Regulating *Campylobacter jejuni* flagellar gene expression: transcriptional and post-transcriptional mechanisms of control

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Abstract

The bacterial pathogen *Campylobacter jejuni* is the leading cause of foodborne gastroenteritis in the developed world. *C. jejuni* flagella are crucial virulence determinants, but the regulation of these complex organelles within different environments is not fully understood. Moreover, regulatory RNAs are important for virulence and flagellar gene expression in many prokaryotes, but their role in *C. jejuni* biology is unknown.

The first aim was to understand flagellar regulation in acidic conditions and what effect this has on virulence. The most acidic pH *C. jejuni* was able to survive was pH 3.6 and acid-shock at this pH and pH 5 increased expression of a subset of flagellar genes and increased invasion of intestinal epithelial cells.

The second aim of this study was to characterise the function of two paralogous small non-coding RNAs (less than 50 nucleotides), NC1 and NC4, which were identified in the *C. jejuni* NCTC11168 transcriptome and are predicted to regulate flagella gene expression. NC1 and NC4 expression was dependent on the flagellar sigma factor, $sigma^{28}$, and post-transcriptionally regulated expression of predicted $sigma^{54}$-dependent *C. jejuni* flagellar gene targets in an *E. coli* based GFP reporter system. However, microarray and phenotypic analysis showed no clear differences in gene expression between *NC1*/*NC4* deletion and over-expression mutants compared to the wild-type strain.

The conclusions are that flagellar gene expression is regulated by acidic conditions and *C. jejuni* invasion of intestinal epithelial cells may be primed in response to acid. In addition, the transcription of NC1 and NC4 is linked to flagella expression and they may function to post-transcriptionally regulate $sigma^{54}$-dependent flagella genes in *C. jejuni*. Although the biological significance of NC1 and NC4 remains unknown, this is the first study to show that non-coding RNAs are potential regulators of gene expression in *Campylobacter*.

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Chapter 1 Introduction to *Campylobacter jejuni* and bacterial flagella

Section 1 *Campylobacter jejuni*

1.1 Brief history

The family Campylobacteraceae includes the genera *Campylobacter*, *Arcobacter* and *Sulfurospirillum* (On 2005), and the genus *Campylobacter* was first classified in 1963 by Selbold (On 2005). Before that time, they were described as *Vibrio*, being spiral-rod shaped cells, and are thought to have been described as early as the 1880's by Escherich (Butzler 2004). Vibrio-like species were reported a number of times, mostly within a veterinary setting causing abortion in livestock (Butzler 2004). The first reported human case of what was later characterised as *C. jejuni* infection originated from milk and was identified by microscopy of patients' faeces and blood culture samples (Levy 1946). In 1977, Skirrow reported successful culture of *C. jejuni* using a selective media in low oxygen concentrations (Skirrow 1977), which is still in use today.

There are 17 formally recognised species of *Campylobacter* with the most recent member, *C. ureolyticus*, being added in 2010 (Vandamme *et al.* 1991). Campylobacters colonise many different niches, including a wide range of animals, aqueous environments and a variety of exposed surfaces. In humans, *C. jejuni* causes up to 90% of *Campylobacter* infections with the remainder of cases being attributed to *C. coli* and the other less prevalent *Campylobacter* species (DEFRA 2012). This thesis focuses on *C. jejuni* subspecies *jejuni*, which is a gram-negative, microaerophilic bacterium that commonly resides in water and colonises most warm-blooded animals (Young *et al.* 2007).

1.2 *C. jejuni* infection and incidence

1.2.1 Clinical symptoms

Human infection most commonly occurs through the ingestion of undercooked, contaminated poultry. The infection results in painful abdominal cramps followed by gastroenteritis producing mild watery diarrhoea after a 24-72 hour incubation period, but symptoms are usually self-limiting lasting for approximately one week (Zilbauer *et al.* 2008). Gastroenteritis can be severe with acute inflammatory bloody diarrhoea and other symptoms may include fever, headache and vomiting (Zilbauer *et al.* 2008). The

severity of gastroenteritis depends on the virulence of the *C. jejuni* strain and on the host immune response.

C. jejuni can cause Guillain-Barré Syndrome (GBS), which is an autoimmune disease occurring after infection and causes a potentially life threatening paralysis. GBS affects less than 0.1% patients who have been infected by the bacterium, but approximately 30% of all GBS cases are caused by *C. jejuni* (van Doorn *et al.* 2008). It occurs because *C. jejuni* lipooligosaccharide (LOS) is a molecular mimic of human peripheral nerve gangliosides (Komagamine and Yuki 2006). Autoreactive antibodies are generated to LOS, which causes systemic inflammation and tissue damage (Komagamine and Yuki 2006). *C. jejuni* also causes Miller Fischer Syndrome, again causing paralysis, in particular within eye and urinary muscles (Yu *et al.* 2006). *C. jejuni* has been associated with reactive arthritis, with 1-5% of patients with *Campylobacter* infection developing loosely defined reactive arthritis symptoms (Pope *et al.* 2007). *C. jejuni* gastroenteritis may also be a risk factor for inflammatory bowel disease, where 4.2% of patients with previous *Campylobacter* infection have developed the disease (DuPont 2008).

1.2.2 Emerging *Campylobacters*

Campylobacter species colonise a number of animals, which are important for the farming industry and so may lead to disease in humans. Different *Campylobacter* species have been reported in cattle, pigs, sheep, chickens, turkeys, cats, dogs and rabbits, and many of these species have also been isolated from humans with gastroenteritis. *C. upsaliensi*s and *C. concisus* are the most prevalent among emerging *Campylobacter* species, but *C. curvus*, *C. fetus* subsp. *fetus*, *C. lari*, *C. hyointestinalis*, *C. insulaenigrae*, *C. mucosalis*, *C. sputorum* biovar sputorum and *C. ureolyticus* have also been isolated from the faeces of humans with gastroenteritis (Man 2011). In addition, emerging *Campylobacter* species have been associated with inflammatory bowel disease, periodontal disease, septicaemia, meningitis and urinary tract infections (Man 2011).

1.2.3 Antibiotic treatment

Antibiotic treatment is given for those with severe symptoms and for those with extraintestinal complications. Resistance to fluoroquinolones was first detected in the 1990's, increasing remarkably since then and is highly prevalent in many countries

(Luangtongkum *et al.* 2009). Macrolide resistance is highly variable depending on the country, but rate of resistance is lower than the fluoroquinolones (Gibreel and Taylor 2006). The macrolide erythromycin and the fluoroquinolone ciprofloxacin are employed most frequently, the latter is commonly used for travellers diarrhoea (Luangtongkum *et al.* 2009), and both are also used to control infection in livestock (Angulo *et al.* 2004). Increasing resistance is a concern and there is a strong possibility that frequent use in animal feed has contributed to antibiotic resistance (Gibreel and Taylor 2006), and so new therapies or interventions are needed.

1.2.4 Incidence

C. jejuni is the leading cause of food-poisoning outbreaks and is the most common cause of gastroenteritis in the UK with approximately 80,000 reported cases in 2011 (DEFRA 2012; Tam *et al.* 2012). It is estimated that ten times as many cases are unreported and so the incidence of *Campylobacter* could be approximately 800,000 cases per year (DEFRA 2012). It is also the most commonly reported zoonotic disease in Europe with 220,209 confirmed *Campylobacter* infections in 2011, and there has been an increase in *Campylobacter* cases over the last five years in both the UK and Europe overall (European Food Safety Authority 2013). The scale of *C. jejuni* infection means that it is a heavy economic burden on developed countries, due to a loss of workforce activity.

Increased understanding of *Salmonella* biology, pathogenesis and infection routes has helped to reduce incidence of *Salmonella* infection to under 15,000 cases per year from approximately 50,000 (DEFRA 2012). However, understanding of *C. jejuni* pathogenesis is still lagging and this, combined with lack of effective control measures within industry, has hindered the reduction of *Campylobacter* infection. Research continues to accumulate knowledge of how *C. jejuni* survives in the environment and causes disease, and genomic approaches will help to understand the variation between *Campylobacter* species. A collaborative group in the UK, including government bodies and industry groups, aims to reduce the number of slaughterhouse chickens externally contaminated with more than 1000 colony forming units per gram *Campylobacter* from 27% (the 2008 baseline) to 10% by 2015 (Wearne 2013). It is anticipated that this would lead to a 30% reduction of human *Campylobacter* cases, with every 1%

reduction resulting in an estimated £9 million saving to the UK economy (Wearne 2013).

1.2.5 Transmission

As *C. jejuni* resides in warm blooded animals, consumption of animal products is a risk factor for infection. *C. jejuni* colonises poultry asymptomatically and rapid poultry slaughtering methods increase product contamination (Hue *et al.* 2010), which contributes to the prevalence of infections from this food source. Although *C. jejuni* can be associated with other livestock, very low levels of *C. jejuni* contaminate the animal products; 0.6% pork and 0.4% beef samples were contaminated with *Campylobacter* in the European Union in 2010 (European Food Safety Authority 2012). *Campylobacter* contamination of broiler chickens in the European Union was remarkably higher at 26.9% (European Food Safety Authority 2012) and is probably due to the slaughtering methods. All known sources of UK *C. jejuni* outbreaks were attributed to the consumption of poultry and, in particular, chicken or duck liver in catered foods, such as paté (DEFRA 2012). It is estimated that 60-80% of clinical cases can be linked to handling or consumption of poultry (DEFRA 2012). *C. jejuni* infection can occur through consumption of unpasteurised or improperly pasteurised milk and dairy products and this is the most common route of transmission in the USA (Jay-Russell *et al.* 2013). *C. jejuni* can also cause outbreaks through contaminated water sources, possibly via sewerage contamination or by *C. jejuni* persisting in amoebae (Snelling *et al.* 2005; Pitkanen 2013).

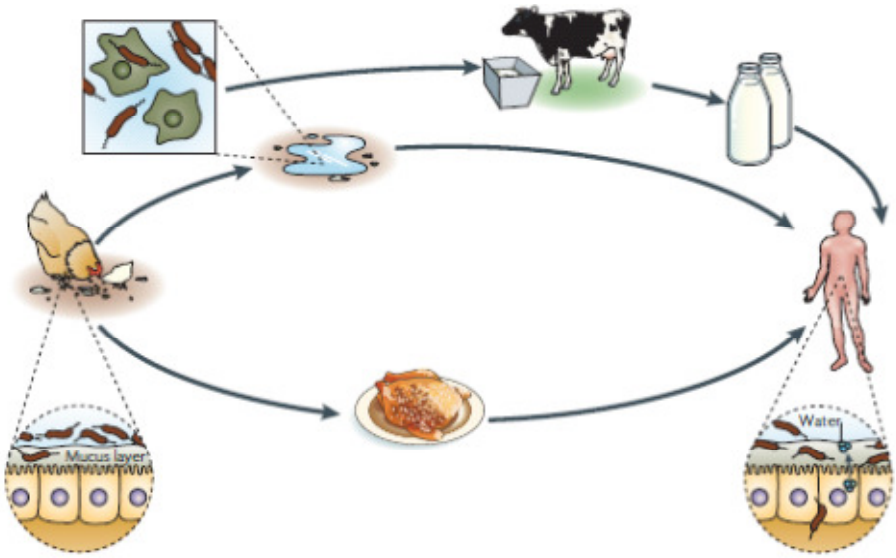


Figure 1.1 Routes of *C. jejuni* transmission.

C. jejuni resides in warm blooded animals, but are considered to be commensal organisms of poultry. Humans can ingest *C. jejuni* through the consumption of contaminated poultry products, animal products and contaminated water sources, where *C. jejuni* may associate with amoebae. Human infection results in a self-limiting gastroenteritis, due to intestinal epithelial damage. Diagram taken from Young et al (Young *et al.* 2007).

1.3 Morphology

C. jejuni are Gram-negative, spiral rods with a cell size that is often less than 2 µm long, but have been reported to be 0.2-0.8 µm wide and 0.5-5 µm long (Ferrero and Lee 1988). *C. jejuni* have a single, unsheathed polar flagellum at one or both poles of the cell and are highly motile. *C. jejuni* cells change shape into a coccoid form under stress, such as limited nutrient availability. These are often considered to be degenerate cells as they exhibit a loss of cell wall integrity and an abundance of extracellular debris (Buck *et al.* 1983). However, the coccoid form could be an alternative physiological state that remains viable but non-culturable, as some were found to have intact membranes and continued, but reduced metabolism (Rollins and Colwell 1986). Other bacteria display this phenomenon and potentially to contribute to disease, through antibiotic resistance and surface attachment (Oliver 2010).

1.4 *C. jejuni* growth requirements

1.4.1 Temperature

Optimal temperature for *C. jejuni* growth is 42°C, which is the body temperature of poultry, but can grow between 30 and 47°C (Jackson *et al.* 2009). However, *C. jejuni* can survive for several months at 4°C in batch cultures (Lazaro *et al.* 1999). It is unknown what mechanisms *C. jejuni* use to survive cold conditions, but long term, low temperature survival of polynucleotide phosphorylase mutants was impaired (Haddad *et al.* 2009). Polynucleotide phosphorylases down-regulate cold-shock protein expression in other prokaryotes, but no cold-shock proteins or other survival strategies have been elucidated in *C. jejuni* (Hazeleger *et al.* 1998; Jackson *et al.* 2009). In response to heat shock, *C. jejuni* express heat shock proteins, including heat shock protein 70 (*hcrA-cj0757*, *grpE-cj0758*, *dnaK-cj0759*) (Thies *et al.* 1999), GroES/EL (*cj1220*/*cj1221*) (Thies *et al.* 1999) and DnaJ (*cj1260c*) (Konkel *et al.* 1998). The RacRS two-component system is required for surviving at 42°C and heat-shock, possibly through the regulation of *dnaJ* expression (Apel *et al.* 2012).

1.4.2 pH

Optimum pH for *C. jejuni* growth is between 6.5 and 7.5. Above pH 9 or below pH 3.6 (Chapter 3) survival of *C. jejuni* is severely reduced and enters the viable, but non-culturable state (Chaveerach *et al.* 2003). In a recent study, oxidative stress proteins were up-regulated in hydrochloric and acetic acid exposed *C. jejuni* strains, but only transcripts for *dps* (*cj1534c*), *ahpC* (*cj0334*), *sodB* (*cj0169*) and *p19* (*cj1659*), were confirmed as being up-regulated in strain NCTC11168 and in acetic acid-shock at pH 5.7 only (Birk *et al.* 2012). Altered gene expression in acid-shocked *C. jejuni* has been reported in a number of experiments and these are discussed in more detail in Chapter 3.

1.4.3 Nutrient uptake

C. jejuni requires amino acids for growth, and favours serine, aspartate, asparagine and glutamine in that order of preference (Stahl *et al.* 2012). Serine is metabolised by SdaA (*cj1624c*), after uptake by the SdaC (*cj1625c*) transporter (Velayudhan *et al.* 2004). Aspartate and glutamate are largely taken up by the Peb1 system (*cj0919c-cj0922c*) (Leon-Kempis Mdel *et al.* 2006) and metabolised by AspA (*cj0087*) and AspB

(*cj0762c*), respectively (Guccione *et al.* 2008). The asparaginase AnsB is required for asparagine metabolism in *C. jejuni* strain 81-176 (Hofreuter *et al.* 2008), but only strains that carry this gene with a preceding signal peptide to allow periplasm export are able to use asparagine as a sole source of amino acid (Hofreuter *et al.* 2008). *C. jejuni* NCTC11168, which has *ansB* (*cj0029*) without the signal peptide shows reduced growth in defined media supplemented with asparagine (Hofreuter *et al.* 2008). If these amino acids have been exhausted, *C. jejuni* can metabolise proline (Wright *et al.* 2009), which is abundant in gut mucin, which is an important nutrient source if the preferred amino acids are limited. *C. jejuni* PutA (*cj1503c*) and PutP (*cj1502c*) for proline uptake and metabolism are homologous to those in *E. coli* (Zhou *et al.* 2008), and are required for *C. jejuni* to colonise the intestine (Hofreuter *et al.* 2012).

C. jejuni is unable to use many carbon sources, as it lacks enzymes from the glycolytic pathway. *C. jejuni* lacks glucokinase and fructose metabolism enzymes and so cannot metabolise glucose or pentose sugars (Stahl *et al.* 2012). However, *C. jejuni* is able to take up the carbohydrate L-fucose using a FucP permease (*cj0486*), and L-fucose metabolism may involve some genes in the *cj0480c-c0j490* region, but their functions remain unknown (Stahl *et al.* 2011). In addition, *C. jejuni* will use pyruvate as the primary carbon source if present in media, but how pyruvate is transported into the cell is unknown (Velayudhan and Kelly 2002). Citric acid cycle intermediates are easily transported and used by *C. jejuni*, and other carbon sources include the short chain fatty acids, acetate and lactate, which are by-products of metabolism by the intestinal microbiota (Stahl *et al.* 2012).

C. jejuni requires transition metals for growth, colonisation and virulence because they have fundamental roles in biology, such as maintaining protein structure, functioning in enzyme catalytic sites or mediating metabolic reactions. For example, many enzymes involved in metabolism contain iron-sulphur clusters, which explain why iron is essential for growth and why there are many uptake and regulation systems. As free iron contributes to Fenton chemistry and damages cells, it is often bound with other molecules, such as siderophores, which are iron containing complexes made by bacteria and specific transport systems for different types of siderophores exist (Miethke and Marahiel 2007). *C. jejuni* can take up siderophores and uptake systems for these and

other iron regulation mechanisms are regulated by Fur, which binds ferrous iron (van Vliet *et al.* 1998; van Vliet *et al.* 2002).

Moreover, copper, zinc and nickel homeostasis is important for *C. jejuni* survival. Bacterial CeuO oxidises toxic cuprous ions after transport into the periplasm by CopA (Osman and Cavet 2008) and *C. jejuni* CeuO (*cj1516*) and CopA (*cj1161*) are required for survival in high copper environments (Hall *et al.* 2008). A ZnuABC (*cj0141c-cj0143c*) transport system is required for survival in low zinc environments, including host niches (Davis *et al.* 2009). A nickel ABC cassette (*cj1580c-cj1584c*) is required for nickel uptake, which may be important for hydrogenase activity during host colonisation (Howlett *et al.* 2012). The *E. coli* general ion transporter, ZupT, has been shown to transport zinc, iron, cobalt and manganese, but the function of the *C. jejuni* homologue has not been investigated (Stahl *et al.* 2012).

1.5 *C. jejuni* virulence factors

To cause disease *C. jejuni* must survive transit through the host gastrointestinal system and reach the intestinal epithelium. Food contaminated with *C. jejuni* is ingested and enters the stomach, where bacteria must survive harsh acidic conditions. *C. jejuni* would then pass into the small intestine and would have to adapt to the change in pH before overcoming host defences. *C. jejuni* must then move to the epithelium and transverse the mucus layer, before invading and colonising epithelial cells. During this process, *C. jejuni* must also manipulate host cells for invasion and evade the innate and adaptive immune system. The molecular basis of *C. jejuni* infection is not well understood, including how it colonises humans and what role it has in the multifaceted diseases of reactive arthritis and inflammatory bowel disease. However, *C. jejuni* possess virulence factors that have been individually shown to contribute to aspects of pathogenicity. Despite our increasing knowledge of these virulence factors, we still do not fully understand how they function in synergy to cause disease.

1.5.1 Capsule

C. jejuni has a polysaccharide capsule that contributes to the development of diarrhoea and resistance to host defences (Bacon *et al.* 2001; Karlyshev *et al.* 2001). Invasion of the intestinal epithelium is important for virulence and the capsule was shown to

contribute to this phenotype (Bacon *et al.* 2001), and was required for virulence in mouse, ferret and wax moth models of infection (Champion *et al.* 2010; Maue *et al.* 2013). *C. jejuni* capsular modifications increased interleukin-17 (IL-17) production from mouse small intestinal CD4+ cells and reduced Toll-like receptor (TLR) activation *in vitro* (Maue *et al.* 2013). Variations in capsular polysaccharide structures are reflected in the different *C. jejuni* serotypes and in the further modifications observed with sugars, *O*-methyl phosphoramidate, glycerol and ethanolamine (McNally *et al.* 2007; Maue *et al.* 2013). This suggests that the capsule and its modifications modulate the immune response and may also dictate strain associated immune responses if capsular modification enzymes differ between strains.

1.5.2 Lipooligosaccharide (LOS)

C. jejuni express oligosaccharides attached to Lipid A (LOS) in the cell wall and the *C. jejuni* Lipid A core structure is different to that of other Gram negative bacteria (van Putten *et al.* 2009). The structure of LOS is highly variable and this, including differences in sialic acid incorporation, can modulate the host immune responses and determine the severity of disease (van Putten *et al.* 2009; Stephenson *et al.* 2013). LOS leads to an inflammatory response as it has been shown to activate TLR4, which leads to cytokine production and phagocytosis (Huizinga *et al.* 2012; Stephenson *et al.* 2013). LOS (*cj1138c-cj1144c*) is required for growth, natural transformation (Marsden *et al.* 2009) and for pathogenesis, as mutations in LOS biosynthesis genes alter *C. jejuni* invasion of human intestinal epithelial cell lines (Fry *et al.* 2000; Javed *et al.* 2012). In addition, LOS is a molecular mimic of eukaryotic glycoproteins and glycolipids (Houliston *et al.* 2011), which generates the autoreactive antibodies responsible for GBS, as described earlier in 1.2.1.

1.5.3 Protein glycosylation

N-linked protein glycosylation is important for *C. jejuni* biology and is conserved in all strains, although this pathway is more common in eukaryotes (Nothaft *et al.* 2012). The *pgl* locus encodes this glycosylation system and mutations in *pgl* genes perturb a number of biological functions, including natural competence, adherence and invasion of epithelial cells and colonisation of hosts (Karlyshev *et al.* 2004; Young *et al.* 2007). Altered protein glycosylation also alters the immune response to *C. jejuni*, and the ability of *C. jejuni* to colonise tissues and invade intestinal epithelial cells, but the

mechanism of this is unclear as most of the glycosylated proteins are located in the periplasm (Young *et al.* 2002). Flagellins are modified by *O*-linked glycosylation, which is described in Section Two.

1.5.4 Cytolethal distending toxin

C. jejuni can produce a cytolethal distending toxin (CDT), which causes cell-cycle arrest (Ge *et al.* 2008). The toxin functions as a complex of three proteins, CdtABC, but CdtB is the active component that is able to localise to the host nucleus, with the aid of CdtAC, and act as a DNase to cause DNA damage (Ge *et al.* 2008). CDT elicits IL-8 production in intestinal epithelial cells (Hickey *et al.* 2000), which is an important inflammatory cytokine released by the intestinal epithelium and leads to epithelial damage and diarrhoea. CDT might also have a role in asymptomatic infections as an immune tolerance or an evasion mechanism, as CDT was shown to be expressed by bacteria colonising chickens, but the chickens did not produce any anti-CDT antibodies (Abuoun *et al.* 2005).

1.5.5 Outer membrane vesicles

C. jejuni membrane blebbing releases outer membrane vesicles (OMV) that can contain many proteins (Lindmark *et al.* 2009). CDT and *N*-linked glycoproteins have been shown to be encapsulated in OMVs and so they may be important in pathogenesis (Elmi *et al.* 2012). Indeed CDT-associated OMVs are biologically active and are able to elicit an inflammatory response from eukaryotic cells (Lindmark *et al.* 2009). OMVs are also cytotoxic to host cell lines and can kill *Galleria mellonella* (insect infection model) (Elmi *et al.* 2012).

1.5.6 *C. jejuni* adherence and invasion mechanisms

C. jejuni has many proteins that have been shown to contribute to host colonisation. CadF (*cj1478c*) forms membrane channels binding fibronectin and is required for maximal binding to and invasion of host cells, and for chick colonisation (Ziprin *et al.* 1999; Monteville *et al.* 2003). FlpA (*cj1279c*) is another surface protein that is required for adherence to chicken cell lines and colonisation of broiler chickens (Flanagan *et al.* 2009).

Lipoproteins JlpA (*cj0983*) and CapA (*cj0628/cj0629*) are required for adherence to host cells. CapA is a membrane bound autotransporter and these are known to promote adherence to host cells and was further shown to be required for colonisation of chicks (Ashgar *et al.* 2007). JlpA binds to host cells and triggers a proinflammatory response through the NF-kB signalling pathway (Jin *et al.* 2003).

Periplasmic proteins Peb1A (*cj0921c*) and Cj1496c are required for invasion of human epithelial cells. Cj1496c is required for chick colonisation and Peb1A contributes to mouse model colonisation (Pei *et al.* 1998; Kakuda and DiRita 2006). Peb1A has been detected in culture supernatants and contains a motif that is common to surface expressed lipoproteins, and so may be accessible to host cells (Leon-Kempis Mdel *et al.* 2006). However, the mechanisms by which periplasmic proteins facilitate colonisation are unknown.

Recently, genes co-expressed with flagellar genes have been found to contribute to host cell invasion and colonisation, and these have been named flagellar co-expression determinants (Feds) (Barrero-Tobon and Hendrixson 2012). In *C. jejuni* 81-176, these genes include *cjj0083* (FedA), *cjj0414* (FedB), *cjj1053* (FedC) and *cjj1647* (FedD), which were shown to be dependent on sigma^{28} for expression, and therefore dependent on expression of upstream flagellar proteins (Barrero-Tobon and Hendrixson 2012). *C. jejuni* Fed mutants were still motile, but were less able to colonise chicks (Barrero-Tobon and Hendrixson 2012).

Certain strains of *C. jejuni* carry the pVir plasmid, which contains genes that are involved in adherence to host cells and is associated with virulence a ferret infection model (Bacon *et al.* 2001). Mutation of pVir *comB3* and *virB11* reduces adherence to INT407 cells (Bacon *et al.* 2002). A plasmid encoding tetracycline resistance (pTet) has been sequenced and was found to be conjugative by expression of a type IV secretion system (Batchelor *et al.* 2004). The type IV-like system, encoded by pVir is possibly required for DNA uptake as it was also found to be conjugative (Bacon *et al.* 2001). Both plasmids carry genes of unknown function, which may be important in host interactions.

It is still unclear how *C. jejuni* enters host cells, but it may be that *C. jejuni* exploits caveolae as caveolin-1 is needed for entry and sequestration of cholesterol inhibits *C.*

jejuni entry (Douillard *et al.* 2008; Watson and Galán 2008). Dynamin, which is needed to form the caveosome, was not required for cell entry, suggesting that *C. jejuni* provides or uses some other mechanism to enter the cell (Hu and Kopecko 2008). Microtubules were shown to be needed for *C. jejuni* internalisation, which may involve tyrosine or phosphoinositide 3 kinases as inhibitors of these enzymes reduced bacterial entry (Hu and Kopecko 2008), but the specific mechanism is unknown. Actin was not required for cell entry (Oelschlaeger *et al.* 1993), but Rho GTPase function in *C. jejuni* internalisation has been reported (Boehm *et al.* 2011). CadF and flagella were also shown to activate Rac-1 and so it is hypothesised that specific *C. jejuni* adhesins cause actin cytoskeleton rearrangements upon contact with host cells (Boehm *et al.* 2011). These conflicting reports mean that the mechanisms of *C. jejuni* host cell entry are still unclear and require further study.

1.5.7 Flagella

C. jejuni is highly motile with a single flagellum at one or both poles of the cell and it is well established that flagella are needed for *C. jejuni* to successfully colonise hosts (Nachamkin *et al.* 1993; Wassenaar *et al.* 1993; Hendrixson and DiRita 2004). Moreover, *C. jejuni* mutations in flagella structure, motor and chemotaxis genes are attenuated for chick colonisation (Hendrixson and DiRita 2004). Therefore, flagella and the associated apparatus need to be complete as well as functional in order to colonise hosts. More detail on the role of flagella in colonisation and protein secretion is given in Section Two.

1.5.8 *C. jejuni*-host interaction models

In vitro models for studying *C. jejuni* host-interactions are derived from cell types with intrinsic differences, which make it difficult to draw conclusions about infection mechanisms by comparing data from different studies. Commonly used cell lines for *in vitro* invasion and adherence experiments, include non-polarised HeLa (cervix epithelial carcinoma) and the derivatives HEp-2 (rat tumours induced by epidermal carcinoma tissue) and INT407 (human embryonic intestine) (Friis *et al.* 2005). Also common are the polarised colonic adenocarcinoma cell lines Caco-2, HT29 (and MTX clone producing mucin) and mucin producing T84 (Lesuffleur *et al.* 1990; Friis *et al.* 2005). However, partially differentiated Caco-2 cells have been recommended as models because they accurately mimic the sites of *Campylobacter* invasion in humans and are

well characterised (Friis *et al.* 2005). Human cell lines can be grown in vertical diffusion chambers to provide more physiologically relevant experimental conditions where apical microaerobic and basolateral aerobic compartments are used (Mills *et al.* 2012).

Porcine (IPEC-J2) and mouse intestinal cell lines (m-IC_{cl2}) have been used for invasion assays using *C. jejuni* and both display small intestine cell characteristics, including cell polarisation, ion transport, tight junctions, a brush border and the expression of intestinal epithelial cell markers, which are common to all species (Bens *et al.* 1996; Parthasarathy and Mansfield 2009; Brosnahan and Brown 2012). Therefore, these may be the most suitable *in vitro* model for studying invasion and adherence, as well as molecular cell entry mechanisms, and could be grown in vertical diffusion chambers to provide more physiologically relevant culture conditions. A chicken hepatocellular carcinoma cell line has also been used for *C. jejuni* adherence assays (Quinones *et al.* 2009), but this may not be the most suitable model considering the attributes of the aforementioned cell lines even though it is derived from the natural host of *C. jejuni*. Although the use of different cell lines makes it difficult to compare and confirm findings, any observations can be taken forward and can form further lines of investigation.

It is important to validate *in vitro* results and provide more relevant experimental conditions when studying *C. jejuni* host-interactions and virulence determinants. *In vivo* infection models currently used for this include ferrets and pigs, and chickens have been used as colonisation models. Recent additions include insect and mouse infection models. The wax moth caterpillar (*Galleria mellonella*) can be used as a high-throughput, cost effective infection model, as *C. jejuni* cause visible disease symptoms (melanisation) and kills the insect (Champion *et al.* 2010). This insect model is an effective system in which to assess the importance of specific *C. jejuni* mutations in virulence and is likely to become a widely used phenotype assay. *C. jejuni* readily colonise mice without causing disease (Bereswill *et al.* 2011), but recent developments using mice colonised with a humanised microbiota do show clinical disease symptoms and have been successfully used for characterising murine immune responses to *C. jejuni* (Bereswill *et al.* 2011). Murine models could be the most relevant for forming

hypotheses about human immune responses, but would then require validation in humans.

Early observation and infection studies using human volunteers provided characterisation of clinical symptoms and an understanding of virulence differences between strains (Black *et al.* 1988). Studies with human volunteers are now performed to examine *Campylobacter* vaccine responses and *C. jejuni* strains 81-176 and CG8421 have been characterised for use as vaccine strains (Tribble *et al.* 2009). During the last decade, there have also been studies in primates attempting to establish models to study vaccine responses.

Using *in vivo* models has the advantage that *C. jejuni* can be studied in physiologically relevant conditions, but establishing a model that exhibits the same symptoms and immune response as humans has been difficult. A variety of *in vitro* models are available, but differences in protocols may confound results and fundamental variations between cell lines make findings difficult to infer mechanisms for other cell types or *in vivo* models. The choice of model would depend on what aspect of host-interaction is being studied and what resources are available to maintain and develop the models.

1.6 Gene regulation

C. jejuni has a relatively small genome containing 1643 open reading frames (Parkhill *et al.* 2000). Along with this, *C. jejuni* also has a limited number of transcriptional regulators. There are only three sigma factors: FliA, RpoN and RpoD and genome analysis has revealed 34 transcriptional regulators that can be grouped into 15 different families based on conserved sequence motifs and similarity with other transcriptional regulators (Wösten *et al.* 2008). As well as transcriptional regulators, transcriptome analysis has identified potential regulatory RNAs that post-transcriptionally control gene expression. Physical genomic rearrangements and small molecules can also regulate gene expression.

1.6.1 Transcriptional regulators

(i) Sigma factors

Sigma (σ) factors are required as part of the RNA polymerase holoenzyme to recognise and bind promoters, and transcribe different gene classes (Kazmierczak *et al.* 2005). RpoD (σ^{70}) is the housekeeping σ factor, which regulates nearly all *C. jejuni* promoters. The consensus sequence TAtAAT at the -10 region (numbers correspond to the transcriptional start site +1) of promoters are recognised by *C. jejuni* σ^{70} (fig. 1.2) (Wösten *et al.* 2008). Recognition of the -10 promoter sequence is well conserved with *E. coli*, but a -35 sequence, which is present in *E. coli*, is not present in *C. jejuni* (Wosten *et al.* 1998a). This coincides with a lack of protein conservation in the -35 binding site with *E. coli* σ^{70} (Wosten *et al.* 1998a).

RpoN (σ^{54}) recognises GGaa-N_6-TTGCTT -24 and -12 sequences (fig. 1.2) and 18 σ^{54}-dependent genes are known to encode proteins involved in and incorporated into flagella, secreted proteins and flagellar glycosylation proteins (Studholme and Dixon 2003; Porcelli *et al.* 2013). A bacterial enhancer is required to bind upstream of σ^{54} promoters (usually approximately 100 bp) and DNA looping has to occur in order for the enhancer to contact the σ^{54}-holoenzyme and catalyze formation of the open complex (Studholme and Dixon 2003). Because σ^{54} is dependent on these additional regulators, it can be more tightly and subtly controlled so that gene expression is precisely and deliberately coordinated. Therefore, σ^{54} is a flexible tool for the regulation of flagella assembly.

FliA (σ^{28}) regulates 26 promoters with -10 CGATwt sequences (Porcelli *et al.* 2013). The regulated genes also encode proteins involved in and incorporated into flagella, as well as secreted proteins. Expression of σ^{54} and σ^{28} is controlled by σ^{70}, with expression of σ^{54} further regulated by at least one specific transcriptional regulator, and with σ^{28} regulated by an anti-σ factor (Wösten *et al.* 2008; Wösten *et al.* 2010). Mutations in *rpoN* and *fliA* renders *C. jejuni* non-motile, but *fliA* mutants still assemble the hook structure (Wösten *et al.* 2008).

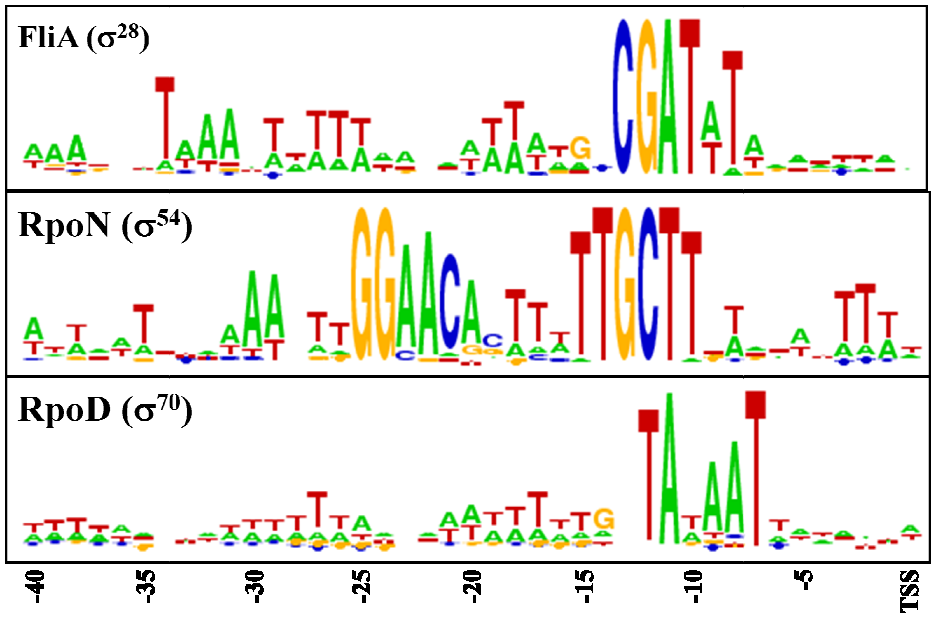


Figure 1.2 Consensus promoter sequences of sigma (σ) ^{70}, ^{54} and ^{28} in *C. jejuni* NCTC11168.

Weblogo sequences (Crooks, Hon et al. 2004) for FliA (σ^{28}), RpoN (σ^{54}) and RpoD (σ^{70}) were made using 26, 18, and 948 promoter sequences, respectively. The degree of conservation is represented by the height of the letters. TSS - Transcription start site. Diagram taken from Porcelli *et al.* (Porcelli *et al.* 2013).

(ii) Other transcriptional regulators

Transcriptional regulators have major functions in all aspects of *C. jejuni* biology and control expression of several regulons. For example, the ferric uptake iron regulator, Fur, is an important regulator of iron metabolism and is involved in the expression of approximately 45 iron acquisition and oxidative stress genes (Holmes *et al.* 2005). Regulation by Fur overlaps with that of PerR, which is a peroxide stress regulator belonging to the Fur family of transcription regulators (van Vliet *et al.* 2002). PerR expression may also be regulated by Fur adding further complexity and flexibility to the network (Holmes *et al.* 2005).

Two component systems can control the expression of multiple genes and operons, where a sensor detects an environmental signal and activates a response regulator. These recognise distinct sequences in promoters and activate or repress gene expression, which allows the cell to adapt to the environment (Jung *et al.* 2012). The response regulator, FlgR, is involved in flagellar assembly activating σ^{54}-dependent transcription by binding to and re-modelling closed DNA complexes to allow transcription (Wosten

et al. 2004). FlgR regulation will be described further in Section 2. There are many other two component systems that control a wide range of biologically relevant functions, which have been reviewed by Wösten *et al* (Wösten *et al.* 2008).

1.6.2 Post-transcriptional regulators of gene expression

(i) Protein regulation

The global, carbon storage regulator CsrA (*cj1103*) was identified in *C. jejuni* and mutants deficient in CsrA expression were attenuated for survival to oxidative stress, biofilm formation and invasion of intestinal epithelial cell lines (Fields and Thompson 2012). In *E. coli*, CsrA acts post-transcriptionally by binding to the 5' untranslated region (5'UTR) of mRNA and inhibiting ribosome access to the ribosome binding site (Romeo *et al.* 2012). The mechanism of *C. jejuni* CsrA (*Cj*CsrA) function is still unclear, but *Cj*CsrA can complement an *E. coli csrA* mutant to a degree, restoring several phenotypes of the mutant (Fields and Thompson 2012).

Cj0706 is predicted to be a post-transcriptional regulator of *flaA* (*Cj1339c*) expression. It is 37% identical to *H. pylori* 26695 FlgZ (HP0958), which binds flaA mRNA, protects σ^{54} from proteolysis and is required for motility (Douillard *et al.* 2008). The structure of Cj0706 can be fitted to the known structure of FlgZ (Caly *et al.* 2010) and shares several FlgZ mRNA binding residues, which suggests it is a good candidate for RNA binding (analysis by Le, unpublished). Moreover, Cj0706 is predicted to interact with σ^{54}, and so could perform similar functions to FlgZ. However, Cj0706 could not be inactivated in strains NCTC11168 or 81-176, and so its role in motility could not be assessed (Le, data unpublished).

(ii) RNA regulation

Non-protein-coding RNAs (ncRNAs) are established post-transcriptional regulators of gene expression, functioning directly on nucleic acids or indirectly on proteins. RNA regulation is required in many biological processes and is important in all kingdoms of life. In prokaryotes, RNA regulation has also been shown to contribute to the expression of virulence factors, and so is of importance in pathogenesis.

Approaches for discovering ncRNAs and their functions are progressing rapidly with advances in RNA sequencing methods generating a wealth of transcriptomic information and revealing novel RNA species. Although ncRNAs are present in many prokaryotes, our understanding of their individual roles and mechanisms largely comes from studies with the Gamma-Proteobacteria, and especially the Enterobacteriaceae. Notably, these have revealed complex ncRNA regulatory networks in *Escherichia*, *Salmonella* and *Pseudomonas* to name a few.

Broad classes of ncRNA exist and can be grouped based on how they function. Perhaps the simplest ncRNAs are *cis*-located riboswitches, which are 5' mRNA aptamers that bind specific metabolites or secondary structures in response to environmental stimuli (Papenfort and Vogel 2010). The result is a conformational change in the 5'UTR of the mRNA that allows access or blocks the ribosome binding site, which alters translation or stability of that particular mRNA (Serganov and Patel 2012). Only a single riboswitch has been identified in *C. jejuni* and this is the thiamine pyrophosphate (TPP) riboswitch, which was identified upstream of the thiamine biosynthesis protein ThiC (*cj0453*) (Gundogdu *et al.* 2007; Chaudhuri *et al.* 2011). However, the function of this riboswitch has not yet been evaluated experimentally in *C. jejuni*.

Bacterial and archeal genomes contain clustered and regularly interspaced short palindrome repeats (CRISPR), which are repeated sequences of DNA that are transcribed together in a single RNA transcript and are subsequently cleaved into short repeats. These then act to prevent phage replication by guiding CRISPR associated proteins (Cas) to recognize and cleave invading foreign DNA, but also have the potential to regulate intrinsic nucleic acids (Makarova *et al.* 2011). *C. jejuni* has a CRISPR-cas system that may be required for virulence, as inactivation of the *cas9* marker gene reduced invasion of human intestinal cell lines (Louwen *et al.* 2012). In addition, CRISPR-cas gene degeneration is correlated with the presence of sialyltransferases that form the antigenic LOS structures in *C. jejuni* GBS strains, and so the CRISPR-cas system could be a marker for GBS causing strains (Louwen *et al.* 2012).

Discrete ncRNAs can interact with proteins and serve as molecular mimics, which compete for regulatory sites of proteins and inhibit their activity (Marzi and Romby 2012). In *E. coli*, the CrsA family of post-transcriptional regulators are regulated by

ncRNAs CsrB/CrsC (Suzuki, Wang et al. 2002), but these and the two-component system that regulates their expression have not been identified in *C. jejuni* (Fields and Thompson 2008).

Non-coding RNAs that interact with other RNAs can be located *in trans* or *in cis* of their target genes. Trans-acting ncRNAs, typically ~50 to 250 nt in length, are commonly located in intergenic regions and are currently the most extensively characterised ncRNAs (Papenfort and Vogel 2010). Trans-acting RNAs function by binding the 5'UTR of the target gene mRNA transcript via complementary, but imperfect, base pairing and largely results in negative regulation of gene expression by interfering with ribosome function or by targeting the complex for degradation (fig. 1.3) (Papenfort and Vogel 2010). However, ncRNAs can also promote gene expression by inhibiting mRNA secondary structures and titrating away RNAse E recruiting proteins that interact with ncRNA-mRNA complexes (fig. 1.3) (Frohlich and Vogel 2009; Gopel *et al.* 2013). Non-coding RNA binding regions are surprisingly short and perhaps explain the involvement of a RNA stabilising protein chaperone, Hfq, which is required for ncRNAs to function in many prokaryotes (Vogel and Luisi 2011). However, *C. jejuni* lacks Hfq and so either has novel RNA stabilising chaperones or ncRNAs are able to act independently of proteins. Putative *C. jejuni hfq* genes *cj0138*, *cj0667* and *cj1103*, have been mutated, but did not result in any pleiotropic effects, suggesting that they were not major ncRNA protein chaperones (Meier *et al.* 2012). Identification of such a protein chaperone would aid in the study of *C. jejuni* ncRNAs.

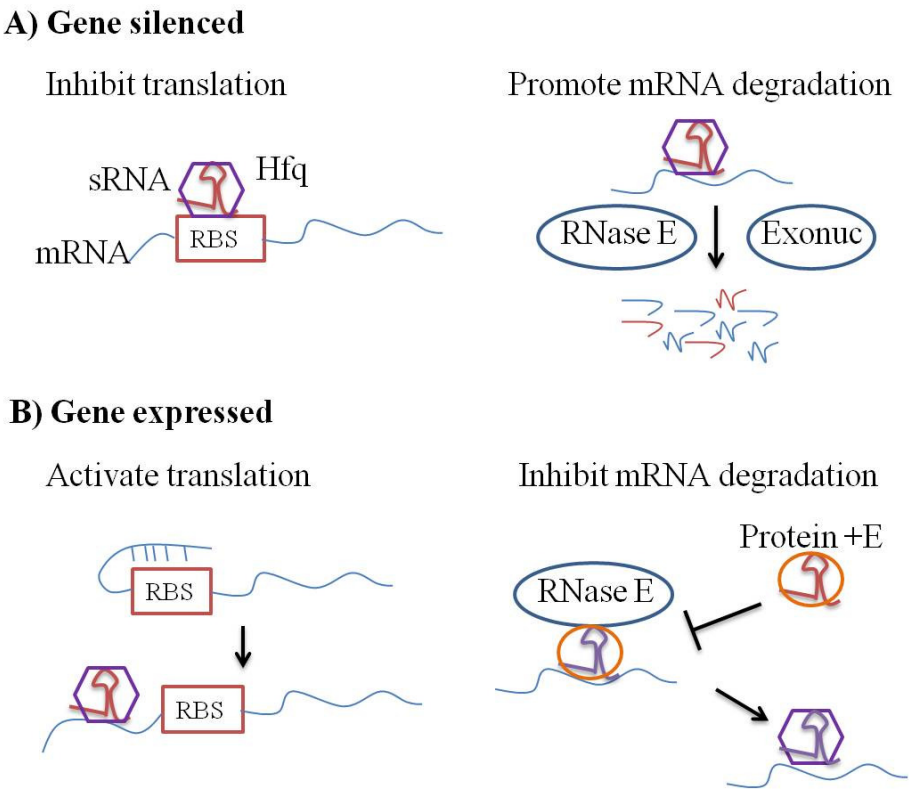


Figure 1.3 Mechanisms of trans-acting non-coding RNA function.

Hfq is a protein chaperone that stabilises interactions between t*rans*-acting ncRNAs (ncRNA) and messenger RNA (mRNA). **A)** *Trans*-acting ncRNAs can silence gene expression by inhibiting translation through blocking the ribosome binding site (RBS) of target gene mRNA or targeting the mRNA for degradation by RNAse E and the exonuclease pathway (Exonuc). **B)** *Trans*-acting ncRNAs can promote gene expression by activating translation through relieving the formation of secondary RNA structures or inhibiting mRNA degradation by titrating away RNAse E recruiting proteins that interact with ncRNA-mRNA complexes (Protein +E).

Differential RNA sequencing of transcriptomes, enriched for primary transcripts, has identified many more ncRNAs in *C. jejuni*. Approximately 30 small ncRNAs (2% of the genome) have been identified and 10 of these have been confirmed by Northern hybridisation (Porcelli *et al.* 2013). Chapters 4-6 examine the function of two of these ncRNAs, but functions for any other *C. jejuni* ncRNAs have not been described so far.

1.6.3 Phase variation

It is well known for physical DNA rearrangements to regulate gene expression. In *C. jejuni* polymeric G- or C- tracts are present in the genome, which may result in slipped-strand mispairing and may affect transcription if the region is in the promoter or there is differential expression of transcriptional regulators. For example, Cj1419c and Cj1420c are often differentially expressed on two-dimensional protein gels and sequence analysis reveals a polymeric G-tract in the *cj1420c-cj1422c* region (personal communication with Francis Mulholland). Expression of the flagellar regulator *flgR* is phase variable (Hendrixson 2006), which adds a layer of regulation, perhaps missing from the lack of a master regulator of flagellar gene transcription.

