INTERACTIONS BETWEEN CAMPYLOBACTERS AND THEIR BACTERIOPHAGES

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ABSTRACT

Campylobacter jejuni is a leading cause of human bacterial enteritis worldwide. Consumption of contaminated poultry meat is considered a major source of infection. The use of virulent bacteriophages as a form of biocontrol to specifically reduce this pathogen in poultry (phage therapy) is a promising intervention that does not rely on antimicrobials and therefore circumvents the emergence of antibiotic-resistant *Campylobacter* strains. In order to achieve this, a better understanding of the mechanisms involved in phage-host interactions at the molecular level would assist in the development of the strategy and the selection of bacteriophages. The main objective of this study was to therefore examine such interactions between *Campylobacter* and its virulent phages. To achieve this, the transcriptional response of *C. jejuni* to phage infection was investigated, along with the role of a Type II restriction-modification system during phage infection of *Campylobacter*. These studies were conducted using the highly phage-sensitive *Campylobacter* strain, *C. jejuni* PT14, in conjunction with a number of group II and III bacteriophages (*Eucampyvirinae*).

Transcriptome studies (RNA-Seq) revealed a phage-induced host response that included a demand for iron and oxygen. This was highlighted by the up-regulation of several siderophore-based iron acquisition genes and down-regulation of genes associated with a number of anaerobic electron transport pathways that utilise alternative electron acceptors to oxygen. In addition, the pattern of gene regulation also suggested apo-Fur regulation of the iron-responsive and flagellar biogenesis genes. This host response has been proposed to occur as a consequence of the reduction of ribonucleotides to form deoxyribonucleotides during phage DNA replication. This process is catalysed by the enzyme ribonucleotide reductase and requires iron and oxygen during the formation of a reactive di-iron centre within the β-subunit of the enzyme.

Unusually knock-out mutants of a Type II restriction-modification system had a negative impact on phage replication. The A911_00150 mutant displayed pleiotropic changes in motility, cell based invasion and the ability to colonise chickens. Transcriptome analysis highlighted down-regulation of the genes required for the synthesis of the bacterial flagellum.

Dedication

This thesis is dedicated to the memory of my loving father, who always taught me the importance of hard work. You are a constant source of inspiration and I know you would have been proud of my achievements.

Everton Erskine Brathwaite

(1949-2002)

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LIST OF ABBREVIATIONS

A_{600}	Absorbance at 600 nm
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BA	Blood agar
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CCDA	*Campylobacter* blood-free selective agar
cDNA	Complementary deoxyribonucleic acid
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CJIE	*Campylobacter jejuni* insertion element
CPS	Capsular polysaccharide
CRISPR	Clustered regularly interspaced short palindromic repeats
CSLC	Carrier state life cycle
° C	Degrees celsius
DNA	Deoxyribonucleic acid
EOP	Efficiency of plating
EU	European Union
FDA	Food and Drug Administration
kb	Kilobase
LOS	Lipooligosaccharide
mRNA	Messenger ribonucleic acid
µl	Microliter

ml	Millilitre
MOI	Multiplicity of infection
MH	Mueller-Hinton
NCTC	National Collection of Type Cultures
nm	Nanometre
NZCYM	New Zealand Casamino Yeast Medium
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
% v/v	Percentage volume per volume
% w/v	Percentage weight per volume
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PGAAP	Prokaryotic Genomes Automatic Annotation Pipeline
RO	Reverse osmosis
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SMRT	Single molecule real-time
TEX	Terminator exonuclease
TTO	Terminal Tagging Oligo
WHO	World Health Organisation

