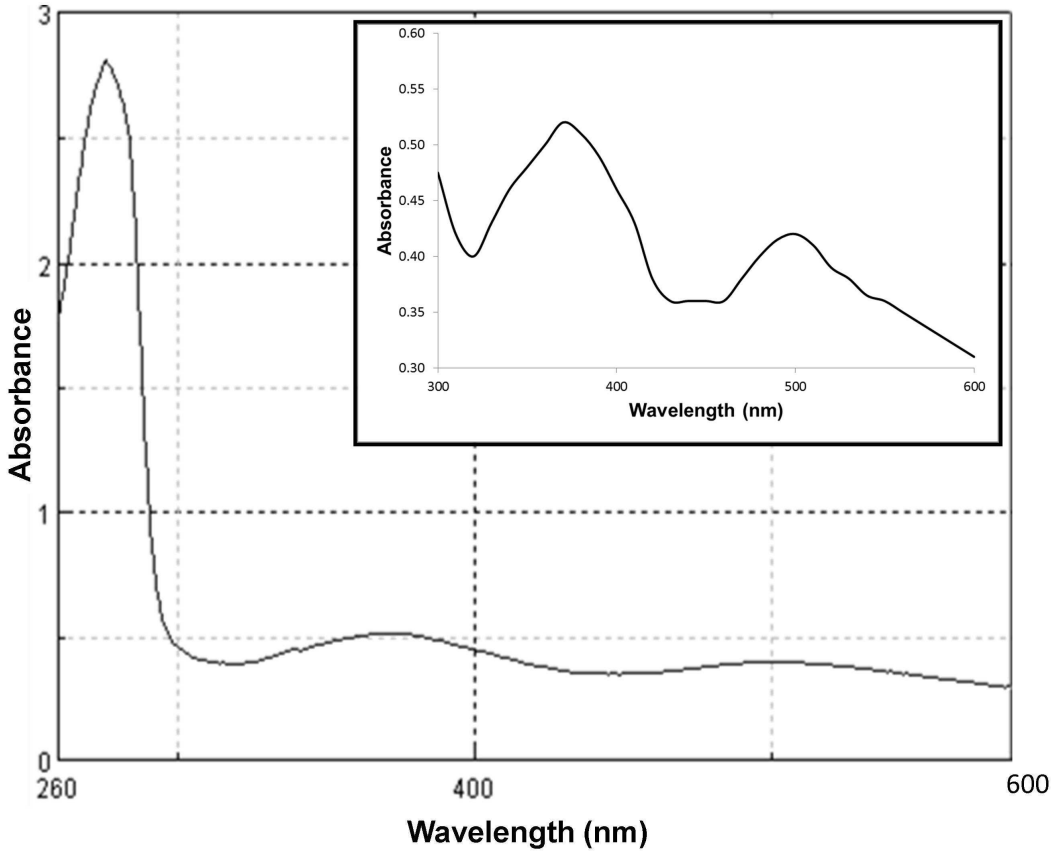


Figure 5.11: Analysis of purified *C. jejuni* PerR by absorbance spectroscopy. Inset shows a zoomed in view of the 300 nm to 600 nm region.

Table 5.4: Calculation of protein concentration from A280

Absorbance at 280 nm [A]	**Extinction coefficient (M^{-1} Cm^{-1}) [ε]**	**Dilution Factor [DF]**	**Concentration (M) [A/ε]*DF**
2.8	12295	1	2.277×10^{-4}

Table 5.5: Calculation of cofactor concentration from peaks at 377 nm and 490 nm

Absorbance [A]	Wavelength (nm)	Extinction Coefficient (M^{-1} Cm^{-1}) [ε]	Path length (Cm) [ı]	Concentration (M) [A = ε ı c]
0.52	377	7300	1	7.12×10^{-5}
0.43	490	9200	1	4.67×10^{-5}
			Average	**5.88×10^{-5}**

An absorbance spectrum of *C. jejuni* PerR reveals two absorbance bands at 377 nm and 490 nm, the absorbance band 280 nm corresponds to the proteins aromatic side chains and indicates a protein concentration of 2.277×10^{-4} (See Table 5.4). The spectrum is consistent with the presence of a metal cofactor present in the protein sample. The metal cofactor is present in approximately a 1 in 4 ratio of cofactor to protein (Table 5.5).

5.3.6 A comparison of the structural characteristics of *C. jejuni* and *B. subtilis* PerR

The sequences of PerR from *B. subtilis* and *C. jejuni* have relatively low sequence homology, as was discussed previously in Section 5.3.1. However the structure of *B. subtilis* PerR has been solved and this was used to generate a model structure of *C. jejuni* PerR using PDB DeepViewer (

Figure 5.12). This suggests that *C. jejuni* and *B. subtilis* PerR may share similar structural characteristics.

Figure 5.12 shows that the metal binding sites of PerR from both species may consist of similar residues. In *B. subtilis* PerR, the zinc binding

metal ion site is annotated as aiding in dimer formation. This zinc dimerization site is co-ordinated by four cysteine residues. If an iron metal ion is co-ordinated by four cysteines it forms a rubredoxin iron centre (

Figure 5.12, box). Rubredoxin iron centres are found in rubredoxin proteins, a group of proteins that have a characteristic red pigmentation when oxidised and colourless when reduced (Weinberg *et al.*, 2004).

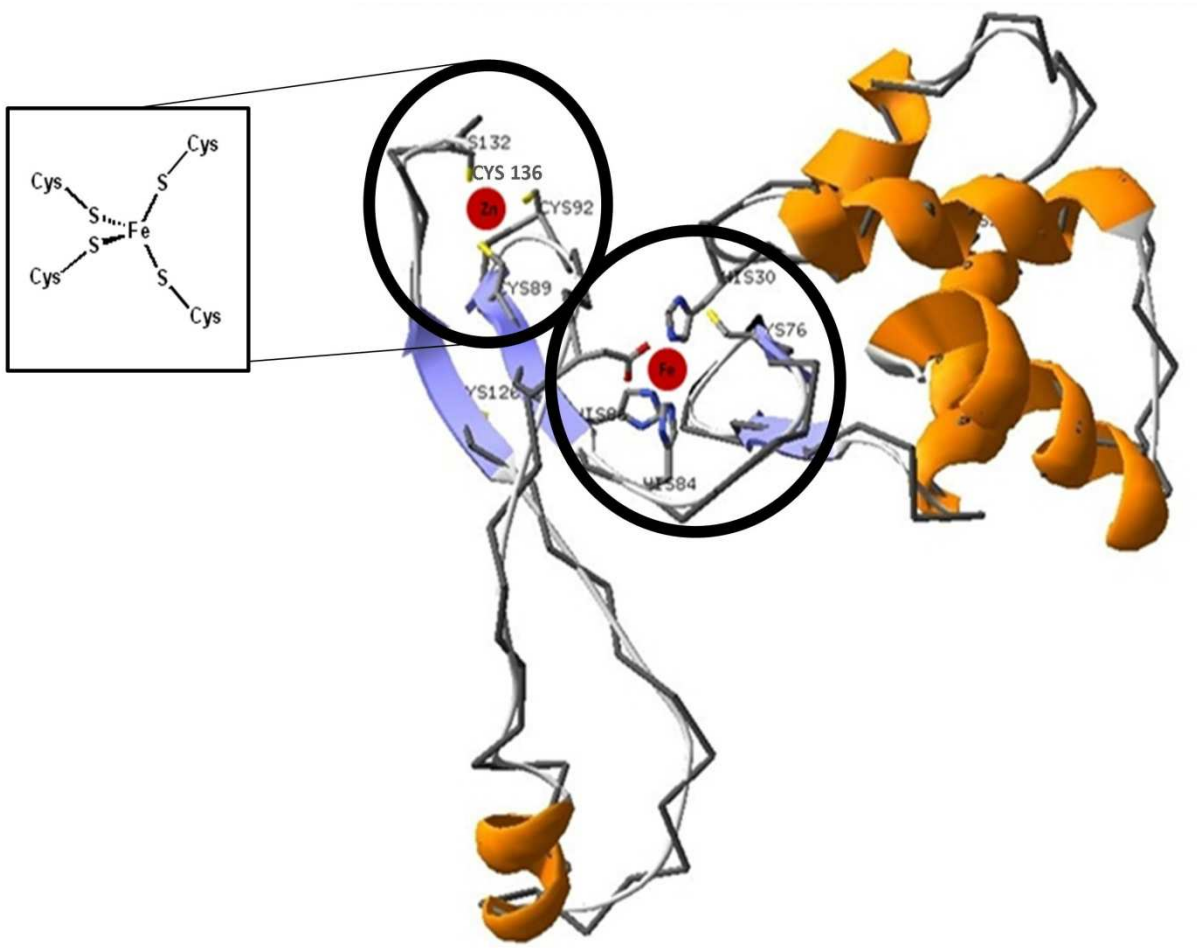


Figure 5.12: The amino acid sequence of *C. jejuni* PerR (black) modelled onto the known structure of PerR from *B. subtilis* (white), metal ion binding sites are labelled according to the *B. subtilis* structure (black circles). The structure of a rubredoxin iron centre is shown boxed.

5.3.7 Attempts to determine the crystal structure of *C. jejuni* PerR using X-Ray Protein Crystallography

The structure of *C. jejuni* PerR is unknown and we initiated attempts to solve the protein structure using X-ray crystallography in order to understand how PerR regulates and binds DNA in *C. jejuni*. Purified *C. jejuni* PerR protein was concentrated to approximately 20 mg/ml for use in crystallisation trials.

Protein was diluted to final concentrations of 5 and 10 mg/ml and used in crystallography trials screens (MD1-01 Structure Screen 1 and MD1-02 Structure Screen 2 and MD1-37 JCSG+ (Molecular Dimensions)) at 4 °C and 16 °C. The majority of the protein in the trial reservoirs precipitated, however after three days of observation two crystal hits were observed as detailed in Figure 5.13. Figure 5.13A shows the presence of multiple small protein crystals known as ‘micro crystals’ imaged at 16 °C but also present in the 4 °C plate. These micro crystals were too small to be used in X-ray crystallography. To focus in on these crystallography conditions sitting drop trays were set up using a range of 26-36 % MPD, 0.1 M MES pH 6.0-7.5 with and without the presence of 0.2 M magnesium acetate. MES was used in place of sodium cacodylate due to the hazardous nature of the compound. Unfortunately all drops precipitated and no further crystals hits were observed under these conditions. Figure 5.13B shows larger crystals, which were present in the same reservoir at both 4 °C and 16 °C. The crystal produced at 4°C was isolated however did not diffract X-rays sufficiently well to enable structure determination.