1.6.4 Small molecules

Small molecules can be regulators of gene expression during the stringent response in low nutrient availability. The characteristic molecules of this response are 5'-diphosphate 3'-diphosphate/5'-triphosphate 3'-diphosphate (ppGpp/pppGpp), which are secondary messengers that down-regulate expression of translation machinery and up-regulate stress response genes by binding directly to RNA polymerase (Boutte and Crosson 2013). *C. jejuni* deficient in SpoT, an enzyme that is involved in the production of ppGpp, shows decreased ppGpp production and increased sensitivity to rifampicin (Gaynor *et al.* 2005). This suggests that ppGpp might regulate gene transcription in *C. jejuni* stress responses, although its interaction with RNA polymerase has not yet been confirmed. Increased biofilm formation by SpoT mutants was associated with alterations in polysaccharide structure and implies that ppGpp may be required for *C. jejuni* survival mechanisms in different niches (McLennan *et al.* 2008).

Section 2 Bacterial flagella

The following section reviews flagellar assembly and function in prokaryotes, and how this compares to *C. jejuni*. Prokaryotic flagella are complex, self-assembling organelles that extend out from the bacterial cell, and the structure of flagella and regulation of flagellar assembly are highly conserved between bacteria. Flagella are crucial for movement for many bacteria and are important for tactic responses that ultimately enable the microorganism to survive in a variety of environments and interact with hosts (Harshey 2003).

Flagella are large macromolecular structures, which requires orchestration of over 50 genes and approximately 20,000 subunits regulated by protein and RNA regulators in a sophisticated network (Chevance and Hughes 2008; Smith and Hoover 2009; De Lay and Gottesman 2012). Bacteria can vary in the number and the arrangement of flagella, but all follow a similar basic structure: a basal body, connecting hook, and the propeller-like filament (Chevance and Hughes 2008). Likewise, a set of core genes contributes to the structure of bacterial flagella and are thought to derive from one or few ancestors (Liu and Ochman 2007).

Understanding flagellar functions and regulation is important for understanding bacterial pathogenesis and may lead to pharmacological interventions in industry or medicine and to the development of vaccines. Knowledge of flagella as self assembling macromolecular machines and biological motors can create opportunities in other areas of science, such as biomedical physics and nanotechnology.

1.7 Structure and assembly of flagella

Bacterial flagella follow a similar design and this is well characterised in *Salmonella enterica* serovar Typhimurium, which provides a model flagellum (fig. 1.4). The basal body comprises the flagella export apparatus (a type III-like Secretion System) and motor-rotor switch complex, which assemble in the centre of the membrane cytoplasmic embedded C and MS rings (Chevance and Hughes 2008). Core genes for the flagellar ring structures include *fliG*, *fliM* and *fliN* for the rotor switch (C ring), and *fliF* for the inner membrane ring (MS ring) (Chevance and Hughes 2008). The motor (*motA* and *motB*) is a core component of the flagellar system, which is associated with the rotor

switch proteins to provide a proton-motive force to power rotation and form stators responsible for rotation of the flagellum (Minamino *et al.* 2008). Interaction with chemotaxis proteins provides directional movement (Chevance and Hughes 2008).

The FlgH peptidoglycan ring (P ring) and FlgI outer membrane ring (L ring) contain multiple subunits and are secreted via the Sec pathway (Jarrell and Mcbride 2008). These proteins/structures are not present in the ancient bacterial lineages of Firmicutes, which lack an outer membrane, and Spirochaetes, which have periplasmic flagella that do not cross the outer membrane. Therefore, FlgH and FlgI are thought to have evolved after the core genes (Liu and Ochman 2007). The core genes *flhA*, *flhB*, *fliI*, *fliP*, *fliR*, *fliQ* encode proteins to form the export apparatus, which then secretes proteins to assemble the rest of the flagella (Chevance and Hughes 2008). The FlgB, FlgC, FlgG and FlgF rod proteins are secreted and form part of the basal body, connecting the external flagellar structures and are associated with proteins that may be involved in flagellar export (Chevance and Hughes 2008).

Core genes for the middle and upper external flagellar structures include *flgE* (main hook subunit), *flgK* and *flgl* (junction proteins between the hook and the filament), and *fliC* (major flagellin subunit) (Liu and Ochman 2007). The hook allows flagella to orientate in different positions so that the bacterium achieves directional movement, and hook length is controlled by export apparatus proteins (Chevance and Hughes 2008). Flagellins form the filament and are polymerised into long chains, called protofilaments, where one flagellum contains 11 protofilaments (Egelman 2010). The filament cap (*fliD*) enables repeating subunits to polymerise forming an elongated filament and is incorporated early in the hook and rod structures, but is removed from the end product (Chevance and Hughes 2008). The final macromolecular structure can extend over many cell lengths and contains a hollow channel from the proximal to the distal end that is a few nanometres in diameter (Jarrell and Mcbride 2008).

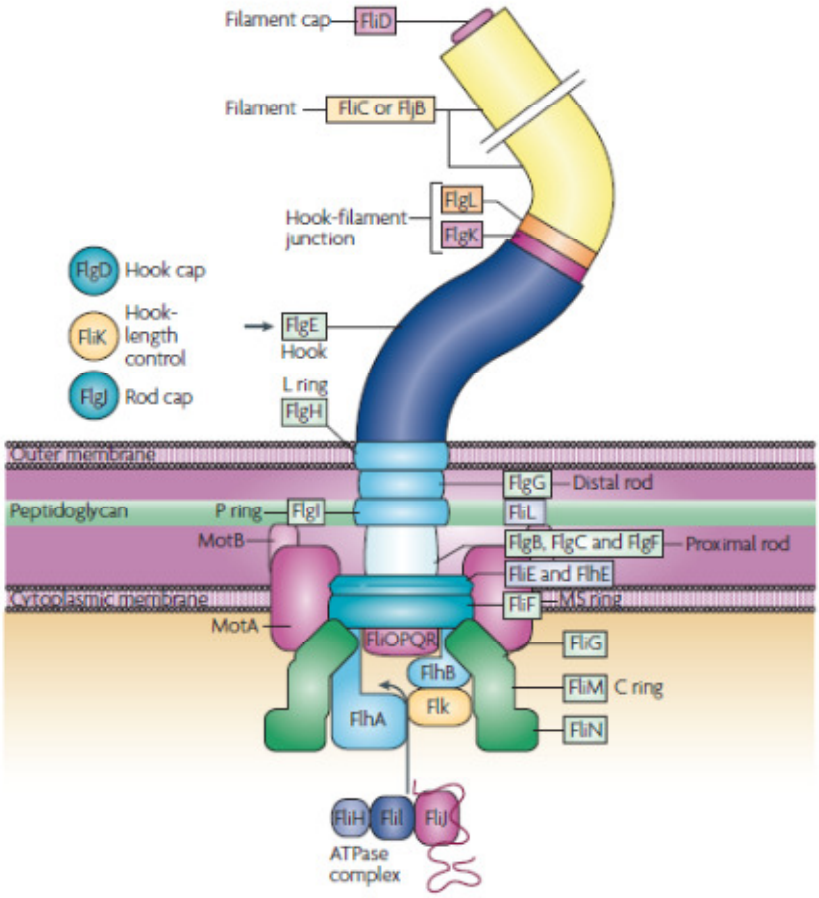


Figure 1.4 Model structure of a bacterial flagellum.

Bacterial flagella consist of three main parts: the basal body, hook and filament. The basal body contains the membrane rings (FliF, FlgH, FlgI); flagella export apparatus (FlhA, FlhB, FliI, FliP, FliR, FliQ), which secretes protein subunits through the flagellar channel for assembly; motor (MotA, MotB) and rotor switch (FliM, FliN, FliG), which provides a proton-motive force to rotate the flagella; and rod proteins (FlgB, FlgC, FlgF, FlgG). Formation of the hook (FlgE), junction proteins (FlgK, FlgL) and filament (FliC) follows. Taken from Chevance and Hughes (Chevance and Hughes 2008).

The basic structure of *C. jejuni* flagella follows the *Salmonella* model, including a motor and switch, membrane-embedded rings, basal body, hook, rod and filament (fig 1.5). Again the *C. jejuni* flagellum is hollow allowing export of flagella proteins for assembly and for secretion of other proteins, but contains seven protofilaments rather than 11 (Egelman 2010). The filament consists of two flagellins: FlaA, the major flagellin, and FlaB, a minor flagellin that constitutes less than 20% of total flagellin units (Guerry *et al.* 1991). FlaC, a protein originally annotated from the genome sequence as a third flagellin, is highly conserved among *C. jejuni* strains (Guerry 2007).

However, it is not incorporated into the filament, but is secreted by the export apparatus, perhaps as an effector protein (Song *et al.* 2004). FlgE2 subunits, which are different to FlgE from *Salmonella* species, form the *C. jejuni* hook, although the *C. jejuni* genome does contain a *flgE* that is not required for motility (Hendrixson and Di Rita 2003). The *C. jejuni* rotor contains an extra rotor switch protein FliY, where other bacteria have either FliY or FliN (Lertsethtakarn *et al.* 2011). Another difference is that extra proteins are required for motility in *C. jejuni*, including FlgP, FlgQ and PflA, which are not incorporated into the flagella (Yao *et al.* 1994; Sommerlad and Hendrixson 2007).

As *C. jejuni* flagella do not contain FliC, the immune response to *C. jejuni* differs to that of many other bacteria. Bacterial flagellin contains microbe-associated molecular patterns, which are recognised by the innate immune system (Vijay-Kumar and Gerwirtz 2009). In humans, extracellular FliC is recognised by TLR5, which is expressed on epithelial cells, macrophages and dendritic cells (Vijay-Kumar and Gerwirtz 2009). Activation of TLR5 on these cells results in a signalling cascade that regulates genes for antibacterial chemicals, chemokines, general stress induced genes and anti-apoptotic factors (Vijay-Kumar and Gerwirtz 2009). Many bacteria activate TLR5 signalling, but *C. jejuni* flagellins do not (Andersen-Nissen *et al.* 2005). A conserved amino acid sequence is required to trigger TLR5 that is lacking in *C. jejuni* FlaA allowing the bacteria to evade this response (fig. 1.6) (Andersen-Nissen *et al.* 2005). Despite this, chemokines are still secreted from *C. jejuni*-invaded intestinal epithelial cells including IL-8, CXCL8-11, and CCL2-5, which target neutrophils, activated T cells, basophils and macrophages (Al-Salloom *et al.* 2003; Johanesen and Dwinell 2006).

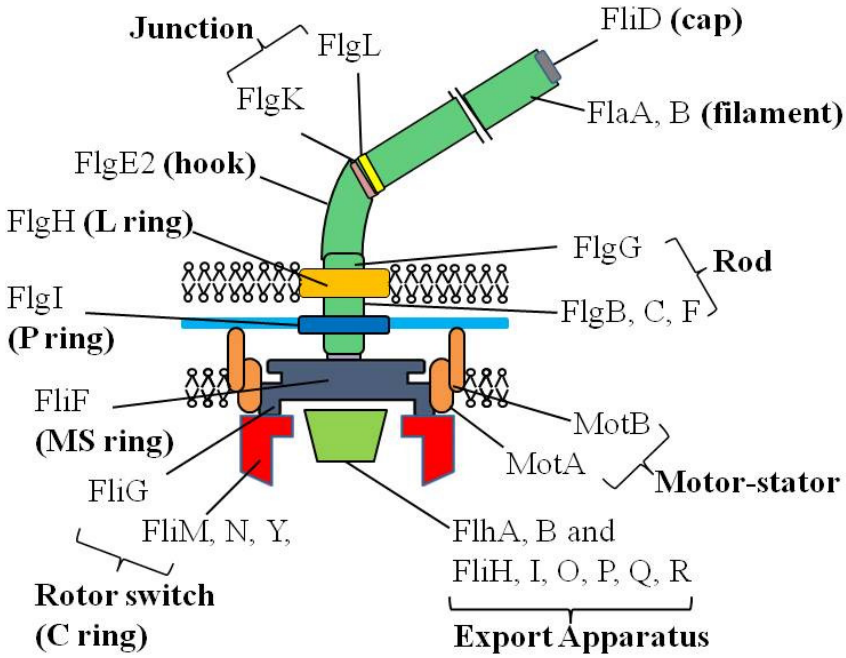


Figure 1.5 Model of the *C. jejuni* flagellum.

C. jejuni flagella are similar to model flagella (based on *Salmonella* species), except for the filament, which comprises seven protofilaments and consists of mainly FlaA subunits with some FlaB subunits, whereas a FliC-like flagellin subunit is present in many other bacteria flagella. The *C. jejuni* hook subunit is encoded by *flgE2* and is different to the *flgE* from other bacteria. The *C. jejuni* rotor switch has an extra protein of unknown function FliY. *C. jejuni* also has extra non-flagellar proteins need for motility and these are FlgP, FlgQ and PflA (not shown).

```
                          ***   *  *  *                *
B.subtilis          86 LQRVRELVVQAGNTGTQDKATDLQSIQDE 114
C.jejuni            88 LDTIKTKATQAAQD--GQSLKTRTMLQAD 114
E.coli              88 LQRIRELTVQASTG--TNSDSDLDSIQDE 114
L.monocytogenes     86 LQRMRQLAVQSSNG--SFSDEDRKQYTAE 112
P.aeruginosa        88 LQRMRDLSLQSANG--SNSDSERTALNGE 114
S.Typhimurium       88 LQRVRELAVQSANS--TNSQSDLDSIQAE 114
S.Enterica          88 LQRVRELAVQSANG--TNSQSDLDSIQAE 114
V.cholerae          88 LQRMRDLALQSANG--TNSASERQALN-E 114
```

Figure 1.6 Sequence alignment of bacterial flagellin and conservation of TLR5 activating residues.

ClustlW2 was used to align protein sequences of bacterial flagellin. Amino acids 89-96 account for most of the TLR5-stimulatory activity of the protein (Smith, Andersen-Nissen et al. 2003). Important residues that stimulate a TLR5 response are indicated by * and those conserved are **blue**.

1.8 Flagellin *O*-linked glycosylation

C. jejuni contains enzymes that glycosylate flagellin and this is well studied in *C. jejuni*, making it, in many respects, the model organism to study *O*-linked glycosylation. The glycans incorporated are pseudaminic acid, mediated by PseABCFGHI enzymes (McNally *et al.* 2006), and legionaminic acid with their derivatives depending on the *C. jejuni* strain (Logan 2006). Pseudaminic acid production is thought to be dependant on vitamin B6 (pyridoxal-5'-phosphate) synthesis as *pdxA* mutants were decreased in both these products (Asakura *et al.* 2013). The mutants also showed impaired motility, which was likely caused by improper flagellin glycosylation (Asakura *et al.* 2013). FlaA is glycosylated on up to 19 sites before export and is essential for flagellar assembly and function (Goon *et al.* 2003; Ewing *et al.* 2009). Flagellar glycans mediate aggregation of *C. jejuni* cells (termed autoagglutination), which is implicated in invasion of host cells (Guerry *et al.* 2006). Autoagglutination possibly contributes to biofilm formation aiding the establishment of microcolonies, and so glycosylation could aid *C. jejuni* survival and infection (Guerry *et al.* 2006).

1.9 Transcriptional hierarchy of flagellar genes

1.9.1 Flagellar regulation in prokaryotes

A common regulation pathway exists in Gram-negative bacteria with peritrichous flagella (Smith and Hoover 2009), with a flagellar regulon comprising different promoter classes, which are temporally regulated during assembly. A master regulator is expressed from a Class I operon at the top the hierarchy. Class II genes are then transcribed leading to expression of a σ factor and regulatory proteins required for the transcription of the subsequent gene class (Mccarter 2006). Successful assembly of the hook-basal body complex then determines when Class III/IV genes are expressed, which finally leads to completion of the filament (Mccarter 2006).

FlhDC is the master regulator in many Beta- and Gamma-Proteobacteria, but it is also the global transcriptional regulator for other genes (fig. 1.7) (Wang *et al.* 2006). It is controlled by environmental signals and promotes the expression of Class II genes via direct contact to σ^{70}-RNA polymerase holoenzyme to activate transcription (Wang *et al.* 2006). The expression of Class III genes is then transcribed by σ^{28}, which is required for

transcription of the late flagellar genes in many bacteria, with 76% of flagellated bacterial species predicted to contain σ^{28}/σ^{D} orthologues (fig. 1.7) (Smith and Hoover 2009).

Associated with σ^{28} is FlgM (the anti-σ^{28} factor) in the cytoplasm, which makes σ^{28} unavailable for transcription and destabilises the σ^{28}-RNA polymerase holoenzyme (Sorensona *et al.* 2004). FliA and FlgM become a substrate for export when the hook-basal body has been fully assembled (Aldridge *et al.* 2006). When *S.* Typhimurium σ^{28} binds FlgM, the N-terminus of FlgM is exposed becoming a suitable substrate for the flagellar export apparatus (Aldridge *et al.* 2006). In the same model, FlgM is separated from σ^{28} as it is secreted out of the cell leaving σ^{28} in the cytosol (Aldridge *et al.* 2006). Therefore, a net decrease in FlgM results in the release of σ^{28}, which is then free to activate transcription of genes.

Variations in gene classes and mechanisms of regulation can be observed or the system may be notably different (fig. 1.7). For example, CtrA is the master regulator found in *C. crescentus*, which is a response regulator that also controls the cell cycle and is controlled by cell-cycle regulators (Collier *et al.* 2007). Many bacteria employ σ^{54} to transcribe Class III/IV genes, and in *C. crescentus* σ^{54} and the enhancer FlbD allows completion of the hook-basal body complex (fig. 2.4) (Collier *et al.* 2007). Some master regulators function as σ^{54} bacterial enhancers and some bacteria have no master regulator, such as FleQ in *Pseudomonas aeruginosa* and FlrA in *Vibrio cholera* (Jyot *et al.* 2002; Syed *et al.* 2009). In these organisms, motility genes are also expressed with virulence genes (Ghosh *et al.* 2006; Syed *et al.* 2009). The FleRS and FlrBC are two-component regulators of *P. aeruginosa* and *V. cholerae* that initialise σ^{54}-dependent transcription of late-phase basal body, hook proteins and σ^{28}, which is required for Class IV flagellin and FlgM expression (fig. 1.7) (Dasgupta *et al.* 2003; Syed *et al.* 2009).

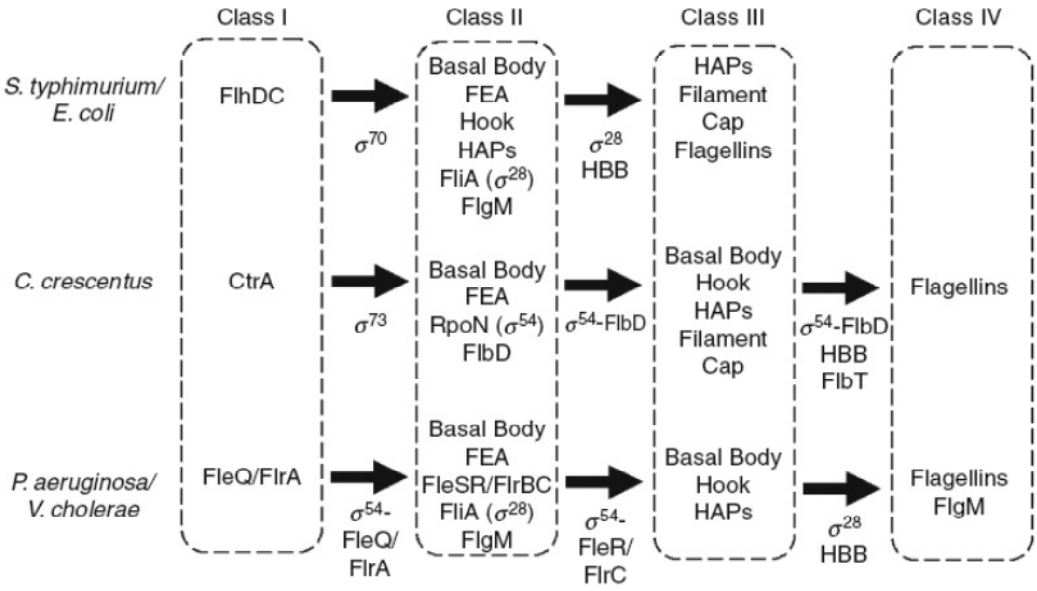


Figure 1.7 Systems for flagellar gene regulation in different bacteria.

Master regulators, encoded by Class I genes, activate transcription of Class II genes. This leads to the transcription of alternative σ factors and important regulatory proteins that control expression of the next classes of genes. Regulation of Class III genes is dependent upon completion of the HBB. Some bacteria transcribe four classes of genes in order to complete the flagellum. Abbreviations: flagellar export apparatus (FEA), hook associated proteins (HAP), and hook-basal body (HBB). Taken from Smith and Hoover (Smith and Hoover 2009).

1.9.2 Flagellar regulation in *C. jejuni*

C. jejuni flagellar genes are not organised into distinct operons, but gene regulation is still provided via temporal control of transcription in a three-tiered system (fig. 1.8). Early phase genes are dependent on σ^{70} for transcription, and include *rpoN*, *fliA*, *flgM*, *flgRS* and *flhF* (Hendrixson 2008). Components of the flagellar export apparatus are also expressed including *flhA, flhB, fliP, fliR, fliO, fliQ* and *fliF*, but these may be constitutively expressed (Hendrixson 2008); genetic analysis has revealed that many of those genes are grouped in regulons that are likely to be involved in growth and metabolism (Hendrixson 2008).

Master regulators have not been ascertained for the Epsilon-Proteobacteria *C. jejuni* and *H. pylori*. However, the FlgRS two-component system regulates the expression of σ^{54}-dependent genes, which then allows transcription of σ^{28}-dependent genes in a similar manner to the *P. aeruginosa*/*V. cholerae* systems. The sensor kinase, FlgS, is thought to be soluble like FleS and FlrB (Wosten *et al.* 2004), but unlike the FleR and FlrC cognate response regulators, the FlgR response regulator for FlgS does not bind DNA and is thought to directly contact the σ^{54}-RNA polymerase holoenzyme instead (Brahmachary *et al.* 2004; Hendrixson 2008). Unusually, both FlgS and FlgR are phase variable via deletion or insertion of a nucleotide within homopolymeric tracts, which results in a truncated non-functional protein (Hendrixson 2006).

After completion of the flagella export apparatus, expression of σ^{54}-dependent genes via FlgR activation results in completion of the basal body, assembly of rod and hook proteins, and σ^{28}, FlgM, and FlaB expression (fig. 1.8) (Hendrixson 2008). The flagellar export apparatus has been suggested to be more than a flagellar protein secretion apparatus and could provide regulatory signals for flagellar biosynthesis. Mutations in *flhB* truncate flagella as a result of down-regulating flagellin expression (Matz *et al.* 2002) and have been shown to form an incomplete export apparatus with decreased expression of σ^{54}-dependent flagellar genes (Joslin and Hendrixson 2009). However, blocking or hindering protein secretion did not effect σ^{54}-dependent flagellar gene expression (Joslin and Hendrixson 2009), which suggests the export apparatus is needed for or to directly provide the signal for FlgS autophosphorylation. It has recently been shown that FlgS interacts with FliF (MS ring) and FliG (rotor protein) and so these must provide the activating signal for FlgS (Boll and Hendrixson 2013). Either the

completion of the flagellar export apparatus marked by completion of the MS ring and rotor, which is sensed as a whole by FlgS, or FliF and FliG form a cytoplasmic domain that interacts with FlgS in the cytoplasm (Boll and Hendrixson 2013). Such regulatory check points may compensate for the requirement of a master regulator.

FlhF is thought to interact with the export apparatus perhaps influencing its location or the number of flagella formed, or monitoring the order and increasing the efficiency of flagellar protein secretion (Balaban *et al.* 2009). FlhF acts as a GTPase that is needed for proper biosynthesis of flagella, as *flhF* mutants were shown to produce flagella in improper numbers and locations (Balaban, Joslin et al. 2009).

In *C. jejuni*, transcription of σ^{28}-dependent genes results in expression of FlaA and the elongation of the filament, but the anti-σ factor FlgM may function differently to the systems previously reviewed. *C. jejuni* FlgM was identified based on homology with *H. pylori* FlgM, which is smaller than that of other bacteria and has a degenerate N-terminus that is usually required for secretion from the export apparatus (Rust *et al.* 2009). Also, most *H. pylori* FlgM remains in the cytoplasm and is found to interact with FlhA in the basal body instead of σ^{28}, possibly by a protein-transfer mechanism (Rust *et al.* 2009). Subsequently, *C. jejuni* FlgM was found to be secreted, but its association with σ^{28} was temperature dependent; σ^{28} and FlgM did not form a complex at 42°C (Wösten *et al.* 2010) meaning that σ^{28} would only be available for σ^{28}-dependent transcription at that temperature. However, flagella are produced at lower temperatures and so other mechanisms must regulate σ^{28} also to allow gene expression. *C. jejuni* FlgM could not be shown to suppress FlaA/B production when hook formation was incomplete and was not secreted in a *flgE* mutant, meaning that a minimum flagellar structure was required for FlgM secretion (Wösten *et al.* 2010).

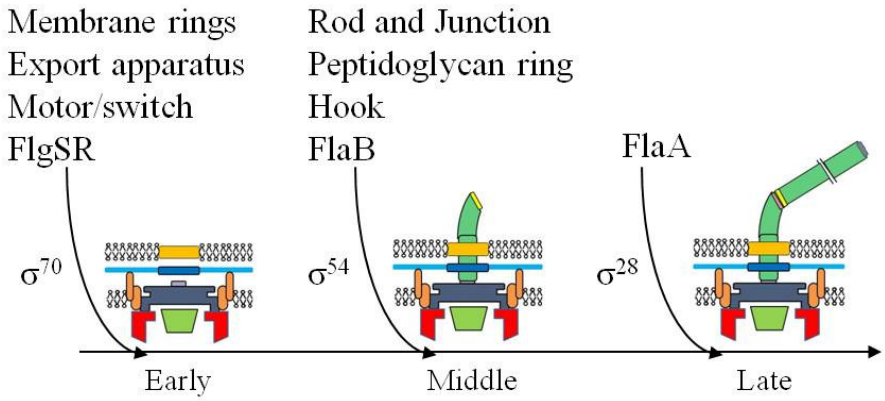


Figure 1.8 Transcriptional hierarchy of flagellar gene expression in *C. jejuni*.

C. jejuni does not have a master regulator of flagellar assembly. Early-phase flagella genes are σ^{70}-dependent including the flagella export apparatus, motor/switch proteins and FlgRS two-component system. The response regulator FlgR activates transcription of middle-phase genes in a σ^{54}-dependent manner. Basal body and hook genes are expressed and assembled before a switch to σ^{28}-dependent transcription after secretion of FlgM. Transcription of the major flagellin, FlaA, by σ^{28} results in elongation of the filament.

1.10 Flagellar functions

1.10.1 Motility

Flagella are able to provide movement by propelling bacteria in different directions and are driven by a biological motor. The motor consists of the motor and rotor switch, which are embedded in the cytoplasmic membrane, and the stators, which provide energy for flagellar rotation and convert energy into torque (Minamino *et al.* 2008). The typical stator system comprises two transmembrane proteins MotA and MotB (four MotA and two MotB), which form ion channels and pump protons across the membrane (Minamino *et al.* 2008). These interact with FliG in the motor switch complex and result in rotation of the flagellum (Thormann and Paulick 2010). Variations of the Mot proteins exist in other bacteria each involving different subunits and substrates, including sodium ions used by *Bacillus subtillus*, *Aeromonas hydrophila* and *Shewanella oneidensis* (Thormann and Paulick 2010).

C. jejuni contains MotA and MotB, but direct investigations on how these function have not been conducted (Lertsethtakarn *et al.* 2011). *H. pylori* MotB was crystalised, which revealed that MotB has binding domains for peptidoglycan, suggesting that stators are

anchored to the cell wall (Lertsethtakarn *et al.* 2011). It remains to be seen whether stators in other bacteria are anchored in the same way. Indeed the motor and flagellar basal bodies appear to differ visually between different bacteria, with Epsilon-Proteobacteria, *C. jejuni* and *H. hepaticus*, displaying quite distinct basal body structures (Chen *et al.* 2011).

1.10.2 Taxis

Taxis, coupled with motility, allows cells to sense more favourable environments and move from toxins or towards epithelia, for example. Chemotaxis, best studied in *E. coli*, occurs through the phosphorylation of Che proteins. When chemoreceptors detect the chemoattractant, they are activated and the scaffold protein, CheW, links the receptors to the Che system. CheA is activated and phosphorylates the response regulator CheY (Wadhams and Armitage 2004). CheZ removes the phosphoryl group from CheY so that CheY can continue to be phosphorylated and respond to the environment (Wadhams and Armitage 2004). The result is that flagellar rotation is altered, which changes the movement of the cell and in bacteria with bidirectional flagella, the cell continuously switches between runs and tumbles with less tumbling leading to migration towards an attractant (Chevance and Hughes 2008). Continuous resetting of the system by methylation adaption gives a mechanism that can respond to chemical concentrations over time, which allows sensing of the environment and movement in the favoured direction (Baker *et al.* 2005).

C. jejuni is attracted to fucose, aspartate, fumarate, formate and pyruvate (Lertsethtakarn *et al.* 2011). In addition to the above Che proteins, *C. jejuni* has unique accessory proteins, including the CheY phosphatases, CheZ and FliY (part of the motor switch) (Lertsethtakarn *et al.* 2011). Although CheZ is present in other bacteria, *C. jejuni* CheZ only shares the active site of these proteins (Lertsethtakarn and Ottemann 2010). FliY is also present in other bacteria and studies in *B. subtilis* have shown that FliY binds and dephosphorylates CheY (Szurmant *et al.* 2004). However, *C. jejuni* FliY does not contain CheY binding motifs and *fliY* mutants are non-motile and so its functions have not been studied (Lertsethtakarn *et al.* 2011). Moreover, other bacteria usually have either FliY or FliN in combination with FliG and FliM as part of the motor switch, whereas *C. jejuni* contains both and the reason for this is unknown. *C. jejuni*

also has another Che coupling protein, CheV, which is thought to perform a similar function to CheW, but its function is not yet known (Lertsethtakarn *et al.* 2011).

1.10.3 Biofilm formation

Bacteria can form biofilms, which are communities of microorganisms attached to a surface and enveloped in an extracellular matrix. Bacteria are likely to be found living in biofilms, rather than as planktonic cells, as biofilms aid survival and persistence (O'Toole *et al.* 2000). Biofilms contribute to bacterial pathogenesis as bacteria in biofilms are more resistant to host defences and antibiotics (O'Toole and Kolter 1998; Fux *et al.* 2005; Yildiz and Visick 2009). Flagella have roles in biofilm formation, for example aflagellate *Vibrio* and *Pseudomonas* species do not form biofilms (O'Toole and Kolter 1998; Yildiz and Visick 2009). In particular, flagella are important in the initial stages of biofilm formation enhancing movement along a surface and promoting attachment to surfaces to form microcolonies (O'Toole *et al.* 2000). Once bacteria are part of the biofilm they lose flagella, but continue to multiply and a mature biofilm may have bacteria that have regained flagella in order to leave the biofilm (Guttenplan and Kearns 2013). Thus, flagellated bacteria are required for the formation and spread of new biofilms, maintaining survival of a bacterial population.

Flagella and motility are required for *C. jejuni* biofilm formation, but defects in flagella do not completely abolish biofilm formation (Kalmokoff *et al.* 2006; Reeser *et al.* 2007; Reuter *et al.* 2010). Flagella may enhance cell-cell interactions and other properties, such as flagellar protein secretion and interaction of flagellar glycoproteins, which then contribute to biofilm formation (Reeser *et al.* 2007). Biofilms do indeed increase the survival of *C. jejuni* in high oxygen, low nutrient environments and at lower temperatures and *C. jejuni* cells in biofilms may enter a viable but non-culturable state to endure stress inducing conditions (Reeser *et al.* 2007; Reuter *et al.* 2010). *C. jejuni* biofilms may have an important role in the persistence of *C. jejuni* in a food setting, as the presence of bacterial and *C. jejuni* autoinducer, which is involved in quorum sensing, was reported in a variety of food matrices (Murphy *et al.* 2006).

1.10.4 Colonisation and invasion

Flagellar dependent motility is required to bring bacteria in contact with host cells (Harshey 2003). However, bacteria have a range of adhesins and macromolecular

structures that interact with host cells, including pili and secretion systems. Although, flagella are not considered as part of this group it may be that *C. jejuni* flagella act as adhesins, but distinguishing their roles in adherence from those in motility has not been possible. In addition, *C. jejuni* flagella secrete effector proteins to enhance colonisation of and survival in host cells (Konkel *et al.* 1999), and so flagella are directly involved in colonisation and invasion performing the role of a type III secretion system. Type III secretion systems are common structures used by bacteria to interact with and deliver effector proteins into host cells to aid invasion and survival, and are evolutionary related to flagella (Blocker *et al.* 2003).

(i) Flagella as an adhesin and invasion determinant

Evidence from the early motility studies showed that centrifugation of strains onto the cell monolayer improved invasion, suggesting that flagella provide motility, rather than adhesion properties (Newell *et al.* 1985; Nachamkin *et al.* 1993; Wassenaar *et al.* 1993). Moreover, flagella did not act as adhesins when sheared and added to bacterial suspensions (Wassenaar *et al.* 1993) and *C. jejuni* with or without flagella showed similar levels of adherence to cell monolayers after centrifugation, although invasion by flagellated *C. jejuni* was higher than aflagellate bacteria (Grant *et al.* 1993). *C. jejuni* were also still invasive when flagella were coated in antibodies, indicating that other adhesins and invasion determinants must contribute to adherence and invasion (Konkel and Joens 1989).

In contrast to these studies, *C. jejuni* with paralysed flagella (*pflA* mutants) have been shown to adhere to host cells, and it was suggested that flagella may primarily mediate *C. jejuni* attachment with the flagella tips and then pseudopods are extended to envelop the bacterium (Konkel *et al.* 1992; Yao *et al.* 1994). *In vivo*, a poorly motile *C. jejuni* variant expressing both *flaA* and *flaB* colonised chicks in high numbers, suggesting that flagella do act as an adhesin regardless of a lack of motility (Wassenaar *et al.* 1993). Moreover, *C. jejuni* expressing *flaB*, but not *flaA* showed at least 100 fold reduced colonisation compared to the wild-type, whereas *C. jejuni* expressing *flaA*, but not *flaB* were as invasive as wild-type (Wassenaar *et al.* 1993). Therefore, FlaA is implicated as a factor for adhesion and host colonisation, but it remains difficult to know whether flagella are important for motility alone or if they are adhesins in their own right.

Nevertheless, flagella are important virulence factors for *C. jejuni*, especially due to the ability to secrete non-flagellar proteins, as described below.

(ii) C. jejuni *secreted proteins*

C. jejuni flagella function as a secretion apparatus for non-flagellar proteins and these are important for virulence. At least eight secreted proteins have been described and were only secreted in the presence of epithelial cells or under certain nutrient-limited conditions, such as serum-free or exhausted media (Friis *et al.* 2007). It may be that Cia expression is triggered on entry into the small intestine, as deoxycholate was found to stimulate Cia protein synthesis (Rivera-Amill and Konkel 1999). Two of these proteins, CiaB (*cj0914c*) and CiaC (*cj1242*) require a minimum flagellar structure for secretion and are needed for human intestinal epithelial cell invasion and colonisation of chickens and pigs (Konkel *et al.* 1999; Konkel *et al.* 2004; Neal-McKinney and Konkel 2012). In contrast, CiaI (*cj1450*), has a type III secretion motif, but does not contribute to *C. jejuni* adherence to or invasion of host cells, but CiaI is required for *C. jejuni* intracellular survival and remodels the *Campylobacter* containing vacuole (Buelow *et al.* 2011). The processes and mechanisms in which Cia proteins are delivered into cells by flagella are still unknown. However, flagellar proteins have not been found to be delivered into host cells, which suggests the mechanism is specific for Cia protein delivery (Neal-McKinney and Konkel 2012).

C. jejuni also secretes other non-flagellar proteins, including FlaC (*cj0720c*) and FspA (*cj0859c*), which are only secreted if the basal body and hook have been formed (Song *et al.* 2004). Purified FlaC binds to host cells and *C. jejuni flaC* mutants are decreased for epithelial cell invasion (Song *et al.* 2004). FspA is found in different forms in different strains of *C. jejuni* and FspA from strain CG8486, a clinical isolate from Thailand, bound to and caused apoptosis of eukaryotic cells, but FspA from 81-176 did not cause apoptosis (Poly *et al.* 2007). Other virulent strains of *C. jejuni* may contain both forms of the protein and so regulation of this protein has important implications for clinical disease (Poly *et al.* 2007). Secreted proteins could potentially be used as vaccine adjuvants as they also elicit host immune responses and patents for the use of non-flagellar secreted proteins and polypeptides have been filed (Guerry-Kopecko and Baqar 2008).

Section 3 Summary and research aims

1.11 Summary

C. jejuni is an important foodborne pathogen that places a large economic burden on developed countries and can cause unpleasant and sometimes life threatening sequelae. *C. jejuni* adapts to survive in many different environments, despite being a fastidious organism, and has virulence determinants that contribute to causing disease in humans. Flagella are important for *C. jejuni* virulence and flagellar structure differs from most bacteria with divergent major flagellin subunits and with extra proteins that are required for motility. Flagellar expression also differs as it does not require control by a master regulator and *C. jejuni* flagella have secretory functions that are unique. Despite its ability to adapt to various conditions and express many survival and virulence mechanisms, *C. jejuni* has a small genome and gene expression is regulated by mainly protein transcriptional regulators including the three σ factors, and by genetic rearrangements.

Questions still remain, such as what are the mechanisms that allow *C. jejuni* to persist in varied and stressful environments, and knowledge gained may be directly applied to implementing cost effective measures to reduce *C. jejuni* in a variety of food matrices and livestock. A reduction of *C. jejuni* in the food chain would translate into reduced prevalence of *C. jejuni* foodborne outbreaks.

In addition, it is known that *C. jejuni* has virulence factors and that they contribute to specific aspects of host infection, but how they work to together to cause disease in humans and why disease is not seen in poultry is unclear. A factor crucial for *C. jejuni* virulence is flagella-dependent motility, but the exact regulation in different environments and what signals triggers flagella expression is unknown.

There is a lack of standardised *in vitro* experiments and limited availability of *in vivo* models generally. New techniques and cell lines mean that physiologically relevant model systems can now be exploited and shared by the community. As recent *in vivo* models become more established, they will be important tools in evaluating *C. jejuni* host-interactions.

A continuing conundrum is that *C. jejuni* has a small genome, but is still able to regulate the expression of many proteins and adapt to many environments. Two of the three σ

factors are dedicated to flagella expression and associated functions, so how does *C. jejuni* regulate gene expression when different conditions are met? To illustrate, *C. jejuni* lacks RpoS (σ^{38}), which activates genes for a general stress response and responds to many stressors in *E. coli* (Hengge 2008). It may be that proteins have evolved to perform more functions to cope with genome reductions (Kelkar and Ochman 2013), or that small molecules (Gaynor *et al.* 2005) and regulatory RNAs may be significant genome regulators in *C. jejuni* (Dugar *et al.* 2013; Porcelli *et al.* 2013). Studies focussed on regulation or expression of specific systems, will ultimately lead to advances in our overall knowledge of genome regulation in *C. jejuni*.

1.12 Aims

The overall aim of this research was to understand flagellar regulation and how it contributes to the pathogenicity of *C. jejuni* infection.

- The first aim was to understand *C. jejuni* responses to acidic pH and how flagellar regulation and function is affected, as acidic environments are encountered by *C. jejuni* during stomach passage and need to be overcome before causing human disease or colonising the avian intestine.
- The second aim was to elucidate the role of small non-coding RNAs in regulating flagellar gene expression and *C. jejuni* virulence. Knowledge about prokaryotic non-coding RNAs, generally, is rapidly expanding, but is still in the early stages of *C. jejuni* research.

Understanding *C. jejuni* flagellar regulation is important because flagella are crucial virulence determinants, but the regulation of this complex and tightly regulated organelle is not fully understood. Furthering knowledge in these areas will enhance our understanding of *C. jejuni* virulence and may create opportunities for interventions preventing *C. jejuni* infections.

Chapter 2 Materials and methods

2.1 Bacterial strains and growth conditions

C. jejuni strains were cultured in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific) under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) at 37°C. For growth on plates, strains were either grown on 1.5% agar plates with Brucella medium, blood medium, or Skirrow medium. Recipes are listed in 2.2.1.

For broth culture, *C. jejuni* single use glycerol stocks were plated on Skirrow agar for 16 h, prior to growth in Brucella broth. Bacteria were recovered in 2 ml pre-warmed Brucella broth and the bacterial suspension was used to inoculate fresh broth at 1:1000 dilution. Cultures were incubated under the above conditions with shaking at 200 rpm for 16 h to form starter cultures for further experiments.

Where appropriate, media were supplemented with 50 μg ml^{-1} kanamycin, 15 μg ml^{-1} chloramphenicol and/or Skirrow antibiotics (10 μg ml^{-1} vancomycin, 5 μg ml^{-1} trimethoprim, 2.5 IU polymyxin-B).

Escherichia coli strains were cultured in Luria-Bertani (LB) medium at 37°C, with broth cultures shaken at 200 rpm. Where appropriate, media were supplemented with 30 μg ml^{-1} kanamycin, 15 μg ml^{-1} chloramphenicol and/or 100 μg ml^{-1} carbenicillin.

2.2 Media

All media were prepared by adding ingredients to 1 litre of water and autoclaving at 69 KPa for 20 minutes. For plates, 1.5% agar (Difco) was added. Antibiotics, dyes or X-gal were added aseptically after autoclaving when necessary.

2.2.1 LB Medium

- 10 g BactoTM tryptone (Difco Laboratories)
- 5 g BactoTM yeast extract (Difco Laboratories)
- 10 g NaCl

2.2.2 SOC

- Tryptone 2.0 g
- Yeast extract 0.5 g
- Glucose (2M) 1.0 ml
- $MgCl_2$ (1M) 0.5 ml
- $MgSO_4$ (1M) 0.5 ml
- NaCl (1M) 1.0 ml
- KCl (1M) 0.25 ml
- Sterile distilled water to 100 ml

2.2.3 Brucella Medium

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

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

- 15 g Proteose peptone
- 2.5 g Liver Digest
- 5.0 g Yeast Extract
- 5.0 g NaCl

Campylobacter selective supplement (Oxoid, final concentration Vancomycin 0.1 mg ml^{-1}, Trimethoprim 50 μg ml^{-1}, Polmyxin B2 500 IU L^{-1}) was added after autoclaving.

2.2.5 Blood medium

- 15 g Proteose peptone
- 2.5 g Liver Digest
- 1% Yeast Extract
- 5.0 g NaCl
- 5% Horse Blood Oxalated (Oxoid)

2.2.6 Antibiotics, dye and X-gal used in media

- Antibiotics and triphenyl tetrazolium chloride dye were made to concentrated solution in water or 50% ethanol and filter sterilised (0.2 μM Sartorius Stedim Biotech) then added to autoclaved media.
- X-gal (bromo-chloro-indolyl-galactopyranoside) was made into solution (1:10 w/v) with dimethyl formamide and then added to autoclaved media.

2.3 Glycerol stocks

To make main glycerol stocks, bacteria were cultured for 16 h in 15 ml Brucella supplemented with the appropriate antibiotics. Cells were precipitated by centrifugation (3, 200 x *g*) and then resuspended in 20% sterile glycerol in Brucella. Stocks were kept at -80°C.

For routine experiments, single use glycerol stocks were prepared from the main stocks. Cells were scraped from the main glycerol stock with a loop, spread on blood plates for 48 h and subcultured on Skirrow plates for 48 h. Cells were recovered in 2 ml Brucella and frozen in 50 μl aliquots at -80°C.

2.4 Nucleic acid manipulations

2.4.1 *C. jejuni* mutations

Motile *C. jejuni* NCTC11168 was used to make all mutant strains. Digestion reactions, primers, and PCR reactions and protocols are listed in tables 2.1, 2.2 and 2.3, respectively.

(i) Gene deletions and inactivations

To make gene deletions and inactivations, antibiotic cassettes were used to replace complete genes or to disrupt the gene on the *C. jejuni* chromosome. The mutation constructs were made in suicide vectors and positive insertions were selected from *E. coli* transformants, which were then sequence checked before being used to transform *C. jejuni* by electroporation.

Kanamycin (kan^R) and chloramphenicol ($chlor^R$) antibiotic cassettes were amplified with primers containing *Bam*HI ends, gel purified, digested with *Bam*HI and PCR purified (Qiagen kit).

The NC1 and NC4 deletion shuttle plasmids were constructed by Bruce Pearson. For the NC1 and NC4 deletions the flanking regions of the genes were amplified with a tag containing a *Bam*HI restriction site. The fragments were joined by an overlap PCR and the joined fragment was purified and ligated to pGEM-T easy. Antibiotic cassettes with *Bam*HI sticky ends were ligated in between the flanking regions contained on pGEM-T easy. A *C. jejuni* double *NC1/NC4* deficient mutant was made by replacing *NC4* with $chlor^R$ in a previously made *C. jejuni NC1*::kan^R mutant.

For *cj0428* and *cj1650* disruptions, the whole gene including the flanking regions was amplified using primers tagged with sequences containing restriction sites for *EcoR*I and *Pst*I (for *cj0428* and *cj1650*). The fragments were gel purified, digested, PCR purified and ligated into pGEM-T easy. An inverse PCR was performed amplifying the gene flanking regions with pGEM-T easy backbone with *Bam*HI restriction sites, but without the majority of the gene. The inverse PCR was PCR purified (Qiagen) and antibiotic cassettes with *Bam*HI sticky ends were ligated to the inverse PCR product.

The *flaAB* deletion suicide plasmid was constructed by Duncan Gaskin. The *flaAB* disruptions were made by amplifying the flanking regions with tags on the 3' end of the 'front' flank and on the 5' end of the 'reverse' flank. The kanamycin cassette was amplified with complimentary tags and all fragments were joined together using overlap PCR. The fragment PCR purified and was blunt-end ligated into pUC19.

(ii) Gene over-expression

Suicide plasmids were constructed and expressed in *E. coli* and positives were identified by colony PCR, isolated and sequences checked before being used to transform *C.*

jejuni. To over-express genes, the genes were amplified with primers with tags containing *Bsm*BI restriction sites. The fragments were PCR purified and digested with *Bsm*BI. The fragments were gel purified and ligated into suicide plasmids digested with *Bsm*BI. The plasmids were designed by Duncan Gaskin to homologously recombine the desired insertion constructs into a pseudogene region; either the *cj0046* (pC46 with chlor^R) or *cj0223* (pK223 with kan^R) region (plasmid maps and sequence are in Appendix 1). Variations of the shuttle vectors have been made, which contain promoters with different expression levels: *metK* (low expression), *fdxA* (moderate expression), *porA* (high expression). The *Bsm*BI site is situated downstream of the promoter and upstream of an antibiotic cassette.