Chapter 1 : Introduction

1.1 CAMPYLOBACTER

1.1.1 Overview of early history

Campylobacters were originally classified in the genus *Vibrio*. Early literature referred to the isolation of "*Vibrio fetus*" from the stomach contents of an aborted lamb (McFadyean & Stockman 1913), "*V. jejuni*" from the jejunum of calves (Jones et al. 1931) and later "*V. coli*" from pigs (Doyle 1944). The first well documented "*vibrio*-related" case of human infection occurred during a diarrhoeal outbreak caused by contaminated milk (Levy 1946) where organisms resembling "*V. jejuni*" were isolated. Kist (1985) however later argued that these organisms were in fact *Campylobacter* spp. He also highlighted the work of Escherich (1886) who had earlier described a spiral form of non-culturable bacteria isolated from stool specimens and mucous from the large intestine of infants who had died from "cholera infantum". King (1957) was the first to successfully culture microaerophilic isolates of *V. fetus* at 42°C. However, this temperature was higher than the optimal growth temperature of traditional vibrios and she provisionally referred to her isolates as "related vibrio". In 1963, the genus *Vibrio* underwent a major taxonomic revision and the new genus *Campylobacter* (Greek for "curved rod") was introduced (Sebald & Véron 1963). The atypical vibrios, *V. fetus* and *V. bubulus*, were placed into this new genus because of their low G+C DNA content, microaerophilic growth requirements and their inability to ferment glucose. *Campylobacter* was finally recognised as a human pathogen after successful isolation from human faeces in 1972 (Dekeyser et al. 1972) and the subsequent development of selective media allowed for routine isolation of campylobacters (Skirrow 1977).

1.1.2 Taxonomy

Following the introduction of the genus *Campylobacter* by Sebald and Véron (1963), there has been extensive revision of this taxon, with many organisms once thought to be campylobacters or *Campylobacter*-like being reclassified (Goodwin et al. 1989; Vandamme, P. et al. 1991; Vandamme et al. 1992). For instance, *C. pylori* and *C. mustelae* were reassigned to the genus *Helicobacter* because they exhibited multiple sheathed flagella, showed differences in their cellular fatty acid composition and were phylogenetically different based on 16S rRNA sequencing (Goodwin et al. 1989). Similarly, several aerotolerant *Campylobacter*-like organisms were re-classified to the genus *Arcobacter* (Vandamme et al. 1992). The *Campylobacteraceae* family includes the genera *Campylobacter, Arcobacter* and *Sulfurospirillum* (Vandamme 2000). Figure 1-1 shows the members of this family along with their phylogenetic relationship based on 16S rRNA homology. The genus *Campylobacter* has been determined to be a member of the epsilon subdivision of the class *Proteobacteria* by partial 23S rRNA gene analysis (Trust et al. 1994) and presently contains 17 species and 6 sub-species (Debruyne et al. 2008) with the type species being *C. fetus.* Many of the campylobacters have been implicated in human disease, however, the two species *C. jejuni* and *C. coli* are recognised as the major causative agents of human *Campylobacter* infections.