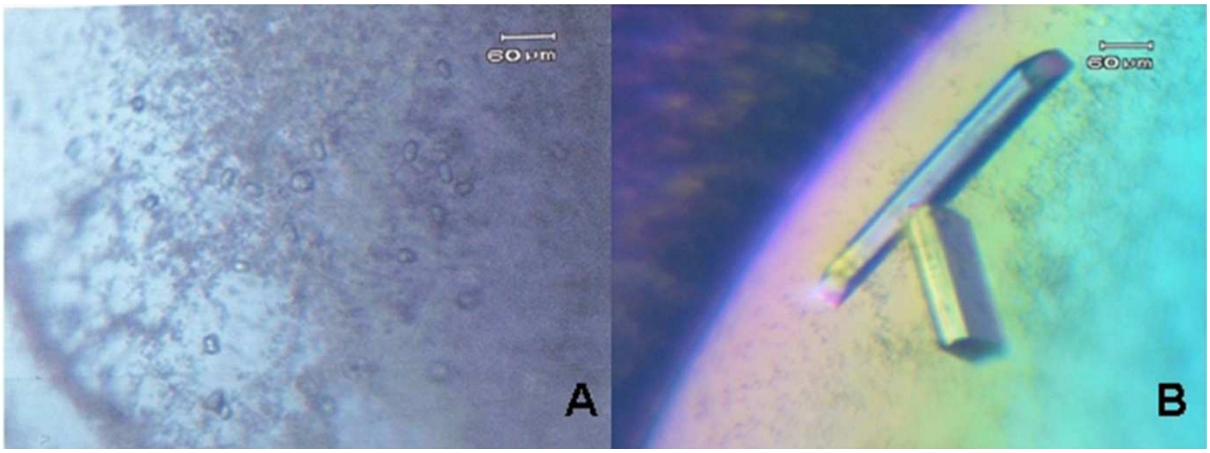


Figure 5.13: *C. jejuni* PerR crystals observed during protein crystallography trial screens at 16 °C. [A] shows micro crystals in a reservoir containing 0.2 M magnesium acetate, 0.1 M sodium cacodylate pH 6.5 and 30 % MPD. [B] shows two large crystals in a reservoir of 0.2 M ammonium dihydrogen phosphate, 0.1 M Tris pH 8.5 and 50 % MPD.

5.4 Discussion

5.4.1 Comparison of *C. jejuni* PerR to known orthologues

In *C. jejuni*, the *perR gene* encodes a metal co-ordinating regulatory protein that is a member of the Fur family of metalloproteins (Bsat *et al.*, 1998; Traore *et al.*, 2006; van Vliet *et al.*, 1999). The PerR protein is widespread and homologues are found across many species of bacteria (Snel *et al.*, 2000). In Figure 5.1A the diversity of some selected PerR homologues protein sequences are explored, using the PerR proteins from *Bacillus subtilis*, *Streptococcus pyogenes*, *Wolinella succinogenes*, *Helicobacter hepaticus*, *C. jejuni* and *Staphylococcus aureus.* PerR protein sequences from these species were explored as they cover a broad diversity of bacteria spanning the Firmicutes to the Proteobacteria (Baar *et al.*, 2003; Bsat *et al.*, 1998; Horsburgh *et al.*, 2001; King *et al.*, 2000; Parkhill *et al.*, 2000). The low sequence homology between the PerR homologues of these bacterial species may be indicative of the broad role played by PerR. It is likely that bacterial gene regulation may have adapted to the niche or lifestyle

of the individual bacterial species (Figure 5.1A), depending on an individual species tolerance to oxidative stress.

The PerR protein sequence is highly conserved amongst the genus *Campylobacter* and the *C. jejuni* species, potentially indicating an important role within *Campylobacter* biology. However in *C. jejuni*, the *perR* gene is not essential gene and *perR* mutants are viable (van Vliet *et al.*, 1999)(Chapter 3). It is possible that PerR has been conserved as PerR-like gene regulation may confers a survival advantage, such as conserving energy by allowing transcription of stress response proteins only when needed or by early detection and response to stress.

In contrast to the other PerR amino acid sequences described in Figure 5.1, *C. jejuni* PerR contains notably higher numbers of cysteine residues compared to the other PerR orthologues, as indicated by red arrows in Figure 5.1B. Unlike *B. subtilis* and other bacterial PerR's which contain four cysteine residues, the *C. jejuni* PerR sequence contains seven cysteine residues (Indicated in Figure 5.1B by arrows). According to structural overlay of the *C. jejuni* PerR protein sequence onto the *B. subtilis* PerR structure (

Figure 5.12) none of these additional *C. jejuni* cysteine residues are predicted to co-ordinate with the second metal binding site annotated in the *B. subtilis* PerR sequence. In *C. jejuni* the second metal binding site is predicted to be co-ordinated by three histidine residues and a lysine residue. It is therefore tempting to predict that due to this excess of cysteine residues, *C. jejuni* PerR may have greater metal binding capability than that of *B. subtilis,* such as an extra metal binding site per monomer or more flexibility in metal binding residues. This adds additional levels of complexity to *C. jejuni* PerR regulation. Although predictions such as these could only be confirmed by experimental studies of *C. jejuni* PerR . The presence of these additional cysteine residues may reflect upon the intimate nature of metal binding and oxidative stress responses in *C. jejuni*, a unique relationship developed by an

organism that has a microaerobic lifestyle combined with aerobic transmission.

5.4.2 Recombinant Purification of the *C. jejuni* Peroxide Regulator

The use of a six-His tag to facilitate *C. jejuni* PerR protein purification from *E. coli* resulted in the protein being lost or expressed in the insoluble fraction as inclusion bodies (Figure 5.2). It is possible that PerR was not recovered due to the protein binding the Nickel-NTA column after the His-tag was cleaved via its own metal binding sites, although this was not confirmed.

Work was continued using native PerR protein, additional types of protein affinity tags were not used, as there was a concern that these too may interfere with the solubility of PerR. Instead the native properties of untagged *C. jejuni* PerR were exploited to purify the protein using a heparin affinity column. Binding of *C. jejuni* PerR to a heparin column is thought to be due to the PerR DNA binding domain; heparin has a strong negative charge and a helical structure, often leading to it being referred to as a 'DNA mimic'. It is therefore possible to use Heparin to selectively bind DNA binding proteins and then elute them.

Purification of native *C. jejuni* PerR was successful, protein was purified to approximately 95% purity (as assessed by SDS-PAGE) (see Figure 5.4, Lanes 11-13) with an average yield of 2 mg/ml per litre of *E. coli*. Figure 5.4, lanes 3 and 4, indicate that some PerR protein yield was lost during purification. Not all PerR protein bound the Heparin column, as faint protein bands can be seen on the gel at approximately 15kDa. This however may represent denatured protein or an *E. coli* protein of a similar molecular weight.

Purified *C. jejuni* PerR protein, was used in biochemical analyses including crystallisation trials. Unfortunately we were unable to generate crystals of *C. jejuni* PerR for diffraction analysis. It is possible that the crystallisation of *C. jejuni* PerR requires anaerobic or microaerobic conditions, as the protein is potentially oxygen sensitive alternatively it may need specific metal ions bound to promote stability (Herbig & Helmann, 2001).

5.4.3 The effect of metal ion homeostasis during PerR purification

Metals form an important part of the biochemistry of PerR proteins. Previous work in *B. subtilis* has shown that metal ion availability can alter the intracellular form of PerR and the sensitivity of bacteria to hydrogen peroxide (Herbig & Helmann, 2001).

Prior to metal addition, purified PerR contained one zinc ion per monomer, meaning one metal binding site was vacant (determined by ICP-MS conducted by Sabina Sarvan). Table 5.3 shows that after the direct addition of metal ions to PerR in solution, the added metal is taken up by this vacant metal binding site. However the additional metal ions lack the binding affinity to displace the zinc ion already bound by *C. jejuni* PerR. This is likely due to the high affinity of the *C. jejuni* PerR binding site for zinc ions.

B. subtilis PerR has been extensively characterised and the crystal structure for this PerR orthologue reveals the presence of a bound zinc ion, co-ordinated by the thiol side chains of four cysteine residues (Cys-96, 99, 136 and 139) (Traore *et al.*, 2006). The sequence alignment of PerR orthologues in Figure 5.1A depicts the lack of PerR sequence homology across various bacterial species. As mentioned previously, there is only 28.7% sequence identity between *C. jejuni and B. subtilis* PerR proteins. However all four cysteine residues associated with this zinc co-ordination site are conserved, not only between *C. jejuni* and *B. subtilis* PerR, but across all of the species discussed in Figure 5.1A, it is likely that this $Zn(Cys)_4$ feature is a characteristic feature of PerR-like metalloregulators. The conservation of these four cysteine residues highlights them as having an important role in protein function. In *B. subtilis*, these residues have been identified as having a role in protein dimerization stability (Traore *et al.*, 2006).

Based on

Figure 5.12 the overlay of the *C. jejuni* PerR sequence onto the structure of *B. subtilis* PerR, it is highly likely that these same cysteine residues form a $Zn(Cys)_4$ site and perform the same function in *C. jejuni* PerR. In PerR, owing to the chemistry of soft Lewis bases, it is likely that there may be a preference of these cysteine residues of PerR to bind zinc

(Zn(II)), a borderline soft Lewis acid (Lippard & Berg, 1994). Additional evidence for the binding of Zn(II) by *C. jejuni* PerR is shown in Table 5.2 and Table 5.3.

Purification of PerR in always yields protein with atleast one zinc ion per monomer, even in the presence of other metals in excess. Whilst ICP-MS can only measure metal concentration, it cannot predict which metal binding site of PerR zinc was bound too.

In an attempt to incorporate specific metal ion species into PerR during expression or purification, metal ions were added to the *E. coli* growth media during PerR protein over expression. However, as shown in Table 5.3 the incorporation of metals into PerR in this manner was largely unsuccessful. With the exception of zinc, very little of the other metals added to the *E. coli* LB growth media were incorporated into the PerR protein being over-expressed, this may be due to the metal ions being utilised by *E. coli* and incorporated in other proteins, or alternatively, the *E. coli* host may not have transported them to the cytoplasm. Additionally the concentration of metal may have not been great enough to result in any increased metal incorporation into PerR. However many of the compounds added to the *E. coli* LB growth media are toxic to *E. coli* growth, so low levels had to be used.

5.4.4 Investigation of the red pigmentation of *C. jejuni* PerR seen during purification

During over expression and purification of *C. jejuni* PerR it became evident that the protein was purified with a red colour. Typically proteins containing iron sulphur clusters are associated with having pigmentation, yet no known orthologues of *C. jejuni* PerR for which structures have been solved, contain a true iron sulphur cluster.

As the structure of *C. jejuni* PerR has not been solved,

Figure 5.12 was used to extrapolate structural data from what is already know about *B. subtilis* PerR. Despite the low sequence identity between *B. subtilis* and *C. jejuni* PerR, several residues are absolutely conserved amongst all known PerR homologues. As discussed previously, it is likely that the metal binding regions of PerR do not fluctuate between

species and it can assumed that *C. jejuni* PerR contains the four cysteine, zinc-binding site of other PerR proteins (Traore *et al.*, 2006).
The red pigmentation of recombinant *C. jejuni* PerR is linked to iron concentration, as indicated by the increase in colour seen between Figure 5.9 and Figure 5.10.
Using the PerR structural overlay (

Figure 5.12) a putative rubredoxin domain was identified within PerR. A rubredoxin domain or rubredoxin iron centre is formed by a polypeptide binding a single iron atom in tetrahedral geometry, coordinated by four cysteinyl sulfur atoms (Sieker *et al.*, 1994). In rubredoxins cysteine ligands occur in pairs with the sequence C-X-X-C at the N and C termini (Yu *et al.*, 1997), as can be seen in the PerR protein sequences shown in Figure 5.1. Crucially, a characteristic of rubredoxin containing proteins is that when in a reduced Fe(II) state the proteins are colourless however they become red upon oxidation to Fe(III), due to sulphur to Fe(II) charge transfer bands (LeGall *et al.*, 1988). This links back to Figure 5.9 where after sonication the lysed *E. coli* began to turn red at the liquid-air interface, potentially signalling the oxidation of *C. jejuni* Fe(II) bound PerR.

Additionally, the absorbance spectrum of *C. jejuni* PerR (Figure 5.11) shows a peak at 490 nm, which has also been observed in oxidised, rubredoxin-like, iron sites in proteins from other species (Weinberg *et al.*, 2004).