The NC1 and NC4 over-expression strains were made by synthesising each of the genes with either their native, *metK* or *fdxA* promoter in a plasmid with *Nco*I restriction sites in between (GeneArt). The plasmid was digested with *Nco*I, and fragments were DyeEx cleaned (Qiagen) and ligated into pC46 (no promoter). The desired inserts were identified by sequencing.

2.4.2 Translational control plasmids

Digestion reactions, primers, and PCR reactions and protocols are listed in tables 2.1, 2.2 and 2.3, respectively.

NC1 and NC4 translational control of their predicted targets was studied using green fluorescent protein (GFP) reporter plasmids, kindly provided by Jörg Vogel and colleagues, which are described by Urban and Vogel (Urban and Vogel 2007). The reporter plasmid containing the 5' untranslated region (5'UTR) of a predicted target fused to *gfp+* was constructed as follows. Complimentary oligonucleotides of the 5'UTR of a target gene, including 10-15 codons, was synthesised with a 5' *Nhe*I restriction site and a 3' *Mph*11031 site (MWG Eurofins). Oligonucleotides were annealed in equal volumes and 2 µl of each 5'UTR insert was mixed with 20 µl pXG-10 (30-70 ng) and digested with *Nhe*I and *Mph*11031. The digest was DyeEx (Qiagen) cleaned and 10 µl was used in a ligation reaction. *E. coli* were transformed using 1.5 µl of the ligation reaction and chloramphenicol resistant transformants were selected for plasmid purification.

The NC1 or NC4 containing plasmids were constructed as follows. A fragment was amplified from pZE12-luc and digested by *Xba*I to create a pZE12 working fragment. NC1 and NC4 complimentary oligonucleotides were synthesised with a 3' *Xba*I restriction site (MWG Eurofins). These were annealed in equal volumes and 2 µl of each was added to a ligation reaction with 8 µl pZE12 working fragment with the addition of 1 µl polynucleotide kinase (NEB). NEB 5' alpha F'lacI^q competent cells *E. coli* were transformed using 1 µl of the ligation and carbenicillin resistant transformants were selected for plasmid purification.

Top10 *E. coli* were then co-transformed with different combinations of reporter and ncRNA containing plasmids. To measure the extent of translational control, the fluorescence of co-transformed *E. coli* was compared to *E. coli* containing the reporter vector alone. Plasmid pJV300 containing a nonsense ncRNA was used as a negative control, which does not regulate *gfp*+ expression (Urban and Vogel 2007).

2.4.3 Ligations

Routine ligations reactions used 1 µl T4 ligase (NEB), 2 µl 10 x T4 ligase buffer, 2 µl plasmid, 8 µl (unless specified differently elsewhere) of cleaned, digested DNA to be inserted and 7 µl water was incubated for 1 h at 20°C or 16 h at 4°C. Ligation reactions were directly used in *E. coli* transformations.

Ligation into pGEM-T easy (Promega) was performed using 1 µl T4 ligase (Promega), 5 µl 2 x Rapid Ligation Buffer, 1 µl pGEM-T easy (50 ng) and a variable volume of insert; the reaction was made up to a final volume of 10 µl with water.

2.4.4 Sequencing

Purified plasmids (50-100 ng) or PCR products (5 ng) and primers (10 mM) were sent to MWG Eurofins for sequencing. Sequences were assembled using Lasergene Seqman Pro (DNASTAR) and were compared to expected *in silico* constructed sequences made using pDraw (Acaclone).

2.4.5 Transformations

(i) Preparation of chemically competent E. coli *cells*

Chemically competent *E. coli* were produced using the calcium chloride method. A 50 µl aliquot of an overnight culture of *E. coli* was inoculated into 5 ml LB broth and

grown at 37°C until the OD_{600nm} reached 0.6. The cells were then centrifuged at 3,220 x *g* for 10 min, the supernatant removed, the pellet resuspended in 1 ml 0.1 M $CaCl_2$ and left on ice for 30 min. The cells were then washed twice in 0.1 M $CaCl_2$ and left on ice for 1 h after the final wash. Finally, the cell suspension was dispensed into 100 µl aliquots, snap frozen on dye ice and stored at -80°C until required.

(ii) E. coli transformation

E. coli Top10 chemically competent cells (Invitrogen) were transformed unless specified elsewhere. Vials were thawed on ice after which DNA was added and incubated for 20 min on ice. Cells were then heat shock at 42°C for 45 sec and then incubated on ice for 2 min. For recovery, 300 µl SOC medium was added and the cell suspension was incubated for 60 min at 37°C with 200 rpm shaking. The cell suspension (50 and 200 µl) was plated on LB agar with antibiotics and X-GAL where appropriate.

(iii) Preparation and transformation of C. jejuni

C. jejuni, from glycerol stocks, were grown on Skirrow plates for 16 h. Lawns of *C. jejuni* were recovered in 2 ml Brucella broth and the cell suspension was centrifuged at 9,200 x *g* for 3 min. The cells were resuspended in 1 ml *Campylobacter* transformation buffer (CTB, 272 mM sucrose and 15% glycerol in demineralised water) and centrifuged as above. A further two washes with CTB were applied. After the final wash, the cells were resuspended in 500 µl CTB and pooled if appropriate. Plasmid DNA (< 1 µg) was added to 100 µl of cell suspension in pre-cooled electroporation cuvettes and electroporated at 2.5 kV, 200 Ω, 25 µF (BioRad). Brucella broth, 200 µl, was added to the cuvettes and then the mix was plated onto blood agar plates to recover in microaerobic conditions at 37°C for 5 h. Lawns were recovered in 800 µl pre-warmed Brucella broth and plated onto Brucella agar plates with the appropriate antibiotics. Plates were incubated in microaerobic conditions. Genomic DNA was isolated from transformants and PCR was used to identify successful disruptions or insertions.

Table 2.1 DNA digestions

Enzymes	Digest reagents	Incubation
*Bam*HI	10 µl Plasmid (0.5-1.0 µg)	37°C, 2 h

(NEB)	60 units enzyme 5 µl 10 x Buffer III Water to final volume 50 µl	
*Bsm*BI (Thermo Scientific)	10 µl pC46 (and antibiotic variants) 2 µl enzyme 5 µl 10 x Buffer Tango 1 µl 50 mM DTT Water to final volume 50 µl	37°C, 1h then add further 1 µl *Bsm*BI and incubate further 1 h
Double *Pst*I-HF, *Eco*RI-HF (NEB)	10 µl pUC19 (~0.5 µg) / purified PCR 60 units *Eco*RI-HF and *Sac*I-HF 5 µl 10 x Buffer IV 5 µl 10 mg ml^{-1} BSA Water to final volume 50 µl	37°C, 2 h
*Nco*I-HF (NEB)	10 µl plasmid (0.5 µg) 60 units enzyme 5 µl 10 x Buffer IV Water to final volume 50 µl	37°C, 2 h
pXG10 with *Nhe*I, *Mph*11031 (Thermo Scientific)	10 µl pXG10 (~70 ng) 2 µl each annealed oligonucleotide 6 µl 10 x Buffer Tango 40 units *Nhe*I 20 units *Mph*11031 Water to final volume 60 µl	37°C, 16 h

pZE12-luc PCR with *Xba*I (Roche)	10 µl PCR (0.5-1.0 µg) 30 units enzyme 5 µl 10 x Buffer A Water to final volume 50 µl	37°C, 2 h

Table 2.2 Polymerase chain reactions.

Performed using a Multigene OptiMax Thermal Cycler (Labnet International).

PCR type	Reagents	Protocol
40 cycle	25 µl Hotstartaq master mix (Qiagen) 0.5 µl each primer* 0.5 µl DNA template < 1 µg RNAse free water to final volume 50 µl	Initial: 95°C 15 min; 40 cycle: 94°C 1 min, 50°C 1 min, 72°C 2 min; Final extension: 72°C, 10 min.
Amplification	10 µl 5 x HF Buffer (NEB) 0.5 µl Phusion DNA polymerase (NEB) 0.5 µl DNA template (< 1 µg) 0.5 µl each primer 1 µl 10 mM dNTP (Invitrogen) Water to final volume 50 µl	Initial: 98°C 1 min; 30 cycle: 98°C 20 sec, 50°C 30 sec, 72°C 30 sec per kilobase; Final extension: 72°C, 10 min.
Check	10 µl Hotstartaq master mix (Qiagen) 0.5 µl each primer 0.5 µl DNA template < 1 µg Water to final volume 20 µl	Initial: 95°C 15 min; 30 cycle: 94°C 1 min, 50°C 1 min, 72°C 2 min; Final extension: 72°C, 10 min.
Colony #	10 µl Hotstartaq master mix (Qiagen) 0.5 µl each primer Variable bacterial cells Water to final volume 20 µl	Initial: 95°C 15 min; 30 cycle: 94°C 1 min, 50°C 1 min, 72°C 2 min; Final extension: 72°C, 10 min.
Overlap	10 µl 5 x HF Buffer (NEB) 0.5 µl Phusion DNA polymerase (NEB) 0.5 µl each fragment (~40 ng) 0.5 µl each primer 1 µl 10 mM dNTP (Invitrogen) Water to final volume 50 µl	Initial: 98°C 1 min; 30 cycle: 98°C 20 sec, 50°C 30 sec, 72°C 30 sec per kilobase; Final extension: 72°C, 10 min.

* Primer working concentration is 10 mM.
# Colony: *E. coli* cells picked with yellow tip and used to inoculate PCR reaction. C. jejuni were pick with yellow tip and suspended in 100 µl water, boiled 5 min 95°C and 1 µl was added to PCR reaction.

Table 2.3 Primers.

Nucleotides in blue are tags containing restriction sites.

Gene deletion	Primer name	Primer Sequence (5' - 3')
chlor	cat for BamHI	GTTTTGGATCCAAGGATATGACTATCTACTG
	cat rev BamHI	GTTTTGGATCCACTCTTCATGTCGATTGATG
cj0428	cj0428 KOF EcoRI	GTTTTGAATTCAATACAGCGCAGGT
	cj0428 KOR PstI	GTTTTCTGCAGTGATTTCAATCAAATTGTAG
	cj0428 BamHI Rev inv	CTAGGGATCCATCTCCGCTTGACTTCTCAT
	cj0428 BamHI Fwd inv	CTAGGGATCCAGTGCTAGCGTTCATAATGA
	cj0428 F check	TGGTAAGCTTACGGATTTTCGT
	cj0428 R check	AGCCGTTCATTTTGTCTATGCT
cj1650	cj01650 KOF EcoRI	GTTTTGAATTCCTTACTTTCTTCAACAAG
	cj1650 KOR PstI	GTTTTCTGCAGGAAATTCTTTCTATGATATG
	cj1650 BamHIRinv	CTAGGGATCCTGCTCCTATAAAATCATTTGCA
	cj1650 BamHIFinv	CTAGGGATCCATTACCATGGGCGAAACTAT
	cj1650 F check	TGCACCTTTGTATTTAAACTCA
	cj1650 R check	TGCATAGAGCCTATGATATG
kan	kan for BamHI	GTTTTGGATCCTATTGACAATACTGATAAGA
	kan rev BamHI	GTTTTGGATCCCTAGGTACTAAAACAATTCA

NC1	NC1oligo1 F	GATGGTTTTAGTGTAGATAC
	NC1oligo2 R	GACGCGGATCCGCGTTGGCGCGCCA TATCGGTTTAAATCTTATC
	NC1oligo3 F	GGCGCGCCAACGCGGATCCGCGTCA TAAATCTTTTAAAGCTTC
	NC1oligo4 R	GGAATGAGTTTTGAAAGCAC
	NC1 F check	TAATTATCTCTTAGGCTTAG
	NC1 R check	GGTAAAATTCCTATAGATTG
NC4	NC4oligo1 F	ATTTAATCTTGCGTCCTATG
	NC4oligo2 R	GACGCGGATCCGCGTTGGCGCGCCA ATTCAAATACTATATCGCTTG
	NC4oligo3 F	GGCGCGCCAACGCGGATCCGCGTCA AGGCTTTTTTTATTTTAATCC
	NC4oligo4 R	TTGATCCAAAACTAGAAAGT
	NC4 F check	TATGAGTCAAAGAAGTGATG
	NC4 R check	AAGTGGTGTTTTTGGAGTAG

Table 2.4 Translational control oligonucleotides and primers.

Nucleotides in: red are of the 5' untranslated region; black are codons; and in blue are sticky ends for ligation.

Gene	Oligo	Sequence (5' – 3')	# aa
Cj0428	428 UTR ATG F	GCATATTTTGAAAGGAGAAAACTATGG	1
	428 UTR ATG R	CTAGCCATAGTTTTCTCCTTTCAAAATATGCTGCA	
Cj0428	428 UTR long F 6	GCATATTTTGAAAGGAGAAAACTATGCAGGTAAATTATAGAG	6
	428 UTR long R 6	CTAGCTCTATAATTTACCTGCATAGTTTTCTCCTTTCAAAATATGCTGCA	
Cj0428	428 UTR long F	GCATATTTTGAAAGGAGAAAACTATGCAGGTAAATTATAGAACGATTAGCTCGTATGAAG	12
	428 UTR long R	CTAGCTTCATACGAGCTAATCGTTCTATAATTTACCTGCATAGTTTTCTCCTTTCAAAATATGCTGCA	
Cj0428	428 UTR long F 20	GCATATTTTGAAAGGAGAAAACTATGCAGGTAAATTATAGAACGATTAGCTCGTATGAATACGATGCTATTAGTGGTCAGTATG	20
	428 UTR long R 20	CTAGCATACTGACCACTAATAGCATCGTATTCATACGAGCTAATCGTTCTATAATTTACCTGCATAGTTTTCTCCTTTCAAAATATGCTGCA	
Cj0582	LysC UTR F	CTTGAGATTTAAGGAACAATATTGTGGAAAACGAGAAAAATTATAGACCAAATGTTGCAG	15
	LysC UTR R	CTGCAACATTTGGTCTATAATTTTTCTCGTTTTCCACAATATTGTTCCTTAAATCTCAAGTGCA	
Cj1026c	FlgP UTR long R	CTAGCTCCTGCTATTGCTAGCATAAAATAAATTTTTTTCATTTTTACACCTTCAAAATATTGTAATTTGCA	12
	FlgP UTR long R	CTAGCTCCTGCTATTGCTAGCATAAAATAAATTTTTTTCATTTTTACACCTTCAAAATATTGTAATTTGCA	
Cj1026c	FlgP UTR ATG F	AATTACAATATTTTGAAGGTGTAAAAATGG	1
	FlgP UTR ATG R	CTAGCCATTTTTACACCTTCAAAATATTGTAATTTGCA	

Cj1338c	FlaB UTR ATG F	CGATGCAATATTTTGAAAGGATTTAAAATGG	1
	FlaB UTR ATG R	CTAGCCATTTTAAATCCTTTCAAAATATTGCATCGTGCA	
Cj1729c	FlgE2 UTR long F	ATAAACGCAAAAGTTTTTTAAAGCCAAAGCGTTAAATTTTTT AAAGCAATATTTTATAAAGGATTTAAGATGATGAGATCACTTTGGTCTGGCGTAAGCGGACTAG	12
	FlgE2 UTR long R	CTAGCTAGTCCGCTTACGCCAGACCAAAGTGATCTCATCATCTTAAATCCTTTATAAAATATTGCTTTAAAAAATTTAACGCTTTGGCTTTAAAAAACTTTTGCGTTTATTGCA	
Cj1650	1650 UTR long F	AGCAATATTTTTGAAAGGTAAACAATGAAAAGTGATTTAGATATATTTAAAAAACACTTAG	12
	1650 UTR long R	CTAGCTAAGTGTTTTTTAAATATATCTAAATCACTTTTCATTGTTTACCTTTCAAAAATATTGCTTGCA	
NC1	NC1 Fwd oligo	AAATCTTTTCAAAATATTGCAATTTGCCCATTTTTGGGCATCTTTT	n/a
	NC1 Rev oligo	CTAGAAAAGATGCCCAAAAATGGGCAAATTGCAATATTTTGAAAAGATTT	
NC4	NC4 Fwd oligo	GAATCTTTTCAAAATATTGCAATCAAGCCCATGAAAATGGGCTTTTTT	n/a
	NC4 Rev oligo	CTAGAAAAAAGCCCATTTTCATGGGCTTGATTGCAATATTTTTGAAAAGATTC	
pXG10	pXG-10 F check	ACGGTCTGGTTATAGGTACA	n/a
pXG10	pXG-10 R check	CATGCCGTTTCATATGATCC	
pZE12-*luc*	PLlacOB	CGCACTGACCGAATTCATTAAAG	n/a
pZE12-*luc*	PLlacOD	GTGCTCAGTATCTTGTTATCCGCTCA	

Oligo – Oligonucleotide
aa – number of codons included in fusion

2.5 DNA purification

DNA was purified for downstream applications and was stored at -20°C as necessary.

2.5.1 PCR purification

A QIAquick PCR Purification Kit (Qiagen) was used according to the manufacturer's instructions to clean DNA. Buffer PB (5 volumes) was added to 1 volume of PCR product, mixed by pipetting and added to a QIAquick spin column in a 2 ml collection tube. The spin column was centrifuged for 1 min (20,000 x *g*), the flow-through discarded and the column washed with 750 µl buffer PE. The column was then centrifuged for an additional 3 min, to remove all traces of wash buffer, and placed into a clean 1.5 ml micro centrifuge tube. DNA was eluted in 50 µl buffer EB and DNA yield and quality was checked using a Nanodrop spectrophotometer (Thermo Scientific) or by agarose gel electrophoresis.

2.5.2 DNA extraction from agarose gels

DNA was separated on 0.8% agarose gels and the desired band was excised from the gel using a clean scalpel blade. Three volumes of buffer QG relative to the mass of the excised band were added. The band was then incubated at 50°C for 10 min. Once dissolved, 1 volume of isopropanol was added, mixed by pipetting and the sample added to a QIAquick spin column in a 2 ml collection tube. The spin column was centrifuged (20,000 x *g*) for 1 min, the flow-through discarded and the column washed with 500 µl buffer QC and 750 µl buffer PE. The column was then centrifuged for 3 min to remove any traces of wash buffer remaining, placed in a clean 1.5 ml microcentrifuge tube and the DNA eluted with 50 µl buffer EB. DNA yield and quality was checked using a Nanodrop spectrophotometer or by agarose gel electrophoresis.

2.5.3 DyeEx

The spin columns were vortexed for 30 sec, the seal lock broken and the lids loosened by a quarter turn. The columns were placed in collection tubes and centrifuged (770 x *g*) for 3 min. The columns were place in clean microcentrifuge tubes and the DNA solution to be purified was pipetted drop by drop into the centre of the gel. The column was centrifuged for 3 min to elute the DNA.

2.5.4 DNA isolation

Genomic DNA was isolated from *C. jejuni* strains after 16 h growth in 25 ml Brucella broth. Cultures were centrifuged (3,220 x *g*) for 20 min and cell pellets were resuspended in 1 ml PBS. The cell suspension was centrifuged for 3 min at 20,000 x *g*. The pellet was resuspended in 400 µl TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA) and lysed with 70 µl 10% SDS and 5 µl 10 mg ml^{-1} proteinase K at 65°C for 10 min. The lysis reaction was incubated for a further 10 min after the addition of 100 µl 5 M NaCl was added and mixed, then 100 µl 10% Hexadecyl trimethyl ammonium bromide in 0.7 M NaCl (both solutions pre-warmed to 65°C). Choloroform/isomayl alcohol (24:1), 500 µl, was added, vortexed for 20 sec and centrifuged for 5 min. The upper aqueous phase was incubated at -20°C for 30 min with 0.6 volumes isopropanol. The mixture was centrifuged for 10 min and washed with 500 µl 70 % ethanol. The DNA pellet was air dried, resuspended in 50 µl TE buffer and incubated for 30 min at room temperature before storing at -20°C.

DNA was also isolated using a DNeasy kit for Blood and Tissue (Qiagen) from *C. jejuni* cells grown 16 h in a maximum of 10 ml Brucella broth. Bacteria were centrifuged (3,200 x *g*, 20 min) and cells were resuspended in 180 µl ATL buffer. The sample was vortexed, 15 sec, and 200 µl AL buffer then 200 µl 100% ethanol were added sequentially with vortexing between stages. The sample was centrifuged through the DNeasy Mini Spin column (6,000 x *g*, 1 min), and the flow through was discarded. The column was washed with 500 µl AW1 buffer (6,000 x *g*, 1 min), then 500 µl AW2 buffer (20,000 x *g*, 3 min). DNA was eluted in 200 µl AE buffer applied twice to the filter membrane (1 min incubation at room temperature, followed by 6,000 x *g*, 1 min).

2.5.5 Plasmid purification

(i) Mini-prep (isolation of up to 30 µg DNA)

E. coli were grown 16 h over night in 5 ml LB broth with the appropriate antibiotics. Cells are pelleted by centrifugation (3,220 x *g*) for 10 min and the E.N.Z.A. Plasmid Mini Kit I (Omega Bio-tek) was used to isolate plasmid. Cells were resuspended in 250 µl Solution I and then lysed 250 µl Solution II at 20°C, and neutralised in 350 µl Solution III. DNA binding columns were prepared with 100 µl Column Equilibration Buffer, which was discarded after centrifugation (20,000 x *g*) for 1 min. The column

was washed with 500 µl HB Wash and 700 µl DNA wash, and then dried by centrifugation for 3 min. DNA was eluted in 75 µl Elution Buffer.

(ii) Midi-prep (isolation of up to 1 mg DNA)

The GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich) was used to isolate plasmids. *E. coli* were grown 16 h over night in 100 ml LB broth with the appropriate antibiotics. Cells are pelleted by centrifugation (3,220 x *g*, 2 x 20 min) and resuspended in 4 ml Resuspension buffer (with RNAse A, final concentration 0.2 µg ml^{-1}). The cell suspension was lysed (4 min, 20°C) and neutralised with the appropriate buffers (4 ml each). The cell lysate was filtered with the provided syringes after the addition of 3 ml Bind solution. Spin columns were prepared by centrifuging (3000 x *g*, 2 min*)* with 4 ml Column Prep buffer and the filtered cell lysate was applied to the column, centrifuged and washed with Wash 1 and Wash 2 buffers (4 ml). The column was removed of excess ethanol by centrifuging for 5 min and the DNA was eluted in 1 ml elution buffer, which added re-applied to the column and eluted once more.

(iii) Midi-prep for very low copy plasmids (isolation of up to 1 mg DNA)

Plasmid XG-10 was isolated using a Midi Kit (Qiagen, UK), according to the 'Very low copy' protocol as it has a copy number of less than five per cell (pSC101 derivative). *E. coli* was grown 16 h in 400 ml LB. Cells were then pelleted by centrifugation at 3,220 x *g* for 20 min in 50 ml batches. The pellets were resuspended in 20 ml buffer P1 containing RNase A at a final concentration of 100 µg ml^{-1}, vortexed and transferred to a Duran bottle. Buffer P2 (20 ml) containing Lyseblue indicator (1:1000 dilution) was added, mixed by inversion until completely blue and incubated for 5 min at 20°C. Buffer P3 (20 ml) was added, mixed by inversion until completely white and incubated for 20 min on ice. The sample was then centrifuged (20,000 x *g*, 4°C) for 45 min, the supernatant was transferred to new centrifuge tubes promptly after centrifugation and centrifuged again for 30 min. DNA was precipitated by adding 42 ml (0.7 volumes) of room-temperature isopropanol and centrifugued for 30 min. The DNA pellet was dissolved in 500 µl TE buffer (pH 8.0) and buffer QF was added to a final volume of 5 ml. A QIAGEN-tip 100 was equilibrated with 4 ml Buffer QBT and allowed to empty by gravity. The DNA in QF buffer from the previous step was applied to the column and allowed to enter the resin by gravity. The column was washed with 2 x 10 ml of buffer QC; the DNA was then eluted in 5 ml buffer QF. DNA was precipitated by

adding 3.5 ml (0.7 volumes) of room-temperature isopropanol, mixing and centrifuging for 30 min. The supernatant was decanted and the DNA pellet washed with 5 ml room-temperature 70% ethanol with centrifugation 15 min. After complete removal of the supernatant, the pellet was air-dried for at 20°C and dissolved in 200 µl TE buffer.

2.6 RNA isolation

To extract total RNA from *C. jejuni*, broth cultures were grown to OD_{600nm} 0.3 and mixed with 0.1 volume of ice-cold 5% phenol in ethanol, and cells were harvested by centrifugation at 7, 200 x *g* for 15 min at 4°C. The pellet was resuspended in 3ml extraction buffer (10mM sodium acetate, 0.15M sucrose, pH 4.8), 333 µl 10% SDS (Gibco) was added and the cell suspension was vortexed. Subsequently 5ml 65°C phenol was added and the cell suspension was incubated at 65 and on ice for 5 min each, and then centrifuged (7, 200 x *g* at 4°C). The upper phase was transferred to a new centrifuge tube, 3 ml room temperature phenol was added and vortexed, and the solution was centifgured again. This step was repeated once with phenol and once with chloroform. Ethanol-sodium acetate (30:1, EtOH-NaAc) was added and incubated 16 h at -20°C. The solution was centrifuged and the pellet was awashed with 75% ethanol. The supernatant was discared and the pellet was air dried. The pellet was resusepedned in 100 µl RNAse, DNase free water (Gibco). A volume of nucleic acid suspension (75-100 µg) was digested with DNaseI (Thermo Scientific, 1 U per 3 µg) for 45 min at 37°C. EDTA (0.1 volumes) was added and the reaction was inactivated for 10 min at 65°C and then incubated on ice 5 min. Removal of DNA was checked by a 40 cycle PCR table 2.2. RNA was extracted with equal volume Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA and vortexed and centrifuged. RNA was precipitated from the upper phase in EtOH-NaAc 16 h at -20°C. The solution was centrifuged and the pellet was washed with 75% ethanol (-20%). The pellet was air dried and resuspended in 100 µl RNAse, DNase free water (Gibco).

2.7 Microarray

2.7.1 RNA reverse transcription, cDNA labelling and chip preparation

Three µg of chromosomal DNA was labelled with Cy3-conjugated dCTP using the BioPrime labelling kit (Invitrogen), with labelling reactions performed overnight at 37 °C. Labelled cDNA was prepared from 15 µg RNA using Stratascript Reverse Transcriptase (Stratagene) for direct incorporation of Cy5-conjugated dCTPs (Amersham), with labeling reactions performed for approximately 16 hr at 42°C. Labelled nucleic acids were cleaned with the Qiaquick purification kit (Qiagen) and dried before being resuspended in water and prepared for hybridisation. Samples were boiled for 2 min, cooled at 18-25°C for 5 min and centrifuged at maximum speed in a microfuge for 2 min to remove any solid particles from the hybridisation mixture. This mixture was put onto the microarray slide and incubated at 60°C for approximately 16 h.

The transcriptome of the *NC4* deletion, *NC1/NC4* deletion and *NC4* over-expressing strains was compared to the wild-type. Labelled cDNA was prepared from 15 µg total RNA using Affinity Script Reverse Transcriptase (Agilent Technologies, UK) for direct incorporation of Cy3- and Cy5-conjugated dUTPs (PA55321, GE Healthcare, UK), with labelling reactions performed for approximately 16 hr at 42°C. Labelled DNA was then cleaned using a DyeEx gel filtration spin column (Qiagen, UK). The samples were then dehydrated using a Savant Speedivac Plus SC2104 (Thermoquest) with attached Vacuum System UV5400A (Thermoquest).

The dried samples were resuspended in 10 µl diethylpyrocarbonate (DEPC) treated water and 25 µl 2 x HiRPM Hybridisation buffer (Aglilent) and 5 µl blocking agent were added (Agilent Hi-RPM Gene Expression Hybridisation Kit). The samples were heated to 103°C (3 minutes) and 40 µl was added onto the array. The array was securely fastened into the gasket and hybridisation occurred overnight in the hybridization oven chamber (Shell Lab) with constant rotation. The array slide was removed from the chamber and submerged in Agilent gene expression wash buffer 1, incubated for 5 min in the buffer 1 on a roller mixer SRTI (Stuart) (room temp., in dark), incubated for 2 minutes in Agilent gene expression wash buffer 2 (37°C, in dark). This was followed by rinses in acetonitrile and stabilisation solution 1 sequentially.

2.7.2 Chip scanning

DNA microarrays were scanned using an Axon GenePix 4000A microarray laser scanner (Axon Instruments) and the data from detected features initially processed using the GenePix 3.0 software.

2.7.3 Analysis

Data were obtained for 1608 genes. For each condition, two independent RNA isolations were hybridised. Each array was manually checked to ensure that detection of each probe was correct. Probe fluorescence intensities were exported into Excel. The data were combined for each biological replicate and Excel macros, including 'Marray,' were used to perform a regression analysis and analyse the statistical significance of changes in gene expression (Holmes *et al.* 2005). Briefly, LOWESS (locally weighted scatter plot smoothing) regression analysis was performed to determine the relationship of the estimated slope and intercept between the Cy3 and Cy5 signals, and so identified genes that had potentially changed in expression level. F-tests were carried out on the hypothesis that there was no change in gene expression, where if rejected then the gene was classified as having increased or decreased expression.

2.7 Nested RT-PCR and detection of ncRNAs

A reverse transcription (RT) reaction was performed using an specific RT primers for NC1, NC4 and NC3 with a 5' extension. A reaction mix containing 500 ng total RNA, 1 µl 10 mM primer and 5 µl nuclease free water (Gibco) was incubated 65°C for 5 min and cooled 10 min at room temperature. Added to this was 2 µl 100 mM DTT, 2 µl Affinity Script buffer, 8 µl 10 mM dNTPs and 1 µl Affinity Script (Aglient). The reaction was incubated at 55°C for 1 h and then inactivated at 70°C for 15 min. A PCR reaction ('Check' protocol, table 2.2) with a specific primer for the extension of the RT primer ('RT tag') and either NC1, NC4 or NC3, followed by gel electrophoresis in 2% low melting point agarose of the product was done to detect the ncRNAs. NC3 (intergenic *Cj1258-Cj1259*) was included as a strain as it is highly transcribed, had a longer sequence and is not predicted to be controlled by σ^{28}. Primers are listed in table 2.5.

Table 2.5 Primers for nested RT-PCR

Nucleotides in red are the added tags for reverse transcription.

Primer name	Primer sequence
NC1 PCR tag	AAATCTTTTCAAAATATTGCAA
NC3 PCR tag	GAATCTTTTCAAAATATTGCAATC
NC4 PCR tag	GAACCGAAAAACATTCATAAG
RT NC1	GCCTTGCCAGCCCGCTCAGACGAGACATC AAAGATGCCCAAAAATGG
RT NC4	GCCTTGCCAGCCCGCTCAGACGAGACATC AAAAAGCCCATTTTCATG
RT NC3	GCCTTGCCAGCCCGCTCAGACGAGACATC AGGGATTTAAGCTAGGCGTG
RT tag	GCCTTGCCAGCCCGCTCAG

2. 8 Northern hybridisations

RNA samples (10 µg) were denatured in RNA loading buffer (95% (v/v) formamide, 0.1% (w/v) xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA) for 5 min at 95°C. They were then separated on 6% acrylamide, 8.3 M urea gels, and transferred to Hybond XL membranes (GE Healthcare) by semi-dry blotter at 25 V for 1 h (Biorad, UK). RNA was UV crosslinked and pre-conditioned in Rapid Hybridisation buffer (GE Healthcare, UK) at 42°C for 1 h, before 5' [γ^{32}P] end labelled probes for NC1 or NC4 were added and incubated for 16 h. NC1 probe: GGCAAATTGCAATATTTTGAA. NC4 probe: TCATGGGCTTGATTGCAAT. Membranes were washed in 5 x SSC/0.1% SDS, 1 x SSC/0.1 SDS and 0.5 x SSC/0.1% SDS. Signals were visualized on a Typhoon 9200 phosphorimager (GE lifesciences) after at least 16 h exposure to a phosphor screen.

2.8 Quantification and determining quality of nucleic acids

RNA and DNA was quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific) to obtain 260/280 and 230/260 ratios. DNA was also visualised on agarose gels if appropriate. RNA quality was checked using the Bioanalyzer 1000 (Agilent).

2.9 Prediction of non-coding RNA gene targets

NC1 and NC4 targets were identified using TargetRNA http://snowwhite.wellesley.edu/targetRNA/ (Tjaden *et al.* 2006), which is no longer available.

2.10 Protein

2.10.1 Cell lysis and fractionation

Bacterial cells were lysed by sonication (MSE) at 10 micron amplitude for 5 x 10 sec, with 30 sec pause on ice, in 1 ml 50 mM Tris-HCL pH 7.5.

To separate the cytoplasmic fraction the lysed whole-cell suspension was centrifuged (17,900 x *g*) for 60 min at 4°C. A sample of the supernatant was kept. To obtain the inner membrane fraction the pellet was resuspended in 400 µl 50 mM Tris-HCL pH 7.5, 1% sarkosyl and solubilised 1h on a rotator (Stuart, 15 rpm). The mix was centrifuged as before to separate the inner membrane fraction (supernatant) from the outer membrane fraction (pellet). The pellet was resuspended in 50 mM Tris-HCL pH 7.5. NuPage SDS loading buffer (Invitrogen) was added to each sample (to a 1 x final concentration) and heated at 95°C for 5 min with β-mercaptoethanol (10%). Samples were stored at 4°C until gel electrophoresis and were re-heated if usage was more than one week after fractionation.

2.10.2 SDS-PAGE

A *C. jejuni* culture was grown in Brucella broth until culture optical density reached OD_{600} 0.3. Bacteria were centrifuged (3,220 x *g*) to precipitate cells, which were subsequently lysed and fractionated. Samples (10-30 µl) were loaded onto pre-made

polyacrylamide gels 4-20% (Expedeon) and run in NuPAGE MOPs SDS running buffer (Invitrogen) at 400 mA.

Gels were stained with Coomassie blue by heating in a microwave (320 W) for 3 x 10 sec, destained (40% isopropanol, 10% acetic acid) by 30 min rocking (Stuart, 30 rpm) and washed with water by 16 h rocking.

2.10.3 Protein concentration

Protein concentrations for 2D gel samples were determined using the 2D Quant Kit (Amersham) according to the manufacturer's instructions. An appropriate amount of sample containing the required protein concentration (0-50 µg) and BSA was used as standard curve (5 different volumes 0-25 µl, 2 mg ml^{-1}), was added to 500 µl precipitant (provided in 2D Quant Kit), vortexed and incubated (3 min, 20°C). To every sample and standards 500 µl co-precipitant was added, vortexed (10 sec), centrifuged (20,000 x *g*, 5 minutes, 4°C). The pellet was resuspended into 100 µl copper solution and 400 µl water. Then, 1 ml working colour reagent (100 part solution A, 1 part solution B from kit), was added to each sample and inverted immediately. After 15-20 min the absorbance was measured at 480 nm (Spectrophotometer Uvikon XL, NorthStar Scientific) and a standard curve was general using the known BSA standards. The assay was performed in duplicate for each sample.

Protein was concentrated to 50 µl or less using a Biomax-5K NMWL membrane filter (Millipore) to contain 100 µg protein (10,000 x *g*, 4°C). Non-bromophenol blue rehydration buffer (450 µl) was added and the sample mixed, then centrifuged using the same membrane filter (10,000 x *g*) until the volume ~50 µl. Then, 400 µl rehydration buffer was added to the sample ensuring all the protein was collected from the membrane filter.

2.10.4 Two-dimensional protein separation

(i) Gel casting

Polyacrylamide gels were cast in-house. For 11 10% gels 344.75 ml 30% Duracryl 0.65% Bis (Genomic Solutions), 257.79 ml 1.5 M Tris gel buffer (1.5 M Tris-HCl, pH 9) and 425.50 ml water were mixed and de-gassed for 10 minutes. Added and mixed to the solution were 10% SDS solution (10.87 ml), tetramethyl ethylene diamine (0.520

ml) and 10% ammonium persulphate (2.64 ml). The SDS-acrylamide solution was poured between two custom-made glass plates in a specially designed chamber (Investigator, Genomic Solutions). Each gel was overlaid with 1 ml water saturated butanol and the gels polymerised after 3-4 h. Each gel was then cleaned to remove the butanol and the excess polyacrylamide gel. The gels were stored in sealed bags with ~50 ml 1/5 diluted Tris gel buffer for up to 2 weeks at 4°C.

(ii) Protein separation by isoelectric point

Sample containing 100 µg protein was made to volume 400 µl with IPG strip rehydration buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 0.1% Bromophenol Blue, 28 mg.ml-1 DTT) and loaded into re-swelling tray. A gel strip (18 cm 3–10NL Immobiline DryStrip, Amersham Biosciences) was laid into the solution. The gel strip was rehydrated at room temperature for minimum 12 h. A moist electrode wick was placed at either end of the IPG gel strip and each well was covered with mineral oil ~2.5 ml. Isoelectrical focussing was performed for 80 kVh at 20°C over 24 hours using the pHaser system (Genomic Solutions). First dimension gel strip can be stored at -80°C.

(ii) Protein separation by molecular weight

Prior to the second dimension the focused gel 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). To reduce and alkylate cysteines the strips were treated first with 8 mg ml^{-1} DTT in the equilibration buffer (9 ml; 30 min with gentle rotation Rotatest shaker, R100/TW, Luckham. Then the strips were transferred into 25 mg ml^{-1} iodoacetamide containing equilibration buffer (9 ml; 30 min with gentle rotation). Polyacrylamide gels were placed in the gel tank, combs removed and wells washed, and the gel strip was placed on to the polyacrylamide, gel-side down). The Investigator 2nd Dimension Running System (Genomic Solutions) was used with cathode buffer (200 mM Tris base, 200 mM Tricine, 14 mM SDS,) and anode buffer (25 mM Tris-acetate buffer, pH 8.3). Electrophoresis was carried out using either a maximum voltage of 500 V or a maximum power of 20 W per gel.

2.10.5 Imaging 2D Gels

The gels were carefully removed from the glass plates. The proteins were fixed (400 ml, 40% methanol, 10% acetic acid, 16 h). Then proteins were stained by Sypro-Ruby (Bio-

Rad, 400ml, 24 h, in the dark). Finally, the gels were de-stained (400 ml, 10% methanol, 6% acetic acid, 4 h). The gels were imaged at 100 μm resolution using the Pharos FX Laser Scanning Fluorescent Imager (Bio-Rad) and a 16-bit image in 65,000 shades of gray was obtained. Voltage of the Photo Multiplier Tube (PMT) was adjusted for each image to improve image quality. The excitation filter used was 532 nm. Gel images were compared using ProteomWeaver analysis software (Definiens). The gels were stored in the de-stain, in the dark at 4°C, for several weeks.

2.11 Routine molecular biology buffers and agarose gels

2.11.1 Phosphate buffered saline (PBS)

PBS tablets (Sigma-Aldrich) were dissolved in 200 ml water and autoclaved. The manufacturer's ingredients were:

- 0.01 M phosphate buffer,
- 0.0027 M potassium chloride
- 0.137 M sodium chloride
- adjusted to pH 7.4

2.11.2 20 x SSC

- 3 M sodium chloride
- 300 mM trisodium citrate
- adjusted to pH 7.0

2.11.3 5 x TBE

- 890mM Tris
- 890mM Boric acid
- 20mM EDTA

2.11.4 TE

- 10 mM Tris
- 1 mM EDTA
- adjusted to pH 8.0

2.11.5 Agarose

Routine gels were made with 0.8% (w/v) agarose powder in 1 x TBE and gels were stained with Safe View (1:20,000 dilution, Applied Biological Materials).

2.12 Phenotypic assays

2.12.1 Viable bacterial counts

To measure numbers of viable bacteria, cultures were grown on plates and colony forming units were counted. Serial 10-fold dilutions were made in sterile PBS and 20 µl of the appropriate dilutions were grown on Brucella-agar plates. Alternatively, 5 µl dilutions were grown on Brucella-agar, which are referred to as 'spot-plates' or 'spotting.' If three or more colonies grew, then this was a positive indicator that there were significant numbers of viable bacteria in the dilution. The number of viable bacteria was taken to be one and so the estimated number of colony forming units (cfu) = 1 x (1000 µl / 5 µl) x dilution factor. Brucella-agar plates were incubated under microaerobic conditions for two days.

2.12.2 Acid-shock survival

Acid-shock was performed by growing *C. jejuni* to mid-exponential phase, and resuspending cells in Brucella broth adjusted with HCl to pH values from 2.0-7.0. Viability assays following acid-shock were performed by determining the number of cfu after incubation for 10 min and 30 min at pH 2.0, 3.0, 3.25, 3.5, 3.75, 4.0, 5.0, 6.0 and 7.0 under microaerobic conditions at 37°C. *C. jejuni* was incubated with non-adjusted Brucella broth as a control. Serial, 10-fold dilutions were made, 5 µl of each dilution was spotted onto Brucella-agar plates and incubated under microaerobic conditions for 48 h days at 37°C. Three independent assays were performed for each pH value, and survival was expressed as the percentage of surviving bacteria relative to the control.

2.12.3 Growth

To monitor growth *C. jejuni* were grown for 16 h in Brucella broth. Fresh media was inoculated to OD_{600nm} 0.05 and aliquots of 200 µl were grown in a 96 well plate in triplicate. Optical density was monitored with a FLUOstar Omega plate reader with atmosphere control unit (BMG Labtech, Germany). *C. jejuni* were grown in

microaerobic conditions with shaking at 500 rpm at 37 or 42°C for 24 h. Experiments were done in triplicate per biological experiment.

2.12.4 Autoagglutination

To monitor autoagglutination, *C. jejuni* was grown for 16 h in Brucella broth and 1 ml was centrifuged at 9,200 x *g* and resuspended in 1 ml PBS. The OD_{600nm} of the bacterial suspension was measured immediately and after 24 h incubation at room temperature in air. Experiments were done in triplicate per biological experiment.

2.12.5 Motility

Motility was assessed using soft agar plate assays. *C. jejuni* strains were grown to OD_{600nm} 0.3 and 5 µl culture was spotted on 0.4% Brucella-agar (Reuter *et al.* 2010). Brucella agar plates were incubated under microaerobic conditions for 48 h. Experiments were done in triplicate per biological experiment.

2.12.6 Aerotaxis

A *C. jejuni* one-shot glycerol was thawed and grown 16 h on Brucella-agar. Cells were recovered in 2 ml PBS and 50 µl was added to a 15 ml centrifuge tube, filled 10 ml with 0.4% Brucella-agar supplemented with triphenyl tetrazolium chloride which converts into a red formazan dye by oxidation. Tubes were incubated at 37 and 42°C in air for 5 days and photographs were taken every 24 h. The distance between the top of the agar and the dye front was measured using ImageJ 1.41o.

2.12.7 Flagella observation

To visualise flagella, *C. jejuni* was grown to OD_{600nm} 0.3 in Brucella broth and a 10% dilution was viewed under x 10,000 magnification using an Eclipse 50i microscope (Nikon UK Limited). Flagella were visualised using the Ryu stain described by Kodaka *et al*. (Kodaka *et al.* 1982). Briefly, two solutions were made: Solution I contained 10 µl 5% phenol solution, 2 g tannic acid, 10 µl saturated aluminium potassium sulphate; Solution II contained 6 g crystal violet in 50 ml ethanol. Solution I and II were mixed in the ratio of 1:10. To the edge of the cover-slip, 5 µl of Ryu stain was applied and left to diffuse into the sample by capillary action. Slides were photographed using a Nikon Coolpix E4500 camera.

2.12.8 Scanning electron microscopy

Approximately 200 µl of sample was pipetted drop by drop onto an Isopore membrane polycarbonate filter (HTTP01300, Millipore), 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 min and the filters were subsequently placed in 2.5% glutaraldehyde in 0.1 M PIPES buffer (pH 7.2) and fixed for 1 h. After washing with 0.1M PIPES buffer (Sigma-Aldrich), 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 3kV. The method was carried out by Kathryn Cross at the Institute of Food Research.

2.12.9 Biofilm formation

C. jejuni was grown for 16 h in Brucella broth. From this, a 3% dilution was made and 1 ml added to a sterile Pyrex disposable culture tubes (Sigma-Aldrich). These were incubated without shaking at 37°C under microaerobic or aerobic conditions for two days to allow biofilms to form. Three technical replicates were used for each strain under each growth condition per biological experiment. After incubation, viability was assessed by enumerating cfu.

For crystal violet staining, the culture was poured out of the tubes and discarded. Tubes, containing the biofilm, were washed with water, and then dried at 60°C for 1 h. One millilitre of 1% crystal violet solution was added and the tubes were incubated on a rocker (Stuart) at 30 rpm, room temperature for 30 min. Crystal violet was disposed into chemical waste with excess unbounded stain washed with water and the tubes were dried at 37°C. Bound crystal violet was dissolved in 20% acetone in ethanol for 10 min and the OD_{590nm} measured.

2.12.10 Invasion

(i) m-IC_{cl2} culture

The murine intestinal crypt-like cell line m-IC_{cl2} (Bens *et al.* 1996) was cultured in m-IC_{cl2} media consisting of Dulbecco's modified Eagle Medium/Ham's F-12 12 g l^{-1} (1:1 v/v; Gibco), 20 mM D-glucose (Sigma-Aldrich), 10 ng ml^{-1} mouse epidermal growth factor (Merck), 5 μg ml^{-1} insulin (Sigma-Aldrich), 60 nM selenium (Sigma-Aldrich, UK), 5 μg ml^{-1} human Apo-transferrin (Sigma-Aldrich), 1 nM triiodothyronine (Merck, UK), 20 mM HEPES (Sigma-Aldrich), 2% fetal calf serum (Gibco), 50 nM dexamethasone (Sigma-Aldrich), 2 mM L-alanyl-L-glutamine (Sigma-Aldrich) at 37 °C in 5% CO_2 atmosphere. Cells of passage 9-15 were used for experiments.

The m-IC_{cl2} cells were grown until confluent on a Type I collagen matrix (C7661, Sigma-Aldrich, UK), in plastic 24-well, flat-bottomed plates (Sarstedt) or on transwell inserts with 8 μm pores (Corning) at 37°C in 5% CO_2 atmosphere. For transwell inserts the transelectrical resistance of membrane was measured with an epithelial voltohmmeter (EVOM2, World Precision Instruments) and cells were considered confluent when resistance was at least 130 Ω.cm^2 (Bens *et al.* 1996).

(ii) Caco-2 culture

Caco-2 medium contained 10% fetal calf serum and 2 mM L-alanyl-L-glutamine in Dulbecco's modified Eagle Medium. Caco-2 cells were grown until confluent and domes had developed on a Type I collagen matrix. Cells of passage 20-25 were used for experiments.

(iii) Invasion

C. jejuni strains were grown to OD_{600nm} 0.3, centrifuged (3,220 x *g* for 10 min), resuspended in m-IC_{cl2} media and 500 μl was added to the cell monolayers (MOI 1,000). Bacterial invasion was allowed for 2 h at 37°C and 5% CO_2. To remove adherent *C. jejuni*, the cell monolayer was washed twice with PBS and incubated in fresh cell culture media containing 500 μg ml^{-1} gentamicin for 1 h (Friis *et al.* 2005). The infected cell monolayer was washed twice with PBS and then lysed with 1% Triton X-100 (Sigma-Aldrich, UK) to release intracellular *C. jejuni*. Viable *C. jejuni* were measured by enumerating cfu using spot-plates. This was also done for the initial inoculum so that the proportion of invaded bacteria could be calculated.