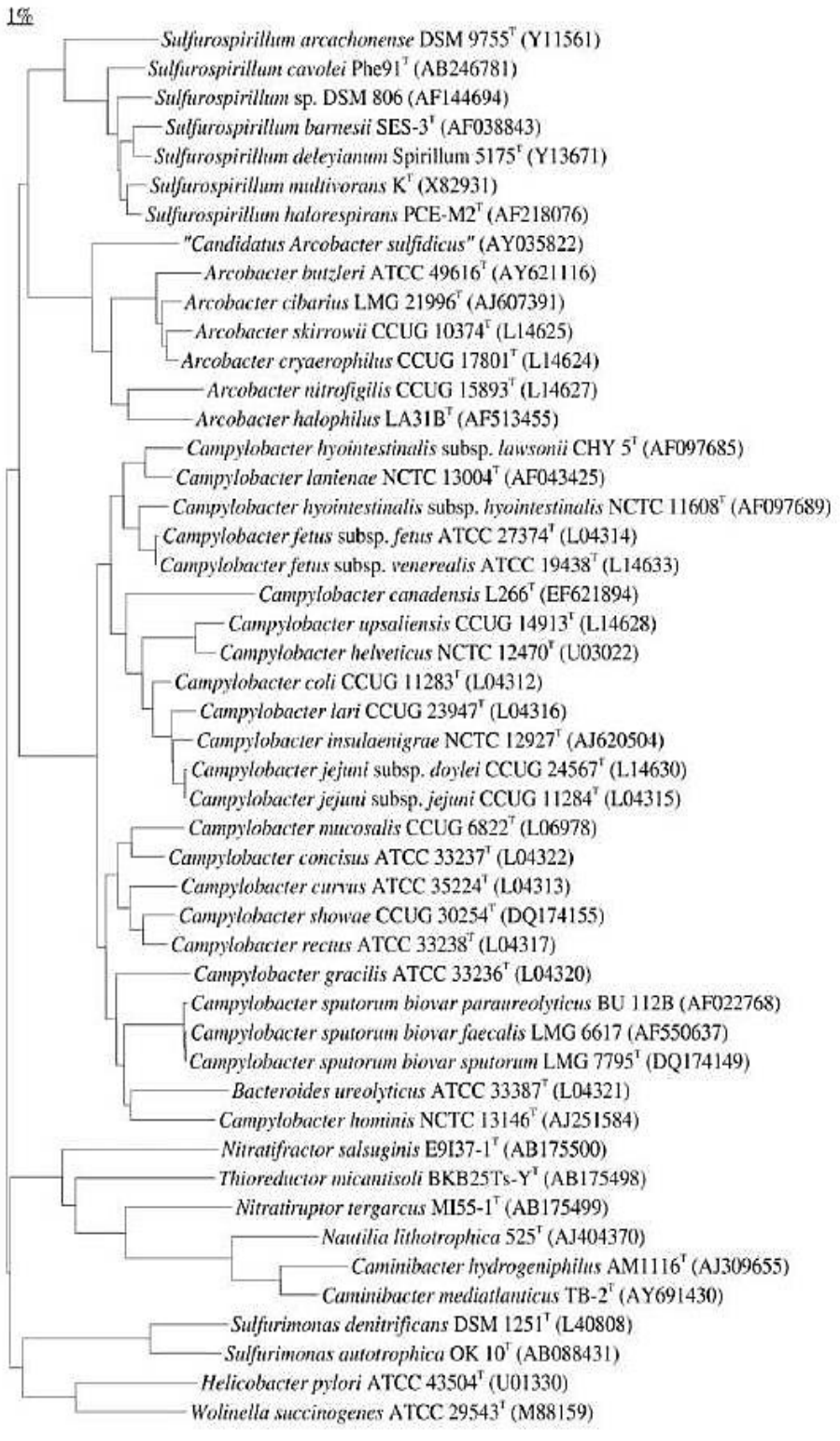


Figure 1-1 Phylogenetic relationship of the *Campylobacteraceae* family based on 16S rRNA sequencing.

(Reproduced from Debruyne et al., 2008)

1.1.3 Physiology

Campylobacters are Gram-negative nonspore-formers that possess a low G+C content (27-47%) and require temperatures ranging from 30-37°C for optimal growth. *Campylobacter* cells are typically curved, S-shaped or spiral rods (0.2 to 0.8 µm wide and 0.5 to 5 µm long) (Vandamme 2000) but some species, such as *C. showae*, produce cells with less curved rods and occasionally *C. jejuni* strains may also produce cells with straight rods (Wassenaar & Newell 2006). *Campylobacter* cells may change to spherical or coccoid forms on entering the late stationary phase of their life cycle or when exposed to environmentally stressful conditions (Ng et al. 1985; Griffiths 1993). *Campylobacter* cells typically display corkscrew-like motility through the use of a single, polar non-sheathed flagellum at one or both ends (Vandamme 2000). However, *C. gracilis* lacks flagella and is therefore non-motile while *C. showae* possesses multiple unipolar flagella (Vandamme & De Ley 1991; Vandamme 2000). *C. jejuni* and *C. coli* are also known to produce spontaneous non-motile variants, especially following frequent subculturing. In *C. jejuni* 81-176, this phenotype has recently been linked to a *motA* gene mutation, which encodes a flagellar motor protein that allows energy to be transmitted to the flagellum (Mohawk et al. 2014).