It is therefore suggested that *C. jejuni* PerR, when recombinantly over-expressed in *E. coli*, can bind Fe(II) in place of Zn(II). It is likely that this is not uniform across all of the PerR protein purified, but may be occurring in apo-PerR, where no zinc is already bound by the four cysteine metal site. The metal content of recombinant PerR is most likely determined by the metal content of the *E. coli* growth media at the point of induction (Dauter *et al.*, 1996; Lippard & Berg, 1994). However, data from ICP-MS analyses suggests *C. jejuni* PerR may have a selectivity for Zn(II).

To provide further evidence of iron-binding, the absorbance spectrum of PerR from *C. jejuni* was measured (Figure 5.11) and closely resembles that of published spectra from other bacterial rubredoxins (Coulter *et al.*,

1999). The absorbance bands at 370 nm and 490 nm are characteristic of an oxidised [Fe(II)Cys_4] ferric site (Lippard & Berg, 1994).

At present it is unclear if this finding is physiologically relevant or an artefact of over-expression and purification in a host bacterial strain. However, previous studies that have used *E. coli* over-expression strains to generate iron-binding proteins have frequently found zinc bound in its place, largely due to zinc binding more tightly to the cysteine co-ordinated sites, as has been previously explained (Dauter *et al.*, 1996; Eidsness *et al.*, 1992). It is therefore not possible to ascertain which metal is biologically significant in *C. jejuni*. Typically, bacteria that contain proteins with rubredoxin domains, such as *C. jejuni* (Rrc, Cj0012c), often limit zinc concentrations relative to iron concentrations to avoid the wrong metal incorporation (Lippard & Berg, 1994).

6 : Chapter Six

Investigating the function of *rrc* (*cj0012c*), a PerR regulated gene

6.1 Background

The proteomic characterisation of recombinantly expressed *C. jejuni* PerR in Chapter 5 led to the hypothesis that PerR may contain a putative rubredoxin protein domain – due to the pigmentation of the protein when in aerobic conditions. Proteins with rubredoxin domains are typically found in sulphur metabolising bacteria. A genome search of *C. jejuni* revealed the presence of one rubredoxin domain containing protein, Rrc (*cj0012c*) (Parkhill *et al.*, 2000). Rrc, a non heme iron protein was previously identified in Chapter 4 as PerR regulated, and is clearly over expressed in a *C. jejuni* Δ*perR* mutant.

Little is known about the role of Rrc in *C jejuni,* it is a novel, multi-domain metalloprotein that shares homology with two proteins. Sequence alignment suggests Rrc may be a fusion of two proteins, rubredoxin oxidoreductase and rubrerythin. The first 32 amino acid residues at the N terminal end of Rrc share 69% identity with rubredoxin oxidoreductase (Rbo), the remaining 183 amino acid residues share homology with a rubrerythin (Rbr) as shown in Figure 6.1 (Yamasaki *et al.*, 2004). Both Rbo and Rbr have been linked to oxidative stress defence in anaerobic bacteria, such as *Desulfovibrio vulgaris* (Lumppio et al., 2001).

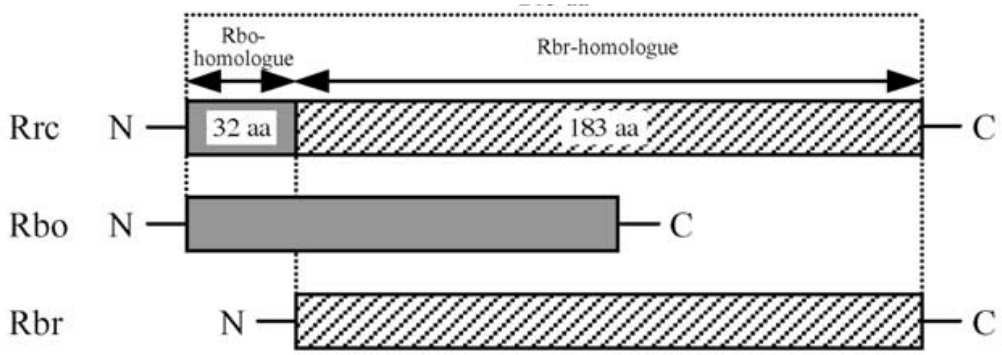


Figure 6.1: Homology of Rrc to the protein domains of rubrerythin and rubredoxin oxidoreductase (Yamasaki *et al.*, 2004).

Rrc has been linked with a role in oxidative stress after the protein was identified as being degraded in the presence of hydrogen peroxide

(Yamasaki *et al.*, 2004). It is conserved in all sequenced *Campylobacter* species to date and has been shown to be under the regulatory control of four different oxidative stress regulation systems in *C. jejuni*, incorporating PerR, Fur, CosR and CprSR regulation (Holmes *et al.*, 2005; Hwang *et al.*, 2011; Palyada *et al.*, 2009; Svensson *et al.*, 2009). Rrc-like proteins have been identified in a range of bacteria including close relatives of *C. jejuni* such as *Campylobacter coli, Wolinella succinogenes* and *Helicobacter winghamensis*.

Due to the potential biochemical similarities between Rrc and PerR, and that little is known about Rrc's function, we investigated the role of Rrc in *C. jejuni* including a potential role in oxidative stress defence.

6.2 Objectives

- To identify the function of *rrc* in *C. jejuni*

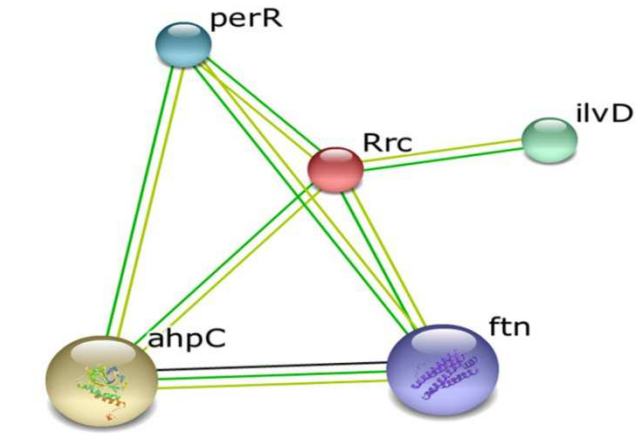
- To characterise a *C. jejuni* Δ*rrc* mutant

- To recombinantly over express, purify and characterise *C. jejuni* Rrc protein.

6.3 Results

6.3.1 Homology and Alignment searches for Rrc

The function of *rrc* in *C. jejuni* is unknown and no homologues of *rrc* have been characterised in other bacteria. In order to investigate the functional role of *rrc* a network analysis was performed using StringDB to highlight proteins that may have associated or related functions To Rrc (Franceschini *et al.*, 2013).



Rrc non-haem iron protein (215 aa) *(Campylobacter jejuni 11168)*

Predicted Functional Partners:

		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
ahpC	alkyl hydroperoxide reductase (198 aa)	•						•		0.925
ilvD	dihydroxy-acid dehydratase (558 aa)	•						•		0.900
perR	peroxide stress regulator (136 aa)	•						•		0.889
ftn	ferritin; Iron-storage protein (167 aa)	•						•		0.881

Figure 6.2: Network map of proteins that interact with *C. jejuni* Rrc. Interactions are colour co-ordinated by relationship, as detailed above. Scores indicate level of confidence, 0.150 (low confidence) to 0.900 (highest confidence) (Franceschini *et al.*, 2013).

Network analysis reveals that *rrc* has high confidence, predicted functional partnerships with four genes in *C. jejuni*, *ahpC, ilvD, perR* and *cfn*, based on gene location. Only *ilvD* (*cj0013c*) is located in the same genomic

neighbourhood as *rrc* in *C. jejuni* (*cj0012c*), whereas *ahpC (cj0334), perR (cj0322*) and *cfn (cj0612c)* are located throughout the genome (Parkhill *et al.*, 2000). This suggests that *rrc* homologues in other bacteria are closely located near these genes. To verify this, the neighbourhood location of *rrc* homologues in comparison to these predicted functional partners was analysed using StringDB (See Figure 6.3) (Franceschini *et al.*, 2013).

Figure 6.3: A StringDB alignment of *rrc* homologs across all bacterial groups. Homologs of *ahpC, ftn* and *perR* are shown if located in proximity to *rrc*.

A StringDB alignment shows *rrc* homologues are found across several groups of bacteria from Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria, Bacteroidetes chiorobi, Thermotogaceae and Elusimicrobia. In all of these groups except Bacteroidetes chiorobi *rrc* is located on the genome with a Fur family protein either directly upstream or downstream on the genome. The majority of these Fur family proteins have strong identify to PerR, however due to the high sequence identity of the Ferric Uptake protein family, in some cases Fur may be identified as a protein ‘similar to

PerR'. Species containing *rrc* and *perR* in close genomic proximity to each other are detailed in Table 6.1

Table 6.1: Species containing *perR* and *rrc* in close genomic proximity based on StringDB analysis (Franceschini *et al.*, 2013). * indicates divergent *perR-rrc* homologues.

Bacteria Name	Group	Description
Clostridium difficile CD196	Firmicutes	Anaerobic, gastrointestinal pathogen
Alkailphilus oremlandii	Firmicutes	Anaerobic, aquatic sediment
Anaerocellum thermophilum	Firmicutes	Anaerobic, hyper-thermophilic, thermal spring
Desulfotomaculum acetoxidans	Firmicutes	Anaerobic, acquatic sediment
Caldicellulosiruptor saccharolyticus	Firmicutes	Anaerobic, thermophilic, thermal spring
Frankia sp. Cc13	Firmicutes	Aerobic, root nodule forming, soil dweller
Frankia sp. EAN1pec	Firmicutes	Aerobic, root nodule forming, soil dweller
*Synechococcus elongatus 7942**	Cyanobacteria	Photoautotrophic, aquatic
Gloeobacter violaceus	Cyanobacteria	Photoautotrophic, aquatic
Fervidobacterium nodosum	Thermotogacea e	Anaerobic, hyper-thermophilic, thermal spring
Elusimicrobium minutum	Elusimicrobia	Anaerobic, insect

		intestinal tract
Desulfovibrio vulgaris hildenborough	Proteobacteria D	Anaerobic, soil dwelling
Desulfovibrio salexigens	Proteobacteria D	Anaerobic, aquatic
Desulfovibrio magneticus	Proteobacteria D	Anaerobic, acquatic sediment
Pelobacter propionicus	Proteobacteria D	Anaerobic, sewage
Pelobacter carbinolicus	Proteobacteria D	Anaerobic, aquatic
Sorangium cellulosum	Proteobacteria D	Anaerobic, soil dwelling
Desulfatibacillum alkenivorans	Proteobacteria D	Anaerobic, aquatic sediment
Syntrophobacter fumaroxidans	Proteobacteria D	Anaerobic, sewage

The species listed in Table 6.1 represents a diverse range of bacteria, many of which are anaerobic, aquatic, extremophiles. Every bacterial species listed has a *rrc* homolog located directly upstream or downstream from a Fur family homolog (PerR). Interestingly *C. difficile*, another gastrointestinal pathogen is amongst those species with PerR and Rrc in close genomic proximity.

6.3.2 The generation of a Δ*rrc* null mutant in *C. jejuni* strain NCTC 11168

To understand the physiological and functional role of Rrc in *C. jejuni* an isogenic *rrc* single mutant was constructed, and is detailed in full in Figure 6.4. Mutagenesis was performed essentially as described for *perR* and *fur* with some changes to the restriction enzymes used.