2.13 Fluorescence measurements by spectroscopy

Bacteria were centrifuged (9,600 x *g* for 3 min) and resuspended in PBS to OD_{600nm} 0.5. The cell suspension (200 µl) was assayed in triplicate in with a FluoStar OPTIMA plate reader and GFP+ was exited at 485 nm and detected at 520 nm.

2.14 Fluorescence measurements by flow cytometry

2.14.1 Sample preparation

(i) E. coli

E. coli was grown in LB broth supplemented with chloramphenicol and/or carbenicillin where appropriate and grown for 8 h with shaking. Cultures were diluted with PBS (1:200) supplemented with propidium iodide (PI, 1:1000 dilution). Fluorescence was measured triplicate in at least three independent experiments by flow cytometry using the Cytomics FC5000. Results were analysed using FlowJo (TreeStar) and at least 10,000 live bacteria were included in the analyses.

(ii) C. jejuni

C. jejuni growth in Brucella broth (Bru) and a defined minimal medium (Min, Gibco) was analysed using flow cytometry. *C. jejuni* ncRNA mutants were used to inoculate either Bru or Min media (table 2.6) and grown for 16 hr to be used as starter cultures for the study. Cultures were diluted with PBS (1:100) supplemented with PI and *C. jejuni* were enumerated by flow cytometry to inoculate fresh cultures at similar numbers. Samples were taken at 1, 2, (1:8 dilution) 5, 8, 12, (1:40 dilution) and 24 h (1:100 dilution) and diluted in PBS for acquisition. To improve visualisation of *C. jejuni*, samples were stained with Syto 12 (1:80 dilution, Invitrogen).

Table 2.6 Contents of minimal medium.

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	18.75	0.25
L-Alanine	89	4.45	0.05
L-Asparagine-H_2O	150	7.5	0.05
L-Aspartic acid	133	665	5.00
L-Cysteine hydrochloride-H_2O	176	17.56	0.0998
L-Cystine 2HCl	313	31.29	0.1
L-Glutamic Acid	147	7.35	0.05
L-Histidine hydrochloride-H_2O	210	31.48	0.15
L-Isoleucine	131	54.47	0.416
L-Leucine	131	59.05	0.451
L-Methionine	149	17.24	0.116
L-Phenylalanine	165	35.48	0.215
L-Proline	115	17.25	0.15
L-Serine	105	525	5.00
L-Threonine	119	53.45	0.449
L-Tryptophan	204	9.02	0.0442
L-Tyrosine disodium salt dihydrate	261	55.79	0.214
L-Valine	117	52.85	0.452
Vitamins			
Ascorbic Acid phosphate	289.54	2.5	0.00863
Biotin	244	0.0035	0.0000143
Choline chloride	140	8.98	0.0641

D-Calcium pantothenate	477	2.24	0.0047
Folic Acid	441	2.65	0.00601
Niacinamide	122	2.02	0.0166
Pyridoxine hydrochloride	206	2	0.00971
Riboflavin	376	0.219	0.000582
Thiamine hydrochloride	337	2.17	0.00644
Vitamin B12	1355	0.68	0.000502
i-Inositol	180	12.6	0.07
Inorganic Salts			
Calcium Chloride ($CaCl_2$) (anhyd.)	111	116.6	1.05
Cupric sulfate ($CuSO_4$-$5H_2O$)	250	0.0013	0.0000052
Ferric Nitrate ($Fe(NO_3)_3$-$9H_2O$)	404	0.05	0.000124
Ferric sulfate ($FeSO_4$-$7H_2O$)	278	0.417	0.0015
Magnesium Chloride (anhydrous)	95	28.64	0.301

2.14.2 Acquisition

The flow cytometer used was a Cytomics FC500 MPL (Beckman Coulter) using blue (488 nm) and red (633 nm) lasers. Side scatter discriminator was set to 1 and events were acquired at low flow for 30 sec (*C. jejuni* experiments) or 60 sec (*E. coli* experiments) for or until a maximum of 10^6 events had been acquired. GFP+ fluorescence excitation maximum is 475 nm (major peak) and maximum emission is 509 nm. Syto 12 (Invitrogen) fluorescence excitation maximum is 499 nm and maximum emission is 522 nm and was detected in the FL1 channel. Propidium Iodide (Invitrogen) fluorescence excitation maximum is 535 nm and emission maximum is 617 nm and was detected in the FL3 channel when analysed individually or in the FL4 channel when in conjunction with Syto 12. GFP and Syto 12 fluorescence was measured using a 525/20 band pass filter and PI was measured using a 615 dichroic short pass filter/620 short pass filter.

2.14.3 Analysis

(i) E. coli

E. coli cells were selected from SS/FS plots and PI negative (live) cells were gated (often 97 % of total population, fig. 2.1). GFP negative and positive *E. coli* were used to determine GFP positive events (fig. 2.1). The median GFP fluorescence of live cells was measured.

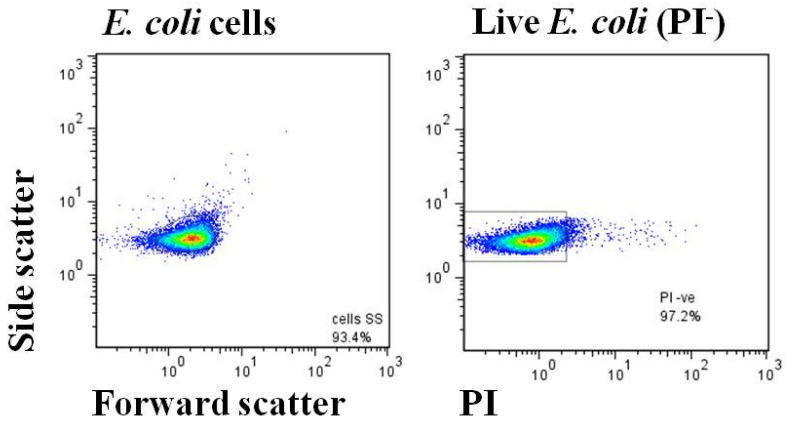


Figure 2.1 Flow cytometry gating strategy for *E. coli*.

E. coli cells were selected using forward and side scatter. The fluorescence intensity (GFP) of propidium iodide negative cells (PI^-) was measured.

(ii) C. jejuni

C. jejuni cells were selected from SS/Fl-1 plots and then PI negative (live) cells were gated. A gate of 10 sec was placed where acquisition was regular (fig. 2.2) and live cells (PI negative) were gated for which the side scatter, forward scatter and counts of live cells were measured. Dead cells (PI positive) were gated separately and numbers were recorded. Different gating had to be used for 37 and 42°C, possibly due to release of DNA by cells at different growth phases.

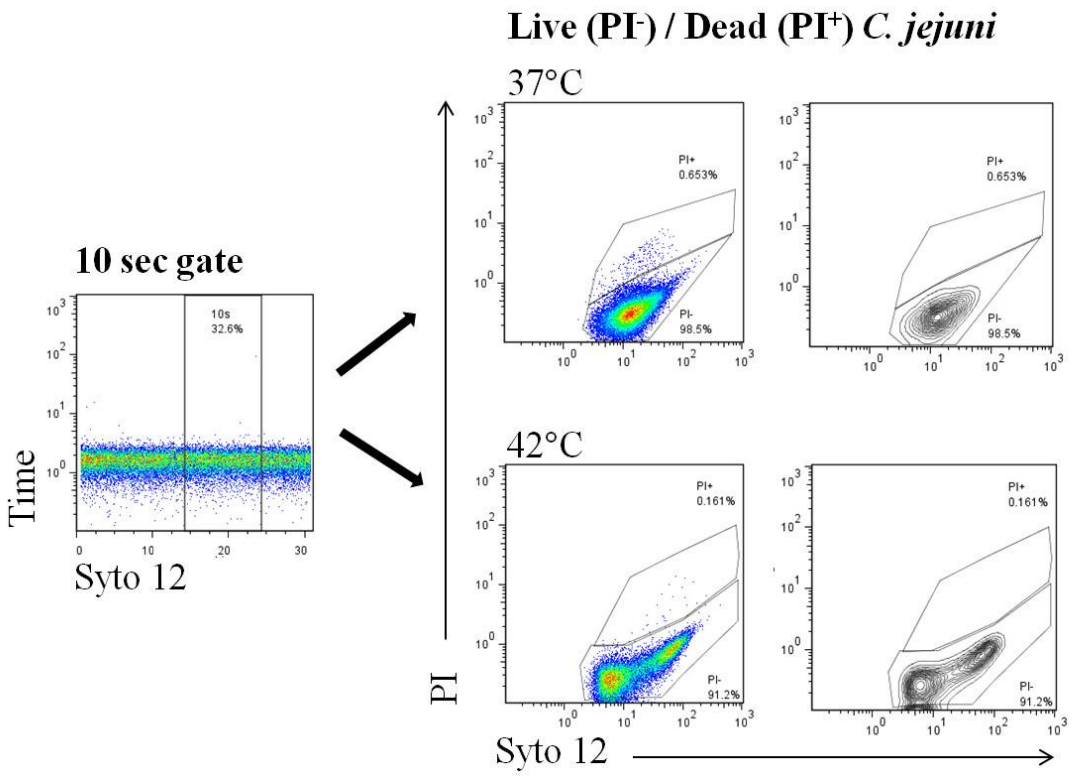


Figure 2.2 Flow cytometry gating strategy for *C. jejuni*.

C. jejuni were stained with Syto 12 to detect the cells and propidium iodide (PI) to select for live/dead cells. To measure numbers of *C. jejuni*, a 10 sec gate was used to define cell numbers. The GFP fluorescence intensity of propidium iodide (PI) negative cells was measured. Dot plots (coloured) and contour plots (black and white) were used to visualise the data.

2.15 Statistics

Statistics were performed using GenStat 15.1.0.8035 (VSN International) and Prism 5.1 (GraphPad). Unless specified otherwise, One-Way Analysis of Variance was used to determine statistical significance of results and were considered to be statistically significant if $P < 0.05$.

Chapter 3 Acid-shock induces flagellar gene expression in *C. jejuni*

Collaborative work:

- Microarray performed and analysed by Ida Porcelli
- Invasion assays with m-IC_{cl2} established with Caroline Weight and Duncan Gaskin
- Based on Le, M. T., Porcelli, I., Weight, C. M., Gaskin, D. J. H., Carding, S. R. and van Vliet, A. H. (2012). Acid-shock of *Campylobacter jejuni* induces flagellar gene expression and host cell invasion. *European Journal of Microbiology and Immunology* **2**(1): 12-19 (Appendix 2).

3.1 Introduction

Ingestion of *C. jejuni*, either by humans or in the avian host, is inevitably followed by bacterial passage though the stomach, before entering the small intestine and causing disease. In the human stomach, the gastric pH can range from approximately 2 to 7 depending on the state of the stomach (empty-full), gastrin production and stomach contents (Dressman *et al.* 1990). In addition, food is retained in the stomach for 30-60 min before the stomach begins to empty (Siegel *et al.* 1988). Thus, *C. jejuni* must have the means to cope with sudden and variable exposures to acidic conditions. However, *C. jejuni* responses to acid stress have not been as extensively characterised as those in the well studied enteric pathogens *Escherichia coli* or *Salmonella* Typhimurium (Audia *et al.* 2001).

Many Enterobacteriaceae, like *E. coli*, have specific acid-resistance systems allowing direct protection against acidic pH, such as the well characterised glutamate decarboxylase system, which metabolises glutamate to increase internal pH (Audia *et al.* 2001). This and other nutrient dependent acid-resistance are regulated by complex networks and are also shared with *S.* Typhimurium (Burton *et al.* 2010). However, while widespread in the Enterobacteriaceae, these specific systems are absent in *C. jejuni* and related organisms.

C. jejuni has not been found to mount a specific acid response, but does have an adaptive tolerance response during stationary growth phase, where in the case of acid adaptation, bacteria are sensitised by mild acid-shock (e.g. pH 5), upon which they display increased survival at low pH (Ma *et al.* 2009). It has been suggested that *C. jejuni* has mechanisms enabling adaptive tolerance responses, which is initiated by a release of extracellular proteins (Murphy *et al.* 2003a; Ma *et al.* 2009).

C. jejuni flagellar gene expression is altered in acidic conditions. *C. jejuni* is known to up-regulate stress response genes and down-regulate capsular polysaccharide biosynthesis genes in response to acid-shock (Reid *et al.* 2008a; Reid *et al.* 2008b). As well as these changes in gene expression, it has also been suggested that σ^{54} may play a role in acid resistance in *C. jejuni* (Hwang *et al.* 2011). Expression of *C. jejuni* flagellar and flagellar-associated genes is tightly regulated, which depends on a transcriptional

hierarchy of σ factors, and σ^{28} and σ^{54} are dedicated to this role (Smith and Hoover 2009).

Exposure to low pH can induce genes involved in virulence phenotypes, as was shown in the related human pathogen *Helicobacter pylori*, where acid-shock increases the expression of genes for the acid-resistance factor urease and the expression of motility-associated genes (Merrell *et al.* 2003). Expression of urease enables *H. pylori* to survive acidic conditions and may aid taxis responses during initial colonisation of the gastric mucosa (Merrell *et al.* 2003). Acidic conditions are sensed by the ArsRS two-component system, which regulates the expression of urease of other genes of unknown function. ArsSR orthologues exist in *C. jejuni*, although their function has not been evaluated experimentally (Muller *et al.* 2009).

The aim the study was to understand how *C. jejuni* gene expression is affected by acid-shock and whether acid-shock has the potential to induce *C. jejuni* virulence phenotypes. The objectives of the study were to:

- Ascertain the most acidic pH in which *C. jejuni* is able survive
- Examine *C. jejuni* gene expression after acid-shock by microarray
- Assess the effect of acid-shock on *C. jejuni* invasion of intestinal epithelial cells.

3.2 Results

3.2.1 The lowest pH that *C. jejuni* is able to survive is pH 3.5

To determine if *C. jejuni* can survive the acidic conditions present in the stomach, bacteria were grown to exponential phase in Brucella broth adjusted with HCl to different pHs ranging from 2.0 to 7.0, with survival being assessed by viability counts. There was no significant loss in viability when *C. jejuni* was incubated for 10 minutes at pH values of 3.5 and higher, but below pH 3.5 there was a rapid loss of viability, with less than 0.1% of viable cells recovered after acid-shock at pH 3.25 (fig. 3.1). When the duration of acid-shock was increased to 30 minutes, the lowest pH at which *C. jejuni* showed no decrease of viability was pH 3.75, where less than 0.01% of viable cells were recovered at pH 3.5 (fig. 3.1).

The acid adaptive tolerance response of *C. jejuni* was assayed by pre-incubating exponential phase bacterial cultures at pH 5.0 and 7.0 prior to acid-shock at pH 2.75. There were no viable cells recovered at pH 2.75 after a mild acid-shock (Ida Porcelli, data not shown), suggesting that *C. jejuni* lacks an acid tolerance response at this growth phase as previously observed (Murphy, Carroll et al. 2003).

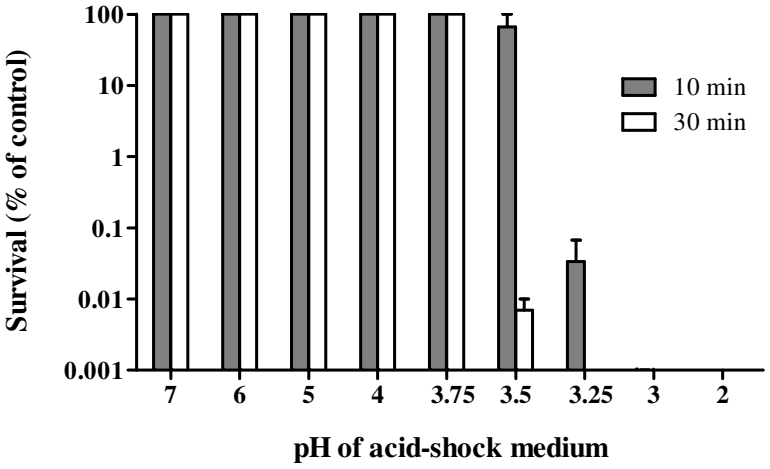


Figure 3.1 The threshold pH for C. *jejuni* NCTC11168 survival is 3.5.

Motile *C. jejuni* was grown to exponential phase and incubated for 10 and 30 min in Brucella broth adjusted to pH 2 to 7. *C. jejuni* was incubated with non-adjusted Brucella broth as a control. Survival was assessed by counting colony forming units after 10 min (solid bars) and 30 min (white bars). Results were expressed as a percentage of surviving bacteria relative to the survival of the control. Error bars denote standard error of the mean and results shown are an average of three biological replicates.

3.2.2 Acid-shock increases the expression of σ^{54}-dependent flagellar biosynthesis genes.

To assess the effect of acid-shock on gene transcription in *C. jejuni*, transcriptomic analyses were performed using *C. jejuni* microarrays to compare transcriptional profiles at pH 7 and at acidic pH (pH 3.6 and pH 5, for 10 and 30 minutes). Levels of RNA were expressed as a ratio of acidic pH/pH 7, and genes were considered differentially expressed if transcript levels were more than two-fold different and if the False Discovery Rate (FDR) was below 0.1. The transcript level of 232 genes was > 2-fold increased and 294 genes were > 2-fold decreased upon acid shock in one of the four tested conditions, with an FDR < 0.1. Of the genes with increased transcript levels, 137 showed a significant change in one of the four conditions, whereas 95 showed increased transcript levels in two or more of the tested conditions. Of the genes with decreased transcript levels upon acid shock, 151 genes were identified in one of the four conditions, whereas 143 showed decreased transcript levels in two or more of the tested conditions. At pH 3.6, 78 genes were differentially expressed during both 10 and 30 minutes acid-shock and at pH 5, 42 genes were differentially expressed during both 10 and 30 minutes acid-shock.

(i) Down-regulated C. jejuni *genes upon acid-shock at pH 3.6 and 5*

Down-regulated genes, as a result of acid-shock, included those encoding 50S and 30S ribosomal proteins (*rpm*, *rpl* and *rps*), which were consistently down-regulated across two or more conditions (table 3.1). Also, genes encoding the F_0F_1 ATPase subunits (*atpDFH*) were down-regulated after acid-shock at pH 3.6, and this was also the case for *sec* protein-export genes (*secAFY*) (table 3.2). Genes encoding leucine biosynthesis enzymes (*leuABC*) were down-regulated after 10 minutes acid-shock at pH 3.6 and pH 5, as were genes encoding Pgl glycosylation enzymes (table 3.3). Genes *cj0018c* (*dba,* involved in sulphur protein production (Grabowska *et al.* 2011), *cj0224* (*argC*, an oxidoreductase), *cj0865* (*lepP*, a peptidase) and *cj0882c* (*flhA*, a flagellar protein) were between two- and 10-fold down-regulated in all conditions. Unknown genes that were down-regulated in all conditions include *cj0331c*, *cj0520 cj0883c*, and *cj0949c*. Overall, these changes suggest that the cells shut down protein synthesis and modification in response to acid stress, due to the change from exponential growth to adaptation for survival.

Table 3.1 Fold down-regulation of *C. jejuni* NCTC11168 ribosomal gene expression in two or more acid-shock conditions.

Green shading indicates significant and greater than two-fold change decrease in gene expression. Significant genes have a false discovery rate < 0.1 from three biological replicates.

Gene	Name	pH 3.6 10 min	pH 3.6 30 min	pH 5 10 min	pH 5 30 min
cj0094	*rplU*	2.79	3.34	2.36	2.56
cj0095	*rpmA*	2.79	5.40	2.03	2.99
cj0155c	*rpmE*	5.67	5.08	4.35	5.36
cj0244	*rpmI*	3.60	4.26	3.75	4.00
cj0245	*rplT*	1.74	1.89	2.42	2.43
cj0330c	*rpmF*	3.17	2.66	2.57	2.92
cj0370	*rpsU*	0.35	3.03	0.67	4.35
cj0450c	*rpmB*	2.12	2.28	3.67	4.06
cj0471	*rpmG*	2.12	2.05	2.64	2.22
cj0473	*nusG*	3.11	2.53	3.88	2.88
cj0474	*rplK*	4.05	2.92	2.75	2.01
cj0475	*rplA*	2.60	3.89	1.75	2.61
cj0476	*rplJ*	2.62	2.99	1.16	1.46
cj0477	*rplL*	2.24	3.81	1.20	2.04
cj0478	*rpoB*	2.55	1.47	2.89	1.07
cj0479	*rpoC*	3.15	2.49	2.66	1.53
cj0491	*rpsL*	4.87	5.11	3.06	4.48
cj0492	*rpsG*	3.82	3.86	3.68	4.41
cj0664c	*rplI*	1.01	1.16	0.69	0.78

cj0710	*rpsP*	4.07	4.34	1.25	2.39
cj0714	*rplS*	4.02	3.25	3.41	3.46
cj0884	*rpsO*	2.97	3.50	1.59	3.44
cj0891c	*serA*	2.17	1.26	1.71	1.72
cj0893c	*rpsA*	1.17	1.26	1.36	2.25
cj0960c	*rnpA*	2.48	3.31	2.16	4.76
cj0961c	*rpmH*	2.31	5.18	1.85	8.32
cj1070	*rpsF*	3.20	2.46	1.86	1.47
cj1072	*rpsR*	3.01	2.762	1.15	1.26
cj1182c	*rpsB*	2.80	3.31	2.63	4.10
cj1479c	*rpsI*	3.68	2.30	8.87	3.47
cj1591	*rpmJ*	3.61	2.33	1.80	1.49
cj1592	*rpsM*	3.51	1.85	2.43	1.25
cj1593	*rpsK*	4.16	2.83	2.77	1.44
cj1594	*rpsD*	3.70	2.49	2.13	1.40
cj1595	*rpoA*	4.70	4.50	2.10	1.52
cj1596	*rplQ*	2.73	2.53	1.54	1.43
cj1611	*rpsT*	8.71	5.96	6.21	7.15
cj1689c	*rp10*	3.03	2.63	2.91	2.06
cj1691c	*rplR*	4.18	2.69	4.43	1.94
cj1692c	*rplF*	3.73	2.76	3.62	2.17
cj1693c	*rpsH*	3.93	2.29	3.28	2.01
cj1694c	*rpsN*	3.77	2.79	3.28	2.38
cj1695c	*rplE*	2.15	1.56	3.65	2.34

cj1696c	*rplX*	2.78	1.77	3.83	2.12
cj1697c	*rplN*	2.98	2.39	4.52	2.85
cj1698c	*rpsQ*	3.29	1.98	4.96	2.50
cj1699c	*rpmC*	3.72	2.20	5.07	2.44
cj1700c	*rplP*	2.20	1.64	4.90	2.68
cj1701c	*rpsC*	2.90	1.83	6.02	3.00
cj1702c	*rplV*	2.49	2.32	5.34	3.54
cj1703c	*rpsS*	3.13	2.31	5.44	3.44
cj1704c	*rplB*	2.51	2.51	4.65	3.73
cj1705c	*rplW*	2.71	1.86	8.85	4.62
cj1706c	*rplD*	3.72	2.75	15.4	7.30
cj1707c	*rplC*	5.30	2.80	17.7	8.84
cj1708c	*rpsJ*	5.36	3.97	14.2	10.83

Table 3.2 Fold down-regulation in secretion proteins (*sec*) and F_0F_1 ATPases (*atp*) gene expression in *C. jejuni* NCTC11168 acid-shocked at pH 3.6 for 10 and 30 min.

Green shading indicates significant and greater than two-fold change decrease in gene expression. Significant genes have a false discovery rate < 0.1 from three biological replicates.

Gene	Name	pH 3.6 10 min	pH 3.6 30 min
cj0235c	*secG*	2.08	1.95
cj0472	*secE*	3.23	2.63
cj0942c	*secA*	2.04	2.07
cj1092c	*secF*	2.94	2.32
cj1093c	*secD*	2.05	2.23
cj1688c	*secY*	3.11	3.21
cj0102	*atpF*	4.02	4.09
cj0103	*atpF*	2.79	3.37
cj0104	*atpH*	2.88	2.45
cj0105	*atpA*	2.58	2.92
cj0106	*atpG*	4.08	2.25
cj0107	*atpD*	2.74	2.31

Table 3.3 Fold down-regulation of glycosylation enzyme and leucine biosynthesis gene expression in *C. jejuni* NCTC11168 after 10 min acid-shocked at pH 3.6 and 5.

Green shading indicates significant and greater than two-fold change decrease in gene expression. Significant genes have a false discovery rate < 0.1 from three biological replicates.

Gene	Name	pH 3.6	pH 5
cj1123c	*pglD*	2.87	2.68
cj1124c	*pglC*	2.95	2.59
cj1125c	*pglA*	2.99	2.21
cj1126c	*pglB*	3.66	2.53
cj1127c	*pglJ*	2.72	2.24
cj1128c	*pglI*	4.73	1.80
cj1129c	*pglH*	2.79	1.56
cj1130c	*pglK*	2.07	1.29
cj1717c	*leuC*	13.7	8.06
cj1718c	*leuB*	19.2	12.5
cj1719c	*leuA*	18.2	12.0

(ii) Up-regulated C. jejuni *genes upon acid-shock at pH 3.6 and 5*

After acid-shock at pH 5, the up-regulated genes included those of heat shock proteins ClpB, DnaK, HrcA, and HtrA (encoded by *cj0509c*, *cj0759*, *cj0757*, *cj1228c* respectively), and the catalase gene (*cj1385, katA*), which were up-regulated between two- and ten-fold compared to non-shocked *C. jejuni*. Genes for respiratory functions, such as *cj0533 and cj0534* (*sucCD*) encoding succinyl-coenzyme A synthase and *cj1537c* (*acs*) for acetyl-coenzyme A synthase were up-regulated under all conditions, the latter being up-regulated by more than 50-fold at pH 3.6. Other genes up-regulated under all conditions were: the lactate oxidase operon *cj0073c-0075c* (Thomas *et al.* 2011); *cj0203* encoding a possible transmembrane transport protein; *cj0486* encoding the fucose permease and *cj0487* required for fucose utilisation, an unknown gene; possible periplasmic protein genes *cj0722c* and *cj0834c*; possible membrane proteins genes *cj0920c* and *cj0987c*; *cj1238* (*pdxJ*) encoding a pyridoxal-phosphate (vitamin B6) biosynthetic protein (Grubman *et al.* 2010); and *cj1503c* (*putA*) a possible proline metabolism enzyme, which was up-regulated by more than 20-fold at pH 3.6 (Table 3.4).

The oxidoreductase genes *cj0535-cj0538* and NADH dehydrogenase (*cj1569c, cj1570c, cj1572c, cj1573c and cj1578c*) genes were up-regulated after 10 minutes acid-shock at pH 5 only. Gluconate dehydrogenase genes (*cj0414, cj0415*) were greatly up-regulated after acid-shock at pH 5 and are known to be stress-responsive (Pajaniappan *et al.* 2008). Genes encoding putative tricarboxylic acid cycle enzymes GltA (*cj1682c*) and AcnB (*cj0835c*) are more than five-fold up-regulated in two or more conditions. Genes *cj1658*, *cj1660* and *cj1661* encoding putative membrane proteins are up-regulated after acid-shock at pH 5. Genes of unknown functions *cj1540-cj1543* are more than three-fold up-regulated at 30 minutes acid-shock at both pH 3.6 only. These changes suggest a general up-regulation in stress responses.

Table 3.4 Fold up-regulation of *C. jejuni* NCTC11168 gene expression after acid-shock at pH 3.6 and 5 for 10 and 30 min.

All genes are significantly regulated and have a false discovery rate < 0.1 from three biological replicates.

Gene	Name	pH 3.6 10 min	pH 3.6 30 min	pH 5.0 10 min	pH 5.0 30 min
cj0073c	*cj0073c*	14.9	12.0	10.7	7.30
cj0074c	*cj0074c*	15.0	12.4	9.02	7.03
cj0075c	*cj0075c*	20.2	19.6	9.50	7.71
cj0203	*cj0203*	11.2	6.96	5.23	2.94
cj0486	*cj0486*	4.37	3.62	2.60	3.31
cj0487	*cj0487*	3.08	2.24	2.70	2.56
cj0533	*sucC*	4.80	7.68	3.79	5.99
cj0534	*sucD*	3.59	4.57	3.30	4.55
cj0772c	*cj0772c*	4.82	5.47	3.07	4.36
cj0834c	*cj0834c*	5.57	4.76	3.36	2.71
cj0920c	*cj0920c*	9.69	5.06	13.2	5.02
cj0987c	*cj0987c*	21.6	14.8	9.56	5.22
cj1238	*pdxJ*	3.09	2.80	2.13	3.12
cj1503c	*putA*	36.7	24.9	3.83	2.88
cj1537c	*acs*	85.2	57.2	7.55	5.83

Flagellar genes that are transcribed from σ^{54}-dependent promoters during the middle phase of flagellar assembly were up-regulated after acid-shock at pH 3.6 and 5 (fig. 3.2). These genes included components of the basal body, hook, junction proteins and associated outer membrane proteins (fig. 3.2). Figure 3.3 shows flagellar gene products that are up-regulated after acid-shock at pH 5 for 10 min in relation to the main structural components of the flagellum. Genes expressed at the early and late phases of flagellar assembly remained mostly unaffected by acid-shock except for *fliQ* expression, which was significantly up-regulated in response to 10 minutes acid-shock at pH 5. The *flaC* and *ciaB* invasion and *peb1A* adhesion determinants (Pei *et al.* 1998; Konkel *et al.* 1999; Song *et al.* 2004) were also significantly up-regulated under one or more acid conditions (fig. 3.2).

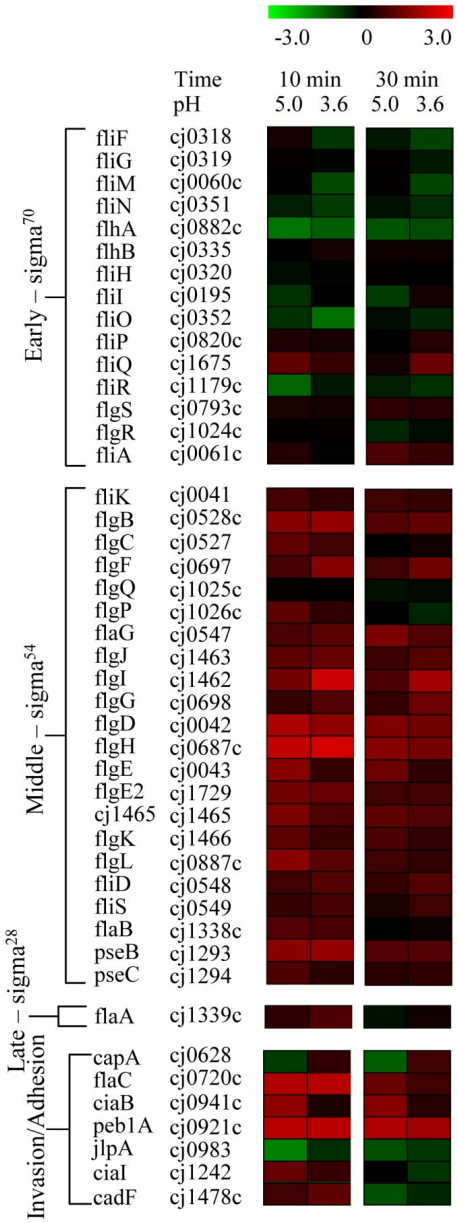


Figure 3.2 Acid-shock at pH 3.6 and 5 increases expression of a subset of flagellar biosynthesis genes in *C. jejuni* NCTC11168.

Genes transcribed by σ^{54} during the middle phase of flagellar assembly are up-regulated by up to three-fold. RNA levels of log phase cells incubated at pH 7 was compared with cells incubated pH 3.6 and 5 for 10 and 30 min. Results are shown for a subset of genes, including flagellar genes and previously identified invasion and adhesion determinants. Flagellar genes are listed and grouped in approximate order of flagellar assembly: early phase genes are controlled by σ^{70}, middle phase by σ^{54}, and late phase by σ^{28}; invasion and adhesion determinants are grouped separately. Up-regulated genes are shown in red and down-regulated genes are shown in green. Maximum colour output represents a threefold change in expression. Results shown are an average of three independent experiments.

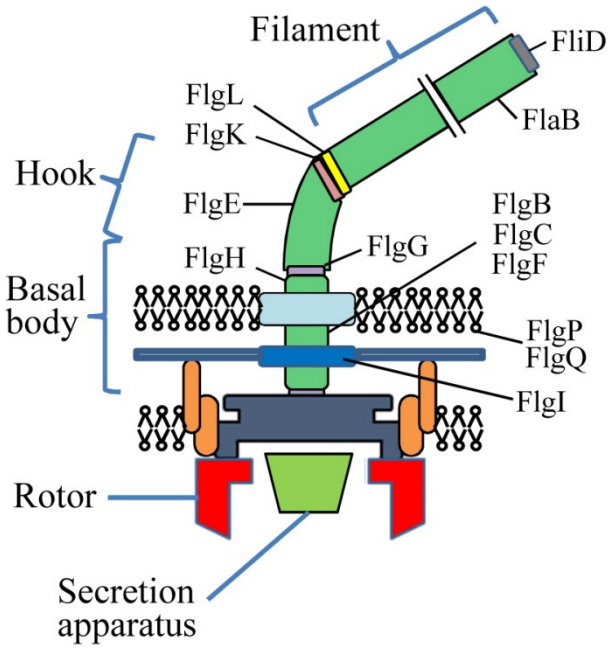


Figure 3.3 Diagram of up-regulated components of the *C. jejuni* NCTC11168 flagellum after acid-shock.

Genes and locations of the flagellar gene products that are up-regulated after acid-shock at pH 5 for 10 min are named. The main structural components of the flagellum are also labelled. Adapted from Wösten *et al.* (Wosten *et al.* 2004).

3.2.3 Acid-shock increases invasion of *C. jejuni* into mouse intestinal crypt (m-IC$_{cl2}$) cells grown on transwell inserts.

To investigate the effect of acid-shock and the associated increase in flagellar gene expression on *C. jejuni* virulence phenotypes, we performed invasion assays with a mouse intestinal crypt cell line (m-IC$_{cl2}$) (Bens *et al.* 1996). This cell line represents crypt-like cells of the small intestine and was used in preference to colon-derived cell lines such as Caco-2. Confluent layers of m-IC$_{cl2}$ cells were grown either on transwell inserts, or on flat-bottomed wells (fig 3.4 A), and were subsequently incubated with *C. jejuni* at MOI 1000. The *C. jejuni* cultures had either been incubated at pH 5 for 30 minutes, or at pH 7 as a control. Following the established gentamicin-killing method for determining numbers of invaded bacteria, m-IC$_{cl2}$ cells were lysed and invasive, intracellular *C. jejuni* were enumerated as colony forming units (cfu).

The transwell model assessed both bacterial intracellular invasion from the lysed monolayer and bacterial translocation through the m-IC$_{cl2}$ cell layer to the basolateral

side of the well. However, the flat-bottomed well model tested for intracellular invasion from the apical side only. An example of *C. jejuni* cfu recovered from the lysed monolayer and the basal lateral side is shown in figure 3.4 B. When comparing control *C. jejuni* cultures, higher invasion levels were seen in the flat-bottomed well model than in the transwell model (fig. 3.4 C). In the flat-bottomed well model, there was no significant effect of acid-shock on the number of *C. jejuni* recovered from m-IC_{cl2} cells (fig. 3.4 C). However, in the transwell model, acid-shock increased the levels of *C. jejuni* invasion up to two logs compared to the control, so invasion levels became comparable to those observed in the flat-bottomed well model (fig. 3.4 C). The number of translocated *C. jejuni* (recovered from the compartment below the transwell insert) was also increased up to two logs after acid-shock, although cfu recovery of acid-shocked *C. jejuni* was very variable across technical replicates (fig. 3.4 B, C).

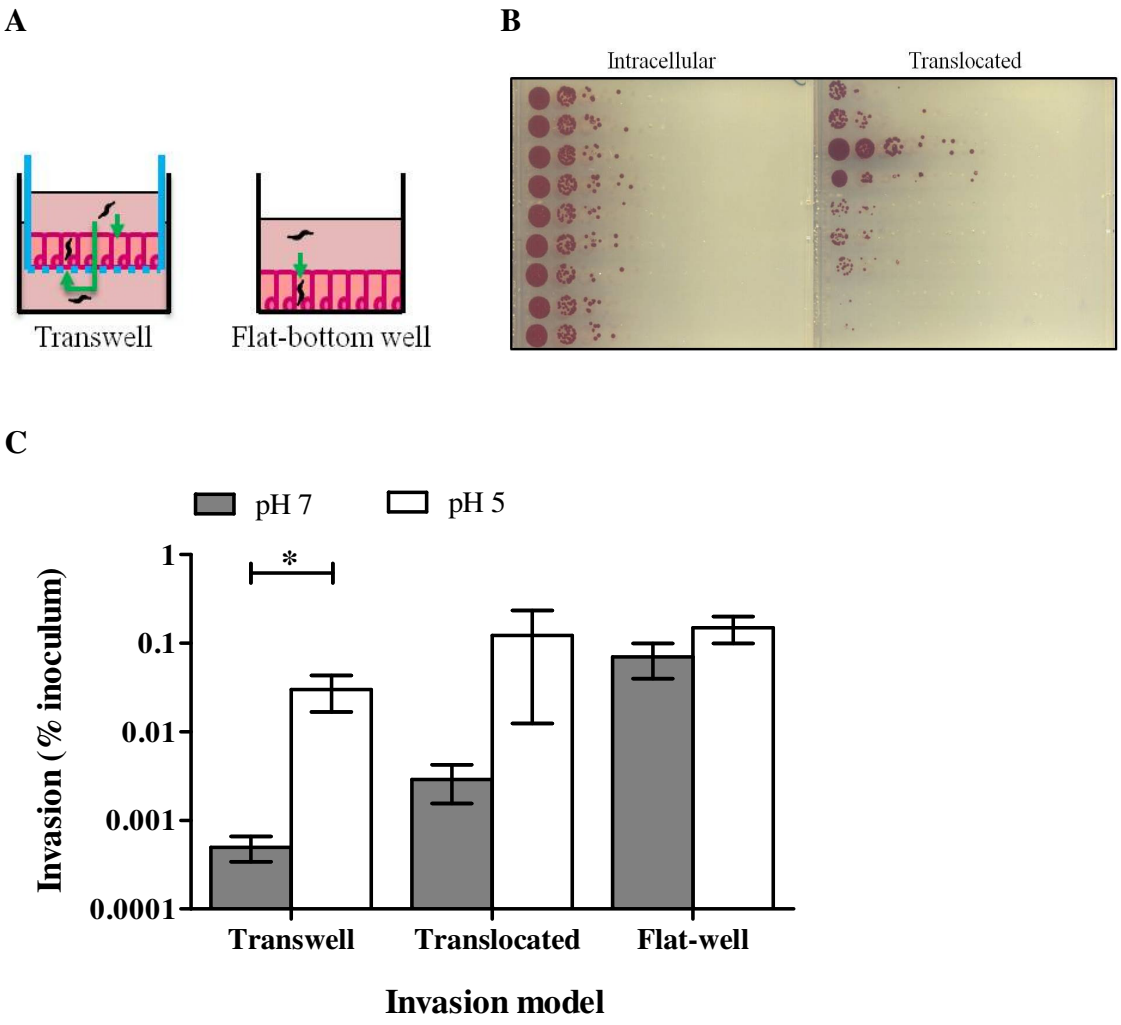


Figure 3.4 Acid-shock increases *C. jejuni* NCTC11168 invasion of mouse intestinal crypt (m-IC$_{cl2}$) cells grown on transwell inserts, but not on flat-bottomed wells.

A) Schematic representations of the 'Transwell' and 'Flat-bottom well' *in vitro* invasion models. Highlighted are possible routes (**green** arrows) of *C. jejuni* invasion: apical and basolateral when m-IC$_{cl2}$ cells are grown on transwell inserts and apical only when grown on flat-bottomed wells. Cells were grown until confluent on transwell inserts or flat-bottomed wells prior to exposure with motile *C. jejuni* (MOI 1000) that had been acid-shocked at pH 5 or incubated at pH 7 for 30 min. After 2 h invasion, a gentamicin-killing wash was performed and m-IC$_{cl2}$ cells were lysed and viable *C. jejuni* cfu determined. **B)** Image of *C. jejuni* colony forming unit spot-counts from inside m-IC$_{cl2}$ cells (Intracellular) and from the basal compartment (Translocated) of transwell assays. **C)** Bar chart showing the percentage of invasive or translocated bacteria relative to the inocula that were recovered from the monolayer grown in transwells (Transwell), the basal compartment of the transwell (Translocated) and from the monolayer grown on flat-bottomed wells (Flat-bottom). *C. jejuni* incubated at pH 7 is represented by solid bars and *C. jejuni* acid-shocked at pH 5 is represented by open bars. Error bars denote standard error of the mean. Transwell experiments comprised 10 technical replicates. Flat-bottomed well experiments comprised three biological repeats each with 6 technical replicates. Asterisk indicates $P < 0.05$ (One-way ANOVA).

3.3 Discussion

When *C. jejuni* colonises a new host, the faecal-oral route of infection will inevitably include exposure to the acidic environments of the stomach in mammals as well as the acidic proventriculus (glandular stomach) in birds (Avila *et al.* 1986). Enteric pathogens require acid-resistance mechanisms for successful transmission to the intestine. In this chapter, *C. jejuni* survival in mild and strong acid-shock conditions was observed and was linked with increased transcription of a subset of flagellar biosynthetic genes and stress responses, as well as a down-regulation in genes involved in cell division and metabolism. Furthermore, acid-shock increased *C. jejuni* invasion of mouse small intestinal crypt (m-IC_{cl2}) cells in a transwell assay.

In this study, the threshold for survival of *C. jejuni* was up to pH 3.5 for 10 minutes acid-shock and up to pH 3.75 for 30 minutes acid-shock (fig. 3.1), indicating that *C. jejuni* can survive physiological acidic conditions of the stomach. Previous work describing the response of *C. jejuni* to acid exposure reported that *C. jejuni* cannot be cultured, but remains viable after prolonged exposure at pH 4 (Chaveerach *et al.* 2003). At pH 3 *C. jejuni* was reported to survive for no more than four minutes exposure, but that high numbers of *C. jejuni* were recovered from the pig stomach, which has a pH range of 3.8 and 4.2, suggesting that pH alone cannot explain this increased survival (Reid *et al.* 2008a).

We also investigated whether *C. jejuni* induced an adaptive tolerance response (ATR) to acid. Exponential phase cultures did not show an ATR to lethal acid-shock at pH 2.75 after 2 hours pre-incubation with pH 5. This is in agreement with the results from Murphy *et al.*. although their pre-incubation step was longer (Murphy *et al.* 2003a).

C. jejuni gene expression in response to acid-shock was analysed at the lower threshold of pH 3.6, and with the mildly acidic condition of pH 5, at both 10 and 30 minutes incubation. Transcriptomic analysis of acid-shocked *C. jejuni* showed that exposure to acidic conditions resulted in increased expression of a subset of flagellar biosynthetic genes. Flagellar gene expression is tightly regulated by a hierarchy of σ factors and begins with σ^{70}-dependent transcription of the inner membrane ring and secretion apparatus (Carrillo *et al.* 2004). Middle phase expression of genes coding for the minor flagellin, basal body and junction proteins is σ^{54} dependent and expression of the major

flagellin gene is dependent on σ^{28} during late phase (Hendrixson 2008). The flagellar genes that were up-regulated are transcribed by σ^{54} during the middle phase of flagellar assembly. This finding is consistent with a recent report where a *C. jejuni rpoN* mutant, which lacks the σ^{54} factor, showed reduced survival at pH 5 (Hwang *et al.* 2011), and is comparable with studies that reported transient expression of many of these flagellar biosynthetic genes upon acid-shock (Reid *et al.* 2008a; Reid *et al.* 2008b). Perhaps a change in expression of flagellar genes may be part of a general stress response. Indeed, down-regulation of genes encoding the cell cycle ATPases and ribosomal proteins was observed, which indicates a cessation of cell division and replication, and an up-regulation of oxidative stress and heat shock proteins. Although *C. jejuni* did not directly encounter these stressors, stress responses have been shown to overlap, which was shown with mutants lacking some of the heat shock proteins (Maurer *et al.* 2005; Pflock *et al.* 2006). However, alongside the reported overlap in responses, there were also a large number of differences compared with previously published datasets (Reid *et al.* 2008a; Reid *et al.* 2008b), which may be a consequence of experimental design and analysis, bacterial growth and the natural variations between strains.

Flagellar genes are linked with acid responses in other bacterial pathogens, including *E. coli* and *H. pylori*. In *E. coli,* flagellar genes are strongly induced in acidic conditions, but few flagellar regulators are up-regulated (Maurer *et al.* 2005). Increased levels of flagellar gene transcription have also been observed in *E. coli* responses to long-term acid exposure, but were not observed after short-term exposure to acidic conditions (Polen *et al.* 2003). In *H. pylori*, acid-shock resulted in increased expression of σ^{54}-dependent flagellar genes, and this correlated with an increase in the number of motile cells and speed of motility (Merrell *et al.* 2003). Thus, exposure to a range of acidic conditions seems to correlate with increased flagellar gene expression among gastrointestinal bacterial pathogens.

Specific sensing mechanisms that bacteria use to sense low pH are currently not well understood in *C. jejuni*. In *C. jejuni* and *H. pylori*, FlgS is a cytoplasmic histidine kinase that regulates flagella assembly (Joslin and Hendrixson 2009; Wen *et al.* 2009). In *H. pylori*, FlgS senses low pH and activates urease expression, which contributes to bacterial survival, although this occurs independently of the two-component response regulator partner FlgR (Wen *et al.* 2009). In *C. jejuni*, our transcriptomic analysis

showed no change in the expression of *flgR* or either of the alternative σ-factors, meaning that the up-regulation of flagellar genes in *C. jejuni* was mediated by an alternative regulatory pathway. It is possible that post-transcriptional regulators, such as other protein regulators or non-coding RNAs contributed to the change in expression of this subset of genes. Non-coding RNAs are abundant in the *C. jejuni* genome (Dugar *et al.* 2013; Porcelli *et al.* 2013) and their function in regulating flagellar gene expression are the subject of the remaining thesis chapters. However, the microarray did not include probes for non-coding RNAs as their sequences were not known at this time and no candidate proteins regulators were identified.

One possible explanation for the observed acid-induced increase in transcript levels of flagellar genes could be that acid-shock prepares *C. jejuni* for invasion or colonisation of the small intestine. Therefore, *in vitro* invasion experiments were performed with acid-shocked *C. jejuni* cultures compared to a pH 7 control, which resulted in increased invasion of mouse intestinal epithelial (m-IC_{cl2}) cells by *C. jejuni* when the m-IC_{cl2} cells were grown in a transwell model (fig. 3.4 C). Interestingly, this phenomenon was not observed when m-IC_{cl2} cells were grown on flat-bottomed wells. This could be due to the elimination of the basolateral route of invasion in cells grown on flat-bottomed wells. Since *C. jejuni* can translocate through epithelial cell monolayers (Grant *et al.* 1993; Douillard *et al.* 2008; van Alphen *et al.* 2008), this may be an important route of invasion for more motile, acid-shocked bacteria. Acid-shock may therefore increase the numbers of *C. jejuni* with flagella, which are required for translocation (Grant *et al.* 1993), enabling more bacteria to translocate the epithelial cell layer and invade cells. However, the numbers of translocated *C. jejuni* varied between individual wells, reflecting perhaps differences in invasion mechanisms or responses to host secreted factors within the bacterial population. Quantification or visualisation of translocated bacteria at different time points after infection would address this.