1.1.4 Growth requirements and biochemical characteristics

Campylobacters generally thrive in microaerophilic environments, requiring low concentrations of oxygen (5-10%) and elevated levels of carbon dioxide (3-5%) for optimal growth. Some species, such as *C. gracilis* and *C. showae*, are capable of growing under anaerobic conditions and they also require H_2

and sometimes formate or fumarate for growth (Wassenaar & Newell 2006). Members of the *Campylobacter* genus are oxidase-positive, with the exception of *C. gracilis*, indole-negative and able to reduce nitrate, except for *C. jejuni* subsp. *doylei*. They are also unable to hydrolyze hippurate, except *C. jejuni* which is known to possess the hippurate hydrolase gene, *hipO*. Campylobacters produce energy through respiration, and because they do not ferment or oxidise carbohydrates, they utilise amino acids and intermediates from the tricarboxylic acid cycle as substrates (Mohammed et al. 2004).

1.1.5 Reservoirs

Campylobacters readily inhabit the intestinal mucosa of many warm-blooded animals (Fhogartaigh & Edgeworth 2009). For instance, *C. coli*, *C. mucosalis* and *C. hyointestinalis* are known to colonise the intestines of pigs (Schultheiss et al. 1989; Young et al. 2000b) while *C. upsaliensis* and *C. helveticus* can be isolated from the intestines of cats and dogs (Mandrell et al. 2005). Campylobacters have also been found to inhabit cold-blooded animals as the strain *C. fetus* was previously isolated from reptiles such as snakes, turtles and lizards (Wang et al. 2013). *C. jejuni* primarily colonises the intestinal mucosa of avian species and appears to have evolved to optimally grow at their internal body temperature of 42°C. *C. jejuni*, *C. coli* and *C. lari* are all recognised as thermophilic *Campylobacter* species because they optimally grow at such a temperature. Campylobacters are considered pathogens when they colonise the intestinal mucosa of mammals (Newell 2001) but in comparison *C. jejuni* and *C. coli* typically behave as commensals in the avian gut (Waldenstrom et al. 2002), with a few strain-specific exceptions (Knudsen et al. 2006) and the

exception of ostriches, where outbreaks of enteritis and avian hepatitis have been reported following infection (van Der Walt et al. 1996; Stephens et al. 1998). More recently, it was also reported that a fast-growing commercial breed of broiler chickens exhibited a strong inflammatory response to *C. jejuni* infection (Humphrey et al. 2014). This response led to intestinal mucosal damage and diarrhoea, which in turn led to subsequent damage of the feet and legs of the chickens as a result of standing on wet litter. It was determined that the disease occurred as a consequence of poor regulation of the inflammatory immune response because the affected chickens failed to express the regulatory cytokine, interleukin-10, which controls inflammation (Humphrey et al. 2014).

1.2 PREVALENCE OF *CAMPYLOBACTER* IN POULTRY

Campylobacters have been readily isolated from broiler carcasses and poultry meat (Newell et al. 2001; Cui et al. 2005; Moran et al. 2009; Ridley et al. 2011). Within this genus, *C. jejuni* and *C. coli* are the main species posing major problems for food safety (Adak et al. 2005; Fhogartaigh & Edgeworth 2009). In 2001, a study in the United States looked at 32 broiler flocks on 8 different farms over a one year period (Stern et al. 2001). Up to 87.5% of the flocks were found to be *Campylobacter*-positive, while only 4 flocks (12.5%) remained free of the pathogen throughout the six to eight week rearing period. Similarly, a survey was conducted in Europe from January to December 2008 to investigate the prevalence of *Campylobacter* in broiler flocks and on broiler carcasses immediately following slaughter (EFSA 2010a). A total of 10,132 broiler batches were sampled from 561 slaughterhouses within 26 European Union Member States as well as Norway and Switzerland. *Campylobacter* was