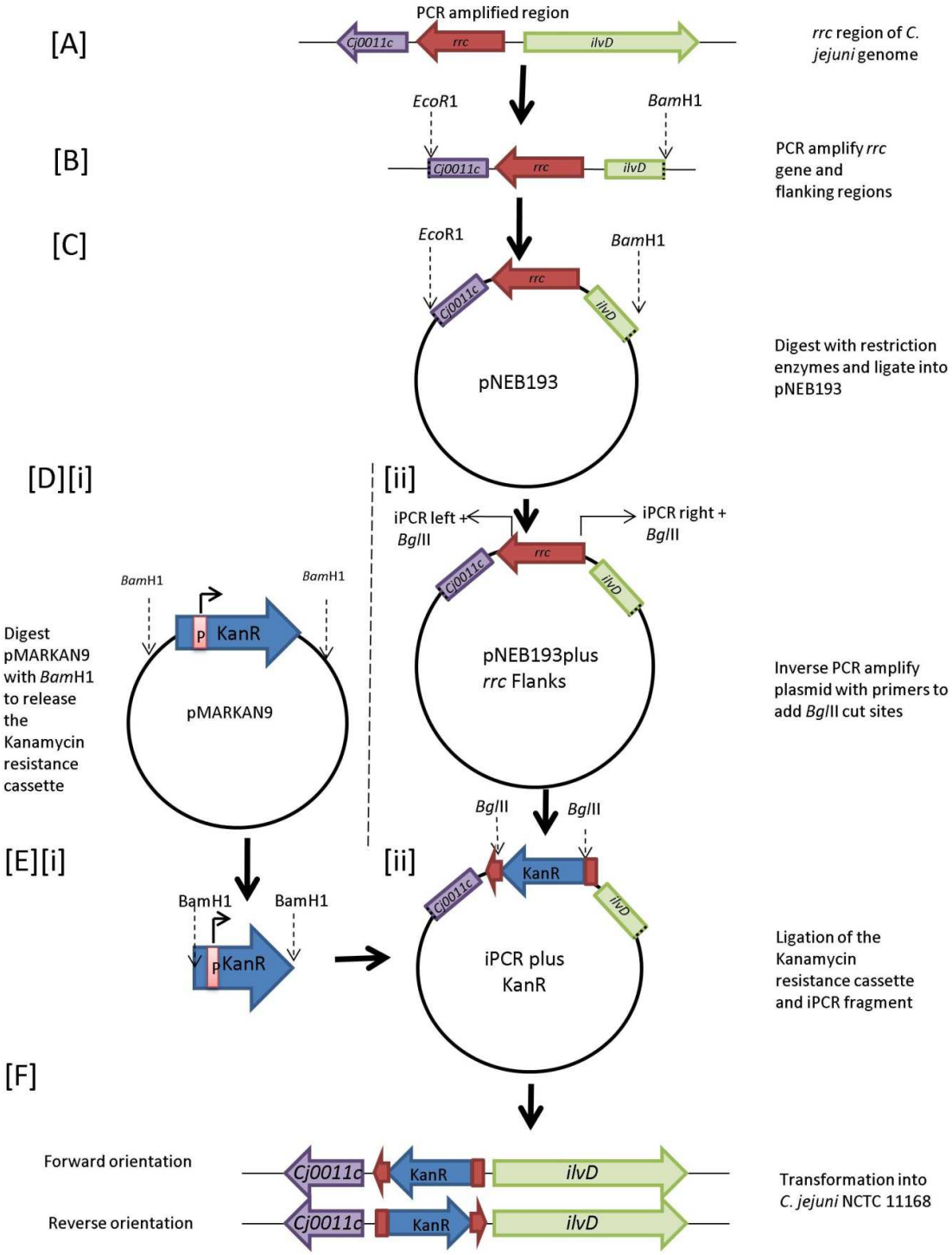


Figure 6.4: Illustration detailing the process of vector construction and *rrc* mutagenesis in *C. jejuni.* [A] The *rrc* loci of the *C. jejuni NCTC 11168* genome. PCR amplification of *rrc* and 500 bp flanking regions, adding 5' and 3' restriction sites. [B] Double restriction digest of the

rrc loci PCR fragment and pNEB193 with _Eco_RI and Bam_HI_, and subsequent ligation of the DNA fragment into cut pNEB193 [C] [i] Restriction digestion of pMARKAN9 with _Bam_HI to excise the Kanamycin resistance cassette. [ii] Inverse PCR (iPCR) amplification of pNEB193 to amplify _rrc_ flanking regions and add 5' and 3' _Bgl_II restriction enzyme sites. [D] [i] The Kanamycin resistance cassette with _Bam_HI sticky ends. [ii] Restriction digest of the pNEB193:_rrc_ inverse PCR product with _Bgl_II then ligation of the kanamycin resistance cassette to the inverse PCR product, forming a new vector 'pINK3'. Two orientations of the kanamycin cassette are possible. [E] Transformation of pINK3 into the _C. jejuni_ NCTC 11168 wild-type genome. The two possible orientations of the kanamycin cassette are shown.

The successful construction of the plasmids detailed in Figure 6.4 was determined by restriction digest and plasmid DNA sequencing. The successful allelic replacement of the wildtype *rrc* gene into an *rrc* mutant in *C. jejuni* NCTC 11168 was confirmed by PCR amplification of genomic DNA from transformed colonies grown on selective media and phenotypic analyses.

A *C. jejuni rrc* mutant was then analysed phenotypically, looking at growth, motility, oxidative stress defence and proteomics.

6.3.3 Physiological characterisation of *C. jejuni* NCTC 11168 Δ*rrc* mutants

6.3.3.1 Growth of *C. jejuni* NCTC 11168 Δ*rrc* compared to the wildtype

Characterisation of *C. jejuni* growth at 37 °C and 42 °C was performed using a FLUOstar Omega incubator under microaerobic conditions, shaking at 600 rpm in a double orbital configuration. All cultures were grown in Brucella broth at an approximate starting A_{600} nm of 0.5 in a final culture volume of 500 µl.

6.3.3.1.1 37 °C

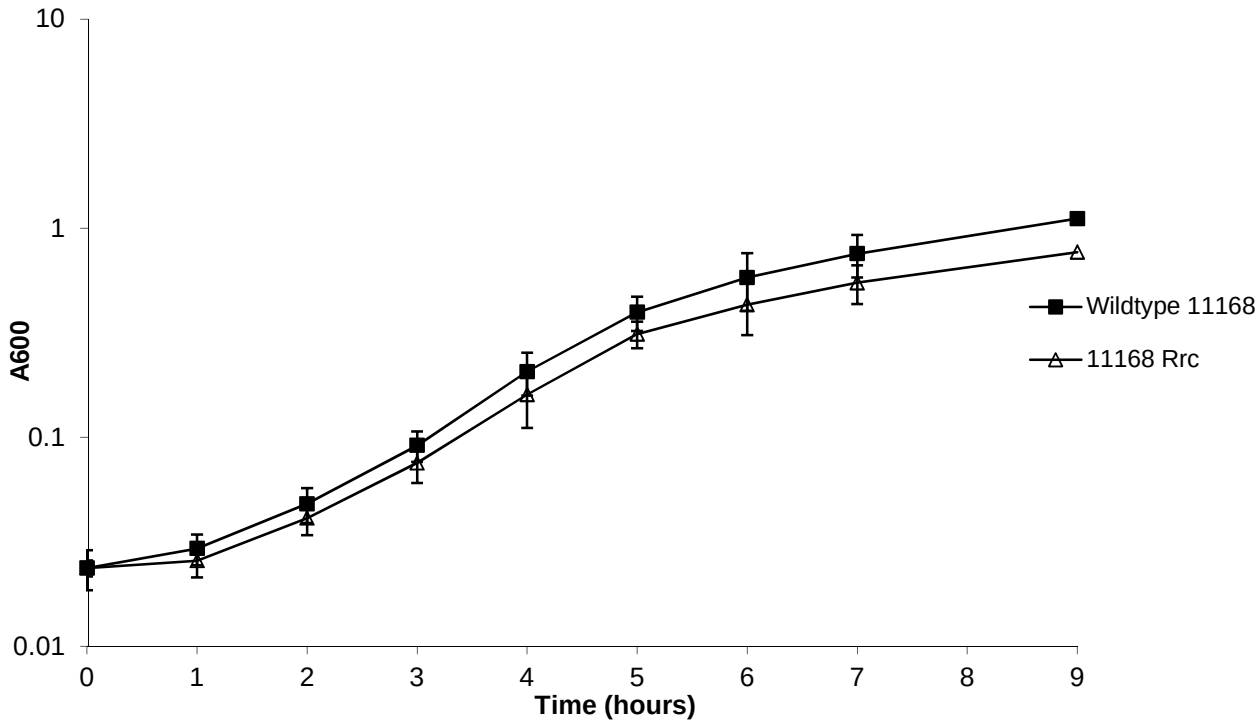


Figure 6.5: Growth of *C. jejuni* NCTC 11168 wildtype and a ∆*rrc* mutant at 37 °C. Experiments were performed in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

A *C. jejuni rrc* mutant has a wildtype growth phenotype at 37 °C, when grown in microaerobic growth conditions (Figure 6.5).

6.3.3.1.2 42 °C

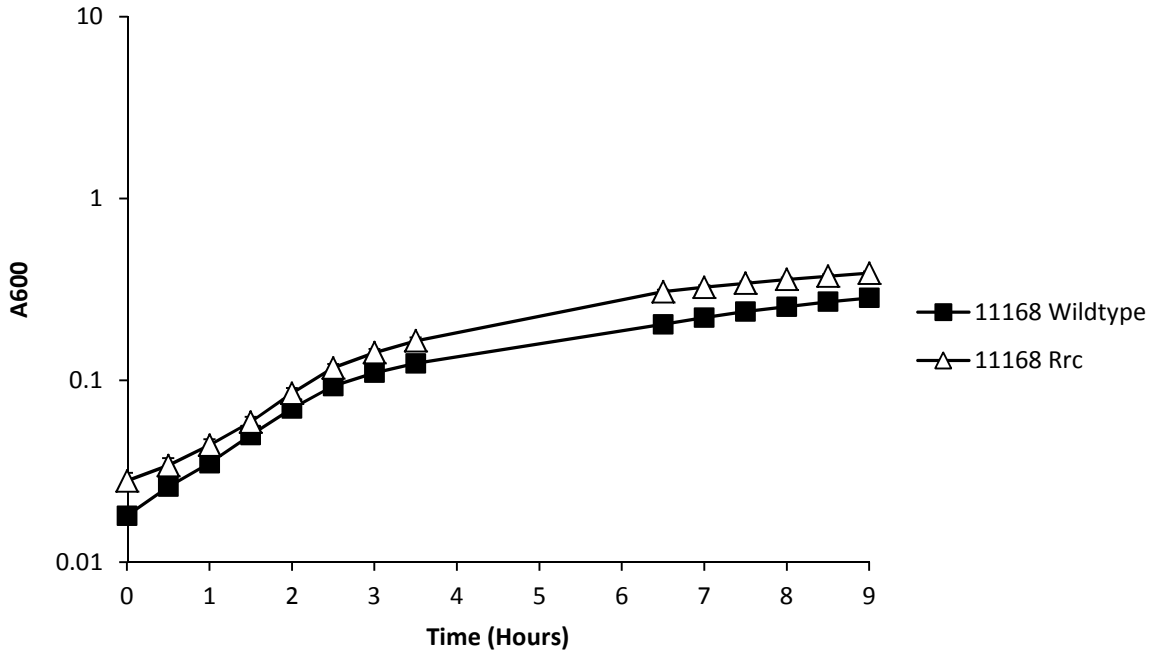


Figure 6.6: Growth of *C. jejuni* NCTC11168 wildtype and a ∆*rrc* mutant at 42 °C. Experiments were performed in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

A *C. jejuni rrc* mutant also demonstrates a wildtype growth phenotype at 42 °C, indicating no role for *rrc* in the normal growth of *C. jejuni* (Figure 6.6).