Another difference was that lower invasion levels were seen in the transwell model than in the flat-bottomed well model when comparing control, non-shocked *C. jejuni* cultures (fig. 3.4 C). Translocated *C. jejuni* that did not subsequently invade m-IC_{cl2} cells may account for this difference. Acid-shock then increased invasion levels because *C. jejuni* were more able to invade m-IC_{cl2} cells or were forced to escape acid-shock. Alternatively, the transwell invasion model was more physiologically relevant due to

greater m-IC_{cl2} cell polarisation, and so invasion levels were, in fact, more realistic than those in the flat-bottomed well model.

C. jejuni was able invade m-IC_{cl2} cells and so this cell line is suitable for use in *C. jejuni* invasion assays, providing a system that represents the mammalian small intestine, rather than the colon. These cells can be used to complement *in vivo* mouse studies (Chang and Miller 2006; Watson *et al.* 2007; Bereswill *et al.* 2011). There is, however, no perfect invasion model for studying *C. jejuni* pathogenesis currently making the choice of model to use for both *in vitro* and *in vivo* work difficult. The majority of *in vitro* studies have used Caco-2, INT407, Hep-2 and HeLa cell lines (Friis *et al.* 2005), which have greatly advanced our knowledge of the molecular mechanisms of *C. jejuni* pathogenesis. However, as *C. jejuni* initially colonises the small intestine, which is inflamed and damaged in *C. jejuni* infected patients (Konkel *et al.* 2001), we propose that m-IC_{cl2} cells may be a more relevant cell line for investigating host cell invasion, as they are morphologically and functionally similar to small intestinal crypts (Bens *et al.* 1996).

To date, this is the first attempt to characterise the capacity of acid-shock to induce *C. jejuni* invasion of intestinal epithelial cells. Acidic conditions may trigger the activation of *C. jejuni* virulence phenotypes in preparation for host cell invasion. One study investigating co-incubation of *C. jejuni* with amoebae demonstrated that incubation of *C. jejuni* under mildly acidic conditions increased its adherence to and invasion of amoebae (Axelsson-Olsson *et al.* 2010). This study also reported that bacterial survival increased after long term acid exposure and that incubation of *C. jejuni* with low pH for an hour increased motility on a swarm plate. However, this was not observed in an earlier study where *C. jejuni* motility was reduced when inoculated onto swarm plates adjusted to different pHs (Szymanski *et al.* 1995). The effect of acidic conditions on *C. jejuni* motility is therefore not clear and needs to be investigated further as motility is required for host cell invasion, which is important for virulence (Grant *et al.* 1993; Nachamkin *et al.* 1993; Hendrixson and DiRita 2004).

In summary, this work shows that *C. jejuni* responds to acid-shock by down-regulating genes involved in cell division and replication and by up-regulating flagellar and stress response genes. Understanding flagellar regulation is key to piecing together aspects of pathogenesis and perhaps devising interventions to prevent morbidity. Acid-shock

increases *C. jejuni* invasion of intestinal epithelial cells when the basolateral invasion route is available. More work is now needed to extend these observations using virulence models. Also, the mechanisms that *C. jejuni* employs to sense acid-shock need to be elucidated to enhance our understanding of *C. jejuni* survival and response to acidic conditions, which is relevant to both the food industries and *C. jejuni* pathogenesis.

Chapter 4 Discovery of small non-coding RNAs NC1 and NC4 in *C. jejuni*

Collaborative work:

Differential RNA sequencing and confirmation of RNAs performed by Ida Porcelli *et al.* (Porcelli *et al.* 2013).

4.1 Introduction

In the last decade, non-protein-coding RNAs (ncRNAs) have been established as post-transcriptional regulators of gene expression. These ncRNAs are not messenger, transfer or ribosomal RNAs and are also referred to as regulatory or functional RNAs in the literature. Regulation by ncRNAs affects many biological processes and ncRNA regulatory networks are present in all kingdoms of life. In prokaryotes, ncRNAs have been shown to contribute to virulence factor expression, and so are important in bacterial pathogenesis (Papenfort and Vogel 2010).

Rapid advances in high-throughput sequencing technologies have opened up the field of ncRNA research. Differential RNA sequencing of primary transcripts was first performed for ncRNA discovery in the related organism, *Helicobacter pylori* 26695 (Sharma *et al.* 2010). Surprisingly over a quarter of all transcription start sites (TSS) were antisense and more than 60 riboregulators have been discovered and validated by Northern hybridisation (Sharma *et al.* 2010). Of note, one ncRNA regulates urease activity *in cis*, which is important for survival in the host (Wen *et al.* 2011) and one antisense ncRNA regulates chemotaxis receptor expression *in trans* (Pernitzsch and Sharma 2012). Thus, it is likely that regulation by ncRNAs is an important mechanism of gene expression in Epsilon-Proteobacteria and that they have relevant roles in bacterial survival and host interactions.

The aims of the study were to discover potential regulatory ncRNAs. The objectives were to:

- Sequence primary RNA transcripts
- Identify ncRNAs that are potentially involved in regulating the expression of virulence factors by bioinformatic analysis
- Confirm expression of potential regulatory ncRNAs.

4.2 Results

4.2.1 Two small non-coding RNAs, NC1 and NC4, are present in the *C. jejuni* genome.

Differential RNA sequencing of *C. jejuni* NCTC11168 transcriptome enriched for primary transcripts revealed the presence of several potential ncRNAs (Porcelli *et al.* 2013). Two of these, designated NC1 and NC4 are transcribed from genes situated in different genomic locations, the *cj0082-Cj0085c* and *cj1633-Cj1634c* intergenic regions respectively (fig. 4.1). NC1 and NC4 are 45 and 47 nucleotides long and have similar sequences (fig. 4.2). Poly G and C tracts may lead to formation of a stem-loop transcription termination structure, suggesting that they are transcribed as discrete RNA species (fig. 4.2).

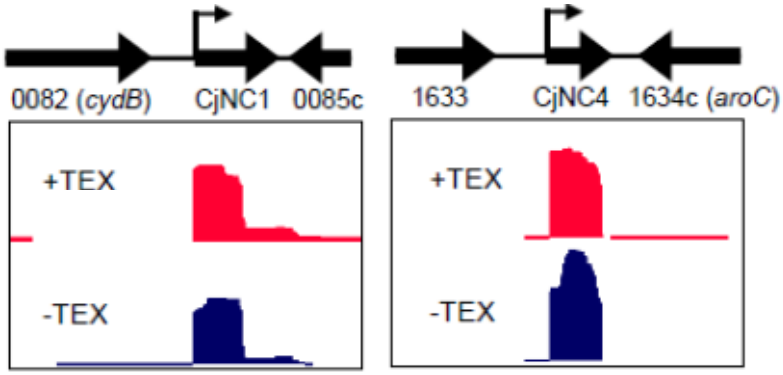


Figure 4.1 NC1 and NC4 are expressed in primary transcripts.

Differential RNA sequencing histograms are shown: the **red** histogram representing transcripts from the cDNA library enriched for primary transcripts by the addition of terminator exonuclease (TEX); and the **blue** histogram representing the non-enriched cDNA library. Above the histograms, the ncRNA and surrounding genes (*cj* numbers for *C. jejuni* NCTC11168) are shown as large arrows indicating the direction of transcription. The small arrows represent the ncRNA promoters. Taken from Porcelli *et al.* (Porcelli *et al.* 2013).

```
                                    stem  loop  stem
                                  >>>>>>      <<<<<<
NC1  AAATCTTTTCAAAATATTGCAATT-TGCCCA-TTTT-TGGGCA-TCTTT
      ||||||||||||||||||||||   |||||  ||| |||||  | |||
NC4  GAATCTTTTCAAAATATTGCAATCAAGCCCATGAAAATGGGCT-T-TTT
                                  >>>>>>>    <<<<<<<
                                   stem  loop  stem
```

Figure 4.2 NC1 and NC4 have similar sequences.

The sequences of NC1 and NC4 are shown. Bases that are predicted to from a stem-loop structure are labelled bases forming the stem are indicated by < or >, which point to toward the top of the stem. Where bases match exactly is indicated by | .

NC1 and NC4 are conserved in other thermophilic *Campylobacter* species including *C. jejuni* subspecies *doylei* and *C. coli*. NC1 is also present in the *C. upsaliensis* genome (fig. 4.3). Conservation of these RNAs suggests a conserved role in *Campylobacter* biology. The promoters of NC1 and NC4 contain consensus σ^{28} (CGATAT) -10 sequences and these are also conserved. This suggests that the ncRNAs are transcribed during flagellar assembly, as σ^{28} is only expressed during this process.

NC1: intergenic region *cj0082* (*cydB*) - *cj0085c*

```
                    -10
Cjej          TTAAACCGATATAAGT-ATAAATCTTTTCAAAATATTGCAATT-TGCCCA--TTTT--TGGGCATCTTTTAA
Cdoy          TTAAACCGATATAAGT-ATAAATCTTTTCAAAATATTGCAATT-TGCCCA--TTTT--TGGACATCTTTTAA
Ccoli         TTAA-CCGATTTAGGA-TTAAATCTTTTCAAAATATTGCAATTTTGCCCATATTTATATGGGCA--TTTTTA
Cups          AATAAACGATATAGTAGATGAATCTTTTCAAAATATTGCAA-T-AA-ACCCAAGTTAAAGCTTGGGTTTTTT
                 *  ***** *     * ********************* *     *     *    *      ****
```

NC4: intergenic region *cj1633* - *cj1634c* (*aroC*)

```
                    -10
Cjej          AACAAGCGATATAGTATTTGAATCTTTTCAAAATATTGCAA-TCAA-GCCCATGAAAATGGGCTTTTTT
Cdoy          AAGAAACGATATAGTATTTGAATCTTTTCAAAATATTGCAA-TCAA-GCCCATGAAAATGGGCTTTTTT
Ccoli         AAGAGACGATATGG-AGTTAAATCTTTTCAAAATATTGCAAATTAAAGCCCA--AAAATGGGCTTTATC
              ** *  ****** * * ** ********************   **  ****  ************ *
```

Figure 4.3 NC1 and NC4 are conserved in different *Campylobacter* species.

Conservation of NC1 and NC4 in *C. jejuni* subspecies *jejuni* NCTC11168 (Cjej), *C. jejuni* subspecies *doylei* 269.97 (Cdoy), *C. coli* RM2228 (Ccoli), *C. upsaliensis* RM3195 (Cups). The -10 promoter is shown in **red**. The non-coding RNA sequences are in **blue** and the predicted stem-loop structure is indicated. Bases conserved in all strains are indicated by * .

4.2.2 NC1 and NC4 are predicted to base pair with σ^{54}-dependent gene mRNA transcripts.

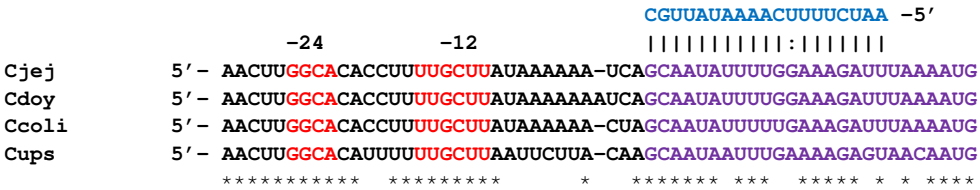
The TargetRNA programme (Tjaden *et al.* 2006) was used to predict gene targets for NC1 and NC4 and six of the highly ranked genes were σ^{54} dependent genes: *cj0243c* (hypothetical gene), *cj0428* (hypothetical gene), *cj1026c* (*flgP*), *cj1338c* (*flaB*), *cj1729c* (*flgE2*), and *cj1650* (hypothetical gene) (table 4.1). Further analysis revealed that NC1 and NC4 predicted interactions with target genes were characteristic of *trans* encoded ncRNAs. NC1 and NC4 were predicted to base pair with regions within the 5' untranslated region (5' UTR) of the target mRNA transcripts, with base-pairing being imperfect and short, incorporating around 20 nucleotides. NC1 and NC4 base-paring regions were predicted to be in close proximity to the translation start site and cover the ribosome binding sites, indicating that the ncRNAs may prevent ribosome binding and, therefore, translation (fig. 4.4). A further σ^{54}-dependent target was added to the predictions, which was *cj0887c* encoding *flgL* (fig. 4.4). The binding region of NC1 and NC4 is predicted to be relatively distant from the translation start site of *flgL* and *flgE2* transcripts, at approximately 25 and 50 nucleotides from the translation start site, respectively (fig. 4.4).

Table 4.1 List of NC1 and NC4 predicted *C. jejuni* gene targets.

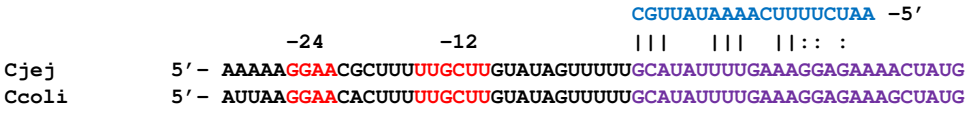
Genes listed are predicted by TargetRNA to be targets of NC1 and NC4 ($P < 0.001$) and are ranked based on P value. * denotes sigma54 dependent genes.

NC1			NC4		
Rank	**Gene**	**P value**	**Rank**	**Gene**	**P value**
1	*cj1338c* (*flaB*)*	3.21×10^{-7}	1	*cj1338c* (*flaB*)*	9.28×10^{-7}
2	*cj0243c**	9.28×10^{-7}	2	*cj0243c**	2.68×10^{-7}
3	*cj1026c* (*flgP*)*	6.51×10^{-5}	3	*cj0878*	8.04×10^{-5}
4	*cj0878*	8.05×10^{-5}	4	*cj0428**	9.95×10^{-5}
5	*cj0428**	9.95×10^{-5}	5	*cj0582* (*lysC*)	1.88×10^{-4}
6	*cj1729c* (*flgE2*)*	1.23×10^{-4}	6	*cj1026c* (*flgP*)*	1.88×10^{-4}
7	*cj0582* (*lysC*)	1.88×10^{-4}	7	*cj0852c*	2.33×10^{-4}
8	*cj0143c*	2.33×10^{-4}	8	*cj1650**	2.88×10^{-4}
9	*cj0852c*	2.33×10^{-4}	9	*cj1729c* (*flgE2*)*	3.56×10^{-4}
10	*cj1650**	2.88×10^{-4}	10	*cj0143c*	4.41×10^{-4}
11	*cj0462*	5.45×10^{-4}	11	*cj0462*	5.45×10^{-4}

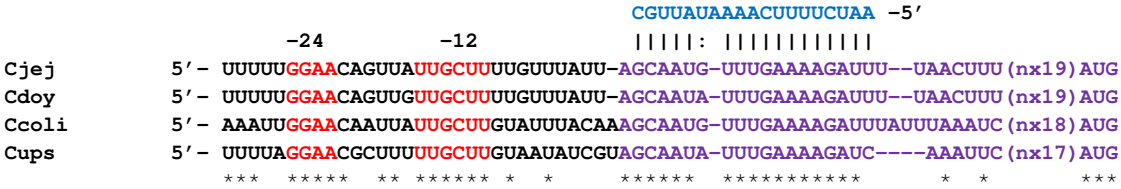
cj0243c (*hypothetical*)



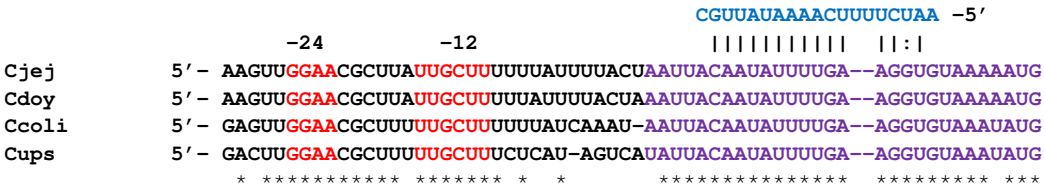
cj0428 (*hypothetical*)



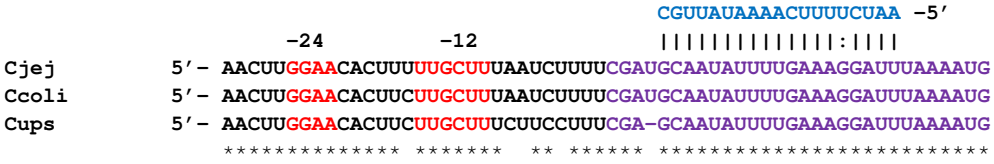
cj0887c (*flgL*)



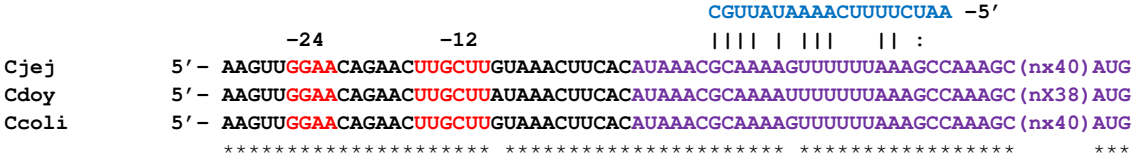
cj1026c (*flgP*)



cj1338c (*flaB*)



cj1729c (*flgE2*)



cj1650 (*hypothetical*)

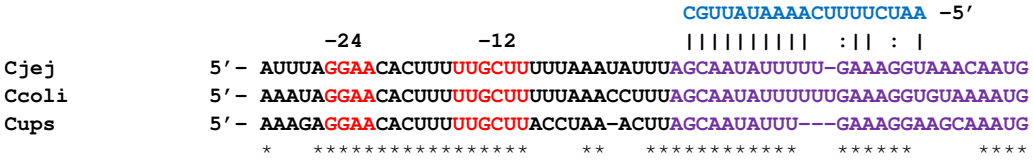


Figure 4.4 NC1 and NC4 are predicted to bind the 5' untranslated region of σ^{54}-dependent flagellar genes.

The predicted 5' untranslated region (5' UTR) of each *C. jejuni* target gene is shown in **purple** and the preceding promoter region is shown with consensus σ^{54} sequences in **red**. The NC1/NC4 binding region is shown in **blue** and where base pairs match to the target 5' UTR is indicated by **|** and partial binding is indicated by **:** .

4.2.3 *NC1* and *NC4* expression is confirmed by Northern hybridisation.

The expression of the ncRNAs was confirmed by Northern hybridisation analysis, where NC1 and NC4 were detected in RNA extracted from *C. jejuni* grown to early, mid-log and late-log phase and the amount of transcript expressed was similar across growth phases (fig. 4.5). However, the probe for NC1 required long exposure to [γ^{32}P] radiation for detection with a band being visible after 24 hours exposure. This suggests that the probe for NC1 was not suitable for the detection of the RNA or that low levels are expressed (fig. 4.5).

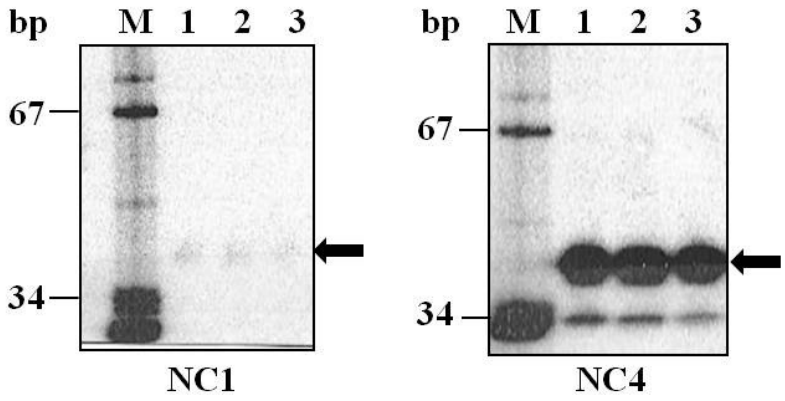


Figure 4.5 NC1 and NC4 are expressed in the *C. jejuni* NCTC11168 transcriptome.

Northern hybridisation was performed using [γ^{32}P] end labelled probes for detection of NC1 and NC4 transcripts in early logarithmic, mid-logarithmic and late logarithmic phases, lanes 1, 2 and 3, respectively. M - RNA marker (pUC Mix Marker, 8, Fermentas) and sizes of relevant fragments are indicated in base pairs (bp). NC1 and NC4 are 45 and 47 nucleotides long, respectively, and are indicated with black arrows. Performed by Ida Porcelli (Porcelli *et al.* 2013).

4.2.4 *NC1* and *NC4* expression is dependent on σ^{28} for transcription.

In order to establish whether NC1 and NC4 are dependent on σ^{28} for transcription as predicted, expression of the RNAs was assessed in a *C. jejuni* wild-type and *fliA* inactivated mutant (*fliA* encodes σ^{28}). Nested reverse transcription-PCR with gel analysis was used to detect NC1 and NC4 in the total RNA extract of these strains. NC1 and NC4 were expressed in the wild-type (fig. 4.6 A), but expression was markedly decreased in the σ^{28} mutant. The expression of NC3 was also assessed as a control as expression is high as it is dependent on σ^{70}, and so would be expressed and detected in both the wild-type and the mutant. NC3 expression was unaffected by the inactivation of *fliA* (fig. 4.6 A). No product was detected in the genomic DNA controls meaning that the primers only detected copy-DNA.

Northern hybridisation showed that NC4 was present in the wild-type, but was absent in the *fliA* inactivated mutant (fig. 4.6 B). NC1 was undetectable by Northern hybridisation after five days exposure to [γ^{32}P] radiation.

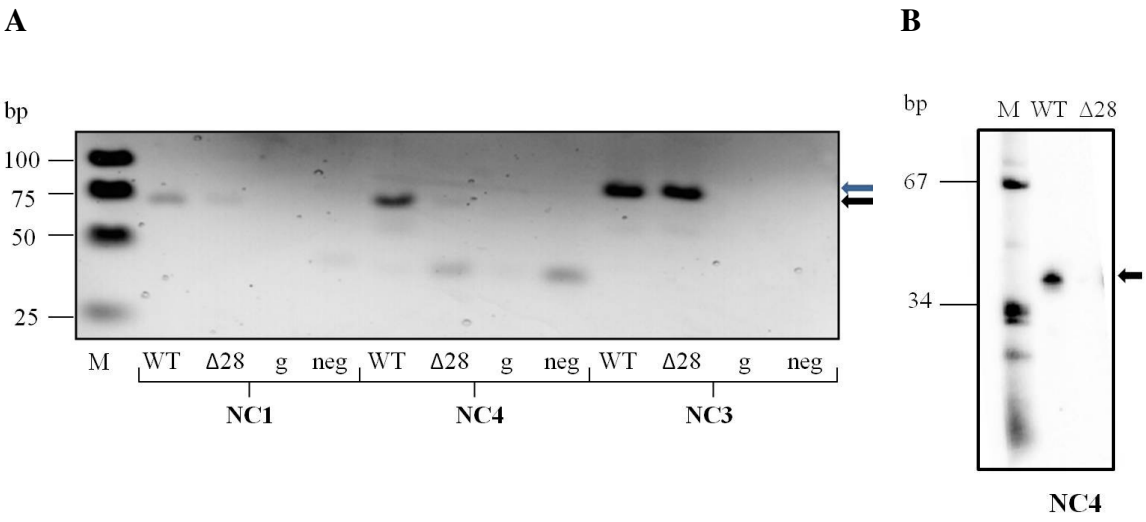


Figure 4.6 *NC1* and *NC4* expression is driven by σ^{28} transcription in *C. jejuni* NCTC11168.

NC1 and NC4 expression in a wild-type *C. jejuni* was compared to that of a *fliA* inactivated mutant, which would be deficient for functional σ^{28}. **A)** NC1 and NC4 expression was assessed in total RNA of the wild-type and the *fliA* inactivated mutant by PCR amplification of reverse transcribed RNA. Reverse transcription and amplification of NC3, a highly transcribed RNA upstream of the *porA* gene with a σ^{70} promoter, was performed as a control. Image has been inverted to show black bands on a light background. **B)** NC4 expression was assessed by Northern hybridisation in total RNA of the wild-type and the *fliA* inactivated mutant. Black arrows indicate NC1 and NC4 and **blue** arrow indicates NC3 positions. Symbols: WT - wild-type; Δ28 - *fliA* inactivated mutant; g - genomic DNA; -ve - primers only; M - Marker: Low Molecular Weight marker for PCR (NEB) or pUC mix marker 8 for Northern hybridisation (Thermo Scientific); bp - base pair.

4.3 Discussion

C. jejuni ncRNAs were discovered by differential RNA sequencing and were subsequently confirmed by Northern analysis (fig. 4.6, (Porcelli *et al.* 2013)). NC1 and NC4 were predicted to target σ^{54}-dependent flagellar genes and so act *in trans* to regulate gene expression. NC1 and NC4 were shown to be dependent on σ^{28} for expression in *C. jejuni* (fig. 4.6), which may mean that they are part of the flagellar regulatory network.

NC1 and NC4 were detected by differential RNA sequencing of *C. jejuni* NCTC11168 transcripts. NC1 and NC4 are conserved in other *C. jejuni* strains (Dugar *et al.* 2013) and in a few thermophilic *Campylobacter* species (fig. 4.3) (Dugar *et al.* 2013) in which they may perform a conserved function. Recent work comparing transcriptomes of *C. jejuni* strains NCTC11168, RM1221, 81-176, 81116 showed that NC4 was expressed by all strains and that expression was high during mid-exponential and stationary phase, but was low after overnight culture (Dugar *et al.* 2013). NC1 was detected and expressed, although probes poorly detected NC1 in Northern blot analysis. NC1 is detected in RNA sequencing so it is likely that NC1 cannot be detected by the probe employed in the assay. The sequences of NC1 and NC4 are similar and so they could perform similar functions or have some functional redundancy. Both the RNAs were expressed during early, mid and late growth phase, indicative of functioning during all stages of *C. jejuni* growth.

Approximately 30 potential *trans*-acting ncRNAs (2% of the genome) have been discovered in *C. jejuni* strains and several candidates have been found on *Campylobacter* plasmids pVir and pTet (Dugar *et al.* 2013; Porcelli *et al.* 2013). These plasmids contain genes for type IV secretion systems and antibiotic resistance (tetracycline resistance on pTet) (Bacon *et al.* 2002; Friis *et al.* 2007), so it is possible that ncRNAs could regulate expression of these systems. Five ncRNAs were predicted by earlier genome screens using the Rfam database (Chaudhuri *et al.* 2011) and four of these were subsequently detected by differential RNA sequencing (Dugar *et al.* 2013). The functions of these ncRNAs have not yet been elucidated.

RNA Illumina and differential RNA sequencing confirmed the presence of other previously described ncRNAs: the TPP riboswitch; signal recognition particle RNA, which is involved in the co-translational targeting of proteins to membranes; the RNA component of RNaseP; and 10Sa RNA, which rescues stalled ribosomes and tags proteins for degradation (Chaudhuri *et al.* 2011; Dugar *et al.* 2013).

NC1 and NC4 were predicted to be dependent on σ^{28} for transcription through the identification of σ^{28} consensus sequences. This was confirmed by the decreased expression of NC1 and NC4 expression in *fliA* inactivated strains (fig. 4.6). Faint bands were observed in semi-quantitative reverse transcription PCR of NC1 and NC4 in *fliA* inactivated *C. jejuni* RNA extracts, but these were not detected by Northern hybridisation of NC4. Leaky transcription from upstream promoters may have resulted in low levels of transcript detected by PCR, which is more sensitive than Northern analysis. The probe for NC1 failed and poorly detected NC1 in previous experiments. As the sequence of NC1 is short and approximately half the RNA forms a stem-loop structure, an alternative probe that did not cross-react with NC4 could not be made.

NC1 and NC4 were predicted to target several genes and many were shared between both the ncRNAs, which was expected as the sequences of NC1 and NC4 are very similar. The base-pairing regions of NC1 and NC4 that do not form the stem-loop structure, are almost identical except for the first nucleotide. Of the 11 highly ranked target genes ($P < 0.001$), six were σ^{54}-dependent genes. The highest ranked target was *flaB* with almost perfect complementary base-pairing and *flaB* is conserved in *C. coli* and *C. upsaliensis* also. The other σ^{54}-dependent target genes were conserved in two or more species of *Campylobacter*, including C. *jejuni* subspecies *jejuni*, *C. jejuni* subspecies *doylei*, *C. coli* and *C. upsaliensis*. The prediction that NC1 and NC4 regulate flagella genes, together with the demonstrated σ^{28}-dependent NC1 and NC4 expression, provide strong indicators for their involvement in flagellar regulation.

The following chapter will examine whether NC1 and NC4 contribute to an observable phenotype. The Epsilon-Proteobacteria seem to have developed their ncRNA repertoires independently of other prokaryotes, as *Campylobacter* and *Helicobacter* species have ncRNAs that are not present outside each genus. It was anticipated that functional studies for the ncRNAs discovered would reveal interesting roles for these potential genome regulators.

Chapter 5 Phenotypes of *C. jejuni* non-coding RNA mutants

5.1 Introduction

The previous chapter showed that NC1 and NC4 are dependent on σ^{28} for expression and are predicted to target σ^{54}-dependent flagellar genes. As these two σ factors are principally involved in flagellar assembly and expression of flagella-associated genes in *C. jejuni*, we hypothesised that NC1 and NC4 are part of the flagellar assembly regulatory network. Because flagellar assembly is tightly controlled and occurs in distinct stages, it was expected that altered expression of these ncRNAs would disrupt this network and alter flagellar assembly and function. Moreover, as NC1 and NC4 expression is dependent on σ^{28}, they would be produced at the late stage of flagellar assembly and then down-regulate translation of target, middle stage σ^{54} genes. Thus, the ncRNAs could act as a negative feedback mechanism to stop unnecessary protein expression after the flagellum has been assembled (fig. 5.1).

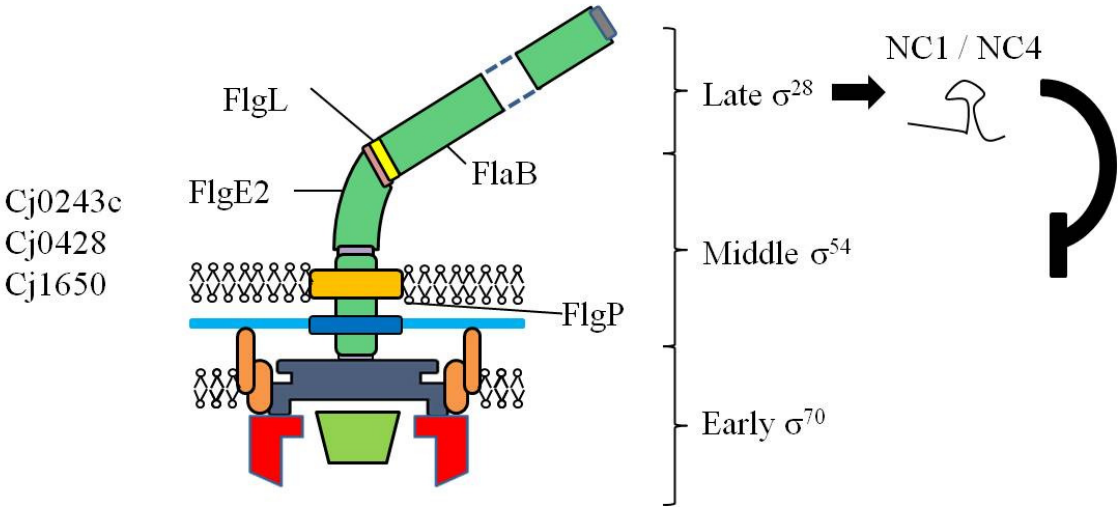


Figure 5.1 Hypothesis: NC1 and NC4 provide a negative feedback system of flagellar protein expression.

Flagellum assembly is regulated by a hierarchy of sigma (σ) factors. NC1 and NC4 would be expressed at the late stage of assembly by σ^{28} and they then target the expression of earlier σ^{54}-dependent genes (*cj0243c* (*hypothetical*), *cj0428* (*hypothetical*), *cj0887c* (*flgL*), *cj1338c* (*flaB*), *cj1026c* (*flgP*), *cj1729c* (*flgE2*), and *cj1650* (*hypothetical*)), during the middle stage of flagellar assembly. Known proteins encoded by these genes are labelled and hypothetical proteins are listed as being expressed in the middle phase of flagella assembly. Thus, NC1 and NC4 could act as a negative feedback mechanism to stop unnecessary protein expression after the flagellum has been assembled.

The predicted target genes for NC1 and NC4 are associated with motility phenotypes. FlgE2 (encoded by *cj1729c*) is the major hook subunit (Hendrixson and Di Rita 2003). Mutating *flgE2* prevents filament formation and *Campylobacter* invasion proteins are not secreted, which prevents motility, and adherence to and invasion of intestinal epithelial cells (Konkel *et al.* 2004). FlgL (encoded by *cj0887c*) is a junction protein and *flgL* mutation also prevents filament formation, motility and Cia protein secretion (Neal-McKinney and Konkel 2012). Membrane ruffling of intestinal epithelial cells was not induced by *C. jejuni flgL* mutants, whereas wild-type and complemented mutants did cause membrane ruffling (Neal-McKinney and Konkel 2012). Therefore, mutating *flgL* would give a visible phenotype forming truncated flagella, and these studies indicate that the role of *flgL* in motility or protein secretion is important for eliciting a host response.

FlgP (encoded by *cj1026c*) is an outer membrane associated protein that is required for motility only, as *flgP* mutants still assemble flagella, but are non-motile (Sommerlad and Hendrixson 2007). FlaB (encoded by *cj1338c*) is the minor flagellin subunit of the filament, whereas FlaA (encoded by *cj1339c*) is the major subunit. *C. jejuni* flagella can function without FlaB, and these bacteria are still motile and can colonise hosts (Wassenaar *et al.* 1993). This means that FlaB deficient *C. jejuni* may be phenotypically indistinguishable from wild-type *C. jejuni*, but phenotypes should be seen as a result of differential regulation of the other target genes. Thus, if NC1 and NC4 did regulate these proposed targets then there may be specific phenotypes that can be expected of NC1 and NC4 mutants.

The aims of this study were to assess the importance of NC1 and NC4 in *C. jejuni* motility and virulence, and whether they contribute to regulation of gene expression. The objectives were to:

- Make *C. jejuni* NC1 and NC4 mutants with single NC1 and NC4 chromosomal deletions, and a double deletion mutant – as NC1 and NC4 are similar, one may compensate for the other in a single deletion mutant;
- Make NC1 and NC4 over-expression mutants because if the ncRNAs silence gene expression, increased levels of NC1 and NC4 would be more likely to decrease expression of the target genes and give rise to a phenotype;
- Examine the growth of *C. jejuni* ncRNA mutants under different conditions;

- Examine motility phenotypes of *C. jejuni* ncRNA mutants in comparison with the wild-type strain and a non-motile mutant with short or no flagellar filament – considering the genes targeted by NC1 and NC4, mutants of the ncRNAs are expected to be phenotypically similar to non-motile *C. jejuni*;
- Examine protein expression of *C. jejuni* ncRNA mutants;
- Examine *C. jejuni* ncRNA mutant gene expression, and in particular the expression of predicted target genes.

5.2 Results

C. jejuni ncRNA mutants were made from a motile, spiral-rod variant of *C. jejuni* NCTC11168. A list of strains used in this chapter is given in table 5.2.1. Mutants were also made in a motile, straight-rod variant and phenotypic tests were performed, but these strains lost motility after passage, and so were re-made. However, this variant was later found by the group to have a growth defect at 42°C and so experiments with these bacteria ceased and the data are not included. As NC1 and NC4 are predicted to target flagellar genes, a range of motility-related assays were performed to investigate the involvement of NC1 and NC4 in flagellar formation and function. Because it was predicted that flagellar regulation would be disrupted in *C. jejuni* ncRNA mutants and result in aflagellate, non-motile bacteria, *C. jejuni* lacking *flaA* and *flaB* was included in motility assays for comparison.

5.2.1 Deletion and over-expression of *NC1* and *NC4* in *C. jejuni* NCTC11168.

NC1 and *NC4* were each deleted and over-expressed in a motile *C. jejuni* NCTC11168 to ascertain their function and contribution to *C. jejuni* biology. Both *NC1* and *NC4* were also deleted to eliminate any possible compensatory actions one ncRNA might have in place of the other. To delete the genes, the flanking sequences of the intergenic regions with antibiotic resistance cassettes were constructed in shuttle vectors, which were used to transform *C. jejuni* by homologously recombining the mutation into the chromosome.

To over-express *NC1* and *NC4*, the genes were synthesised downstream of the *fdxA* promoter (moderate expression strength) with the *Nco*I restriction site at either side (GeneArt). The gene was excised from the synthesised plasmid and cloned into the shuttle vector pC46. Plasmid C46 contains flanking regions of the pseudogene *cj0046* with a chloramphenicol resistance cassette. This plasmid was then used to transform *C. jejuni* as described before. Detailed descriptions of NC1 and NC4 mutations are described in chapter 2. Shuttle plasmid constructs were verified by sequencing and *C. jejuni* mutants were verified by PCR. *C. jejuni NC4* mutants were also confirmed by Northern hybridisation analysis (fig. 5.2). The strains used in this chapter are listed in table 5.1.

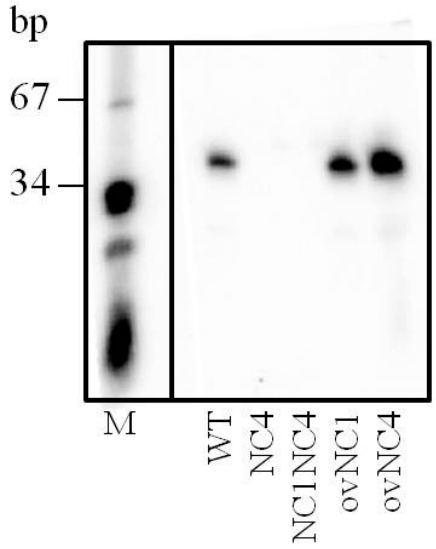


Figure 5.2 Confirmation of *NC4* deletion from *C. jejuni* NCTC11168.

Northern hybridisation was performed using [γ^{32}P] end labelled probes to detect NC4 transcripts in *C. jejuni* RNA extracts from bacteria grown to mid-logarithmic phase. NC4 is 47 nucleotides long. Each lane contains RNA from one strain of *C. jejuni*: WT - wild-type; NC4 - *NC4* deletion; NC1NC4 - double *NC1*, *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression. Lane M contains an RNA marker (pUC Mix Marker, 8, Fermentas) and sizes of relevant fragments are indicated in base pairs (bp).

Table 5.1 *C. jejuni* NCTC11168 strains used in phenotype assays.

Symbol	Description
WT	Wild-type *Campylobacter jejuni* NCTC11168
NM	Non-motile, *flaAB* inactivation, kan^R
NC1	*NC1* deletion, kan^R
NC4	*NC4* deletion, kan^R
NC1NC4	*NC1* deletion, kan^R and *NC4* deletion, $chlor^R$
ovNC1	*NC1* over-expression in *cj0046* region, P*fdxA*, $chlor^R$
ovNC4	*NC4* over-expression in *cj0046* region, P*fdxA*, $chlor^R$

kan^R - kanamycin resistance cassette
$chlor^R$ - chloramphenicol resistance cassette
P*fdxA* – promoter of *fdxA* used for over-expression (van Vliet 2001).

5.2.2 Growth of *C. jejuni NC1/NC4* deletion and over-expression strains is similar to the wild-type strain.

Growth of *C. jejuni* NC1 and NC4 deletion and over-expressing strains was monitored at 37 and 42°C for 24 hours. All NC1, NC4 and double NC1/NC4 deletion strains and the NC1 and NC4 over-expressing strains grew at the same rate as the wild-type strain at both temperatures (fig. 5.3). Growth rate of the non-motile *flaAB* inactivation strain was increased at both 37 and 42°C, which was expected because the strain would not have to expend energy producing flagella.

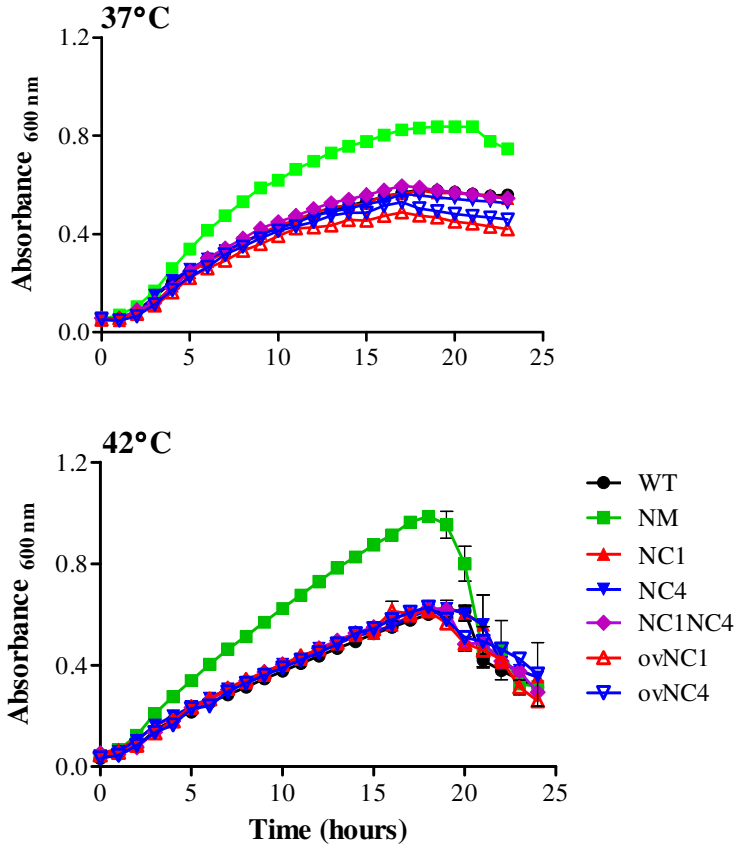


Figure 5.3 Growth of *C. jejuni* NCTC11168 NC1/NC4 deletion and over-expression strains is similar to wild-type at 37 and 42°C.

The growth of *C. jejuni* NCTC11168 strains was monitored at 37 and 42°C by measuring the optical density of batch cultures over time. Error bars represent standard error of the mean of data from three biological replicates. Some error bars were too low to be plotted. Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.3 Autoagglutination of *C. jejuni NC1/NC4* deletion and over-expression strains is similar to the wild-type strain.

C. jejuni cells clump together in static broth cultures and this is known as autoagglutination. Autoagglutination is a common indicator of the presence of flagella or changes in the flagella surface (Friis *et al.* 2007). For autoagglutination experiments, *C. jejuni* cultures, grown to mid-exponential phase, were resuspended in phosphate buffered saline and the change in optical density was measured after 24 hours at 20°C in

air. The average initial optical density was 0.6-0.7 for all strains except for the *flaAB* inactivation mutant, which was ~1.0 (not shown). The NC1 deletion, NC4 deletion, NC1 over-expression and NC4 over-expression *C. jejuni* strains all autoagglutinated to the same extent as the wild-type as the final optical density of cell suspensions was reduced to approximately 20% of the initial optical density e (fig 5.4). The final optical density of double NC1/NC4 deletion mutant cell suspensions was 35% of the initial optical density, but this was not significantly different to the wild-type ($P > 0.05$). The non-motile strain did not autoagglutinate and final optical density remained at approximately 80% of the initial optical density (fig 5.4).

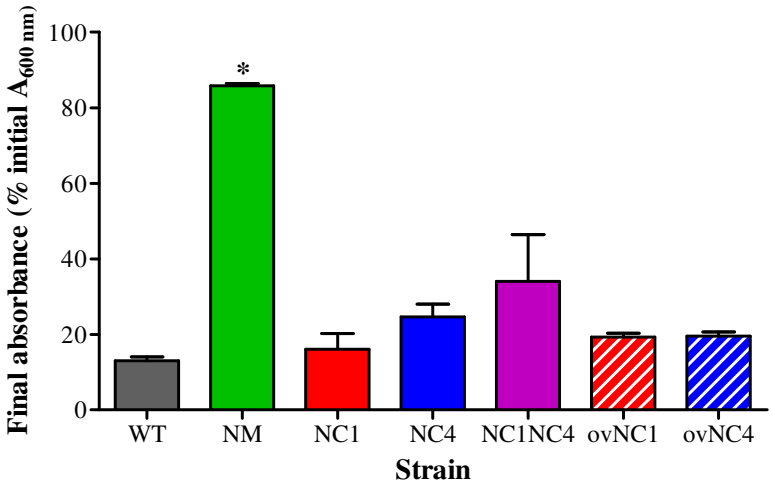


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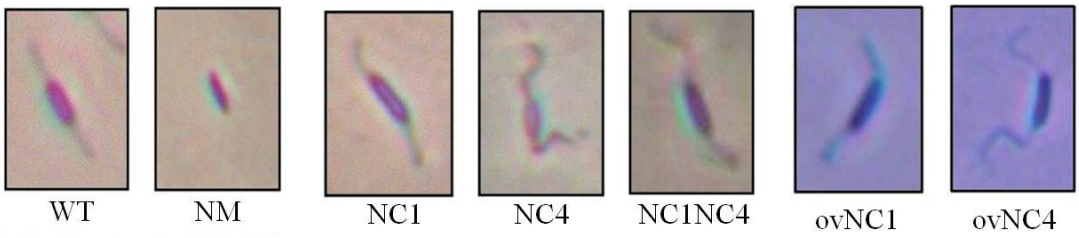
Figure 5.4 Autoagglutination of *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains is similar to the wild-type.

To measure autoagglutination (AAG), *C. jejuni* were resuspended in phosphate buffered saline and the optical density at 600 nm was measured immediately and after 24 hours. The average initial optical density was 0.6-0.7 for all strains except for NM, which was ~1.0. Results are shown as the percentage final optical density of the initial optical density (% initial OD_{600} nm). Error bars represent standard error of the mean of data from three biological replicates. Asterisk indicates $P < 0.05$ relative to WT (One-way ANOVA). Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.4 *C. jejuni NC1/NC4* deletion and over-expression strains express flagella.

C. jejuni and their flagella were directly visualised by light microscopy using a crystal violet based stain. All *NC1* and *NC4* mutant strains had flagella that were similar to the wild-type, except for the non-motile strain, which did not have flagella (fig. 5.5 A). The *C. jejuni NC1* and *NC4* deletion and the wild-type strains were visualised by scanning electron microscopy. All strains had flagella and had similar cell morphologies (fig. 5.5 B).