isolated from the pooled caecal contents of 71.2% of the slaughtered broilers and on 75.8% of broiler carcasses, with such samples being collected from the neck and breast skin immediately after chilling (EFSA 2010a). A high prevalence of *Campylobacter* has also been generally associated with non-conventional broiler production, such as in free-range and organic broiler rearing systems (Kazwala et al. 1993; Rivoal et al. 1999; Heuer et al. 2001; Colles et al. 2008; Colles et al. 2011). A study in France found that 85.7% of faecal samples taken from a batch of broiler chickens raised in a free-range system were *Campylobacter*-positive (Rivoal et al. 1999) while another study in Denmark isolated *Campylobacter* spp. from 100% of organic broiler flocks (Heuer et al. 2001). Rivoal et al. (1999) also investigated the molecular diversity of *Campylobacter* isolates collected during rearing and slaughter of free-range broiler chickens and found that poultry carcasses were contaminated with the same *Campylobacter* strains that were present in the caeca of the broilers. The prevalence of antimicrobial resistant *Campylobacter* spp. from conventional and organic production systems was also investigated and it was found that a large number of isolates from the conventional system were resistant to the nine antimicrobial substances tested compared to the organically raised poultry (Luangtongkum et al. 2006). However, El-Shibiny et al. (2005) reported that although organic flocks in the UK contained largely antibiotic-sensitive campylobacters, multiply resistant *Campylobacter* isolates were recovered from free-range flocks which was of concern since use of antimicrobials as growth promoters had been banned in the European Union since 1998.

In temperate countries, *Campylobacter* colonisation of poultry flocks has generally been reported to vary by season and geographical location, with higher infection rates being reported in the summer and autumn months as compared to the winter (Kapperud et al. 1993; Wallace et al. 1997; Refrégier-Petton et al. 2001; Kovats et al. 2005; Rushton et al. 2009). A study in Great Britain investigated the influence of season and geography on the prevalence of *Campylobacter* subtypes in housed broiler flocks between 2004 and 2006 (Jorgensen et al. 2011). They found that prevalence of positive batches was significantly higher in the northern part of Great Britain compared to southern and central areas ($p < 0.001$), and was significantly higher in July ($p = 0.01$), August ($p = 0.005$) and September ($p < 0.001$) compared to other times of the year, being more pronounced in the southern areas. Some countries in the Europe Union showed a similar seasonal pattern of *Campylobacter* prevalence as well, with the highest levels of infection also being experienced during the months of July to September (EFSA 2010b).

Commercial broiler flocks, reared under controlled conditions, have been found to show an age dependent increased risk of *Campylobacter* colonisation. Broiler flocks do not generally become colonised until 2-3 weeks of age (Shane 2000; Evans & Sayers 2000). This period has been termed the "lag phase" and under field conditions very young birds do not appear to become colonised. Protective mechanisms have been considered to explain this observation, which include the presence of maternal antibodies (Sahin et al. 2001), the use of antibiotic feed additives and competition among gut flora (Newell & Wagenaar 2000). It also appears that intact egg shells may be

permeable to *Campylobacter* since it has been possible to experimentally infect eggs by immersion in a suspension of the organism (Neill et al. 1985; Allen & Griffiths 2001). This suggests that contact with faecal material on the outer shell could result in penetration. Earlier studies have also revealed that *C. jejuni* could be isolated from the inner shell and membrane of eggs but not the egg contents (Doyle 1984; Neill et al. 1985). On this basis, vertical transmission of *Campylobacter* from parent flock to progeny has been proposed (Cox et al. 2012) but was previously thought to be a very rare event (Sahin et al. 2003; Fonseca et al. 2006) and remains a highly controversial issue amongst researchers (Newell & Fearnley 2003). On the other hand, the role that horizontal transmission plays in the spread of *Campylobacter* within flocks has been demonstrated. Once a flock becomes colonised, bird to bird transmission rapidly occurs and up to 100% of the flock can be colonised within a few days (Shreeve et al. 2000; van Gerwe et al. 2009).

Various transmission routes have been suggested within the environment of the broiler house. These include aerosols caused by poor air-ventilation systems that circulate air from potentially contaminated areas as well as transmission from wildlife that occupy the surroundings of the broiler house such as rodents, insects, livestock and domestic pets (Moreno et al. 1993; Newell & Fearnley 2003; Bull et al. 2006). However, commercial broilers within the confined space of the broiler house are also coprophagic and therefore the faecal-oral route is probably the main route of transmission within an already infected flock. Nevertheless, once colonised, it is unclear the actual length of time broiler flocks remain colonised and how long they are capable of

shedding *Campylobacter* in their faeces; but it is thought to last for the duration of their lifespan, which is typically less than 47 days (Newell & Fearnley 2003).