6.3.4 Motility

C. jejuni NCTC 11168 wildtype and ∆*rrc* strains were grown in Brucella broth culture over night, 5 µl of culture from each strain was placed onto the surface of a soft agar plate (0.4 % agar). Plates were incubated microaerobically for 37 °C for two days..

Motility was measured using ImageJ analysis software (Schneider *et al.*, 2012) to measure bacterial movement based on photographs taken after 24 and 48 hours. The movement of *C. jejuni* NCTC 11168 wildtype and ∆*rrc* strains across the semi-solid (0.4%) agar plates were compared to a non motile control, 11168∆*flaAB.*

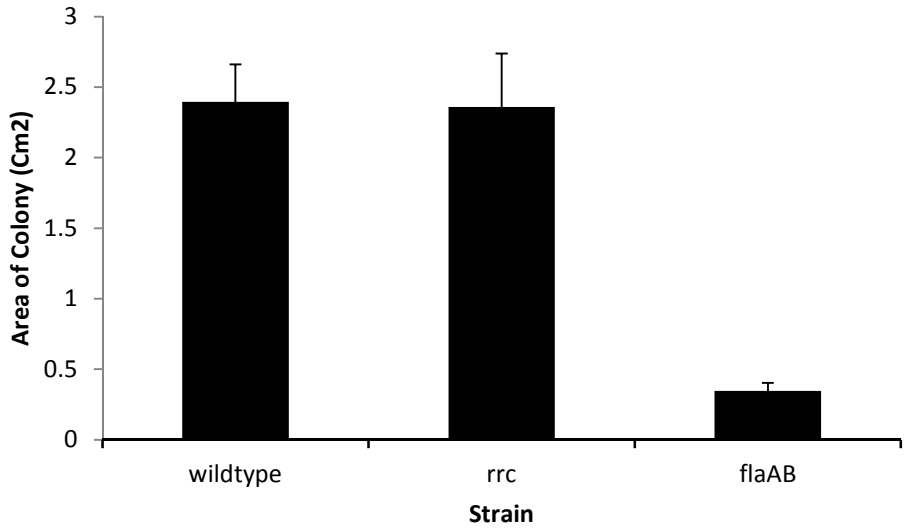


Figure 6.7: The motility of *C. jejuni* 11168 wildtype, Δ*rrc* and Δ*flaAB*. The graph shows the average distance of each bacterial halo after three days as determined by ImageJ image analysis software (Schneider *et al.*, 2012). Experiments were performed in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

An Δ*rrc* mutant in *C. jejuni* NCTC 11168 shows wildtype movement (Figure 6.7), indicating no role for *rrc* in bacterial motility and movement in *C. jejuni*.

6.3.4.1 Oxidative Stress

As StringDB analyses (Figure 6.3) showed a potential functional relationship between *rrc* and several oxidative stress defence proteins in *C. jejuni,* we investigated whether Rrc had a role in oxidative stress defence.

As in Chapter 3, *C. jejuni* NCTC 11168 strains were exposed to oxidants on solid media by means of a plate inhibition assay. Filter paper disks were placed onto the surface of a plate of bacteria, and spotted with 10 µl of oxidant, 3% hydrogen peroxide (H_2O_2) in water (H_2O) (v/v), 3% cumene hydroperoxide (CHP) in dimethyl sulfoxide (DMS) or phosphate buffer saline (PBS) as a negative control. Sensitivity of *C. jejuni* to oxidants is indicated by a zone in which no bacterial growth is seen around the filter paper disk. Zones of no growth were measured using ImageJ image analysis software (Schneider *et al.*, 2012).

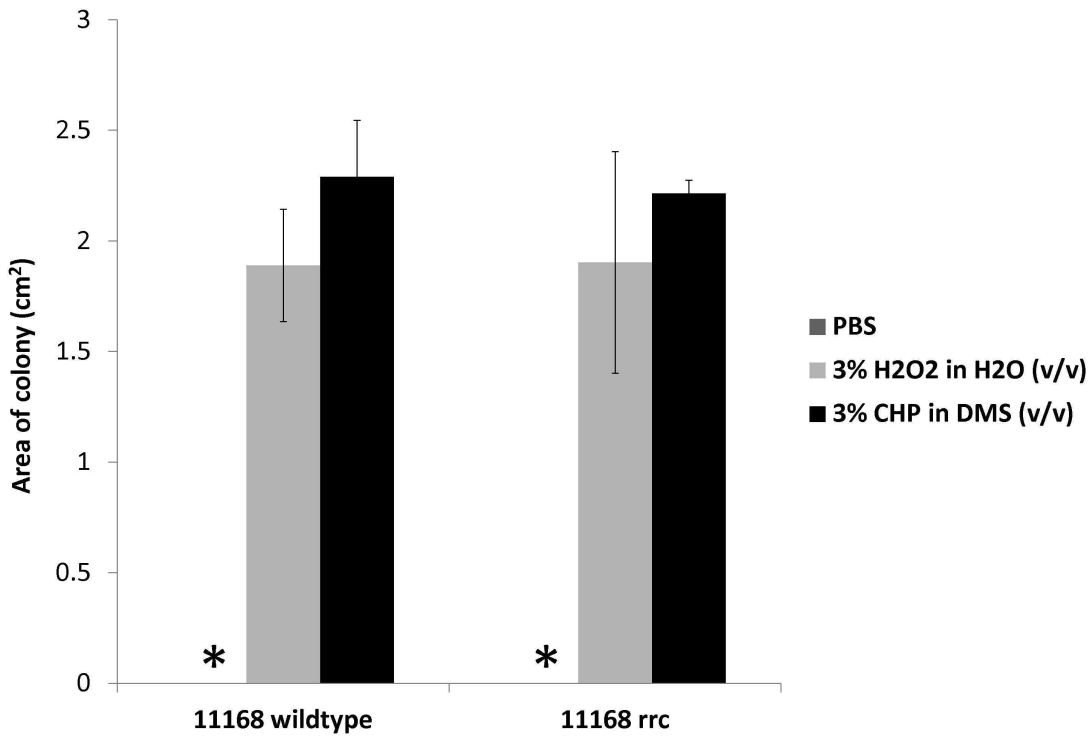


Figure 6.8: A *C. jejuni* NCTC 11168 *rrc* mutant shows wildtype survival to hydrogen peroxide (H_2O_2) and cumene hydroperoxide (CHP) in disk assay format. Experiments were performed in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

When exposed to two different sources of oxidative stress, (3% hydrogen peroxide and 3 % cumene hydroperoxide) a *C. jejuni* NCTC 11168 Δ*rrc* mutant had no reduced ability to survive compared to the wildtype strain (Figure 6.8).

6.3.4.2 Aerobic Tolerance

To explore the effect of *rrc* mutation on the aerobic tolerance of *C. jejuni* broth cultures were grown microaerobically overnight at 37 °C. The A_{600} of these cultures were fixed to 0.4 using PBS and then moved into an aerobic 37 °C incubator. The *C. jejuni* cultures were monitored for cell

viability by plating serial dilutions of the cultures at 0, 3, 6 and 9 hours aerobic exposure.

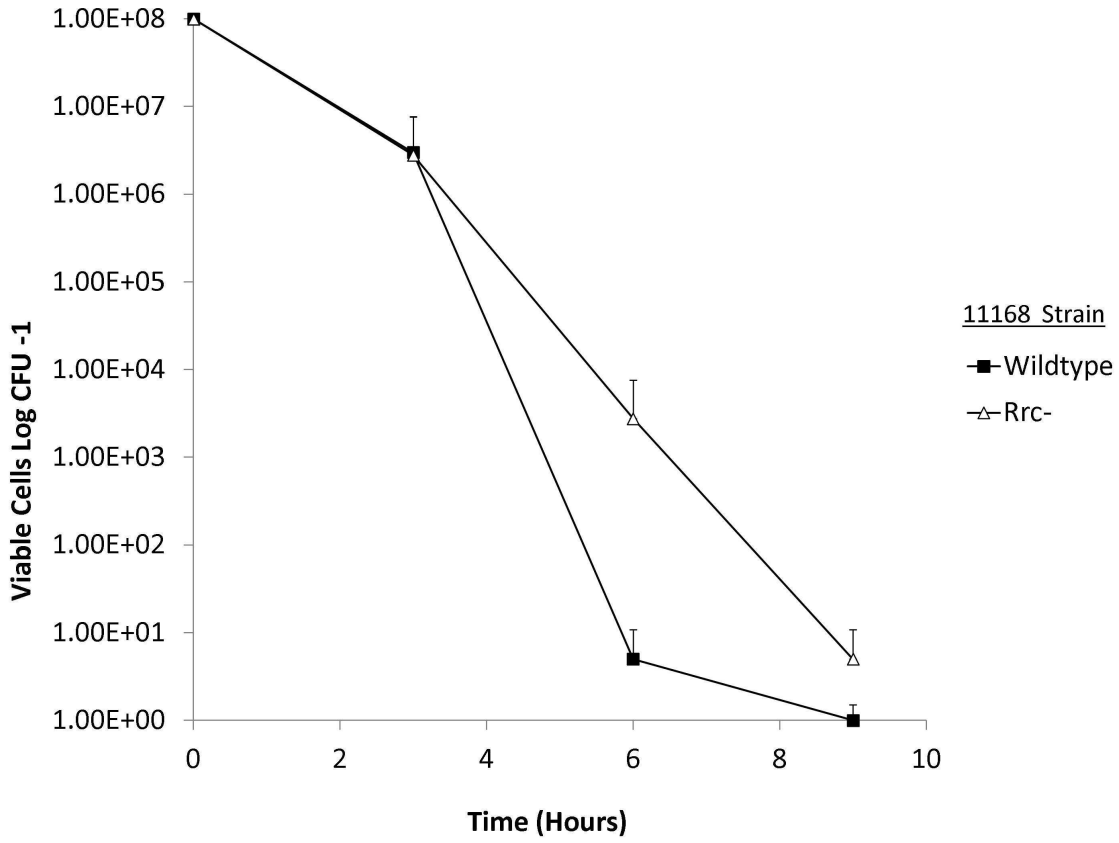


Figure 6.9: A *C. jejuni rrc* mutant is more tolerant than the wildtype strain to aerobic conditions. Experiments were performed in triplicate and error bars indicate standard error of the mean (GraphPad Prism).

A *C. jejuni* Δ*rrc* mutant is more tolerant to aerobic conditions than the wildtype strain. The number of viable cells of the Δ*rrc* mutant strain decreased slower than the wildtype after exposure to aerobic conditions for 6 hours. The increased aerobic tolerance of the *C. jejuni* Δ*rrc* mutant compared to the wildtype is reminiscent of the increased aerobic tolerance of a *C. jejuni* Δ*perR* mutant (as seen in Chapter 3), although the phenotype is not as defined in Δ*rrc*.

6.3.5 Analysis of the proteomic profile of *C. jejuni* NCTC 11168 Δ*rrc* by 2D Gel Electrophoresis

As there were no significant phenotypic changes in a *C. jejuni* NCTC 11168 Δ*rrc* mutant the proteome of Δ*rrc* was characterised to determine deletion of the gene had any effects on protein expression, which may indicate a function. Two dimensional gel electrophoresis was performed as described previously in Chapter 4. Proteins were initially separated by isoelectric point and then by molecular weight.