A



B

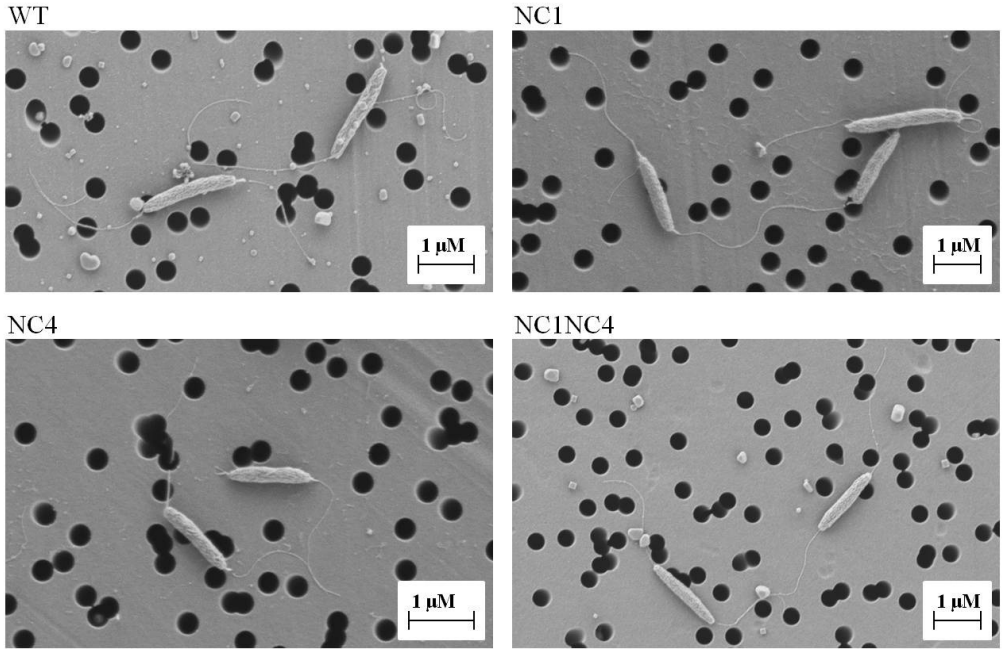


Figure 5.5 *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains express flagella.

A) *C. jejuni* strains were observed by light microscopy using a crystal violet based stain. The non-motile strain did not have flagella. Images are representative of three biological replicates. **B)** Wild-type *C. jejuni* and the NC1 and NC4 deletion strains were observed by scanning electron microscopy (performed by Kathryn Cross). Images are representative of two biological replicates. Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.5 Motility of *C. jejuni NC1/NC4* deletion and over-expression strains is similar to the wild-type strain.

Motility was measured using a swarm plate assay, where soft agar was inoculated with *C. jejuni* strains and the diameter of spread after 48 hours, in microaerobic conditions, was measured. All *C. jejuni NC1*, *NC4* and double *NC1/NC4* deletion strains and the NC1 and NC4 over-expression strains were motile to the same degree as the wild-type (fig. 5.6). As expected the *flaAB* mutant was non-motile and did not spread out from the point of inoculation, and this was significantly different to the wild-type ($P < 0.05$).

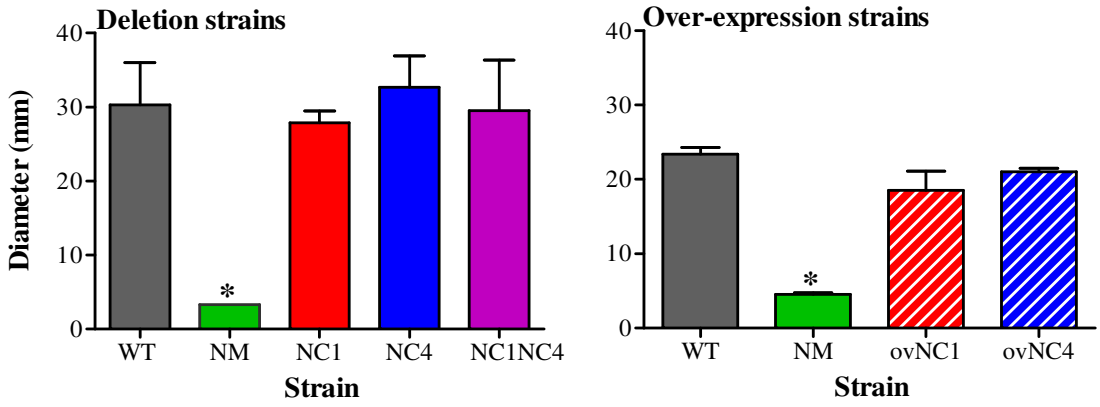


Figure 5.6 Motility of *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains is similar to the wild-type.

Motility of each strain was assessed by measuring the spread of bacteria on soft agar plates and is given in millimetres (mm). Error bars represent standard error of the mean of data from three biological replicates. Asterisk indicates $P < 0.05$ relative to WT (One-way ANOVA). Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.6 *C. jejuni NC1/NC4* deletion and over-expression strains have similar taxis profiles to the wild-type strain.

In addition to the swarm plate assay, a tube-based assay that examines energy taxis was used to test flagellar function as flagella are required for taxis (Reuter *et al.* 2010; Reuter and van Vliet 2013). Brucella soft agar medium supplemented with triphenyl tetrazolium chloride (TTC), which is converted into a red formazan dye by respiration,

was set in a centrifuge tube. The agar was inoculated with *C. jejuni* strains at the top of the tube and these were incubated in air at 37°C. *C. jejuni* move away from the air-surface interface in the agar to migrate away from the high oxygen environment and so the length of migration, indicated by the TTC dye front, was measured after 48 hours, as in the swarm plate assay (fig. 5.7 A). The *C. jejuni NC1*, *NC4* and double *NC1/NC4* deletion strains and the *NC1* and *NC4* over-expression strains displayed similar motility-dependent taxis profiles to the wild-type (fig. 5.7 B). The *NC4* deletion mutant showed decreased taxis compared to the wild-type as the migration of the TTC dye front was 80% of that of the wild-type strain, but this decrease was not statistically significant ($P > 0.05$). The *NC4* over-expression strain showed an average 20% increase in migration compared to the wild-type, but the data were variable and this increase was not significant ($P > 0.05$) (fig. 5.7 B). As expected the non-motile strain showed significantly reduced migration compared to the wild-type (fig. 5.7) (Reuter and van Vliet 2013).

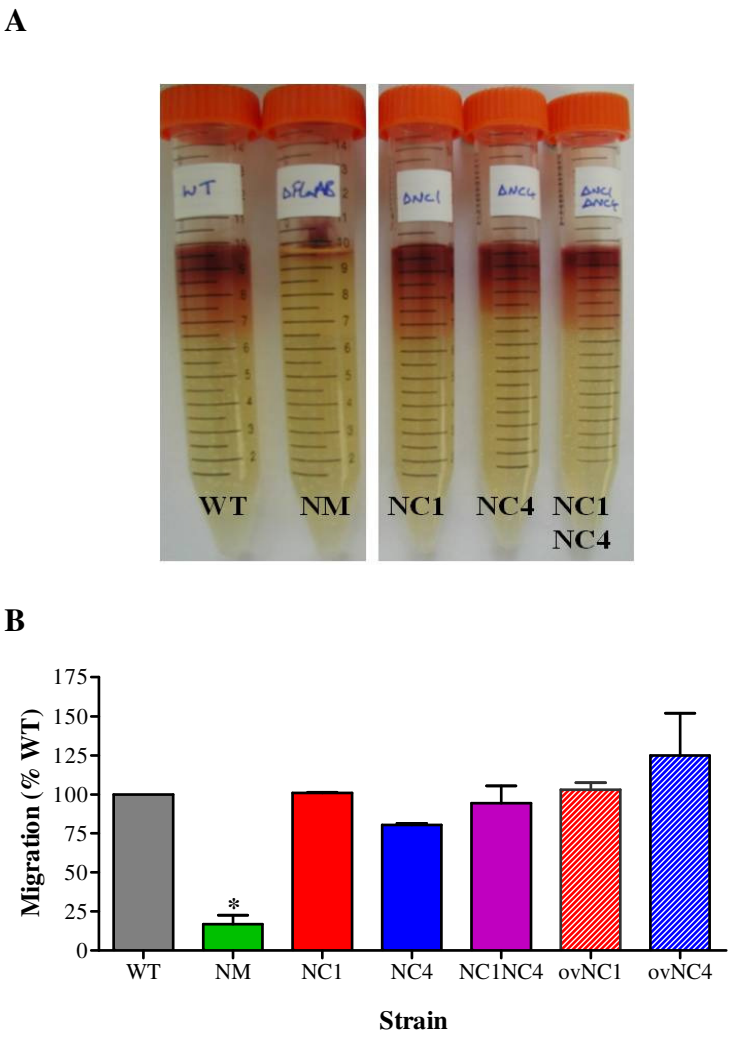


Figure 5.7 *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains have a similar taxis profile to the wild-type.

A) Example energy taxis assay using Brucella soft agar medium supplemented with triphenyl tetrazolium chloride (TTC), which turns red in the presence of live bacteria. *C. jejuni* was added to the medium at the top of the tube and migration of TTC front was measured after 48 hours incubation at 37°C in air. **B)** Migration of *C. jejuni* non-coding RNA mutant strains expressed as percentage of the wild-type (% WT) and error bars represent standard error of the mean of data from three biological replicates. Asterisk indicates $P < 0.05$ relative to WT (One-way ANOVA). Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.7 *C. jejuni NC1/NC4* deletion and over-expression strains form similar amounts of biofilm to the wild-type.

Flagella contribute to biofilm formation in *C. jejuni* (Kalmokoff *et al.* 2006; Reeser *et al.* 2007; Reuter *et al.* 2010). To ascertain whether *NC1* and *NC4* contribute to this aspect of flagellar function, biofilm assays were performed. *C. jejuni* strains were grown to stationary phase, and cultures were diluted in fresh media and incubated in air or under microaerobic conditions for two days. The biofilms formed were then stained with crystal violet, dissolved in a solvent and the amount of biofilm staining could then be determined by measuring the absorbance of the dissolved crystal violet in solution. The amount of biofilm formed by *NC1*, *NC4* and double *NC1/NC4* deletion strains was not significantly different from the wild-type in air or in microaerobic conditions (fig. 5.8). The amount of biofilm formed in air by the *NC1* and *NC4* over-expression strains was slightly increased compared to the wild-type (fig. 5.8), but this increase was not significant ($P > 0.05$, Two-way ANOVA) and data were variable. The non-motile mutant formed significantly less biofilm in air than the wild-type as expected.

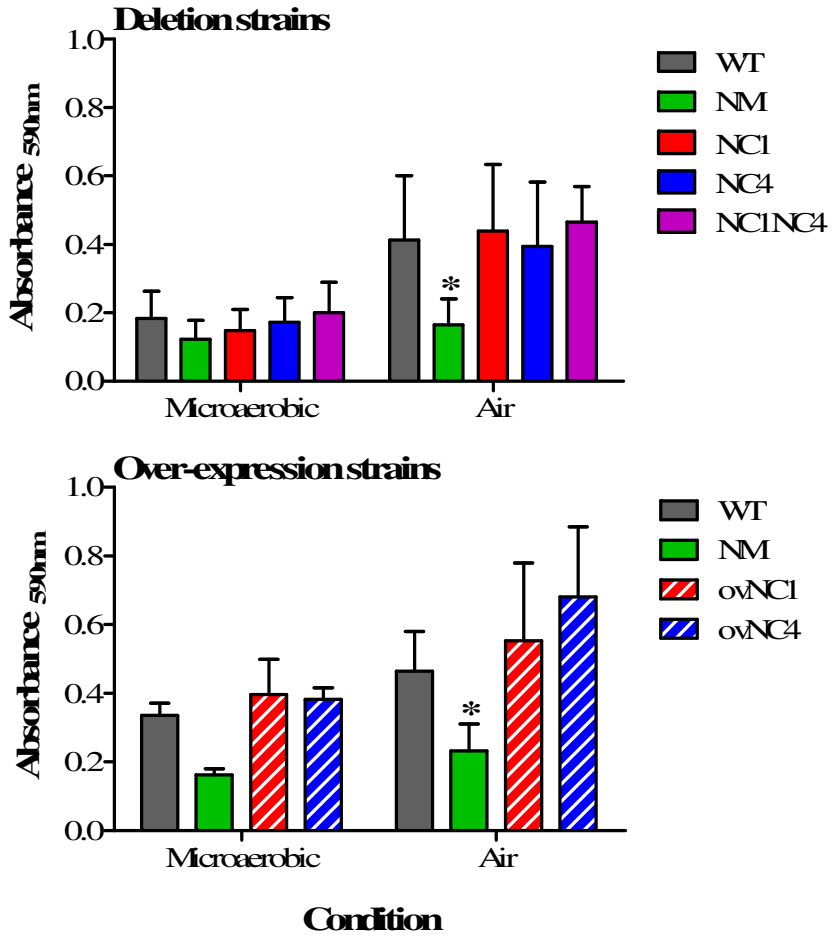


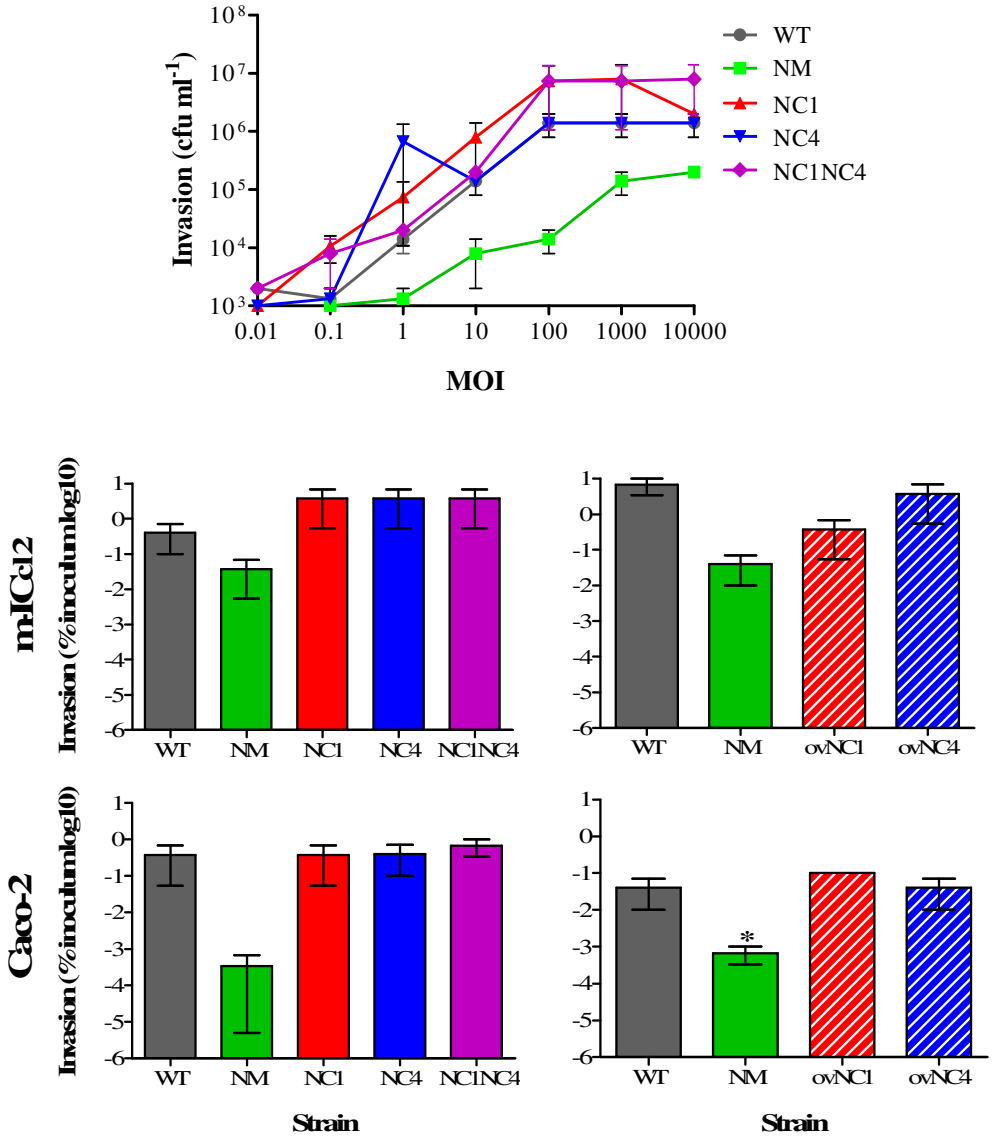
Figure 5.8 *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains form similar amounts of biofilm to wild-type.

The amount of biofilm formation by two-day old static cultures of each strain in air or under microaerobic conditions was measured using a crystal violet stain. The level of staining was determined by dissolving the crystal violet in solvent and measuring absorbance at 590 nm. Error bars represent standard error of the mean of data from four biological replicates. Asterisk indicates $P < 0.05$ relative to WT (Two-way ANOVA). Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.8 *C. jejuni NC1/NC4* deletion and over-expression strains invade intestinal epithelial cell monolayers to a similar extent as the wild-type strain.

Flagellated *C. jejuni* can invade host cells and this is an important part of host colonisation, which can lead to disease (Grant *et al.* 1993; Wassenaar *et al.* 1993; Hendrixson and DiRita 2004). To ascertain whether *NC1* and *NC4* contribute to this aspect of flagellar function, invasion of intestinal epithelial cell monolayers by the ncRNA mutant strains was assessed. A confluent monolayer of m-IC_{cl2} (Chapter 3) (Bens *et al.* 1996) and Caco-2 cells was grown on the bottom of flat-bottomed wells on top of a collagen matrix and infected with the strains of *C. jejuni* at a multiplicity of infection (MOI) of 1000. An MOI of 1000 was chosen for Caco-2 based on existing literature (Russell and Blake 1994; MacCallum *et al.* 2005). An MOI of 1000 was chosen for m-IC_{cl2} as this gave the maximal invasion for all strains (fig. 5.9 A). After a gentamicin wash, cells were lysed and intracellular bacteria were enumerated. *NC1*, *NC4* and double *NC1/NC4* deletion and *NC1* and *NC4* over-expressing strains were all able to invade m-IC_{cl2} and Caco-2 monolayers (fig. 5.9 B). Invasion of Caco-2 monolayers by all ncRNA mutant strains was consistently at the level of the wild-type, whereas invasion by the non-motile strain was at least 100-fold reduced, which was significant in one experiment ($P < 0.05$, fig. 5.9 B). Invasion of m-IC_{cl2} monolayers by *NC1*, *NC4* and double *NC1/NC4* deletion strains was 10-fold increased compared to the wild-type, but this was not statistically significant ($P > 0.05$, fig. 5.9 B). Invasion of m-IC_{cl2} monolayers by the *NC1* over-expression strain was 10-fold reduced compared to the wild-type, but this was also not significant ($P > 0.05$, fig. 5.9 B). Increased numbers of *C. jejuni* invaded m-IC_{cl2} monolayers in comparison with Caco-2 monolayers. Most notably the non-motile strain was 100-fold more invasive of m-IC_{cl2} monolayers than Caco-2 monolayers. As expected, the non-motile strain was less able to invade IEC monolayers (Novik *et al.* 2010).

A



B

Figure 5.9 *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains invade intestinal epithelial cell monolayers to a similar extent as the wild-type.

Invasion assays were performed, where *C. jejuni* were allowed to invade m-IC_{cl2} for 2 h and Caco-2 monolayers and intracellular bacteria were enumerated by colony counts after a gentamicin wash. **A)** Invasion of m-IC_{cl2} monolayers by *C. jejuni* strains in increasing multiplicities of infection (MOI). Results are expressed in colony forming units per millilitre (cfu ml^{-1}). **B)** Invasion of m-IC_{cl2} and Caco-2 monolayers by *C. jejuni* strains using an MOI of 1000. Results are expressed as percentage of invaded bacteria with respect to the inocula on a log scale (% inoculum log10). Error bars represent standard error of the mean of data from three biological replicates. Asterisk indicates $P < 0.05$ relative to WT (One-way ANOVA). Strains: WT - wild-type; NM - non-motile *flaAB* inactivation; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion; ovNC1 - *NC1* over-expression; ovNC4 - *NC4* over-expression.

5.2.9 Protein expression of *C. jejuni* NC1/NC4 deletion and over-expression strains is similar to the wild-type strain.

C. jejuni strains were grown to exponential phase at 37 and 42°C. For protein analysis, cells were separated into cytoplasm, inner membrane or outer membrane fractions. Proteins were denatured and separated using SDS-PAGE. There were no differences detected in the protein profiles of *C. jejuni* double *NC1/NC4* deletion and *NC4* over-expressing strains compared to the wild-type at both temperatures (fig. 5.10). However, a low molecular weight protein was present in the inner and outer membrane fractions (fig. 5.10). The protein differed in molecular weight in all samples, but this was not associated with temperature, strain or cell fraction (fig. 5.10).

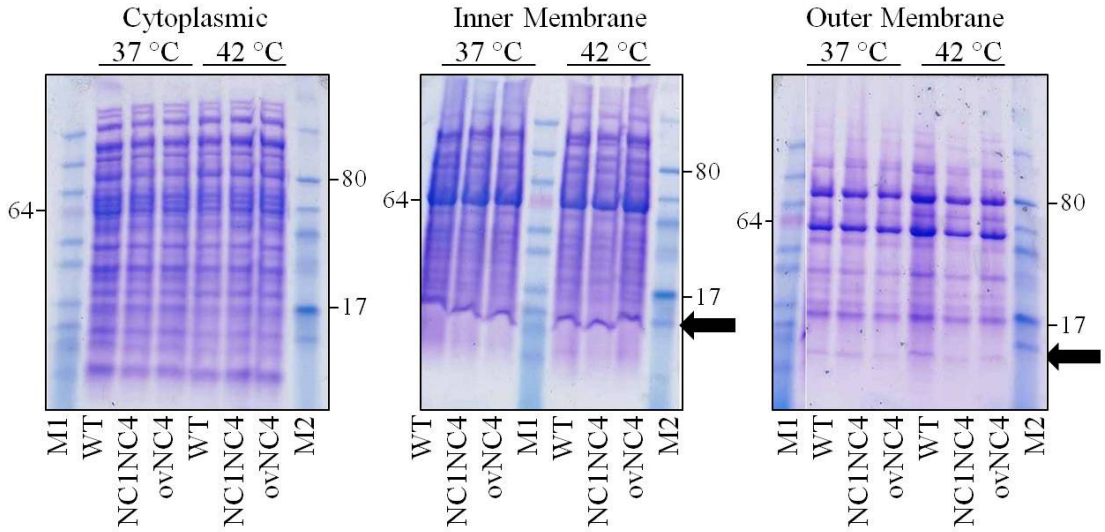


Figure 5.10 *C. jejuni* NCTC11168 *NC1/NC4* deletion and over-expression strains have protein profiles that are similar to the wild-type.

C. jejuni strains were grown to exponential phase at 37 and 42°C under microaerobic conditions. For protein analysis, cells were fractionated so that samples contained proteins from the cytoplasm, inner membrane or outer membrane. Proteins were denatured, separated using SDS-PAGE and stained with Coomassie blue. The black arrow indicates a protein band that varies in molecular weight in all samples. Images are representative of two biological replicates. Strains: WT - wild-type; NC1NC4 - double *NC1* and *NC4* deletion; ovNC4 - *NC4* over-expression. M1 - NEB Broadstain marker, M2 - Invitrogen Benchmark Ladder, molecular weight of bright bands are given in kDa.

5.2.10 Two-dimensional protein profiles of *C. jejuni NC1/NC4* deletion strains are similar to the wild-type strain.

To further analyse protein expression of all *NC1*, *NC4* and double *NC1/NC4* deletion strains, proteins of whole cell lysates were separated by two-dimensional gel electrophoresis (Shaw *et al.* 2012), separated according to isoelectric point (pH 3-11) in the first dimension and by molecular weight in the second (fig. 5.11 A). Three differences in protein expression were observed between deletion mutants and the wild-type, but were not consistent between all strains. In the *NC1* and *NC4* deletion mutants, flagellin was up-regulated in the both of the mutants (fig. 5.11 B). Although these differences were seen in both deletion mutants and in two biological replicates, it is possible that they were due to differences in glycosylation or oxidation states. Also these differences were not observed in the double *NC1/NC4* deletion strain. In the double *NC1/NC4* deletion strain, there was a shift in the position of the nickel iron hydrogenase subunit, HydA (fig. 5.11 C) and there was differential expression of *cj1419* and *cj1420* (fig. 5.11 C), which are known to be phase variable (personal communication with Francis Mulholland). The proteins were not selected for further analysis.

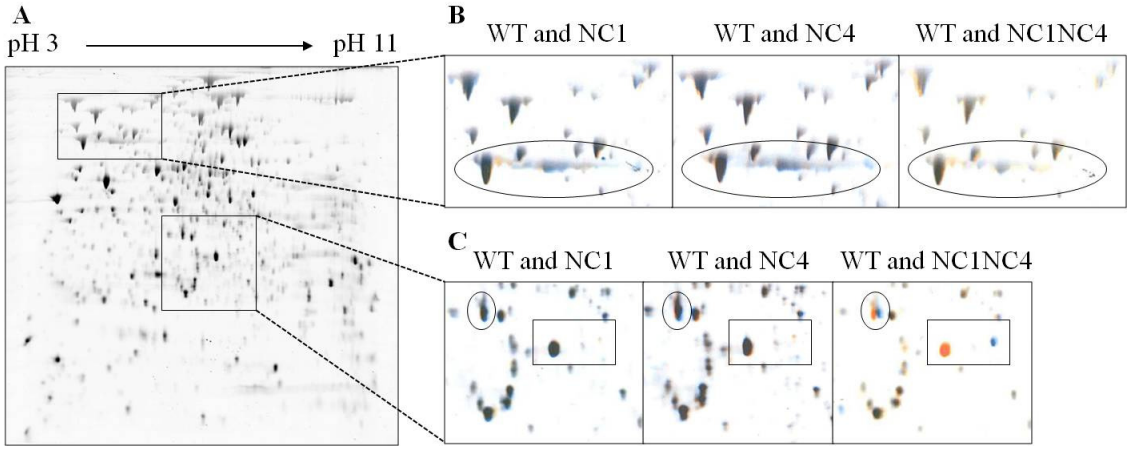


Figure 5.11 Two-dimensional protein profiles of *C. jejuni* NCTC11168 *NC1/NC4* deletion strains are similar to the wild-type.

C. jejuni were grown to mid-log phase and proteins from whole cell lysates were separated by isoelectric point (pH 3-11) and by molecular weight. Proteins were strained with Sypro-Ruby and imaged with a phosphorimager. **A)** An example two-dimensional gel and reference for the expanded images; each spot is a protein (WT strain). **(B)** An expanded region of the gel showing changes in flagellins (circular selection). **(C)** An expanded region of the gel showing a change in a putative oxidioreductase (rectangle selection) and hypothetical, phase variable proteins, Cj1419 and Cj1420. Each panel shows overlaid images from a mutant strain (proteins coloured blue) with the wild-type (proteins coloured orange). The spot appears black, when the protein is expressed in both strains. Images are representative of two biological replicates. Strains: WT - wild-type; NC1 - *NC1* deletion; NC4 - *NC4* deletion; NC1NC4 - double *NC1* and *NC4* deletion.

5.2.11 The *C. jejuni* NCTC11168 NC1 over-expression strain has altered cell surface properties compared with the wild-type strain

C. jejuni strains were analysed by flow cytometry to ascertain whether strains exhibited changes in morphology or whether different populations arose over time and in different conditions that were not detected by previous assays. Strains were analysed during growth in Brucella (rich) or defined media at 37 and 42°C. Forward Scatter (FS, indicates changes in cell size) and Side Scatter (SS, indicated changes in cell granularity or size) were monitored over time.

SS of the double *NC1/NC4* deletion strain and the *NC4* over-expressing strains was similar to that of the WT in both rich and defined media at 37 and 42°C. In defined media at 37 and 42°C, SS of all strains decreased between 12 and 24 hours growth from approximately 1.9 to 1.4, whereas the SS profile of all strains grown in rich media at

37°C remained constant. In rich media at 42°C, SS of the wild-type strain and *NC1/NC4* deletion mutant decreased between 12 and 24 hours growth. In defined media there was an approximately 0.2 decrease in SS up to eight hours growth, whereas there was a 0.1 to 0.3 SS increase in rich media (fig. 5.12).

One notable difference was that the *NC1* over-expression strain had a different SS that was consistently reduced and always below 1.6, compared to the wild-type strain, which was observed at all growth conditions, although all strains showed elevated SS in defined media. Another difference was that the SS of the *NC4* over-expression strain remained high after 24 hours growth in rich media at 42°C, whereas that of the wild-type and other strains were decreased (fig. 5.12). FS of all strains was similar to the wild-type across all conditions over time (data not shown). As the *NC1* over-expression strain differed in SS, but not FS, it may be that NC1 has altered cell surface properties compared to wild-type *C. jejuni*.

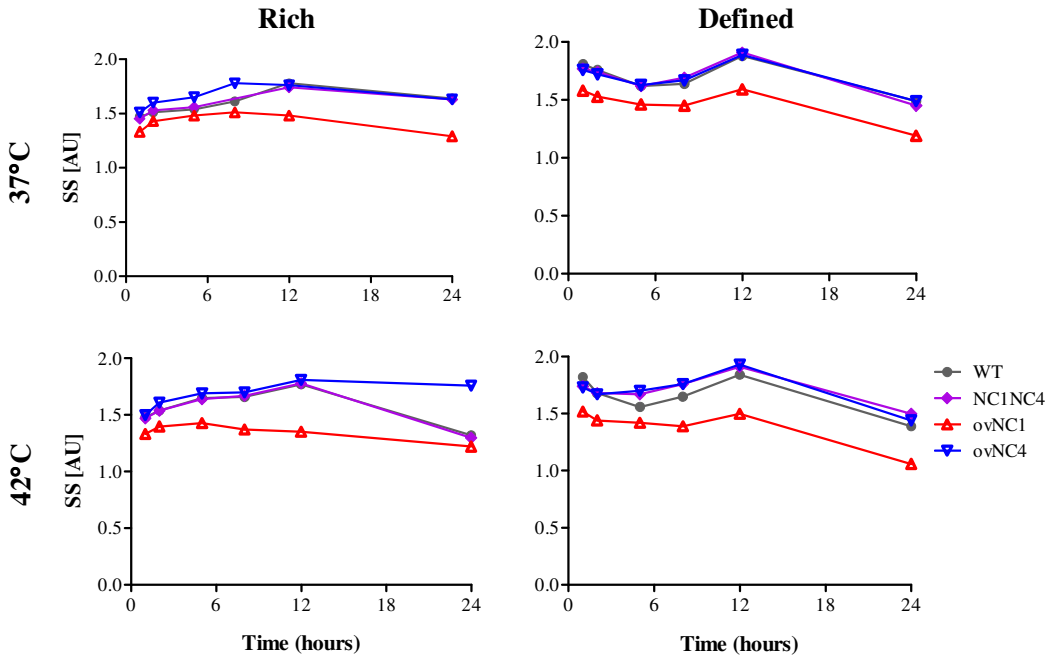


Figure 5.12 *C. jejuni* NCTC11168 *NC1* over-expression strain has a different side scatter profile to that of the wild-type.

C. jejuni strains were analysed by flow cytometry under forward scatter and side scatter in a Brucella (rich) or defined media at 37 and 42°C. Values are given in arbitrary units [AU] and at least 10,000 events were acquired. Strains: WT - wild-type, NC1NC4 - double *NC1* and *NC4* deletion mutant, ovNC1 - *NC1* over-expression mutant, ovNC4 - *NC4* over-expression mutant.

5.2.12 Transcriptomic profile of *C. jejuni NC1/NC4* deletion and over-expression strains are similar to the wild-type strain.

Transcriptomic analysis was performed on total RNA extracted from an *NC4* deletion, a double *NC1/NC4* deletion and an *NC4* over-expression mutant, and compared to the wild-type. Agilent 8 x 15,000 probe microarrays covering 1608 open reading frames were used. Gene expression was considered significantly different if genes were more than two-fold up- or down-regulated and $P < 0.05$ (Holmes *et al.* 2005).

Overall there were up to six genes that had significantly altered expression levels in each strain and two or more of these were genes manipulated as part of the experiment (fig. 5.13, table 5.2). In the *NC1/NC4* double deletion mutant, the pseudogene *cj0565* was down-regulated and a small RNA, which has been annotated as CjNC8, was up-regulated. Down-regulation of *cj0566* was also seen (fig. 5.13 A), but this was not significant. No genes were down-regulated in the *NC4* deletion mutant and only genes *cj1295-cj1298* were up-regulated (fig. 5.13 B). In the *NC4* over-expression mutant, no genes were down-regulated and only *cj1435c* and *cj1436c* were up-regulated (fig. 5.13 C). A summary of all the changes in gene expression are included in table 5.2. Expression of the predicted target genes in the ncRNA mutants was similar to wild-type *C. jejuni* and any minor changes in gene expression compared to the wild-type were not significant (table 5.3).

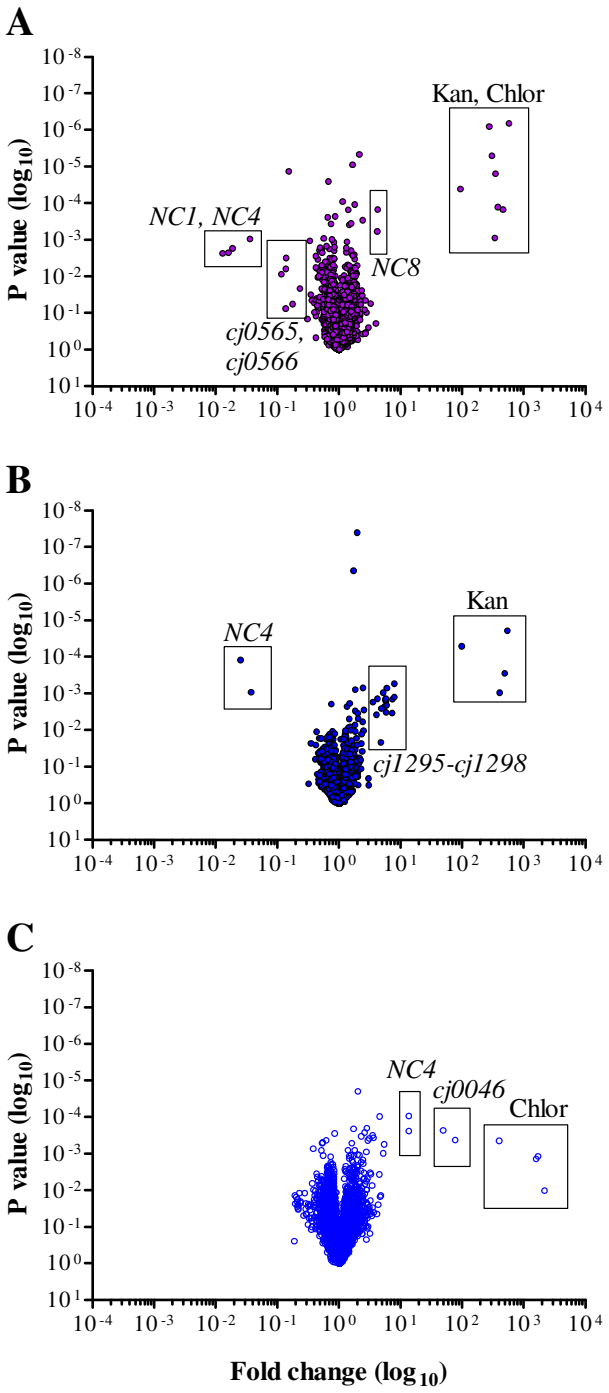


Figure 5.13 *C. jejuni* NCTC11168 *NC1* and *NC4* deletion and over-expression strains show similar gene expression profiles to the wild-type.

Microarrays were performed to compare gene expression of a double *NC1*, *NC4* deletion **(A)**, NC4 deletion **(B)** and NC4 over-expression strain **(C)** to the wild-type. A summary of changes is represented as volcano plots, where the fold change in gene expression of each gene probe (single point) is plotted against the statistical significance of the change (P value). Each gene has more than one probe and results shown are from two biological replicates. Notable changes in gene expression are labelled.

Table 5.2 Summary of all significant changes in gene expression of *C. jejuni* NCTC11168 *NC1* and *NC4* deletion and over-expression strains.

Changes were considered to be significant if there was at least a two-fold change in gene expression and if $P < 0.05$.

NC4		**NC1NC4**		**ovNC4**	
Gene	**Fold change**	**Gene**	**Fold change**	**Gene**	**Fold Change**
NC4	-33.1	*NC1*	-61.1	*NC4*	13.7
KAN	384	*NC4*	-56.6	*CAT*	1470
cj1295	4.97	*cj0565*	-8.18	*cj0046*	62.9
cj1296	6.16	*KAN*	375	*cj1435c*	3.38
cj1297	6.08	*CAT*	388	*cj1436c*	5.07
cj1298	6.25	*NC8*	4.33		

NC4 - NC4 deletion mutant
NC1NC4 - double NC1 and NC4 deletion mutant
ovNC4 -NC4 over-expression mutant
KAN - kanamycin cassette
CAT - chloramphenicol cassette

Table 5.3 Changes in expression of NC1 and NC4 predicted gene targets in *C. jejuni* NCTC11168 *NC1* and *NC4* deletion and over-expression strains.

	NC4		NC1NC4		ovNC4	
Gene	**Fold change**	**P value**	**Fold change**	**P value**	**Fold change**	**P value**
Cj0243c (hypothetical)	-1.45	0.72	-1.32	0.20	1.07	0.73
cj0428 (hypothetical)	-1.09	0.51	-1.33	0.07	1.63	0.06
cj0887c (*flaD*)	-1.26	0.37	1.08	0.59	-1.08	0.24
cj1026c (*flgP*)	-1.08	0.44	1.05	0.45	1.24	0.11
cj1338c (*flaB*)	-1.48	0.30	-1.37	0.50	-1.06	0.14
cj1729c (*flgE2*)	1.01	0.45	-1.09	0.50	1.14	0.36
cj1650 (hypothetical)	-1.05	0.60	1.04	0.70	1.26	0.26

NC4 - *NC4* deletion mutant
NC1NC4 - double *NC1* and *NC4* deletion mutant
ovNC4 - 4 over-expression mutant

5.3 Discussion

To assess whether NC1 and NC4 have an important role in regulating flagellar assembly or function, the ncRNAs were both deleted and over-expressed in *C. jejuni* and these mutated strains were examined using phenotypic assays. Altered expression of NC1 and NC4 was expected to have notable effect on flagellar assembly or function, as it is a tightly regulated process, and so any interference in the regulatory network should be observable. However, the flagella of *C. jejuni* ncRNA mutants were similar to the wild-type both morphologically and in function.

In summary, there was an absence of a clear motility phenotype when the ncRNAs were deleted or over-expressed. *C. jejuni* still possessed flagella and were motile (fig. 5.5 and 6). Functional flagella assays for energy taxis (fig. 5.7), biofilm formation (fig. 5.8), and intestinal epithelial cell invasion assay (fig. 5.9) revealed differences between wild-type and *NC1* and *NC4* mutant strains with invasion of m-IC_{cl2} monolayers only, which were not statistically significant. Protein profiles of cell fractions from *NC1* and *NC4* deletion and over-expression strains were similar after they were grown at either 37 or 42°C (fig. 5.10). Two-dimensional resolution of proteins from *NC1* and *NC4* deletion mutants showed few changes in protein expression that were not consistent between strains (fig. 5.11).

We hypothesised that NC1 and NC4 may function as a negative feedback mechanism for down-regulating protein expression after the flagellum has been assembled. If NC1 and NC4 do act as a negative regulator of excess protein, then it is possible that this would not produce a detectable phenotype. Extracellular proteins would be lost to culture supernatant leaving the flagellum intact. Even though NC1 and NC4 were over-expressed, there may not have been enough ncRNA to down-regulate protein expression to an observable level, especially with one that is highly expressed for multi subunit structures, such as FlgE2. Likewise, it is possible that NC1 and NC4 down-regulation of *flgE2* may be subtle and may not completely switch off allowing the formation of fully formed and functional flagellum, although the purpose of such a process is unclear. Stronger over-expression of NC1 and NC4 may be required to change gene expression to a level that is detectable in phenotype assays. Methods to study secreted flagellar proteins are not currently available as these proteins may precipitate with bacterial cell pellet.

However, a fundamental phenotype that was expected to be affected was motility as *flgP* was predicted to be target of NC1 and NC4. *C. jejuni* flagella are paralysed in *flgP* inactivated mutants (Sommerlad and Hendrixson 2007) and so bacteria are non-motile. *NC1* and *NC4* deletion and over-expression strains were all motile, which suggests that functional FlgP was still expressed. Again, the ncRNA over-expression may not have produced enough RNA to down-regulate all protein expression in *C. jejuni*. The stoichiometry of FlgP to each flagellum is unknown and it may be that very low levels of protein are associated with each flagellum.

It is possible that the functions of the some target flagellar proteins are not essential for flagellar function and so would not be detected in phenotype assays. For example, FlaB is not essential for motility and the flagellum is still extended with FlaA subunits (Wassenaar *et al.* 1993). Ideally, the composition of flagellins should be elucidated in the *NC1* and *NC4* mutants, but this is difficult as they are extracellular proteins and protein sequences are highly similar. The hypothetical gene targets have yet to be attributed to function.

The *cj0428* deficient strain again has no motility phenotype (data not shown), although this gene is regulated with *flhA*, which is regulated with flagella (Carrillo *et al.* 2004). Recent studies found that *cj0428* was up-regulated in *C. jejuni* that cause disease in germ free mice (Bell *et al.* 2013) and was up-regulated in iron limited conditions (Holmes *et al.* 2005). Therefore, if *cj0428* expression is affected in NC1 and NC4 mutants, then a phenotype might be demonstrable in colonisation studies. However, an *in vitro* study demonstrated that *cj0428* was not important for adhesion to INT407 cells (John 2012), so if mutating *cj0428* has no observable phenotype *in vitro*, then NC1 and NC4 regulation would not be detectable either.

The functions of the other hypothetical genes *cj0243c* and *cj1650* remain unknown. The protein encoded by *cj1650* is 27% identical to *H. pylori* HP1076, which is a protein chaperone for a flagellar export chaperone FliS (Lam *et al.* 2010). In *C. jejuni*, it is co-regulated with *flgR* and *flgS* and *rpoN* and so is thought to be secreted (Guerry-Kopecko and Baqar 2008). However, there is a contradictory report of both the up- and down-regulation of *cj1650* in an *rpoN* (σ^{54}) mutant (Chaudhuri *et al.* 2011).

It was thought that cell invasion would be abrogated if altered ncRNA expression disrupted flagellar assembly or flagella function, due to a change regulation of the target genes. There were minimal differences observed in invasion of intestinal epithelial cells by the *NC1* and *NC4* deletion and over-expression strains. The deletion mutants were more able to invade a mouse small intestinal cell line and the NC1 over-expresser was less able to invade this cell line. However, these differences were not statistically significant across three independent experiments, and so more experiments should be performed. Furthermore, colonisation studies may reveal a role for ncRNAs *in vivo*. The environment inside the host is very difficult to replicate, especially the nutrient availability, and it may be that NC1 and NC4 are required in a niche where growth conditions and host factors impact upon bacterial survival.

As phenotypic assays of NC1 and NC4 mutants did not reveal any remarkable differences in comparison with wild-type, experiments were done to ascertain whether NC1 and NC4 were regulating gene expression at the transcription level. Many ncRNAs function by degrading mRNA of target genes and this can be detected by transcriptome analysis. Microarrays performed with *C. jejuni NC4* deletion, *NC4* over-expression and the double *NC1/NC4* deletion mutant revealed very similar transcription profiles when grown to mid-log growth phase (table 5.3). Moreover, there were no significant changes in predicted target transcript levels (table 5.4). One conclusion may be that NC1 and NC4 do not function by degrading mRNA. However, expression of ncRNAs may be transient and may be dependent on specific growth conditions and so it is possible that transcripts collected from a single time-point, in a single growth condition would miss changes in RNA levels. Furthermore, the undoubted heterogeneity of a sampled population may have masked changes in transcript levels. Measuring RNA levels of homogeneous populations at various growth phases or conditions would increase the power of this investigation.

Motility in other prokaryotes is indeed regulated by ncRNAs and complex ncRNA networks have been shown to regulate motility in *E. coli*. Several ncRNAs have been shown to interact with the 5'UTR of *flhDC* mRNA, which encodes the master regulator of flagellar assembly (De Lay and Gottesman 2012). These ncRNAs can either be positive or negative regulators of flagellar assembly and they can also undergo positive or negative regulation in response to the environment. Indirect action through other

regulators, such as the ArcB/A two-component system (De Lay and Gottesman 2012) or CsrA post-transcriptional protein regulator (Lucchetti-Miganeh *et al.* 2008), adds further complexity to the network.

Epsilon-Proteobacteria do not have master regulators of flagellar assembly and so have fundamental differences in the mechanisms that initiate flagellar assembly (Smith and Hoover 2009). This means that any contributing ncRNA networks will also differ considerably. Other evidence for ncRNA regulation of motility is demonstrated in Hfq mutants. Hfq is a protein chaperone that stabilises *trans*-encoded ncRNA-mRNA complexes in many bacteria. For example, a *Salmonella* Typhimurium *hfq* mutant is non-motile due to reduced flagellin synthesis (Sittka *et al.* 2008) and in the Cyanobacterium *Synechocystis sp.* PCC 6803, a *hfq* mutant loses type IV pili and twitching motility (Dasgupta *et al.* 2003). The Epsilon-Proteobacteria, however, lack Hfq and so novel RNA-RNA or RNA-protein interactions may be involved.

Because Epsilon-Proteobacteria have fundamental differences in their biology, ncRNA predictions and functions may not always be applicable when inferred from Gamma-Proteobacteria. Our understanding of ncRNAs outside the Enterobacteriaceae remains in its infancy, but as more studies regarding ncRNA phenotypes and lack of phenotypes are published, a clearer picture of the importance of ncRNAs in all prokaryotes can be formed.

In future experiments, it would be interesting to examine the motility phenotypes in a range of different conditions *in vitro*, but this could be time consuming and may not be relevant to *in vivo* conditions. Culture condition has been found to be important for the expression of certain ncRNAs. For example, RhyB, an ncRNA in *E. coli*, is highly expressed under iron-limited conditions, which then down-regulates expression of succinate dehydrogenase gene expression (Masse and Gottesman 2002). However, in rich broth culture very low, if any, RhyB is expressed and its target genes are expressed in its absence (Masse and Gottesman 2002). Therefore, changing culture conditions can reveal ncRNA functions, but finding the correct condition may be difficult without prior knowledge. It may be that NC1 and NC4 are necessary for survival in and infection of hosts, so colonisation of animal models by the *NC1/NC4* deletion and over-expression mutants should be evaluated. If NC1 and NC4 are essential for host colonisation, then it

would still be important to know what the ncRNAs are responding to in this environment to understand what is regulating flagella.

In addition, during phenotype assays population differences are not taken into account. Single cell analysis assays may be useful in detecting different population groups within a culture. Using flow cytometry to analyse growth of *C. jejuni* NC1 and NC4 mutants in media and temperature over time revealed that all strains had a similar growth rate to the wild-type and had similar side scatter (SS) and forward scatter measurements over time. However, the NC1 over-expresser had a consistently lower SS than the wild-type, even though the changes in SS followed the same pattern as that of the wild-type (fig. 5.12). A lower SS may mean a difference in cell surface properties or size. Further imaging studies are required to investigate the morphological and cell surface properties of the NC1 over-expression strain in more detail.

In the next chapter, the interaction between NC1 and NC4 and their predicted targets were investigated to further ascertain their function at the molecular level.

Chapter 6 NC1 and NC4 function by post-transcriptionally regulating expression of flagellar genes

Collaborative work:

Work in figures 6.6 and 6.7 was largely completed by Mart van Veldhuizen under the supervision of My Thanh Le.