1.3 HUMAN *CAMPYLOBACTER* INFECTIONS

1.3.1 Clinical manifestations of infection

Campylobacter is generally recognised as the leading cause of human bacterial gastroenteritis worldwide (Fhogartaigh & Edgeworth 2009), with *C. jejuni* and *C. coli* representing the main sources of infection (Adak et al. 2005; Fhogartaigh & Edgeworth 2009). Infectious doses of *C. jejuni* as low as 500-800 bacteria have been reported to be sufficient to cause illness in healthy adults (Robinson 1981; Black et al. 1988). The mean incubation period following infection in humans is estimated to range from 1 to 7 days (Blaser & Engberg 2008), although this depends on the infectious dose as well as the immune status of the individual. Clinical manifestations of *Campylobacter* infections vary throughout the world, with individuals from industrialized nations usually exhibiting more severe symptoms than those from the developing world, where high rates of asymptomatic carriage are observed (Oberhelman & Taylor 2000). In industrialized countries, symptoms vary from slightly loose stools to more severe mucoid diarrhoea, which may last for three to five days and is sometimes accompanied by blood, along with abdominal pain, cramps and fever (Wassenaar & Blaser 1999). At the peak of the illness, individuals may experience 8 to 10 bowel movements a day (Blaser et al. 1983). In most non-immune individuals, symptoms are usually accompanied by an acute inflammatory response which results in leukocytes and

erythrocytes being recovered from stools (Blaser et al. 1979), even when loose and not exceptionally bloody (Wassenaar & Blaser 1999).

Although most *Campylobacter* infections are self-limiting and last only a few days, complications may arise due to direct spread from the gastrointestinal tract which may lead to local infections such as cholecystitis, pancreatitis, peritonitis as well as gastrointestinal haemorrhaging (Allos 2001). Extraintestinal manifestations of *Campylobacter* infection have also been reported and include toxic hepatitis and myocardial injury (Braun et al. 2008). In addition, bacteraemia is usually detected in less than 1% of patients suffering from campylobacteriosis, which predominantly affects the young, old and immunocompromised (Allos 2001; Nielsen et al. 2009). Severe systemic illness is rare but may lead to sepsis and even death (Allos 2001). In Europe, mortality due to infection was recorded for 2012 at 0.03% of the 111,464 confirmed campylobacteriosis cases, with a total of 20 deaths being reported for the United Kingdom (EFSA 2014b).

A number of post-infectious complications, which are auto-immune in nature, are associated with *Campylobacter* infections including reactive arthritis, Miller Fisher syndrome and most importantly, Guillain-Barré syndrome (GBS). GBS is an acute disorder of the peripheral nervous system and is characterised by progressive motor weakness in more than one limb, which is usually symmetrical and accompanied by the loss of some tendon reflexes (Asbury & Cornblath 1990). The disease is generally self-limiting and patients usually regain their muscle strength within two to three weeks, with partial or

complete recovery taking place over a few months. However, 15-20% of GBS patients may be left with severe long term neurological defects (Nachamkin et al. 1998). *C. jejuni* is a major antecedent infectious agent in GBS development, where evidence from Japanese patients with GBS showed that the infecting *C. jejuni* strains belonged predominately to the O serotype O:19 (Kuroki et al. 1993). Although the risk of developing GBS may increase following exposure to *C. jejuni* type O:19, it has been estimated that if 20% of GBS-associated *C. jejuni* isolates are serotype O:19 then there is a 1 in 158 chance of developing GBS following infection with this particular serotype (Nachamkin et al. 1998). A number of other O serotypes have also been associated with *C. jejuni* strains from GBS patients from other parts of the world, such as the O:41 *C. jejuni* strains in South Africa (Lastovica et al. 1997). It was previously estimated that the chance of developing GBS following a *C. jejuni* infection was approximately 1 in 1000 infections (Nachamkin et al. 1998).