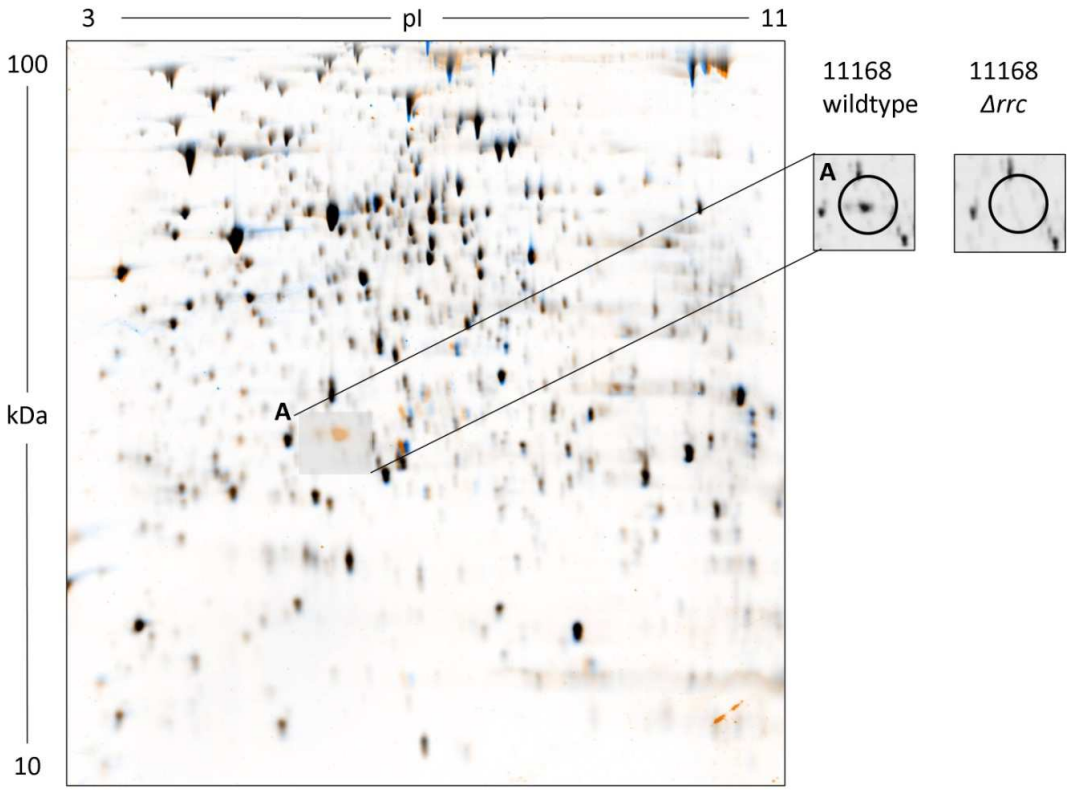


Figure 6.10: Overlaid 2D gel images depicting the proteome of *C. jejuni* NCTC 11168 wild-type (orange) compared to a *C. jejuni* Δ*rrc* mutant (blue). *C. jejuni* were grown in Brucella broth at 37 °C under microaerobic conditions (5 % O_2, 10 % CO_2 with shaking) until late log phase. Differentially expressed proteins are highlighted.

Proteomic analysis of a Δ*rrc* revealed no significant changes compared to the wildtype. The only protein expression that varied was that of Rrc itself, shown in Box A, as would be expected of an Rrc knockout strain.

6.3.6 Over expression of *C. jejuni* Rrc protein recombinantly expressed in *E. coli*

C. jejuni rrc was amplified, cloned and over expressed as described previously for PerR in Chapter 5. Recombinant *C. jejuni* Rrc was cloned into vector pET28a to form vector pINK1 and expressed in *E. coli* Bl21(DE3). Recombinant *C. jejuni* Rrc was over expressed with a removable 6-His tag, comprised of six histidine residues added to the C-terminal end of the protein.

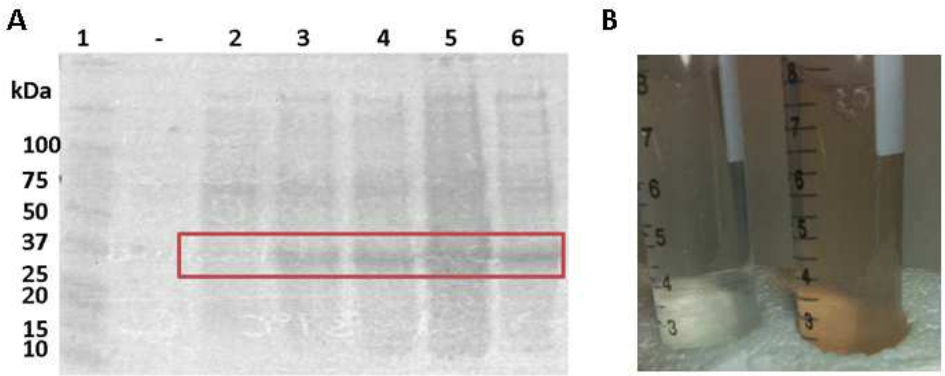


Figure 6.11: A= SDS-PAGE analysis of whole cell *E. coli* over-expressing *C. jejuni* Rrc (Red box) at 0, 2 and 4 hours post IPTG induction (Lanes 2-4). Lanes 5 and 6 show the distribution of Rrc across the insoluble and soluble cell lysate fractions, respectively. Lane 1 shows a standard molecular weight ladder (Precision Plus (Biorad)), sizes are in kDa. B = Falcon tubes of Buffer A (left) and soluble *E. coli* extract containing recombinant *C. jejuni* Rrc (right).

Figure 6.11A shows the successful over expression of a protein with an approximate molecular weight of 30 kDa, at 2 and 5 hours after IPTG induction. Lane 6 shows the soluble expression of this protein and Figure 6.11B shows the red pigmentation of *E. coli* cell extract, as is characteristic of oxidised rubredoxin domain containing proteins.

6.4 Discussion

6.4.1 Predicted functions for Rrc based on genomic loci

Rrc was suggested to have a close functional relationship to PerR, AhpC, Ftn and IlvD in *C. jejuni,* based on evidence from a StringDB analysis (Figure 6.2) (Franceschini *et al.*, 2013). In *C. jejuni*, IlvD is encoded by *cj0013c* and as such is located directly downstream from Rrc, although no functional relationship between these two proteins has been described the predicted link is likely to be based upon the genes close proximity to each other in the *C. jejuni* genome (Parkhill *et al.*, 2000).

AhpC and Ftn are both oxidative stress responsive proteins in *C. jejuni*, indicating that Rrc may also have a role in oxidative stress in *C. jejuni*. Ferritin is an iron storage protein and functions as a protective defense against oxidative stress by storing intracellular iron to prevent Fenton chemistry and the production of reactive oxygen species in the presence of oxygen. However ferritin may also have roles in the provision of metal ions to Rrc as it is an iron binding protein (Butcher *et al.*, 2010; Yamasaki *et al.*, 2004).

Interestingly PerR is suggesed to be functionally tied to Rrc in Figure 6.2. Rrc is regulated by PerR, amongst other regulators, in *C. jejuni* (as is discussed in Chapter 4), however PerR is the only regulator functionally linked to Rrc by StringDB analysis. StringDB connects PerR and Rrc by neighbourhood, yet they are not located in the same genomic region the *C. jejuni*, Rrc is encoded by *cj0012c* and PerR by *cj0322* (Parkhill *et al.*, 2000). However, the genome of *C. jejuni* is less ordered than other bacterial species and as such has less functional gene groupings and fewer operonic gene structures (Parkhill *et al.*, 2000). Upon analysing the genomic location of *rrc* homologues in other bacterial species (Figure 6.3) *rrc* is frequently located in series with homologues of PerR, or other highly similar members of the Fur Family. Close genomic proximity does not prove a functional relationship between two genes but may hint at co-regulation.

Many of the bacterial species in Table 6.1 are anaerobic and isolated from extreme or inhospitable habitats. It is tempting to postulate that if a functional link between PerR and Rrc existed then perhaps it would have a role in survival under extreme stress, or survival in conditions unsuitable for growth, such as during *C. jejuni* transmission through aerobic environments, clearly further work is required to establish the role of Rrc.

6.4.2 Biochemical characterisation of *C. jejuni* Rrc

A red pigmented protein of approximately 30 kDa was successfully over expressed in *E. coli*. Although the protein was not identified by mass spectrometry, the red pigmentation and close predicted molecular weight (*C. jejuni* Rrc is 22.8 kDa) provide evidence that the protein was Rrc. Following this protein expression no further biochemical protein characterisation was conducted due to the extensive biochemical work conducted by Pinto *et al*, 2011, who determined both the electronic and redox properties of each metal binding site in Rrc via UV-vis, EPR and resonance Raman spectroscopies. Rrc was shown to contain two $FeCys_4$ sites similar to the putative $FeCys_4$ site in *C. jejuni* PerR, with reduction potentials of +240 and +185 mV versus NHE and a μ-oxo-bridged diiron site (reduction potentials of +270 and +235 mV) (Pinto *et al.*, 2011).

6.4.3 A functional role for Rrc in *C. jejuni*

A *rrc* mutation in *C. jejuni* produced few phenotypical changes compared to the wildtype strain. The deletion of *rrc* did not affect bacterial growth at 37 °C (Figure 6.5) or 42 °C (Figure 6.6) nor did it affect bacterial motility (Figure 6.7). An Δ*rrc* mutant had wildtype phenotypes when exposed to low levels (3%) of oxidative stress generating compounds hydrogen peroxide and cumene hydroperoxide (Figure 6.8). The Δ*rrc* mutant did show increased survival in aerobic conditions compared to the wildtype strain (Figure 6.9). However proteomic analyses revealed no explanation for this phenotype, and showed that the deletion of *rrc* had no effect on protein expression in *C. jejuni* under normal growth conditions, other than the removal of *rrc* expression itself (Figure 6.10).

Pinto et al, 2011 identified that Rrc protein has a significant NADH-linked hydrogen peroxide reductase activity of 1.8 ± 0.4 lmol H_2O_2 min^{-1} mg^{-1}, breaking hydrogen peroxide down into water (Pinto *et al.*, 2011), which is consistent with Rrc having a role in oxidative stress detoxification in *C. jejuni*.

7 Final Conclusions

The work presented in this thesis has expanded upon previous knowledge of *C. jejuni* biology. Firstly, it has been shown that a *C. jejuni* Δ*perR* mutant has a greater capacity for detoxifying oxidative stress than was previously known, showing a 10-fold increase in resistance to hydrogen peroxide than had previously been reported, (resistance to 3% hydrogen peroxide (in water v/v/), whereas we demonstrate resistance to 30% hydrogen peroxide). The high resistance of a Δ*perR* mutant to oxidative stress is through the de-repression and therefore over abundance of a number of oxidative stress detoxification proteins, which have been indentified in this study (KatA, AhpC, Rrc, TrxB). Whilst a Δ*perR* mutant is not the wildtype strain, it is representative of the level of gene expression the wildtype strain is capable of mediating when under oxidative stress conditions, when PerR gene repression would be relieved.