6.1 Introduction

In the previous chapter, it was shown that the altered expression of ncRNAs NC1 and NC4 did not result in a detectable phenotype in *C. jejuni* and that mRNA degradation may not be the mechanism of NC1 and NC4 function. An alternative ncRNA control mechanism common in prokaryotes is translation inhibition by the blocking of ribosome binding.

The aim of this study was to use an established system for measuring translational control of gene expression developed in *E. coli* to assess translational control of *C. jejuni* flagella genes by NC1 and NC4 (fig. 6.1) (Urban and Vogel 2007). Transcriptional fusions of ncRNA target genes to a reporter gene expressed on a plasmid were used to investigate whether NC1 and NC4 regulate gene expression by controlling translation. Green fluorescent protein (GFP) was used as the reporter of translation, which can be measured directly and easily *in vivo* with increased fluorescence being directly proportional to protein expression; hence measuring GFP fluorescence would be a direct measure of translation. Another plasmid is used to express an ncRNA so that combinations of plasmids containing *gfp* transcriptional fusions and ncRNAs can be expressed in *E. coli* and fluorescence can be measured (Top10, Invitrogen). The *gfp* transcriptional fusions and the ncRNA are constitutively expressed so that changes in translation can be measured.

Another advantage of using this system is that a *C. jejuni* ncRNA and one specific target can be studied in isolation of other targets and expression of the ncRNA is high, whilst that of target is low, increasing the opportunity to detect the any regulation. Moreover, genetic tools for *E. coli* are readily available and the ability to replicate plasmids gives greater flexibility for genetic manipulations over *C. jejuni*, for which plasmids are difficult to use and where polar effects of chromosomal insertions may be a problem.

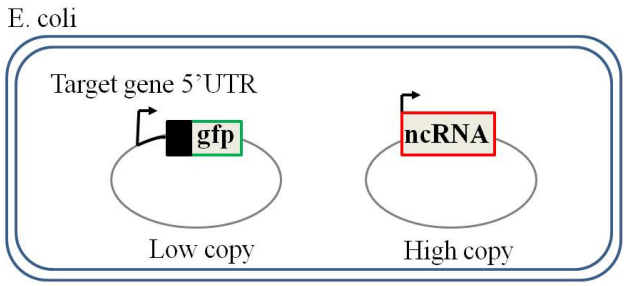


Figure 6.1 Translational control method using *E. coli*.

Schematic of experimental method: the 5' untranslated region (UTR) of predicted target genes was fused to *gfp* under the control of a constitutive promoter in a low copy plasmid. NC1, NC4 and a non-sense RNA were also constitutively expressed in a high copy plasmid. The target gene-*gfp* fusion plasmids were then combined with the NC1 or NC4 expressing plasmids in *E. coli* and fluorescence was measured by flow cytometry or spectroscopy.

The objectives were to:

- Fuse the 5'UTRs of predicted flagellar target genes and at least one non-flagellar gene to *gfp* under the control of a constitutive promoter in a low copy plasmid expressed in *E. coli* and measure fluorescence by flow cytometry or fluorescence plate reader;
- Express NC1, NC4 and a non-sense RNA in a high copy plasmid expressed in *E. coli*;
- Co-transform *E. coli* with a combination of one *gfp* fusion plasmid and one ncRNA expression plasmid and measure fluorescence by flow cytometry or using a fluorescence plate reader.
- Ascertain if regulation by NC1 and NC4 was specific and was dependent on complementary base-pairing by mutating the ncRNAs to disrupt regulation and subsequently compensating the mutations in *E. coli*;
- Establish whether *E. coli* Hfq affects NC1 and NC4 regulation of target genes by conducting translational control assays in *hfq* mutants;
- Assess translational control in *C. jejuni* by expressing the *gfp* transcriptional fusions in the chromosomes of *C. jejuni NC1* and *NC4* deletion and over-expression mutants, and measure fluorescence by flow cytometry or fluorescence plate reader.

As NC1 and NC4 were predicted to be trans-acting ncRNAs (Chapter 4), it was expected that the ncRNAs would inhibit translation of the predicted target genes as this is their typical mechanism of action (Papenfort and Vogel 2010). Therefore, *target-gfp* expression would be down-regulated, resulting in reduced expression of GFP-protein fusions.

6.2 Results

6.2.1 Regulation of *cj0428-gfp* by NC1 and NC4 can be evaluated in *E. coli*.

(i) NC1 and NC4 repress fluorescence of cj0428-gfp *in* E. coli

Initial experiments were performed with a transcriptional fusion of the hypothetical gene *cj0428* to *gfp*, which was expressed in *E. coli*. This was the first target gene that was successfully fused to *gfp* and so was used to test the *E. coli* system. *E. coli* containing the *cj0428-gfp* fusion did fluoresce and fluorescence was 10-fold greater than GFP negative *E. coli* (fig. 6.2). When a non-sense RNA was expressed with the fusion plasmid, *E. coli* fluorescence was of a similar level to the fusion expressed alone, but expression of both NC1 and NC4 with the fusion plasmid repressed fluorescence (fig. 6.2). Repression by NC1 was less than that of NC4. With each assay, *E. coli* positive for GFP and negative for GFP expression was included as a control.

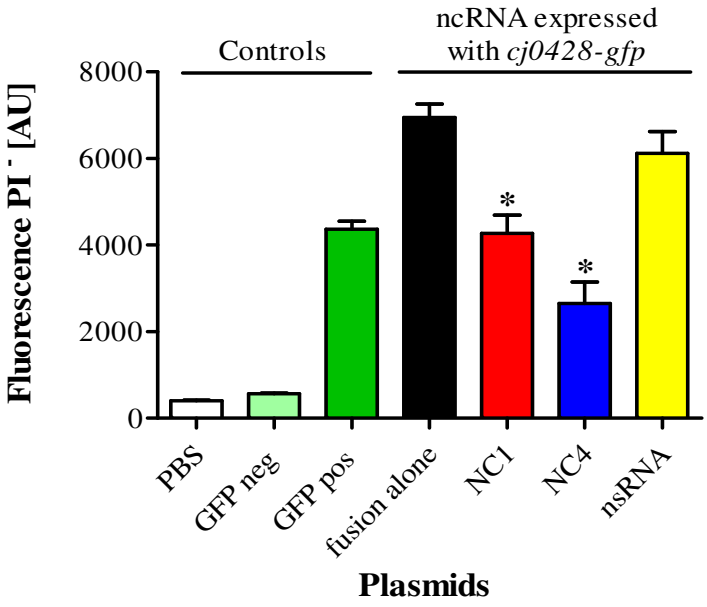


Figure 6.2 NC1 and NC4 repress fluorescence of *C. jejuni* NCTC11168 *cj0428-gfp* in *E. coli*.

A plasmid containing the transcriptional fusion of the *C. jejuni cj0428* 5' untranslated region to *gfp* was constructed. This plasmid was used to transform *E. coli*, along with different combinations of plasmids expressing NC1, NC4 or a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h and flow cytometry was used to measure fluorescence of live bacteria that excluded propidium iodide (PI^-). Error bars represent standard error of the mean of three independent experiments. Results are represented as fluorescence intensities in arbitrary units [AU]. Asterisks represent $P < 0.05$ relative to the fusion alone (One-way ANOVA). Fluorescence of standard controls is also shown: a phosphate buffered saline blank (PBS); *gfp* negative *E. coli* containing a plasmid with *gfp* without an ATG start codon (pXG-10), and *gfp* positive *E. coli* containing a plasmid which expresses *gfp* (pXG-1).

(ii) Fluorescence of cj0428-gfp *requires inclusion of the cj0428 coding region*

Urban and Vogel recommended including 10-30 aa of the target gene into the *gfp* fusion construct to aid GFP folding and, therefore, fluorescence (Urban and Vogel 2007)). To test whether GFP fluorescence is dependent on the length of protein included in the transcriptional fusion, *cj0428-gfp* fusions containing different lengths of protein coding region were constructed (there are 128 codons in the *cj0428* gene). The *cj0428-gfp* fusion that included 5, 12 (construct used for initial experiments) and 20 codons of the *cj0428* coding region were fluorescent and were regulated by NC1 and NC4 (fig. 6.3). Repression of *gfp* expression was similar across *E. coli* with the different constructs

also. Both NC1 and NC4 repressed fluorescence, but NC1 consistently gave less repression than NC4 (fig. 6.3). This was similar to observations from the initial experiment, where NC1 and NC4 decreased fluorescence to different degrees (fig. 6.3). Omission of any *cj0428* coding region, except a start codon required for GFP production, resulted in constructs that gave low fluorescence (fig. 6.3), indicating that *cj0428-gfp* fusions required additional amino acids for a fully functional protein.

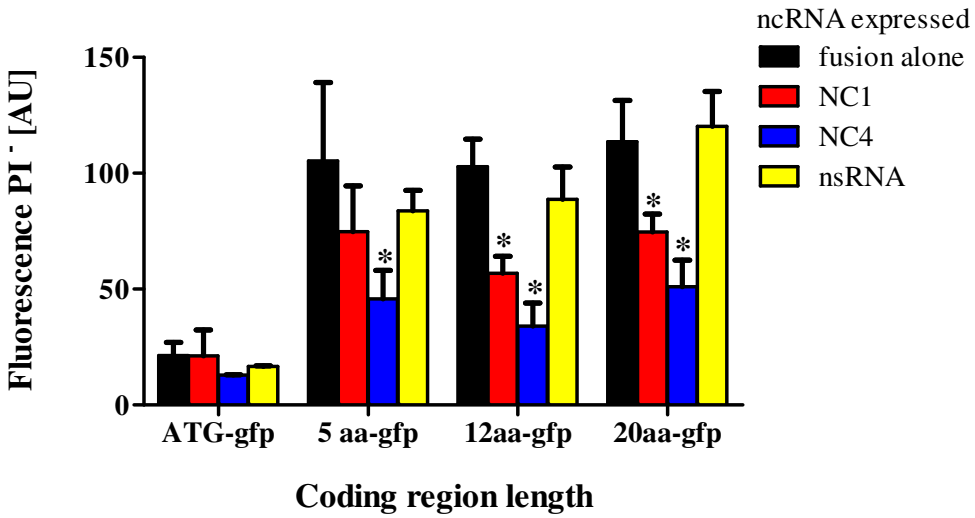


Figure 6.3 NC1 and NC4 repress fluorescence of *C. jejuni* NCTC11168 *cj0428-gfp* fusions that include different lengths of c*j0428*.

Transcriptional *C. jejuni cj0428-gfp* fusions including the start codon only (ATG-*gfp*), 5 codons (5aa-*gfp*), 12 codons (12aa-*gfp*) and 20 codons (20aa-*gfp*) were made. These were used to transform *E. coli*, along with different combinations of plasmids expressing NC1, NC4 or a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h in broth cultures and flow cytometry was used to measure fluorescence of live bacteria that excluded propidium iodide (PI^-). Results of two biological replicates are shown and represented as fluorescence intensities in arbitrary units [AU]. Asterisks represent $P < 0.05$ relative to the fusion alone (One-way ANOVA).

6.2.2 NC1 and NC4 repress fluorescence of *cj0428-gfp*, *flgP-gfp*, *flgE2-gfp* and *cj1650-gfp* in *E. coli*.

NC1 and NC4 translational control of other predicted targets was studied using the system in *E. coli* as described above. Six *target-gfp* fusions constructed including *cj0428-gfp*, *lysC-gfp* (*cj0582*), *flgP-gfp* (*cj1026c*), *flaB-gfp* (*cj1338c*), *flgE2-gfp* (*cj1729c*), *cj1650-gfp*. The *cj0428-gfp* fusion was the most fluorescent in *E. coli* and fluorescence was repressed by NC1 and NC4 to the greatest degree (fig. 6.4 A and B). NC4 repressed fluorescence of *cj0428-gfp* by 2.6 fold and NC1 repressed fluorescence by 2.3 fold ($P < 0.05$, fig. 6.4 B). Introduction of a non-sense RNA did not significantly alter fluorescence of *E. coli* containing *cj0428-gfp*. Both NC1 and NC4 significantly repressed fluorescence in *E. coli* containing *flgE2-gfp*, both giving ~1.5 fold repression ($P < 0.05$, fig. 6.2.3).

Both NC1 and NC4 also reduced *gfp* expression of another *target-gfp* fusion of a hypothetical gene, *cj1650-gfp*. NC1 and NC4 repressed fluorescence in *E. coli* containing *cj1650-gfp* by 1.3 and 3.3 fold, respectively, but only repression by NC4 was significant ($P < 0.05$, fig. 6.4). Fluorescence in *E. coli* containing *flgP-gfp* was repressed by both NC1 and NC4 by 1.2 and 1.4 fold respectively, but only NC4 repression was significant ($P < 0.05$, fig. 6.4).

E. coli the containing *flaB-gfp* fusion did not fluoresce (not shown) and so regulation by NC1 and NC4 could not be evaluated. GFP was also undetectable by Western blot suggesting that the protein was not expressed (data not shown).

LysC was a predicted target for NC1 and NC4 control (table 4.1), but is a σ^{70}-dependent gene, whereas the other predicted targets were σ^{54}-dependent genes. The *lysC-gfp* fusion did fluoresce and NC1 and NC4 did not alter fluorescence of the *lysC-gfp* fusion (figure 6.4 C), which means that NC1 and NC4 are likely to control σ^{54} dependent genes only.

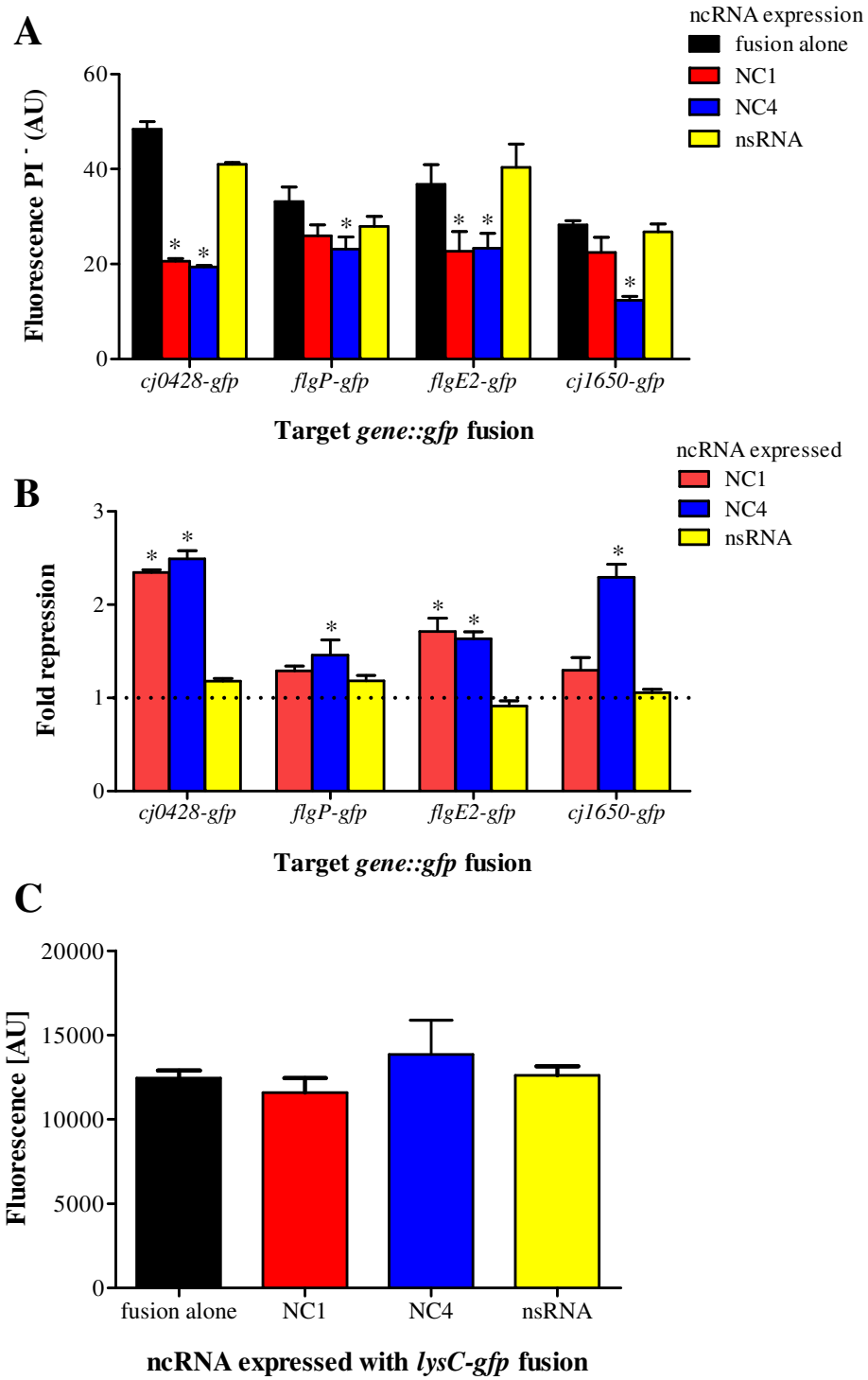

A
ncRNA expression
fusion alone
NC1
NC4
nsRNA
Fluorescence PI - (AU)
60
40
20
0
cj0428-gfp
flgP-gfp
flgE2-gfp
cj1650-gfp
Target gene::gfp fusion
B
ncRNA expressed
NC1
NC4
nsRNA
Fold repression
3
2
1
0
cj0428-gfp
flgP-gfp
flgE2-gfp
cj1650-gfp
Target gene::gfp fusion
C
Fluorescence [AU]
20000
15000
10000
5000
0
fusion alone
NC1
NC4
nsRNA
ncRNA expressed with lysC-gfp fusion

Figure 6.4 NC1 and NC4 repress fluorescence of *C. jejuni* NCTC11168 gene targets in the *E. coli* reporter system.

Transcriptional fusions of *C. jejuni* flagella gene 5' UTRs to *gfp* (labelled as *gene-gfp*) were constructed on plasmids in *E. coli*, including *cj0428*, *cj1026c* (*flgP*), *cj1729c* (*flgE2*) and *cj1650*, and a non-flagellar target *cj0582* (*lysC*). These were used to transform *E. coli*, along with different combinations of plasmids expressing NC1, NC4 or a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h and flow cytometry was used to measure fluorescence of live bacteria that excluded propidium iodide (PI^-). **A)** Results represent fluorescence intensities in arbitrary units [AU]. **B)** Results represent fold repression of fluorescence compared to *E. coli* expressing the fusion alone (fold repression of *E. coli* containing the fusion alone is one and shown by the dotted line). **C)** NC1 and NC4 do not control fluorescence in *E. coli* containing *lysC-gfp*. Bacteria containing the appropriate plasmids were grown 16 h and a fluorescence plate reader was used to measure fluorescence intensities in AU. Error bars represent standard error of the mean of three biological replicates. Asterisks represent $P < 0.05$ relative to the fusion alone (One-way ANOVA).

6.2.3 NC1 and NC4 specifically repress fluorescence of flagellar *gene-gfp*.

To verify that NC1 and NC4 regulation of their targets is due to specific base pairing to the target 5' UTR, a region of the NC1 and NC4 binding nucleotides was mutated and fluorescence was evaluated using the *E. coli* system. Specific mutations of NC1 and NC4 were made for three target genes *cj0428*, *flgE2* and *cj1650*. Subsequently, the mutations were rescued by making the compensatory mutation in the 5' UTR. The ribosome binding site was excluded from mutations to allow translation of *gene-gfp* fusion. Diagrams of mutations made are shown in figure 6.5.

Mutated NC1 and NC4 were no longer able to repress fluorescence of *cj0428-gfp*, *flgP-gfp* or *cj1650-gfp*, whereas wild-type NC1 and NC4 were able to repress fluorescence, as was observed in earlier experiments. Regulation of *cj0428-gfp* fluorescence by wild-type NC1 was variable in these experiments, but regulation by NC4 was similar to the previous experiment (fig. 6.4). Both wild-type NC1 and NC4 repressed fluorescence of *E. coli* containing *flgE2-gfp* by ~1.8-fold (fig 6.6 B), which was increased compared to the previous experiment (fig. 6.4 B). Regulation of *cj1650-gfp* fluorescence by wild-type NC1 and NC4 (fig 6.6 C) was similar to the previous experiment (fig. 6.4).

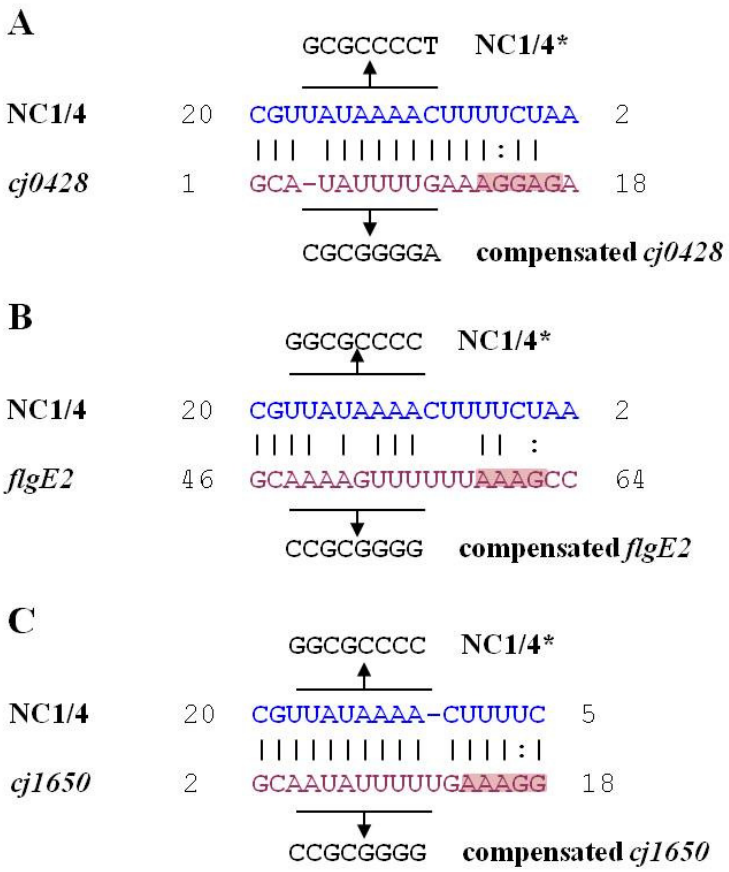


Figure 6.5 Diagram of mutations made in *NC1/NC4* to disrupt regulation and of mutations made in target gene 5' untranslated target regions to restore regulation.

Each panel shows the predicted binding region of *C. jejuni* NCTC11168 NC1/NC4 non-coding RNAs (top strand, **blue**) to their target gene mRNA (bottom strand, **purple**): A) *cj0428*, B) *flgE2* and C) *cj1650*. The ribosome binding site is shaded. The mutations made in the plasmids used for previous translational control experiments are shown above the top strand for those made in NC1 and NC4 and below the bottom strand for those made in the mRNA. The names of the mutations are indicated for reference to subsequent figures. To locate position of the mutations, nucleotide numbers (from +1 transcript) is given. Where base pairs are complementary is indicated by | and possible partial binding is indicated by **:** .

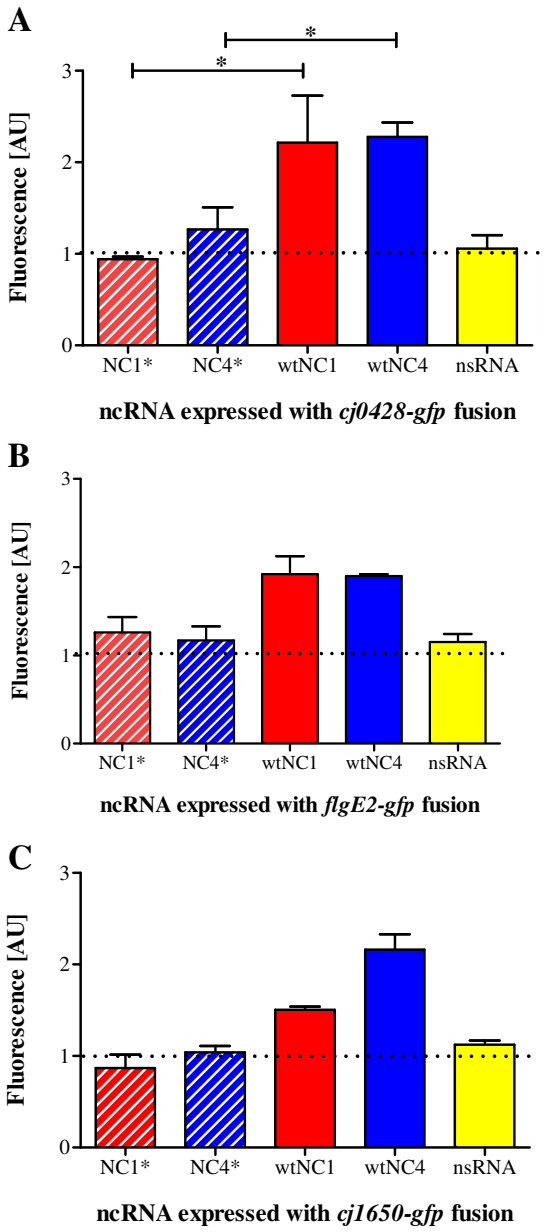


Figure 6.6 Mutating NC1 and NC4 sequences disrupts regulation of *C. jejuni* NCTC11168 flagellar *gene-gfp* fusions in *E. coli*.

Transcriptional fusions of *C. jejuni cj0428* 5' UTR, *flgE2* 5' UTR, or *cj1650* 5' UTR (**A**, **B**, and **C** respectively) to *gfp* were constructed in plasmids. These were used to transform *E. coli*, along with different combinations of plasmids expressing mutated (*) or wild-type (wt) NC1, NC4 and a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h in broth cultures and flow cytometry was used to measure fluorescence of live bacteria that excluded propidium iodide. Results represent fold repression of fluorescence compared to *E. coli* expressing the fusion alone (fold repression of *E. coli* containing the fusion alone is one and shown by the dotted line). Error bars represent standard error of the mean of three biological replicates. Asterisks above a bar represent $P < 0.05$ between the indicated results.

The compensatory mutations were then made in the target 5' UTR, which were expected to restore repression by the ncRNAs if NC1 and NC4 specifically regulate their targets. Compensatory mutations in *cj0428-gfp* restored regulation by the mutated NC1 and NC4 ncRNAs. In fact, levels of repression were higher than previously seen, with mutated NC1 giving approximately four-fold repression and mutated NC4 giving up to nine-fold repression (fig. 6.7 A). Wild-type NC4 still repressed fluorescence of compensated *cj0428-gfp* to a similar degree as with wild-type *cj0428-gfp* in the previous experiment (fig. 6.6 A), which was unexpected as the RNAs should not interact with each other. However, it is clear that the mutated NC1 and NC4 ncRNAs were more able to repress fluorescence of the compensated *cj0428-gfp* (fig. 6.7 A). Compensatory mutations in *flgE2-gfp* restored repression to 1.4-fold repression by mutated NC1, and to 1.7-fold by mutated NC4 (fig. 6.7 B), which is similar to the level seen in the previous experiment (fig. 6.6 B). As expected, wild-type NC1 and NC4 ncRNAs did not repress fluorescence of the compensated *flgE2-gfp* fusion (fig. 6.7 B).

Co-transforming *E. coli* plasmids with compensated *cj1650-gfp* and mutated or wild-type ncRNA plasmids did not demonstrate altered levels of fluorescence compared to *E. coli* with the vector alone (fig. 6.7 C, fig. 6.8), meaning that restoration of regulation could not be fully evaluated. Furthermore, fluorescence of all *E. coli* cultures containing compensated 5'UTR-*gfp* fusion vectors was reduced compared to fusion with the wild-type 5' UTR, which was most notable when comparing *cj1650-gfp* to compensated *cj1650-gfp* (fig. 6.8).

In summary, mutation of NC1 and NC4 disrupted fluorescence regulation of the *gene-gfp* fusions and this was, at least, partially restored by compensatory mutations in the mRNA 5' UTR. This means that NC1 and NC4 are post-transcriptional regulators of gene expression in this experimental system.

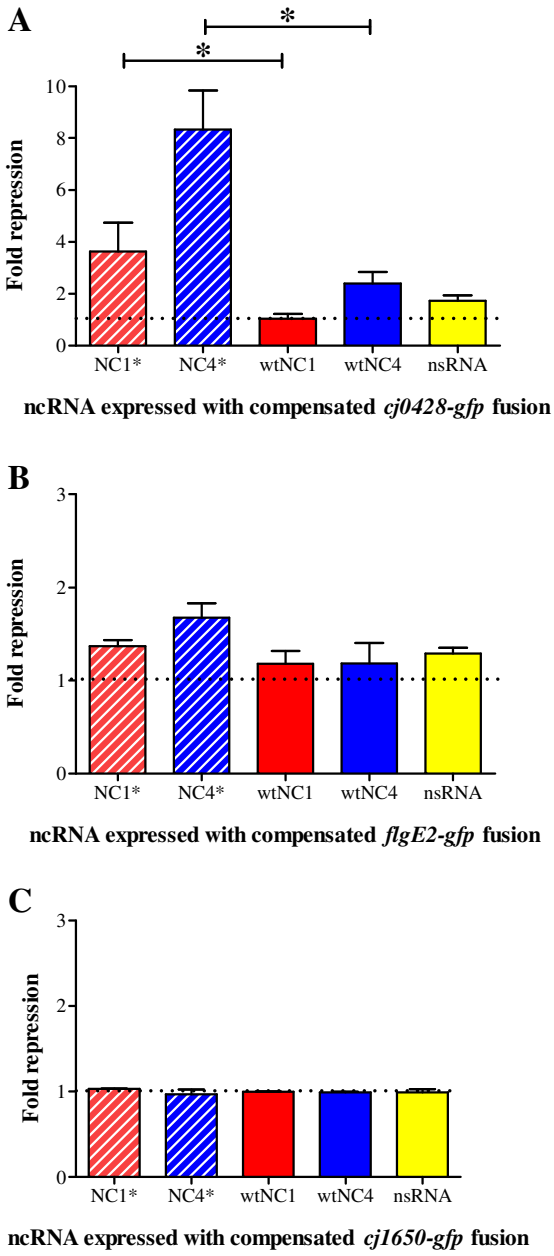


Figure 6.7 Compensatory mutations in *C. jejuni* NCTC11168 flagellar *gene-gfp* fusions restores regulation by mutated NC1 and NC4 in *E. coli*.

Compensated mutations in *cj0428-gfp*, *flgE2-gfp*, or *cj1650-gfp* (**A**, **B**, and **C** respectively) were constructed in plasmids used to transform *E. coli*, along with different combinations of plasmids expressing mutated (*) or wild-type (wt) NC1, NC4 and a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h in broth cultures and flow cytometry was used to measure fluorescence of live bacteria that excluded propidium iodide. Results are represented as fold repression of fluorescence compared to *E. coli* expressing the fusion alone (fold repression of *E. coli* containing the fusion alone is one and shown by the dotted line). Error bars represent standard error of the mean of three biological replicates. Asterisks above a bar represent $P < 0.05$ between the indicated results.

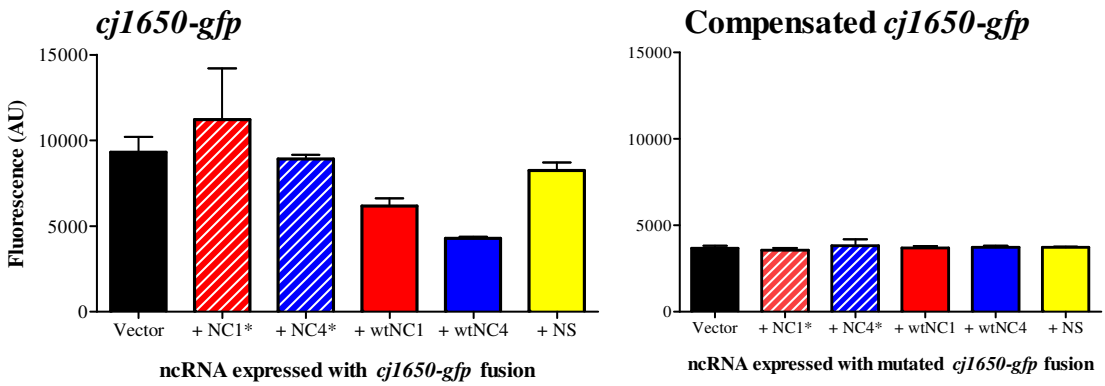


Figure 6.8 Fluorescence regulation of *cj1650-gfp* and compensated *cj1650-gfp* by wild-type mutated NC1 and NC4 in *E. coli*.

A transcriptional fusion of *C. jejuni cj1650* 5' UTR to *gfp* (*cj1650-gfp*) was constructed in a plasmids. These were used to transform *E. coli*, along with different combinations of plasmids expressing mutated (*) or wild-type (wt) NC1, NC4 and a non-sense RNA (nsRNA). A compensatory mutation to the mutated ncRNAs in the *cj1650-gfp* fusion was made (compensated *cj1650-gfp*) and combined with the ncRNA expression plasmids. Bacteria containing the appropriate plasmids were grown 16 h in broth cultures and a fluorescence plate reader was used to measure fluorescence. Fluorescence intensity is in arbitrary units [AU]. Error bars represent standard error of the mean of three biological replicates.

6.2.4 NC1 and NC4 do not require Hfq for regulation in *E. coli*

As *C. jejuni* does not express an Hfq-like, RNA-RNA chaperone protein, we performed translational control experiments with one target, *cj0428-gfp*, in an *E. coli hfq* inactivated mutant. This would assess whether Hfq, present in *E. coli*, may have artificially allowed NC1 and NC4 interaction with target mRNA in our *E. coli* system. An *E. coli hfq* mutant and the parental *E. coli* strain with the wild-type *hfq* were obtained from the Keio collection (Yale, USA). NC1 and NC4 both repressed fluorescence of *hfq* inactivated *E. coli* expressing *cj0428-gfp* to a similar degree as *E. coli cj0428-gfp* containing wild-type *hfq* (fig. 6.9). In addition, regulation was similar to that seen in previous experiments with *E. coli* Top10, but those inactivated in *hfq* were impaired in growth (data not shown) and this may have impacted on the lower fluorescence levels observed (fig. 6.9).

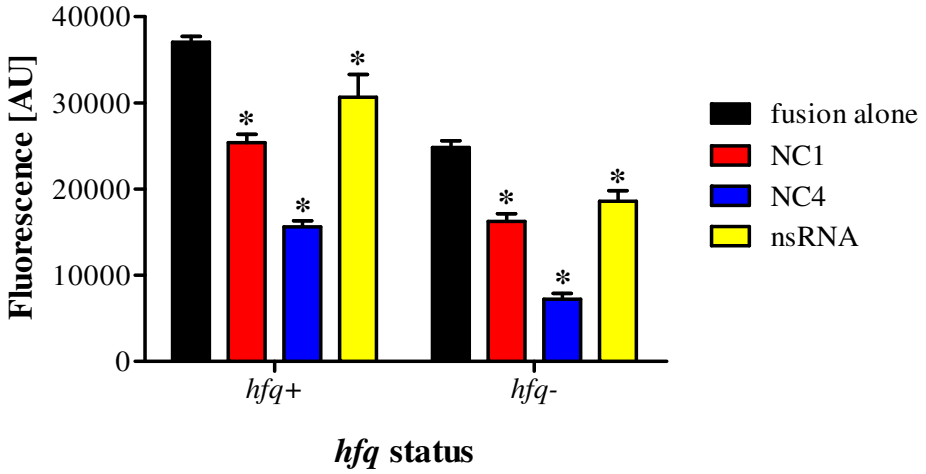


Figure 6.9 NC1 and NC4 repress fluorescence of *cj0428-gfp* in *hfq* inactivated *E. coli*.

An *hfq* deficient *E. coli* (*hfq*-) and the parental stain with wild-type *hfq* (*hfq*+) were co-transformed with a plasmid containing *cj0428-gfp* and a non-coding RNA containing plasmid, either NC1, NC4 or a non-sense RNA (nsRNA). Bacteria containing the appropriate plasmids were grown 16 h in broth cultures and a fluorescence plate reader was used to measure fluorescence in triplicate. Results are represented as fluorescence intensities in arbitrary units [AU]. Error bars represent standard error of the mean of three biological replicates. Asterisk represents $P < 0.05$ relative to the fusion alone (Two-way ANOVA).

6.2.5 Regulation of *cj0428-gfp* by NC1 or NC4 cannot be demonstrated in *C. jejuni*.

A transcriptional *cj0428-gfp* fusion was made and cloned into the *C. jejuni* chromosome using the pC46 suicide plasmids as described in Chapter 4. The *cj0428-gfp* fusion was used because it gave high fluorescence levels and was clearly regulated in *E. coli*. Two different promoters were used to control expression levels of *cj0428-gfp* fusion, as the levels of fluorescence obtained in *C. jejuni* were not known. Thus, the *cj0428-gfp* construct was fused to the weak expression *metK* promoter (P*metK-cj0428-gfp*) and to the moderate expression *fdxA* promoter (P*fdxA-cj0428-gfp*) in the suicide plasmids. These were then used to transform wild-type *C. jejuni* and single NC1 and NC4 deletion *C. jejuni* strains. The promoter-*gene-gfp* constructs were homologously recombined into *cj0046* or *cj0223*. A list of the *C. jejuni* strains made is given in table 6.1.

Table 6.1 *C. jejuni* NCTC11168 strains used in *cj0428-gfp* translational control experiments.

Notation	Genotype	Description
WT	-	Wild-type *C. jejuni*
WT NC PmetK46	*cj0046*::P*metK*::*cj0428*::*gfp*::chlor	*C. jejuni* with no ncRNA mutations and low *cj0428*::*gfp* expression
NC1 PmetK46	*NC1*::kan, *cj0046*::P*metK*::*cj0428*::*gfp*::chlor	*C. jejuni* with NC1 deletion and low *cj0428*::*gfp* expression
NC4 PmetK46	*NC4*::kan, *cj0046*::P*metK*::*cj0428*::*gfp*::chlor	*C. jejuni* with NC4 deletion and low *cj0428*::*gfp* expression
WT NC PfdxA46	*cj0046*::P*fdxA*::*cj0428*::*gfp*::chlor	*C. jejuni* with no ncRNA mutations and moderate *cj0428*::*gfp* expression
NC4 PfdxA46	*NC4*::kan, *cj0046*::P*fdxA*::*cj0428*::*gfp*::chlor	*C. jejuni* with NC4 deletion and moderate *cj0428*::*gfp* expression
WT NC PfdxA223	*cj0223*::P*fdxA*::*cj0428*::*gfp*::kan	*C. jejuni* with no ncRNA mutations and moderate *cj0428*::*gfp* expression
ovNC4 PfdxA223	*cj0046*::P*fdxA*::NC4::cat, *cj0223*::P*fdxA*::*cj0428*::*gfp*::kan	*C. jejuni* with NC4 over-expression and moderate *cj0428*::*gfp* expression

Kan - kanamycin cassette
Chlor - chloramphenicol cassette
P*metK* - promoter of the *metK* gene, low expression
P*fdx*A - promoter of *fdx*A gene, moderate expression
cj0046 and *cj0223*- pseudogenes used for chromosomal insertions.

The P*metK-cj0428-gfp* fusion did fluoresce in *C. jejuni* with no ncRNA mutations, but the level of fluorescence was low and less than two-fold greater than that of wild-type *C. jejuni*, which autofluoresce (fig. 6.10 A). *C. jejuni* single NC1 and NC4 deletion strains with P*metK-cj0428-gfp* were fluorescent, but were similar to the fluorescence of *C. jejuni* P*metK-cj0428-gfp* with no ncRNA mutations (fig. 6.10 B). Fluorescence of *C. jejuni* containing P*fdxA-cj0428-gfp* was much greater than that of P*metK-cj0428-gfp* (fig. 6.10 A). However, fluorescence of an NC4 mutant containing the P*fdxA-cj0428-gfp* was similar to *C. jejuni* P*fdxA-cj0428-gfp* with no ncRNA mutations (fig. 6.10 C). *C. jejuni* with P*fdxA-cj0428-gfp* homologously recombined into *cj0223* also gave increased fluorescence compared to *C. jejuni* with P*metK-cj0428-gfp* in *cj0046* (fig. 6.10 A). However, fluorescence of NC4 over-expressing *C. jejuni* with P*fdxA-cj0428-gfp* in *cj0223* was similar to *C. jejuni* with the same fluorescence construct with no ncRNA mutations (fig. 6.10 D).

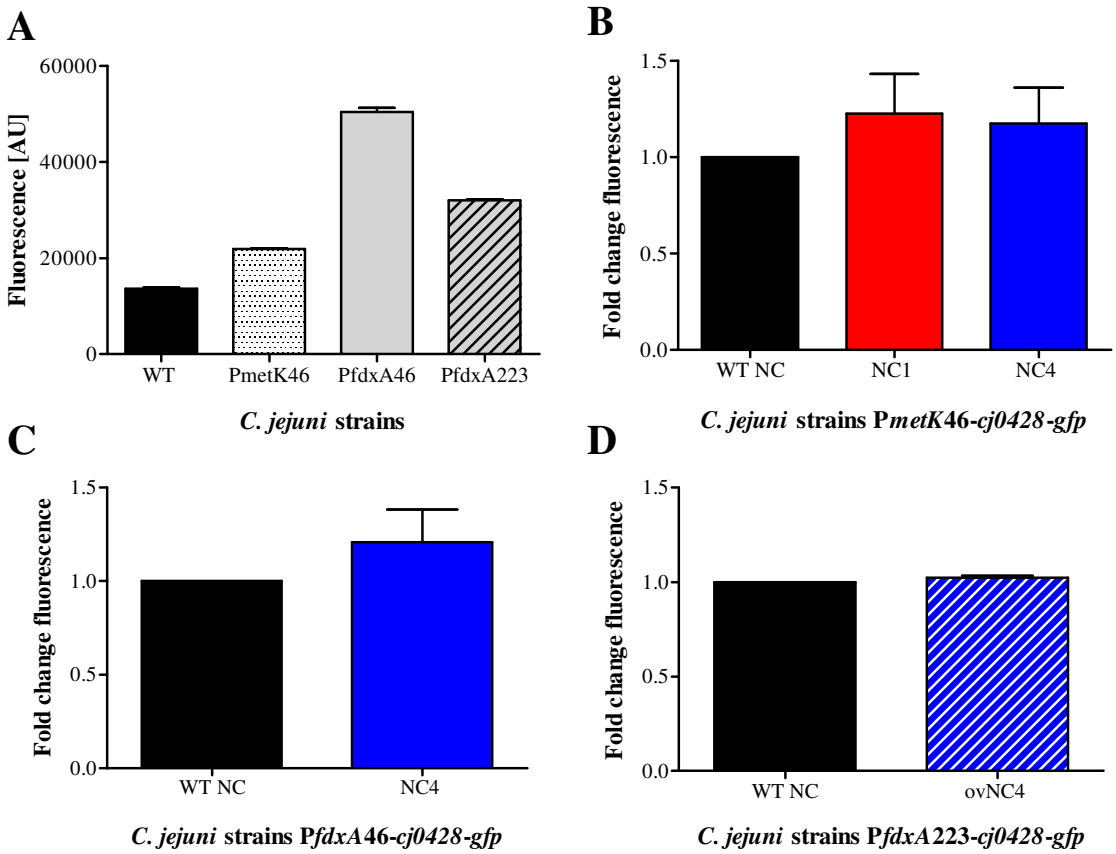


Figure 6.10 Transcriptional c*j0428-gfp* fusions in *C. jejuni* NCTC11168 fluoresce, but do not show regulation by NC1 and NC4.

Bacteria were grown to mid-logarithmic phase in broth cultures and fluorescence was measured using a fluorescence plate reader. Results are represented as fluorescence intensity in arbitrary units [AU] or fold repression of fluorescence compared to *C. jejuni* expressing wild-type ncRNAs and *cj0428-gfp* under the appropriate promoter. Error bars represent standard error of the mean of three independent experiments. **A)** *cj0428-gfp* was expressed under the *metK* (weak) and *fdxA* (moderate) promoter in the *cj0046* pseudogene region of wild-type *C. jejuni* (PmetK46 and PfdxA46 respectively). *cj0428-gfp* was also expressed under the *fdxA* promoters in the *cj0223* pseudogene region (PfdxA223) of *C. jejuni* with wild-type non-coding RNA (ncRNA) expression. Fluorescence of a wild-type, non-gfp+ expressing *C. jejuni* (WT), was also measured. **B)** PmetK46-*cj0428-gfp* in single NC1 and NC4 deletion strains of *C. jejuni* (NC1 and NC4, respectively), and a strain with wild-type ncRNAs (WT NC). **C)** PfdxA46-*cj0428-gfp* in a *C. jejuni* NC4 deletion strain (NC4) and a strain with wild-type ncRNAs (WT NC). **D)** PfdxA223-*cj0428-gfp* in a *C. jejuni* NC4 over-expression strain (ovNC4) and a strain with wild-type ncRNAs (WT NC).

6.3 Discussion

This is the first study to demonstrate that *C. jejuni* ncRNAs target and control translation of gene expression *in trans*. NC1 and NC4 both controlled expression of *cj0428*, *flgP*, *flgE2*, and *cj1650* in an *E. coli* heterologous reporter system (fig. 6.2-6.9). Mutation of NC1 and NC4 nucleotides predicted to be involved in the base-pairing with target gene mRNA disrupted the regulatory action of the ncRNAs, demonstrating that regulation of target gene expression by NC1 and NC4 is specific (fig. 6.6-6.7).

As we have a limited number of genetic tools for *C. jejuni*, in particular the lack of plasmids, we used *E. coli* to express the *C. jejuni* target gene-reporter fusions and assess translational control by NC1 and NC4, which allowed for more flexible genetic manipulations. The *E. coli* system, using the fluorescence of GFP as a reporter, was developed for use in *E. coli* and has now been used to investigate post-transcriptional gene regulation by a several ncRNAs (Levine *et al.* 2007; Urban and Vogel 2007; Corcoran *et al.* 2012).

Using this system, our experiments showed that NC1 and NC4 post-transcriptionally regulated σ^{54}-dependent flagella genes (fig. 6.4 A and B) and not a σ^{70}-dependent predicted target gene (fig. 6.4 C). The σ^{70} target *lysC*, which encodes an aspartokinase, was predicted by TargetRNA programme to be regulated by NC1 and NC4. Also, *lysC* was ranked as being a more likely target above others that were shown to be regulated by NC1 and NC4 in this study (Chapter 4, table 4.2.1). Therefore, current software may not be accurate in predicting ncRNA targets, especially for bacteria outside Enterobacteriaceae.

Fluorescent reporter fusions of *C. jejuni cj0428* and *flgE2* 5' UTRs to *gfp* were successfully expressed in *E. coli* and when NC1 and NC4 were co-expressed with these reporter fusions, fluorescence was reduced (fig. 6.4 A and B). This means that NC1 and NC4 inhibit expression of these genes by preventing translation of mRNA, since reporter fusions and ncRNAs were controlled by constitutive promoters. For *cj0428-gfp* regulation, the number of amino acids included in the *cj0428-gfp* fusion did not alter the trend of NC1 or NC4 regulation (fig. 6.3). In addition, NC4, but not NC1, significantly repressed translation of the *flgP-gfp* and *cj1650-gfp* fusions in *E. coli.* Regulation of *cj0428-gfp* by NC1 and NC4 was maintained in an Hfq deficient mutant (fig. 6.9),

which means that Hfq is not required for NC1 and NC4 function. This was expected as *C. jejuni* does not express an Hfq-like protein, and so *C. jejuni* ncRNA function either requires an unknown protein chaperone or novel mechanisms of RNA-RNA interaction.