Production of serum anti-ganglioside antibodies is frequently triggered as a result of this infection (Ho et al. 1998). Gangliosides contain one or more sialic acid (N-acylneuraminic) residues linked to an oligosaccharide core and are generally classified by letters according to the number of residues present. For instance, M (-mono), D (-di), T (-tri) and Q (-quad). The ganglioside GM1 is found in peripheral nerve tissue and a previous study found that almost 38% of GBS patients had high titres of anti-GM1antibiodies, which was linked with a poor clinical outcome at 6 and 12 months following the development of GBS (Ilyas et al. 1992). Miller Fisher syndrome, on the other hand, is usually associated with the presence of anti-GQ1b antibodies (Willison & O'Hanlon

1999). Pathogenesis of *Campylobacter*-mediated GBS is thought to involve molecular mimicry between the lipooligosaccharide (LOS) of *C. jejuni* and peripheral nerve gangliosides as a result of the presence of sialic acid in the LOS of *C. jejuni* (Xiang et al. 2006).

1.3.2 Epidemiology

Surveillance of *Campylobacter* infections has shown an increasing trend in reported cases over the past 30 years, with initial increases most likely linked to improved diagnosis. In England and Wales between 1989 and 2011 there were 1,109,406 confirmed cases, with 64,582 of them reported in 2011 alone (Nichols et al. 2012). Within the European Union, *Campylobacter* was also the most commonly reported zoonosis between 2005-2012 with 214,268 confirmed cases in 2012 and an incidence rate of 55.49 cases per 100,000 population (EFSA 2014a). These figures included 72,578 confirmed cases from the United Kingdom for 2012. In addition, two studies conducted within the UK (IID1 in 1993-1996 and IID2 in 2008-2009) that looked at the incidence and etiology of infectious intestinal disease (IID) (Wheeler et al. 1999; Tam et al. 2012), found *Campylobacter* to be the most common bacterial pathogen amongst general practice cases. Similarly, there has been an increased incidence of *Campylobacter* infections within the United States since 2008 with the Foodborne Diseases Active Surveillence Network (FoodNet) reporting an incidence of 14.3 confirmed cases per 100,000 population for 2012, resulting in approximately 1.5 million cases for that year (CDC 2013). This figure remains below the national health objective for 2020 which is aiming for no more than 8.5 cases per 100,000 population. *Campylobacter*

infections exhibited the second highest incidence rate of all pathogens reported to FoodNet after *Salmonella* (16.42 per 100,000). In comparison, *Salmonella* was the second highest zoonotic agent to be reported in the EU with 22.2 cases per 100,000 population (EFSA 2014a). This may be linked to the success of control measures for *Salmonella*, including programmes to control *Salmonella* in domestic fowl (*Gallus gallus*) populations which has resulted in the reduced occurrence of this pathogen in eggs (EFSA 2014a). Ultimately the incidence of occurrence of *Campylobacter* infections as well as other zoonoses is generally linked to the number of laboratory-confirmed cases, however under-reporting and under-ascertainment of infections is a major issue for most countries (Gibbons et al. 2014). The true incidence of infection may actually be up to 7-8 times higher than documented case numbers.

The first documented case of a campylobacteriosis outbreak directly linked to consumption of chicken meat was amongst a group of soldiers in the Netherlands, where 72% of 123 soldiers were infected (Brouwer et al. 1979). Symptoms included fever, vomiting, abdominal cramps and diarrhoea, which were accompanied with blood in four of the cases. A number of recent *Campylobacter* outbreaks associated with different food sources have been documented (Black et al. 2006; Inns et al. 2010; Gardner et al. 2011). However the majority of human campylobacteriosis cases are not part of recognised outbreaks and exhibit different epidemiological features (Tauxe 1992). For instance, in temperate countries there is a strong seasonal trend of *Campylobacter* infections, with outbreaks usually occurring during spring and autumn months (Tauxe 1992) while sporadic cases are typically seen during