Secondly, we have shown the PerR regulator to have some function in the aerobic tolerance of *C. jejuni*. This is a potentially significant finding, as whilst mechanisms of aerobic tolerance have not been defined in *C. jejuni*, the identification of PerR as a potential regulator of these mechanisms narrows the search for the proteins involved in surviving aerobic exposure. It is likely that some of the genes repressed by PerR promote aerobic tolerance, as is evident by the increased aerotolerance of the Δ*perR* mutant. As previously discussed, the aerobic tolerance of *C. jejuni* likely is essential part of *C. jejuni*'s success as a pathogen, and likely contributes to the numerous cases of *C. jejuni* food poisoning each year. Without the ability to tolerate aerobic conditions *C. jejuni* would not survive transmission into the human food chain.

This study also characterised the cross talk between the Fur and PerR regulators in *C. jejuni* gene transcription. We reveal that the only role for Fur in the mediation of oxidative stress defenses in *C. jejuni* is in the overlapping regulation of genes with PerR (katA, trxB). It has been previously postulated that Fur is capable of recognising and binding PerR boxes found upstream of

PerR regulated genes, a potential explanation for the crosstalk between these two regulators (van Vliet *et al.*, 2002). This could explain the apparent regulation of catalase expression by Fur, increased expression of catalase was observed in the double ΔfurΔperR mutant compared to the single Δ*perR* mutant, but there was no expression of catalase in a single Δ*fur* mutant. This is suggestive that Fur may have some affinity for PerR boxes in *C. jejuni*, although further investigation of this phenomenon is required.

To date no mechanism of oxidative stress sensing has been described for PerR in *C. jejuni*, although the crystal structure of *C. jejuni* PerR protein is still to be solved. In *B. subtilis* the mode of sensing ROS in PerR is thought to be mediated by the oxidation of the metal coordinating residue His-37, and then the subsequent formation of 2- oxo-histidine through reaction with the ROS. However, in PerR from *S. pyogenes* His-37 is not involved in metal ion coordination, and it is likely that *S. pyogenes* PerR has an alternate method of sensing ROS (Makthal *et al.*, 2013). As there is no crystal structure for *C. jejuni* PerR, the location of His-37 can not be determined and as such the mode of ROS sensing cannot be determined. However this study has provided biological insights into the *C. jejuni* PerR protein. We have demonstrated the ability of PerR to bind several metal ions and have proposed that *C. jejuni* PerR is able to coordinate iron in the four-cysteine, metal binding domain, which is usually associated with zinc binding and dimerization. The coordination of iron by four cysteines forms a putative rubredoxin domain (as discussed in Chapter 5). A protein domain that in other bacterial species roles in the donating electrons to oxidative stress defense enzymes and also the reduction of Fe^{3+} to Fe^{2+}(Coulter & Kurtz, 2001). This poses interesting questions regarding the binding of iron by PerR, such as does the *C. jejuni* PerR regulator also have a functional role in oxidative stress detoxification? It is unclear whether the binding of iron by the PerR dimerization domain is physiologically relevant, and may be an artefact of recombinant purification of *C. jejuni* PerR in *E. coli.*

We have identified that Rrc, the Rubrerythin-rubredoxin oxidoreductase like protein of *C. jejuni* is closely associated with PerR in

other bacteria, suggesting it may also be closely associated with *C. jejuni* PerR (although a functional link between the two proteins has yet to be identified). We attempted to characterise the role of Rrc in *C. jejuni* by investigating the phenotype of a Δ*rrc* mutant. We found no phenotypical variations between Δ*rrc* and the wildtype strain, other than a slight increase in aerobic tolerance in the *rrc* null strain that we have not been able to explain.

Overall there are still questions regarding the survival and proliferation of *C. jejuni* in conditions not permissible for growth. It is clear that *C. jejuni* is an adaptive and diverse organism and much remains to be elucidated before we can begin to combat the high rates of human infection with *C. jejuni.*

8 Future Directions

The oxidative stress phenotype of a *C. jejuni* Δ*perR* mutant has been characterised and the phenotype of this strain represents the full, unregulated oxidative stress responses that *C. jejuni* are capable of producing, giving insight into the level of stress *C. jejuni* can survive. It would be interesting to further characterise the role of PerR in *C. jejuni's* survival throughout transmission in an aerobic environment, and determine if deletion of PerR confers any reduced ability to colonise or infect a *C. jejuni* host. Equally, it would be interesting to expand upon the ability of PerR to survive antibiotics exposure and establish the protection conferred, if any, by a Δ*perR* mutation on the resistance of *C. jejuni* to bactericidal killing by antibiotics.

From these investigations it is clear that the Δ*perR* mutant responds better to oxidative stress than the wildtype strain, as many of the oxidative stress response proteins are already being expressed in high levels. However, further work is needed to characterise the oxidative stress responses of the *C. jejuni* wildtype and elucidate the physiologically relevant signals to relieve PerR gene repression in a wildtype strain. Further investigations are required into the role of PerR in *C. jejuni* to decipher why PerR regulation persists despite the beneficial nature of the *perR* gene deletion for survival.

To date there are still many questions governing gene regulation in *C. jejuni*. In particular there is little overlap between comparative transcriptional studies, something that needs to be addressed before consensus regulons can be agreed upon for regulators such as PerR and Fur in *C. jejuni.* Equally, deciphering and further characterising the interplay of Fur and PerR in *C. jejuni* would be beneficial, however the regulatory activity of these proteins is complicated by the binding and bioavailability of metal ions, in particular iron has been shown to have large regulatory effects in *C. jejuni* (Palyada et al., 2004; van Vliet et al., 2002*)*.

Although we have provided some insights into *C. jejuni* PerR's biochemistry, the crystal structure of the protein still remains to be

elucidated. A crystal structure has recently been solved for *C. jejuni* Fur, showing substantial changes from other characterised Fur proteins, which may hint towards *C. jejuni* PerR also having unique features. The crystal structure of Fur family regulators allows us to understand the effect that metal binding has on protein conformation and DNA binding activities. Future studies should seek to solve the crystal structure of PerR, equally metal content analyses of *C. jejuni* Fur and PerR need to be performed under to fully elucidate the effect of metal binding on gene regulation by Fur and PerR in *C. jejuni*.

To date the majority of metal content work has been via exogenously added metal ions to recombinantly purified protein, future work should aim to discover the physiologically relevant roles of metal in protein extracted from *C. jejuni* itself. Equally, future metal analysis on *C. jejuni* PerR is required to confirm the binding of iron in the metal site predicted to be co-ordinated by four cysteines in an attempt to fully explain the red pigmentation of PerR protein during recombinant purification, and also to ascertain whether this has any physiological significance.

The long term goal of *C. jejuni* PerR research is to elucidate the mechanism of PerR's signal induced switch that governs the repression or transcription of DNA, and this process still remains to be elucidated.

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10 Appendix

10.1 Chapter Four: Supplementary Data

Genes that were identified as being differentially regulated in microarray analyses performed by *Reuter et al* on *C. jejuni* NCTC 11168 Δ*fur,* Δ*perR* or Δ*fur*Δ*perR* strains, yet eliminated as members of the Fur or PerR regulons are detailed below. Genes are grouped by the mutant strain(s) they were identified in.

Δ*fur* growth altered genes:
Cj0009, Cj0021c, Cj0030, Cj0033, Cj0052, Cj0059c, Cj0076c, Cj0093, Cj0099, Cj0110, Cj0125c, Cj0168c, Cj0183, Cj0208, Cj0223, Cj0225, Cj0226, Cj0229, Cj0251c, Cj0297c, Cj0300c, Cj0301c, Cj0308c, Cj0312, Cj0328c, Cj0329c, Cj0346, Cj0348, Cj0364, Cj0403, Cj0404, Cj0408, Cj0411, Cj0417, Cj0427, Cj0448c, Cj0449c, Cj0485, Cj0500, Cj0509c, Cj0510c, Cj0530, Cj0531, Cj0534, Cj0535, Cj0536, Cj0551, Cj0553, Cj0554, Cj0566, Cj0567, Cj0599, Cj0662c, Cj0663c, Cj0689, Cj0690c, Cj0729, Cj0734c, Cj0736, Cj0757, Cj0758, Cj0762c, Cj0770c, Cj0771c, Cj0772c, Cj0776c, Cj0793, Cj0832c, Cj0833c, Cj0843c, Cj0844c, Cj0864, Cj0879c, Cj0903c, Cj0909, Cj0919c, Cj0920c, Cj0940c, Cj0947c, Cj0964, Cj0997, Cj1022c, Cj1023c, Cj1024c, Cj1031, Cj1040c, Cj1045c, Cj1060c, Cj1072, Cj1099, Cj1173, Cj1183c, Cj1184c, Cj1211, Cj1258, Cj1265c, Cj1266c, Cj1267c, Cj1309c, Cj1318, Cj1326, Cj1327, Cj1328, Cj1329, Cj1333, Cj1335, Cj1336, Cj1337, Cj1340c, Cj1341c, Cj1342c, Cj1353, Cj1360c, Cj1369, Cj1370, Cj1380, Cj1381, Cj1393, Cj1413c, Cj1424c, Cj1428c, Cj1430c, Cj1451, Cj1460, Cj1484c, Cj1485c, Cj1486c, Cj1502c, Cj1503c, Cj1508c, Cj1540, Cj1541, Cj1543, Cj1558, Cj1567c, Cj1569c, Cj1570c, Cj1571c, Cj1572c, Cj1574c, Cj1575c, Cj1576c, Cj1583c, Cj1584c, Cj1589, Cj1602, Cj1609, Cj1610, Cj1619, Cj1621, Cj1622, Cj1624c, Cj1625c, Cj1647, Cj1664, Cj1711c, Cj1726c, Cj1727c.

Δ*perR* growth altered genes:

Cj0013, Cj0120, Cj0149c, Cj0270, Cj0333c, Cj0423, Cj0424, Cj0425, Cj0565, Cj0625, Cj0626, Cj0747, Cj0884, Cj1159c, Cj1225, Cj1236, Cj1345c, Cj1667c.

Δ*fur*Δ*perR* growth altered genes:
Cj0010c, Cj0169, Cj0200c, Cj0519, Cj0520, Cj0722c, Cj0987c, Cj1186c, Cj1189c, Cj1305c, Cj1399c, Cj1400c, Cj1401c, Cj1402c, Cj1403c, Cj1500, Cj1513c, Cj1616, Cj1618c, Cj1626c, Cj1721c.

Δ*fur* and Δ*perR* growth altered genes:
Cj0037c, Cj0073c, Cj0074c, Cj0075c, Cj0167c, Cj0454c, Cj0533, Cj0748, Cj0834c, Cj1358c, Cj1421c, Cj1488c.

Δ*fur,* Δ*perR* and Δ*fur*Δ*perR* growth altered genes:
Cj0145, Cj1533c, Cj0036, Cj0391c, Cj0481, Cj0486, Cj0487, Cj1296, Cj1297, Cj1314c, cj1315c, Cj1316c, Cj1450, Cj1489c, Cj1490c, Cj1656c, Cj1682.