Five *C. jejuni* flagella gene 5'UTRs fused to *gfp* were constructed on plasmids, but only four were successfully expressed in *E. coli*. The transcriptional *flaB-gfp* fusion was not fluorescent in *E. coli*, and so regulation of this gene could not be evaluated. Western blots showed that GFP protein was not expressed in *E. coli* (data not shown). Although, *E. coli* transformed with the *flgP-gfp* fusion were fluorescent, fluorescence was low. If additional codons were included in the fusion, no fluorescence was detected, which was possibly due to the inclusion of an outer membrane signal peptide motif at the start of the fusion protein.

Expression of mutated NC1 and NC4 in the *E. coli* system resulted in a loss of fluorescence regulation. This was restored to a certain extent by making the compensatory mutations in the target mRNA (fig. 6.7 and 6.8). This means that translation is specifically regulated by NC1 and NC4 and that they directly interact with target gene mRNA via complementary base-pairing. RNA-RNA shift assays are needed to confirm binding of NC1 and NC4 to target gene mRNA. Compensatory mutations were less successful in that regulation was not always completely rescued and fluorescence of these fusions was lower than fusions with non-mutated 5'UTRs. Perhaps this was because the mutations interfered with ribosome binding preventing expression of the protein, even though mutations were designed to avoid the ribosome binding site. In these experiments, a third of the ncRNA binding region was mutated; it would be interesting to make site directed mutations to further investigate the level of binding needed for regulation, as little is known about Epsilon-Proteobacteria ncRNAs and their functions.

Generally, levels of fluorescence repression of one gene were variable between different experiments, for example 1.5-2 fold repression. This may have been due to the growth phase of *E. coli.* Although, *E. coli* were grown for similar lengths in batch culture before measuring fluorescence and optical density was adjusted to OD_{600} = 1, growth was not strictly standardised. *E. coli* colonies were stored on plates for variable lengths of time and then used to inoculate broth cultures; fluorescence would often be decreased compared to a colony from a recent transformation (data not shown). Fluorescence

repression varies between different genes and has been shown in *Salmonella* and *E. coli* (Urban and Vogel 2007; Corcoran *et al.* 2012).

Based on the *E. coli* system, target *gene-gfp* fusions were expressed in *C. jejuni* to examine translational control of these targets by NC1 and NC4 in the original organism. Expression of NC1 and NC4 was altered in the chromosome and the ncRNA target gene *cj0428-gfp* fusion was expressed within *C. jejuni* pseudogenes under different strength promoters: *metK*, a weak promoter and *fdxA* a moderate strength promoter. Fluorescent GFP was successfully expressed from the chromosomal inserts as they were more fluorescent than wild-type *C. jejuni* (fig. 6.10 A). The promoters were functional as fluorescence of *C. jejuni* containing the *cj0428-gfp* fusion was greater under the *fdxA* promoter than the *metK* promoter, when expressed in both the *cj0046* and *cj0223* pseudogene regions.

Despite successfully expressing the *cj0428-gfp* fusion in *C. jejuni*, regulation by NC1 or NC4 was not observed. This may have been because expression of the *cj0428-gfp* fusion was too high to allow regulation by the ncRNAs. A strain of *C. jejuni* expressing *cj0428-gfp* under the *metK* promoter in an NC1 or NC4 over-expression mutant was not constructed. Perhaps this strain or one that uses a single copy promoter for *cj0428-gfp* expression would allow regulation by NC1 and NC4. Alternatively, NC1 and NC4 could be placed under a stronger promoter, such as the *porA* promoter to increase ncRNA expression. Another reason for the lack of NC1 and NC4 regulation, may be that all the strains contained a wild-type copy of the *cj0428* 5'UTR, which may titrate NC1 and NC4 away from the *cj0428-gfp* fusion. Making the additional mutation of deleting the *cj0428* 5'UTR would be desirable to examine this theory. In addition, known translational regulators could be tested to ensure that translational control can be evaluated by this method in *C. jejuni*.

In summary, although phenotypes of *C. jejuni* NC1 and NC4 mutants were not detected, it was demonstrated that NC1 and NC4 post-transcriptionally regulate expression of their targets in *E. coli*. The method used has been successful in demonstrating and validating ncRNA-target interactions (Urban and Vogel 2007; Corcoran *et al.* 2012; De Lay and Gottesman 2012). NC1 and NC4 function may regulate flagellar gene expression in *C. jejuni* (Chapter 4), but the experimental methods may need refining and may only be relevant in specific conditions. Development of transcriptional reporter

systems, as well as construction of all the relevant *C. jejuni* mutants is needed so that systematic experiments could be performed. These experiments would build upon the work in this investigation and would help to further enhance the range of genetic tools for *C. jejuni* studies.

Chapter 7 Thesis summary and discussion

C. jejuni flagella are crucial virulence determinants and much is known about the structural components and its formation, but the regulation of this complex organelle within different environments and how this contributes to virulence is not fully understood. Non-coding RNAs are also important for virulence and flagellar gene regulation in many bacteria, but their role in *C. jejuni* biology is unknown. Understanding regulation of *C. jejuni* virulence may create opportunities for interventions that prevent *C. jejuni* infections and may reveal novel regulatory mechanisms that further our knowledge of *C. jejuni* biology as a whole.

The first aim was to assess *C. jejuni* gene expression in response to acid, which is an inevitable stressor during faecal-oral transmission. A subset of flagellar genes and invasion of intestinal epithelial cells was up-regulated after mild acid-shock. Transition through an acidic environment may trigger expression of flagella so that greater numbers of *C. jejuni* are more likely to reach and colonise the intestinal epithelium. This is significant because it enhances our understanding of *C. jejuni* virulence and survival in host niches.

In addition, the up-regulation of a subset of flagellar genes was not matched by changes in expression of the main flagellar gene regulators, which is significant because it suggests that other mechanisms of flagellar regulation may be important during pathogenesis of *C. jejuni* infections, such as post-transcriptional regulation and this influenced the remaining scope of the thesis. Studies on the mechanisms of flagella gene expression are needed and although the FlgRS two component system is known to be a central regulator in flagella expression, flagellar regulation in response to changing environments is still unclear.

A limited number of invasion assays were performed and more are required to substantiate the outcomes observed and the invasion model could be improved by using cell monolayers or tissue in vertical diffusion chambers. The *Galleria* infection model could be used to screen survival of *C. jejuni* after exposure to different pHs and this could be followed by colonisation studies in animals.

Wider questions remain about *C. jejuni* acid responses, such as what mechanism of acid sensing and what responses and adaption mechanisms do *C. jejuni* have to acid stress? Also, is flagellar regulation a specific response to acid and, if so, what then is the

response to the more basic environments, such as that of the small intestine? Understanding *C. jejuni* adaptations to pH generally are important in knowing how it survives in hosts and food matrices.

The remainder of the thesis focused on the functions of ncRNAs NC1 and NC4. During the last decade ncRNAs have become established as in integral regulators of gene expression and virulence in bacteria. Recent transcriptome research has revealed ncRNAs in *C. jejuni* (Dugar *et al.* 2013; Porcelli *et al.* 2013). However, only two ncRNAs, NC1 and NC4, were predicted to interact with other genes, many of which were flagella genes. NC1 and NC4 were then shown to post-transcriptionally control expression of these target genes *in trans* in an *E. coli* experimental system. Expression of these ncRNAs was regulated by a flagella-associated alternative sigma factor so it was expected that NC1 and NC4 were important for regulating flagella assembly and function. However, no phenotype could be detected so the biological significance of NC1 and NC4 regulation remains unknown.

From these studies on ncRNAs, it is now known that ncRNAs encoded in the *C. jejuni* genome are expressed by the bacteria and regulate target genes in an established translational control assay, which is a novel finding. This is the first insight into ncRNA functions in *C. jejuni*, but even with experiments on *H. pylori* ncRNAs, our understanding of these regulators is still in the early stages. This warrants more research into the importance and mechanisms of NC1, NC4 and other ncRNA function. Understanding of different ncRNA regulatory networks may help to explain how complex organisms function and adapt to their environment from seemingly limited genetic information, which is the case for *C. jejuni*.

Questions remain about what other conditions the ncRNAs are expressed in and whether they contribute to biologically relevant phenotype, such as *C. jejuni* virulence. Therefore, to continue this work, the conditions of NC1 and NC4 expression need to be established, such as those under limited nutrient availability or in animal colonisation studies. If NC1 and NC4 contribute to a colonisation phenotype, the condition should be replicated *in vitro* to test relevant phenotypes of the NC1 and NC4 deletion and over-expression strains.

Moreover, NC1 and NC4 mechanism of function, as seen in *E. coli*, requires verification in *C. jejuni*. For this, tools for studying the molecular mechanisms of ncRNA interactions need to be developed for Epsilon-Proteobacteria and these will be key to furthering investigations in this area. Developing methods and approaches for examining post-transcriptional control in *C. jejuni* would directly benefit this work. Transcriptional *gfp* fusions were expressed in *C. jejuni*, but further work needs to be completed to examine whether regulation can been seen in *C. jejuni*. LacZ reporter systems could be developed as this is a method widely used for measuring post-transcriptional regulation in bacteria. A *lacZ* vector with a *C. jejuni* origin of replication is available (Wosten *et al.* 1998b), which would limit the number of mutations made to the chromosome. Controlling the expression level of the vector and/or transcriptional fusions would give a system analogous to the GFP system. These may also help develop approaches for investigating ncRNA function in other bacteria that do not maintain plasmids and require tools for chromosomal manipulations and gene expression.

The functions of other *C. jejuni* ncRNAs remain unknown and so studies of other ncRNAs in *C. jejuni* are needed to reveal their functions, if any. The discovery of a protein chaperone, functionally analogous to Hfq, could greatly progress research as it opens up the possibility of co-immunoprecipitating ncRNA and their targets, which has been a successful approach (Sittka *et al.* 2008). This would also provide knowledge of other protein chaperones that could potentially stabilise RNA-RNA interactions in other bacteria. However, it may be that Epsilon-Proteobacteria have adapted a novel mechanism of RNA-RNA interaction that does not involve a protein chaperone, and this would equally be interesting and may uncover alternative mechanisms to Hfq in other bacteria that do not express Hfq, such as *Streptococcus* species (Chao and Vogel 2010).

No targets were predicted for the other ncRNAs using the TargetRNA program, but development of the program was based on knowledge of Enterobacteriaceae regulators, and so may have missed *C. jejuni* targets and may not be suitable to make predictions in bacteria outside this family. Non-coding RNAs have been shown to be important in many aspects of bacterial biology, so there is the potential for these ncRNAs to be required for fundamental biological functions. However, if NC1 and NC4 and other ncRNAs are functionally redundant, then this would be an interesting outcome and would contribute to our knowledge of the proportion of ncRNAs that are functional

compared to those that are redundant. This may also lead to further questions about genome evolution in Epsilon-Proteobacteria and why they have excluded these RNAs from their regulatory network.

The initial aims of this work were to investigate regulation of *C. jejuni* flagellar responses to environmental conditions and investigations were shifted to focus on a possible novel mechanism of flagellar gene regulation. The conclusions are that flagellar gene expression is regulated by acidic conditions and causes an increase in *C. jejuni* invasion of intestinal epithelial cells. In addition, the expression of NC1 and NC4 is linked to flagella expression and they may function to post-transcriptionally control expression of σ^{54}-dependent, flagellar genes. This is the first description of small RNA-mediated gene regulation in a *Campylobacter* species, although its biological significance remains unknown. Regulation of *C. jejuni* flagella may be more complex than previously thought and ncRNAs are potential post-transcriptional regulators of gene expression in *C. jejuni*.

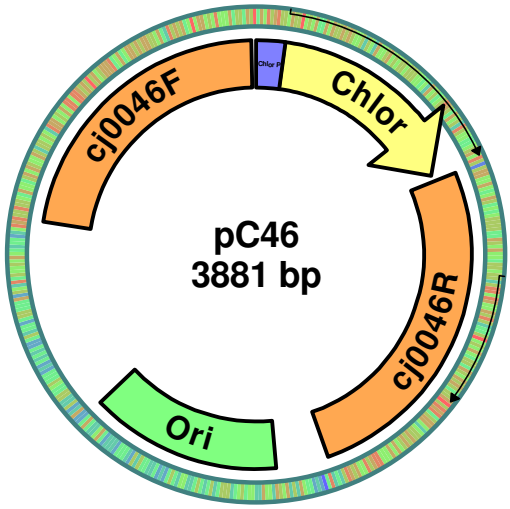
Appendices

Appendix 1 Plasmid sequences and maps of pC46 and pK223

Constructed by Duncan Gaskin.

A) pC46

Map



Key: F – front fragement; R – rear fragment; Chlor – Chloramphenicol cassette; Ori – Origin.

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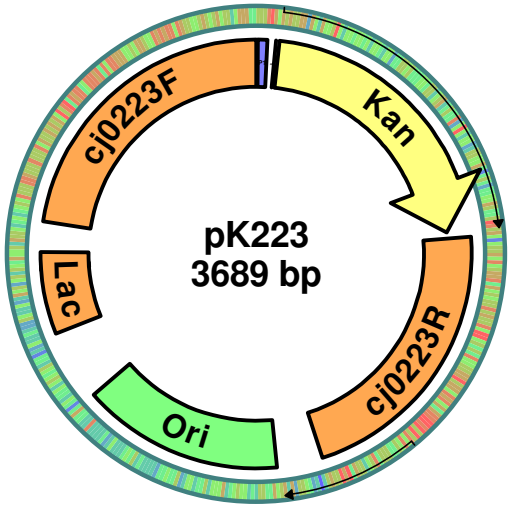
B) pK223

Map



Key: F – front fragement; R – rear fragment; Kan – kanamycin cassette (short version with promoter, P1 and a ribosome binding site); Ori – Origin.

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Appendix 2 Publication containing work from Chapter 3

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ACID-SHOCK OF *CAMPYLOBACTER JEJUNI* INDUCES FLAGELLAR GENE EXPRESSION AND HOST CELL INVASION

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Received: January 13, 2012; Accepted: January 14, 2012

The bacterial pathogen *Campylobacter jejuni* is the leading cause of foodborne gastroenteritis in the developed world, with the organism being transmitted by ingestion of contaminated and undercooked poultry. Exposure to acid is an inevitable stressor for *C. jejuni* during gastric passage, yet the effect of low pH on *C. jejuni* virulence is still poorly understood. Here, we investigate the effect of acid-shock on *C. jejuni* viability, gene expression and host-cell invasion. *C. jejuni* strain NCTC 11168 survived acid exposure at pH 3.5 and above for up to 30 min without a drop in viability, and this exposure induced the expression of flagellar genes transcribed from σ^{54}-dependent promoters. Furthermore, acid-shock resulted in increased *C. jejuni* invasion of m-IC_{cl2} mouse small intestine crypt cells grown on transwells, but not when the cells were grown on flat-bottomed wells. This suggests that *C. jejuni* might be invading intestinal epithelial cells at the basolateral side, possibly after paracellular passage. We hypothesize that acid-shock prior to intestinal entry may serve as a signal that primes *C. jejuni* to express its virulence gene repertoire including flagellar motility genes, but this requires further study in the context of an appropriate colonization or disease model.

Keywords: *Campylobacter*, acid resistance, motility, invasion

Introduction

The bacterial pathogen *Campylobacter jejuni* is a major cause of foodborne gastroenteritis in the developed world, with infection often associated with the consumption of undercooked poultry products [1]. The UK Department for Environment, Food and Rural Affaires (DEFRA) reported over 55,000 cases in the UK in 2008 alone, although this is thought to be a substantial underestimation of the true incidence, due to under-reporting by the community [2]. Clinical symptoms of *C. jejuni* infection are acute (2–7 days) and are illustrated by watery or bloody diarrhea, nausea, fever and abdominal pains, although the disease is usually self-limiting in humans [1]. Secondary complications of *C. jejuni* infection include the autoimmune diseases Guillain–Barré and Miller Fisher syndrome that result in paralysis, and also reactive arthritis and inflammatory bowel disease [1, 3, 4].

Ingestion of *C. jejuni*, either by humans or in the avian host, is inevitably followed by bacterial passage though the stomach before entering the small intestine and causing disease. In the human stomach, the gastric pH can range from approximately 2 to 7 depending on the state of the stomach (empty or full), gastrin production and stomach contents [5]. In addition, food is retained in the stomach for 30–60 min before the stomach begins to empty [6]. Thus, *C. jejuni* must have the means to cope with sudden and variable exposures to acidic conditions. However, *C. jejuni* responses to acid stress have not been as extensively characterized as those in the well-studied enteric pathogens *Escherichia coli* or *Salmonella* Typhimurium [7].

Many bacteria have specific acid-resistance systems allowing direct protection against acidic pH, like the well characterised glutamate decarboxylase system in *E. coli*, which metabolizes glutamate to increase internal pH [7]. This and other acid-resistance systems dependent on growth media are regulated by complex networks and are also shared with *S.* Typhimurium [8]. However, while widespread in the Enterobacteriaceae, these specific systems are absent in *C. jejuni*. *C. jejuni* is known to upregulate stress-response genes and down-regulate capsular polysaccharide biosynthesis genes in response to acid-shock [9, 10]. As well as these changes in gene expression, it has also been suggested that the alternative sigma factor σ^{54} (encoded by the *rpoN* gene), might play a role in acid resistance in *C. jejuni* [11]. *C. jejuni* has been reported to mount an adaptive tolerance response (ATR) during the stationary growth phase, where, in the case of acid adaptation, the bacteria are sensitized by mild acid-shock (e.g. pH 5), upon which they display increased survival at low pH [12]. It has been suggested that *C. jejuni* has mecha-

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nisms enabling ATRs, which are initiated by the release of extracellular proteins [12, 13].

Exposure to low pH can induce genes involved in virulence phenotypes, as was shown in the related human pathogen *Helicobacter pylori*, where acid-shock increases the expression of genes for the acid-resistance factor urease and the expression of motility-associated genes [14]. This enables the bacteria to survive in acidic conditions and may aid taxis responses during initial colonization of the gastric mucosa [14]. Similarly, *E. coli* displayed increased expression of flagellar genes and motility upon acid-shock, as well as an up-regulation in other stress responses [15, 16].

In this, study, we have investigated the effect of acid-shock on the gene expression and survival of *C. jejuni*. We show that *C. jejuni* can survive acid exposure at pH 3.5 and above for up to 30 min without a drop in viability, and that this exposure induces the expression of motility genes transcribed from σ^{54}-dependent promoters. Furthermore, we demonstrate that acid-shock increases invasion of *C. jejuni* in mouse small intestine crypt cells when grown on transwells but not when grown on flat-bottomed wells.

Materials and methods

Bacterial strains and growth conditions

The motile *C. jejuni* strain NCTC 11168 [17] was used for all experiments. *C. jejuni* was cultured in a MACS-MG-1000 controlled atmosphere cabinet (Don Whitley Scientific) under microaerobic conditions (85% N_2, 5% O_2, 10% CO_2) at 37 °C. For growth on plates, strains were grown on Blood Agar Base 2 with Skirrow supplements (10 μg ml^{-1} vancomycin, 5 μg ml^{-1} trimethoprim, 2.5 IU polymyxin-B). Broth culture was carried out in Brucella broth (Becton, Dickinson & Company).

Acid-shock and viability assays

Acid-shock was effected by growing *C. jejuni* to mid-exponential phase, and resuspending cells in Brucella broth adjusted with HCl to pH values from 2.0 to 7.0. Viability assays following acid-shock were performed by determining the number of colony forming units (cfu) after incubation for 10 and 30 min at pH 2.0, 3.0, 3.25, 3.5, 3.75, 4.0, 5.0, 6.0 and 7.0 under microaerobic conditions at 37 °C. *C. jejuni* was incubated with non-adjusted Brucella broth as a control. Serial 10-fold dilutions were made, 5 μl of each dilution was spotted onto Brucella-agar plates and incubated under microaerobic conditions for 2 days at 37 °C. Three independent assays were performed for each pH value, and survival was expressed as the percentage of surviving bacteria relative to the control.

Nucleic acid isolation for microarray

For analysis of *C. jejuni* gene expression after acid-shock, log-phase *C. jejuni* cells were incubated at pH 3.6 and 5.0 for 10 and 30 min as described previously. Cell suspensions were subsequently mixed with 0.1 volume of ice-cold 5% phenol in ethanol, and cells were harvested by centrifugation at 3220 *g* for 15 min at 4 °C. RNA was isolated as described previously [18]. Chromosomal DNA was isolated from wild-type *C. jejuni* cultures grown to mid-exponential phase using Qiagen genomic-tip 100/G gravity columns according to the manufacturers' protocol. Concentration and quality of chromosomal DNA was determined by Nanodrop instrument (Thermo Scientific), and quality of RNA was determined by an Agilent 2100 Bioanalyser (Agilent Technologies).

Transcriptomic analysis

A DNA microarray was constructed as described by Holmes et al. [18]. Three micrograms of chromosomal DNA was labelled with Cy3-conjugated dCTP using the BioPrime labelling kit (Invitrogen), with labelling reactions performed overnight at 37 °C. Labelled cDNA was prepared from 15 mg RNA using Stratascript Reverse Transcriptase (Stratagene) for direct incorporation of Cy5-conjugated dCTPs (Amersham), with labelling reactions performed for approximately 16 h at 42 °C. Labelled nucleic acids were cleaned with the Qiaquick purification kit (Qiagen) and dried before being re-suspended in water and prepared for hybridization. Samples were boiled for 2 min, cooled at 18–25 °C for 5 min and centrifuged at maximum speed in a microfuge for 2 min to remove any solid particles from the hybridization mixture. This mixture was spotted onto microarray slides and incubated at 60 °C for approximately 16 h. Details of the labelling and hybridizations are available on http://www.ifr.ac.uk/safety/microarrays/protocols.html. The slides were washed and dried as described by Holmes et al. [18]. Microarrays were scanned and analysed as described in Lucchini et al. [19], and data were obtained for 1608 genes. For each condition, three independent RNA isolations were hybridized.

Cell culture

The murine intestinal crypt-like cell line m-IC$_{cl2}$ [20] was cultured in m-IC media consisting of Dulbecco's modified Eagle Medium/Ham's F-12 12 g l^{-1} (1:1 *v/v*; Gibco), 20 mM D-glucose (Sigma), 10 ng ml^{-1} mouse epidermal growth factor (Merck), 5 μg ml^{-1} insulin (Sigma,), 60 nM selenium (Sigma), 5 μg ml^{-1} human Apo-transferrin (Sigma), 1 nM triiodothyronine (Merck), 20 mM HEPES (Sigma), 2% fetal calf serum (Gibco), 50 nM dexametha-

sone and 2 mM L-alanyl-L-glutamine (Sigma) at 37 °C in a 5% CO_2 atmosphere. Cells of passage 9–15 were used for experiments.

Invasion assays

The m-IC_{el2} cells were grown until confluent on transwells with 8 mm pores (Corning) or on flat-bottomed wells in plastic 24-well plates (Sarstedt) at 37 °C in a 5% CO_2 atmosphere. For growth on transwells, the trans-epithelial electrical resistance of the monolayer was monitored by the EVOM2 epithelial voltohmmeter (World Precision Instruments) to measure confluence. To compare the effect of acid-shock on invasion, *C. jejuni* cultures were incubated for 30 min at pH 5.0 (acid-shock) or 7.0 (control) as described previously. Both transwell and flat-bottomed well models were infected with *C. jejuni* in m-IC media to MOI (multiplicity of infection) 1000 for 2 h at 37 °C and 5% CO_2 to allow bacterial invasion. To remove adherent *C. jejuni*, m-IC_{el2} cells were washed twice with phosphate buffered saline (PBS) and incubated in fresh m-IC_{el2} media containing 500 mg ml^{-1} gentamicin for 1 h [21]. To quantify bacterial invasion, the infected m-IC_{el2} cells were washed twice with PBS and then lysed with 1% Triton X-100. Serial 10-fold dilutions were made, 5 μl of each dilution was spotted onto Brucella-agar plates and incubated under microaerobic conditions for 2 days at 37 °C. For transwell experiments, the numbers of bacteria in the basal compartment (translocated bacteria) were also counted. Results were expressed as percentage of invasive or translocated bacteria relative to the inoculum. Transwell experiments comprised 10 technical replicates. Flat-bottomed well experiments comprised three independent experiments, each containing six technical replicates. Statistical analyses were performed using IBM SPSS Statistics 19.

Results

The lowest pH that C. jejuni *is able to survive is pH 3.5*

To determine whether *C. jejuni* can survive the acidic conditions present in the stomach, we incubated bacteria grown to exponential phase in Brucella broth adjusted with HCl to different pH values ranging from 2.0 to 7.0, with survival being assessed by viability counts. There was no significant loss in viability when *C. jejuni* was incubated for 10 min at pH values of 3.5 and higher, but below pH 3.5 there was a rapid loss of viability, with less than 0.1% of viable cells recovered after acid-shock at pH 3.25 *(Fig. 1)*. When the duration of acid-shock was increased to 30 min, the lowest pH at which *C. jejuni* showed no decrease of viability was pH 3.75, where less than 0.01% of viable cells were recovered at pH 3.5 *(Fig. 1)*. Acid ATR was assayed by pre-incubating exponential-phase cells of *C. jejuni* at pH 5.0 and 7.0 prior to acid-shock at pH 2.75. There were no viable cells recovered at pH 2.75 (data not shown), suggesting that *C. jejuni* lacks an ATR at this growth phase as previously observed [22].

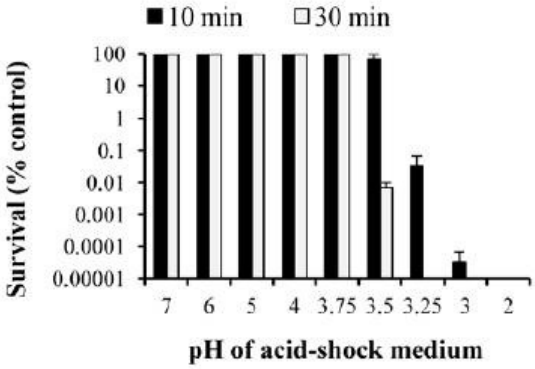

Fig. 1. The lowest pH that *C. jejuni* NCTC 11168 is able to survive is 3.5. Motile *C. jejuni* was grown to exponential phase and incubated for 10 and 30 min in Brucella broth adjusted to pH 2.0–7.0. *C. jejuni* was incubated with non-adjusted Brucella broth as a control. Survival was assessed by cfu after 10 min (solid bars) and 30 min (white bars). Results were expressed as a percentage of surviving bacteria relative to the control. Error bars denote standard error of the mean, and the results shown are the average of three independent experiments.

Acid-shock increases the expression of σ^{54}-dependent flagellar biosynthesis genes

To assess the effect of acid-shock on gene transcription in *C. jejuni*, we performed transcriptomic analyses using *C. jejuni* microarrays to compare transcriptional profiles at pH 7.0 and at acidic pH (pH 5.0 and 3.6, for 10 and 30 min). Levels of RNA were expressed as the ratio of the acidic pH/pH 7.0, and genes were considered differentially expressed if transcript levels were more than twofold different and if the false discovery rate (FDR) was below 0.1. The transcript level of 232 genes was increased more than twofold and that of 294 genes was decreased more than twofold upon acid shock in one of the four tested conditions, with an FDR <0.1. Of the genes with increased transcript levels, 137 showed a significant change in one of the four conditions, whereas 95 showed increased transcript levels in two or more of the tested conditions. Of the genes with decreased transcript levels upon acid-shock, 151 were identified in one of the four conditions, whereas 143 showed decreased transcript levels in two or more of the tested conditions.

Down-regulated genes, as a result of acid-shock, included those encoding 50S and 30S ribosomal proteins (*rpm*, *rpl* and *rps*), which were consistently down-regulated across two or more conditions. Also, genes encoding the F_0F_1 ATPase subunits (*atpDFH*) were down-regulated after 30 min shock at both pH 5.0 and 3.6, and this was also the case for *sec* protein-export genes (*secAFY*). Genes encoding leucine biosynthesis enzymes (*leuABC*)

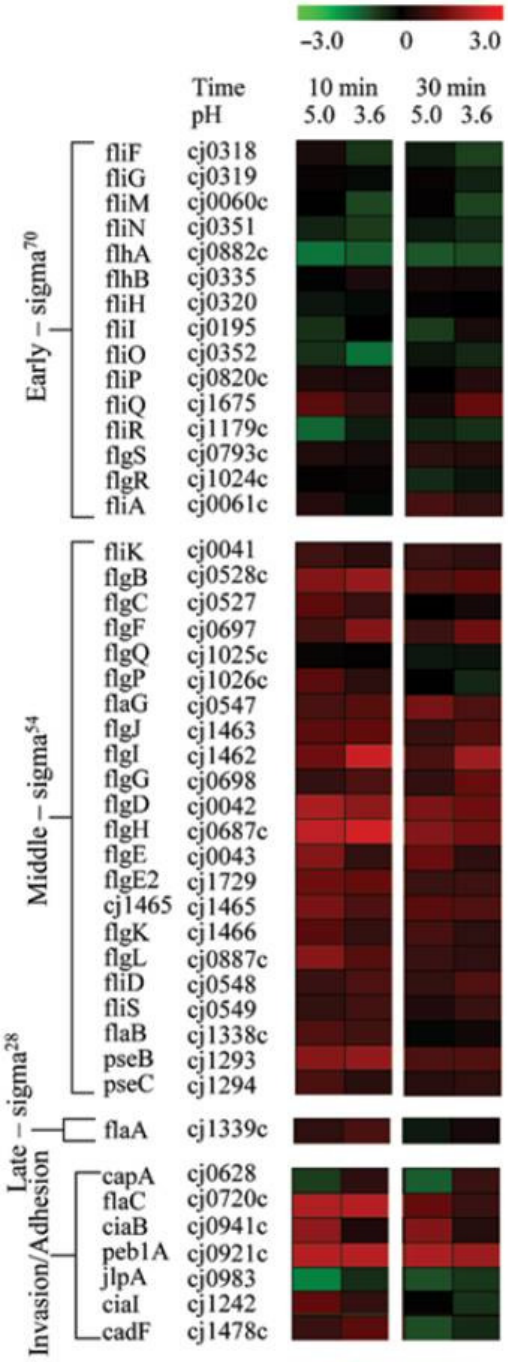

Fig. 2. Acid-shock at pH 5.0 and 3.6 increases the expression of a subset of flagellar biosynthesis genes and, in particular, those transcribed by σ^{54} during the middle phase of flagellar assembly. RNA levels of log phase *C. jejuni* NCTC 11168 cells incubated at pH 7.0 were compared with those of cells incubated at pH 5.0 and 3.6 for 10 and 30 min. Results are shown for a subset of genes, including flagellar genes and previously identified invasion and adhesion determinants [40, 41]. Flagellar genes are listed and grouped in the order of flagellar assembly: early phase genes are controlled by σ^{70}, middle-phase genes by σ^{54}, and late-phase genes by σ^{28}; invasion and adhesion determinants are grouped separately. Up-regulated genes are shown in red, and down-regulated genes are shown in green. Maximum colour output represents a threefold change in expression. Results shown are averages of three independent experiments.

were down-regulated at the acid-shock of pH 5.0 only, as were genes for glycosylation enzymes (*pglABC*). Other *pgl* locus genes, including *pglHIJKI*, were down-regulated after 30 min acid-shock at pH 5.0 only. Overall, these changes suggest that the cells shut down protein synthesis and modification in response to acid stress, as a result of the change from exponential growth to survival mode.

Up-regulated genes, as a result of acid-shock, included the catalase gene (*katA*) and the heat shock proteins encoded by *clpB*, *dnaK*, *hrcA* and *htrA*, which were up-regulated after 10 minutes acid-shock at both pH 5.0 and 3.6. Genes for respiratory functions, such as succinyl-CoA synthesis (*sucCD*) and proline metabolism (*putA*), were up-regulated under all conditions, and the oxidoreductase genes (*oorABCD*) were up-regulated after 10 min acid-shock. Gluconate dehydrogenase genes (*gndAB*) were up-regulated after 10 min acid-shock at pH 5.0 and 3.6, and NADH (nicotinamide adenine dinucleotide hydride) dehydrogenase (*nuoBGHJK*) genes were up-regulated after 10 min acid-shock at pH 5.0 only. Also, the lactate oxidase operon *cj0075c-0073c* [23] was up-regulated across all acid conditions, suggesting a general stress response upon acid exposure.

Flagellar genes that are transcribed from σ^{54}-dependent promoters during the middle phase of flagellar assembly [24, 25] were up-regulated after acid-shock at pH 5.0 and 3.6 *(Fig. 2)*. These genes included components of the basal body, hook, junction proteins and associated outer membrane proteins, as shown in *Fig. 3*. Genes expressed at the early and late phases of flagellar assembly remained mostly unaffected by acid-shock except for *fliQ* expression, which was significantly up-regulated in response to 10 min acid-shock at pH 5.0. The *flaC* and *ciaB* invasion and *peb1A* adhesion determinants were also significantly up-regulated under one or more acid conditions *(Fig. 2)*.

Acid-shock increases invasion of C. jejuni *into mouse intestinal crypt (m-IC$_{cl2}$) cells grown on transwell inserts, but not when m-IC$_{cl2}$ cells are grown in flat-bottomed wells*

To investigate the effect of acid-shock and the associated increase in flagellar gene expression on *C. jejuni* virulence phenotypes, we performed invasion assays with a mouse intestinal crypt cell line (m-IC$_{cl2}$) [20]. This cell line represents the small intestine and was therefore used in preference to colon-derived cell lines such as CaCo-2. Confluent layers of m-IC$_{cl2}$ cells were grown either on transwell in-

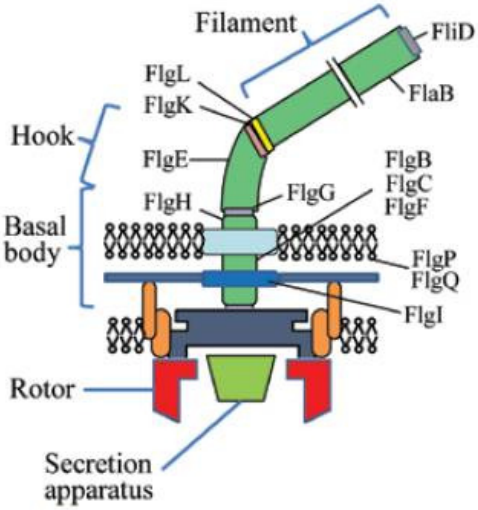

Fig. 3. Diagram showing the main structural components of the *C. jejuni* flagellum modified from Wösten et al. [24]. Only the gene names and locations of the flagellar gene products that are up-regulated after acid-shock at pH 5.0 for 10 min are shown.

serts or on flat-bottomed wells *(Fig. 4a)*, and subsequently incubated with *C. jejuni* at MOI 1000. The *C. jejuni* cultures had either been incubated at pH 5.0 for 30 min, or at pH 7.0 as a control. Following the commonly used gentamicin killing approach [21], m-IC_{cl2} cells were lysed, and invasive intracellular *C. jejuni* were enumerated as cfu.

The transwell model assessed bacterial invasion from both the apical and basolateral sides (after translocation through the m-IC_{cl2} cell layer), whereas the flat-bottomed well model tested for apical invasion only *(Fig. 4a)*. When comparing control *C. jejuni* cultures, higher invasion levels were seen in the flat-bottomed well model than in the transwell model *(Fig. 4b)*. In the flat-bottomed well model, there was no significant effect of acid-shock on the number of *C. jejuni* recovered from m-IC_{cl2} cells *(Fig. 4b)*. However, in the transwell model, acid-shock increased the levels of *C. jejuni* invasion by 1–2 log compared to the control, so invasion levels became comparable to those observed in the flat-bottomed well model *(Fig. 4b)*. The number of translocated *C. jejuni* (recovered from the compartment below the transwell insert) was increased up to 2 log after acid-shock, although cfu recovery was very variable across technical replicates (data not shown).

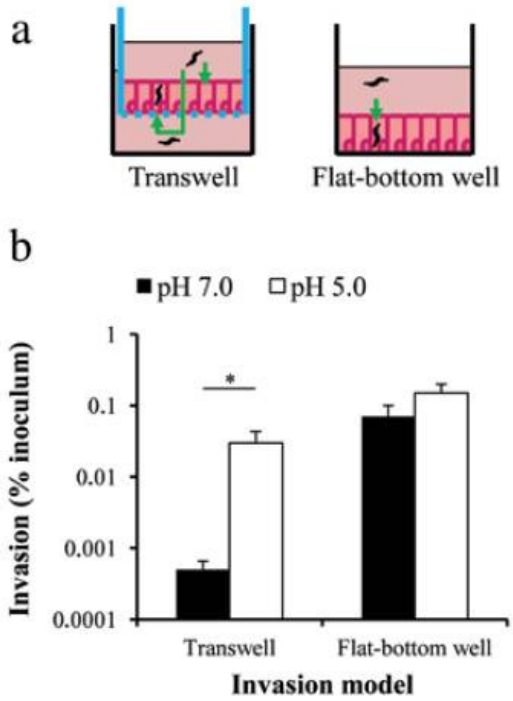

Fig. 4. Acid-shock increases *C. jejuni* NCTC 11168 invasion of mouse intestinal crypt (m-IC_{cl2}) cells grown on transwell inserts, but not on flat-bottomed wells. Increased invasion is seen when m-IC_{cl2} cells are grown on flat-bottomed wells. (a) Schematic representations of the invasion models highlighting possible routes (green arrows) of *C. jejuni* invasion: apical and basolateral when m-IC_{cl2} cells are grown on transwell inserts; and apical only when grown on flat-bottomed wells. Cells were grown until confluent on transwell inserts or flat-bottomed wells prior to exposure to motile *C. jejuni* (MOI 1000) that had been acid-shocked at pH 5.0 or incubated at pH 7.0 for 30 min. Following a killing wash with gentamicin, m-IC_{cl2} cells were lysed and viable *C. jejuni* cfu determined. (b) Results were expressed as percentage of invasive or translocated bacteria relative to the inoculum. *C. jejuni* incubated at pH 7.0 is represented by solid bars and *C. jejuni* acid-shocked at pH 5.0 is represented by open bars. Error bars denote standard error of the mean. Transwell experiments comprised 10 technical replicates. Flat-bottomed well experiments comprised three independent experiments of six technical replicates. Astersk indicates $P \leq 0.05$ (Mann–Whitney U test).

Discussion

When *C. jejuni* colonizes a new host, the fecal–oral route of infection will inevitably include exposure to the acidic environments of the stomach in mammals as well as the acidic proventriculus (glandular stomach) in birds [26]. Enteric pathogens require acid-resistance mechanisms for successful transmission to the intestine. In this study, we have shown that *C. jejuni* can survive mild and strong acid-shock conditions, and that this is linked with increased transcription of a subset of flagellar biosynthetic genes and stress responses, as well as a down-regulation in genes involved in cell division and metabolism. Furthermore, acid-shock increases *C. jejuni* invasion of intestinal epithelial cells in a transwell model, and mouse small intestinal crypt (m-IC_{cl2}) cells can be used as a *C. jejuni* invasion system representing the mammalian small intestine.

Previous work describing the response of *C. jejuni* to acid exposure reported that *C. jejuni* could not be cultured, but remained viable after prolonged exposure to

pH 4.0 [27]. *C. jejuni* was reported to survive for no more than 4 min exposure at pH 3.0, but that high numbers of *C. jejuni* were recovered from the pig stomach, which has a pH range of 3.8–4.2, suggesting that pH alone cannot explain this increased survival [9]. The aims of this study were to ascertain the lowest pH that *C. jejuni* could survive under defined experimental conditions (Brucella broth adjusted to different pH values using HCl), and to use microarrays to access the response of *C. jejuni* to non-lethal acid-shock. In this study, the threshold for survival of *C. jejuni* was pH 3.5 after 10 min acid-shock and pH 3.75 after 30 min acid-shock *(Fig. 1)*, implying that *C. jejuni* can survive the physiological acidic conditions of the stomach. Using this threshold, *C. jejuni* gene expression in response to acid-shock was analysed at the lower threshold of pH 3.6, and with the mildly acidic condition of pH 5.0, at both 10 and 30 min of incubation. We also investigated whether *C. jejuni* induced an ATR to acid. Exponential-phase cultures did not show an ATR to lethal acid-shock at pH 2.75 after 2 h pre-incubation with pH 5.0. This is in agreement with the results from Murphy et al., although their pre-incubation step was longer [22].

Transcriptomic analysis of acid-shocked *C. jejuni* showed that exposure to acidic conditions resulted in increased expression of a subset of flagellar biosynthetic genes. Flagellar gene expression is tightly regulated by a hierarchy of σ factors and begins with σ^{70}-dependent transcription of the inner membrane ring and secretion apparatus [25]. Middle-phase expression of genes coding for the minor flagellin, basal body and junction proteins is σ^{54} dependent, and the expression of the major flagellin gene is dependent on σ^{28} during late phase [25]. The flagellar genes that were up-regulated are transcribed by σ^{54} during the middle phase of flagellar assembly. This finding is consistent with a recent report in which a *C. jejuni rpoN* mutant, which lacks the σ^{54} factor, showed reduced survival at pH 5.0 [11], and is comparable with more recent studies on the response of *C. jejuni* to acid-shock, which was reported to result in transient expression of many of the flagellar biosynthetic genes that we report as up-regulated [9, 10]. Perhaps, as suggested [9, 10], a change in expression of flagellar genes may be part of a general stress response. Indeed, a down-regulation of genes encoding the cell cycle ATPases and ribosomal proteins was observed, which indicates a cessation of cell division and replication as well as an up-regulation of oxidative stress and heat shock proteins. Although *C. jejuni* did not directly encounter these stressors, specific stress response proteins may also have roles in general stress responses as shown with mutants lacking some of the heat shock proteins [9, 10]. However, alongside the reported overlap in responses, there were also a large number of differences compared with previously published datasets [9, 10], which may be a consequence of experimental design and analysis, bacterial growth and the natural variations between strains.

Flagellar genes are linked with acid responses in other bacterial pathogens, including *E. coli* and *H. pylori*. In *E. coli*, flagellar genes are strongly induced in acidic conditions, but few flagellar regulators are up-regulated [16]. Increased levels of flagellar gene transcription have also been observed in *E. coli* responses to long-term acid exposure, but not after short-term exposure to acidic conditions [15]. In *H. pylori*, acid-shock resulted in increased expression of σ^{54}-dependent flagellar genes, and this correlated with an increase in the number of motile cells and speed of motility [14]. Thus, exposure to a range of acidic conditions seems to correlate with increased flagellar gene expression among gastrointestinal bacterial pathogens.

How many bacteria sense low pH is currently not well understood, especially in the Epsilon subdivision of the Proteobacteria, such as *C. jejuni*. In *C. jejuni* and *H. pylori*, FlgS is a cytoplasmic histidine kinase that regulates flagella assembly [28, 29]. In *H. pylori*, FlgS senses low pH and activates urease expression, which contributes to bacterial survival, although this occurs independently of the two-component response regulator partner FlgR [29]. In *C. jejuni*, our transcriptomic analysis showed no change in *flgR* regulation, making it possible that the up-regulation of flagellar genes in *C. jejuni* is mediated by an alternative regulatory pathway.

One possible explanation for the observed acid-induced increase in transcript levels of flagellar genes could be that acid-shock prepares *C. jejuni* for invasion or colonization of the small intestine. Therefore, *in vitro* invasion experiments were performed with acid-shocked *C. jejuni* cultures compared to a pH 7.0 control, which resulted in increased invasion of m-IC_{el2} cells by *C. jejuni* when the m-IC_{el2} cells were grown in a transwell model *(Fig. 4b)*. Interestingly, this phenomenon was not observed when m-IC_{el2} cells were grown on flat-bottomed wells. This could be due to the elimination of the basolateral route of invasion in cells grown on flat-bottomed wells. Since *C. jejuni* can translocate through epithelial cell monolayers [30–32], this may be an important route of invasion for more motile acid-shocked bacteria. Acid-shock may therefore increase the numbers of *C. jejuni* with flagella, which are required for translocation [32], enabling more bacteria to translocate the epithelial cell layer and invade cells. However, the numbers of translocated *C. jejuni* varied between individual wells, reflecting perhaps differences in invasion mechanisms or responses to host-secreted factors within the bacterial population. Quantification or visualization of translocated bacteria at different time points after infection would address this.

Another difference was that lower invasion levels were seen in the transwell model than in the flat-bottomed well model when comparing non-shocked control *C. jejuni* cultures *(Fig. 4b)*. Translocated *C. jejuni* that did not subsequently invade m-IC_{el2} cells may account for this difference. Acid-shock then increased invasion levels because *C. jejuni* were more able to invade m-IC_{el2} cells or were forced to escape acid-shock. Alternatively, the transwell invasion model was more physiologically relevant due to greater m-IC_{el2} cell polarization, and so invasion levels were, in fact, more realistic than those in the flat-bottomed well model.

This is the first report of the use of m-IC_{el2} cells in *C. jejuni* invasion experiments. These cells can be used to complement *in vivo* mouse studies [33–35]. There is, however, no perfect invasion model for studying *C. jejuni* pathogenesis, making the choice of model to use for both *in vitro* and *in vivo* work difficult. Currently, the majority of *in vitro* studies use Caco-2, Int407, Hep-2 and HeLa cell lines [21], which have greatly advanced our knowledge of the molecular mechanisms of *C. jejuni* pathogenesis. However, as *C. jejuni* initially colonizes the small intestine, which is inflamed and damaged in *C. jejuni* infected patients [36], we propose that m-IC_{el2} cells may be a suitable addition to the cell line "toolbox" for investigating host cell invasion, as they are morphologically and functionally similar to small intestinal crypts [20].

This is the first attempt to characterize the capacity of acid-shock to induce *C. jejuni* invasion of intestinal epithelial cells. Acidic conditions may trigger the activation of *C. jejuni* virulence phenotypes in preparation for host cell invasion. One study investigating co-incubation of *C. jejuni* with amoebae demonstrated that incubation of *C. jejuni* under mildly acidic conditions increased its adherence to and invasion of amoebae [37]. The same study also reported that bacterial survival increased after long-term acid exposure and that incubation of *C. jejuni* with low pH for 1 h increased motility on a swarm plate. However, this was not observed in an earlier study, where *C. jejuni* motility was reduced when inoculated onto swarm plates adjusted to different pH values [38]. The effect of acidic conditions on *C. jejuni* motility is therefore not clear and needs to be investigated further, as motility is required for host cell invasion, which is important for virulence [39].

In summary, this work shows that *C. jejuni* responds to acid-shock by down-regulating genes involved in cell division and replication and by up-regulating flagellar and stress response genes. Acid-shock increases *C. jejuni* invasion of intestinal epithelial cells when the basolateral invasion route is available. More work is now needed to extend these observations using virulence models. Also, the mechanisms that *C. jejuni* employs to sense acid-shock need to be elucidated to enhance our understanding of *C. jejuni* survival and response to acidic conditions, which is relevant to both the food industry and *C. jejuni* pathogenesis.

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