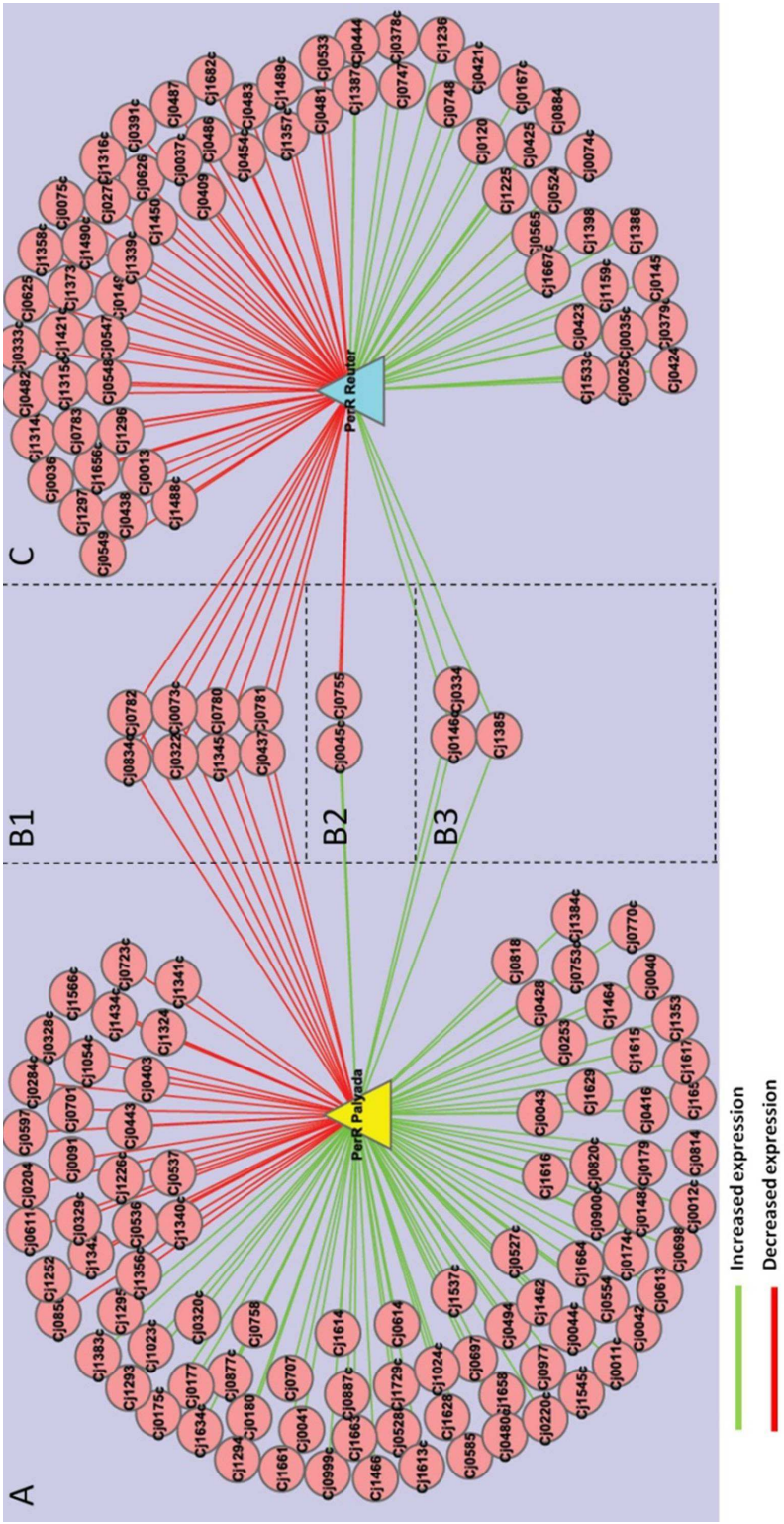


Figure 4.2: A network analysis comparing the genes identified as having altered transcription in two separate microarray analyses of *C. jejuni perR* mutants. Genes identified as being differentially regulated in a *C. jejuni* NCTC 11168 *perR* mutant are segregated based on which study they were identified in.

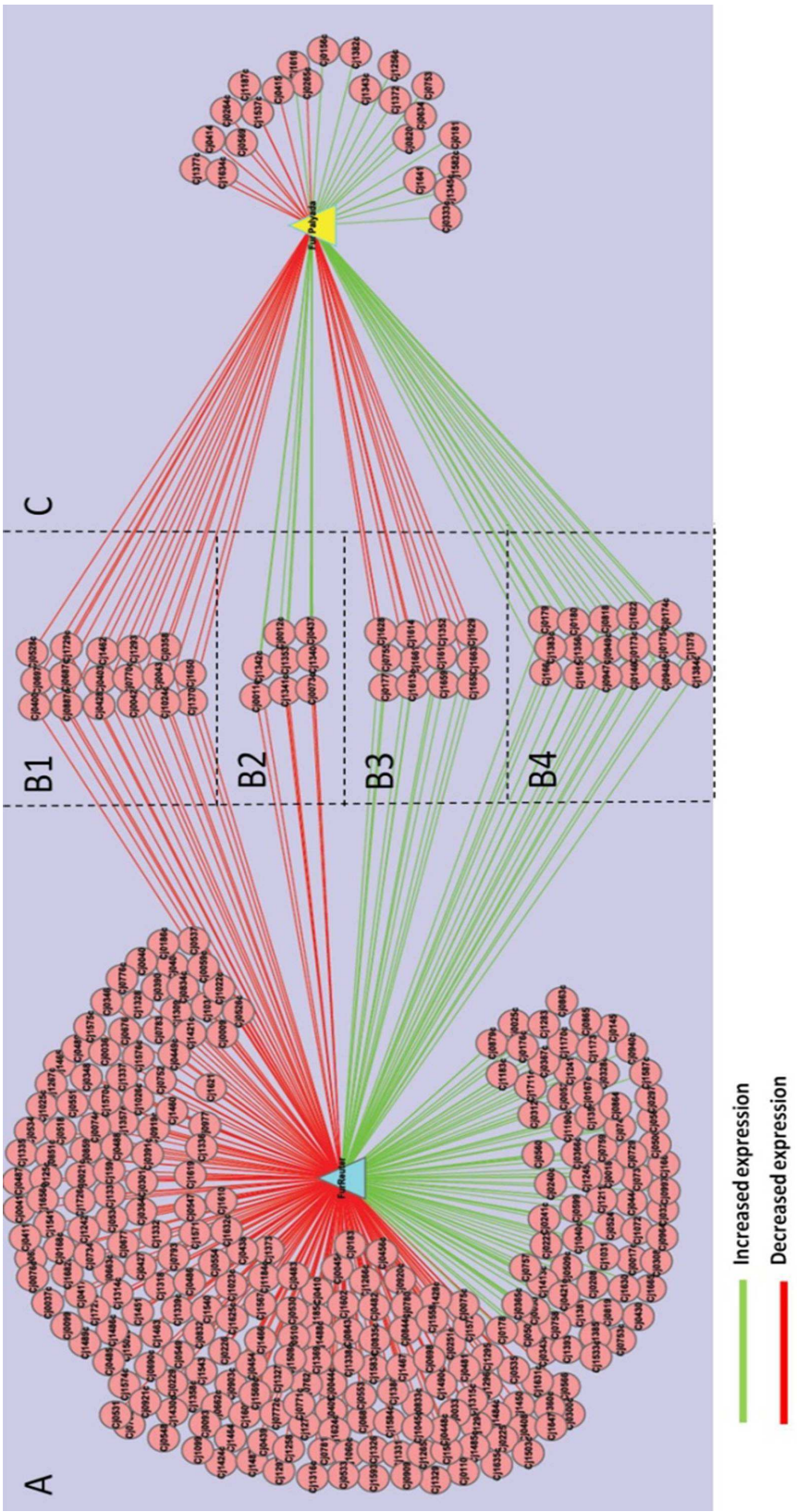
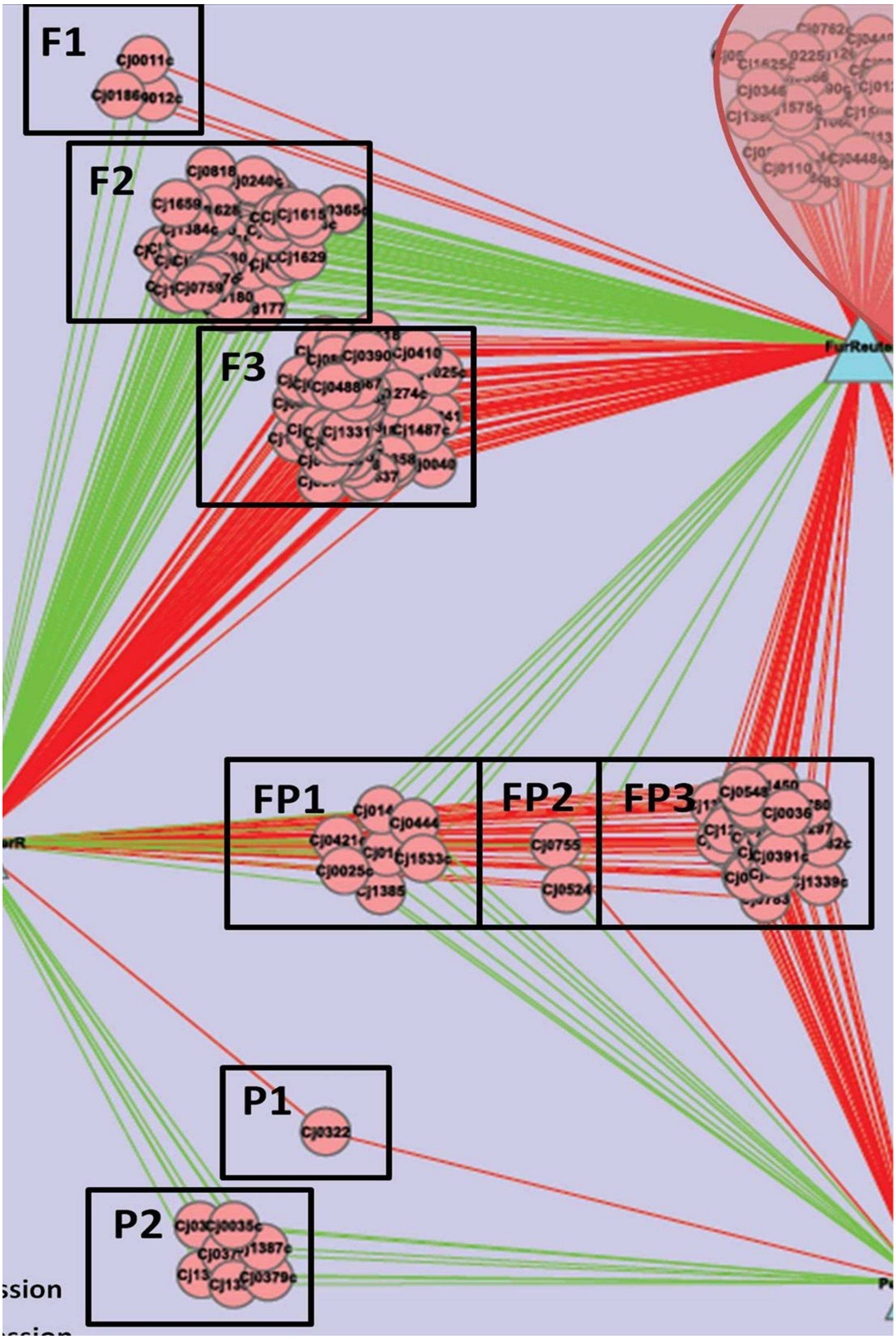


Figure 4.3: A network analysis comparing the genes identified as having altered transcription in two separate microarray analyses of *C. jejuni Δfur* mutants. Genes identified as being differentially regulated in a *C. jejuni* NCTC 11168 Δ*fur* mutant are segregated based on which study they were identified in.



Section of Figure 4.4: A Cytoscape network map comparing differentially regulated genes in single Δ*fur* and Δ*perR* mutants to the differentially regulated genes in a Δ*fur*Δ*perR* double mutant from the Reuter *et al* study.