the summer (Altekruse et al. 1999). Over the past three decades, several case-control studies have been conducted throughout many developed countries to identify risk factors associated with sporadic illnesses. The findings of such studies were generally consistent, indicating a major source of infection was either the handling of raw poultry (Hopkins & Scott 1983) or consumption of undercooked poultry and poultry products (Kapperud et al. 1992; Eberhart-Phillips et al. 1997; Neimann et al. 2003; Friedman et al. 2004). Other important risk factors included consumption of raw or unpasteurised milk (Hopkins et al. 1984; Neimann et al. 2003), foreign travel (Norkrans & Svedhem 1982; Schorr et al. 1994; Eberhart-Phillips et al. 1997; Neimann et al. 2003) and transmission from pets and domestic animals (Norkrans & Svedhem 1982; Hopkins et al. 1984; Eberhart-Phillips et al. 1997; Neimann et al. 2003; Friedman et al. 2004). However, the population attributable fraction determined for all of these variables combined typically does exceed more than 50%.

1.3.3 Treatment of *C. jejuni* infections

Initial treatment of symptoms of campylobacteriosis generally includes rehydration and electrolyte replacement. Primarily, an oral glucose or starch-based electrolyte solution would be recommended, however if the patient is comatose or severely dehydrated then fluids would be administered intravenously (Guerrant et al. 2001; Thielman & Guerrant 2004). Although *Campylobacter* infections are usually self-limiting, some patients may exhibit high fever or bloody diarrhoea, may experience more than 8 bowel movements in 24 hours or their symptoms may persist for more than 1 week following

diagnosis. In such cases, as with cases with immunocompromised individuals, antimicrobial treatment has been shown to reduce the duration of the illness and shedding of *Campylobacter* in the faeces (Ternhag et al. 2007). Erythromycin has been found to be particularly effective against the disease in children if administered early in the course of illness (Anders et al. 1982; Pai et al. 1983; Salazar-Lindo et al. 1986) while other macrolide antibiotics as well as quinolones such as ciprofloxacin are usually prescribed for adults (Dryden et al. 1996).

1.3.4 Antimicrobial resistance

A major problem associated with using antibiotics to treat illness is the development of resistant bacterial strains. The emergence of quinolone-resistant and, to a lesser extent, macrolide-resistant *Campylobacter* strains in the food chain has become a major concern worldwide as these strains impact on clinical management of campylobacteriosis (Engberg et al. 2001). High rates of resistance of *Campylobacter* to antibiotics such as tetracycline, amoxicillin, ampicillin, metronidazole and the cephalosporins have previously been observed, while resistance to erythromycin has been low despite several years of use (Allos 2001). Patients infected with antibiotic-resistant strains have been found to experience prolonged illness (Smith et al. 1999) and this may present adverse implications for immunocompromised individuals. In Europe, quinolone resistance was observed as early as the 1990's when Endtz et al. (1991) suggested that the emergence of quinolone-resistant *C. jejuni* and *C. coli* human isolates coincided with the increasing use of fluoroquinolones, such as ciprofloxacin and enrofloxacin, in human and veterinary medicine.

They screened human and poultry *Campylobacter* isolates from 1982-1989 for quinolone resistance and found that prevalence of resistant strains increased over time from both sources. The presence of fluoroquinolone-resistant *Campylobacter* strains in food animals is now recognised as an important public health issue (Nelson et al. 2007), especially in developing countries where antibiotic use may not be as strictly monitored. *Campylobacter* fluoroquinolone resistance often occurs due to mutations in the genes encoding DNA gyrase (*gyrA*) at positions Thr-86, Asp-90 and Ala-70 (Griggs et al. 2005).

Antimicrobial resistance in *Campylobacter* isolates from human, animals and food was evaluated in the EU for 2012, with data submitted from 26 EU member states (EFSA 2014b). High levels of resistance to ampicillin, ciprofloxacin, nalidixic acid and tetracyclines were observed for *Campylobacter* isolates of human origin. However, resistance to erythromycin in human *C. jejuni* isolates was generally low but moderately high for isolates of *C. coli*. Very high levels of resistance to ciprofloxacin were also observed in human *Campylobacter* isolates, with co-resistance to ciprofloxacin and erythromycin being reported for almost one in six *C. coli* isolates. The prevalence of resistance to ciprofloxacin, erythromycin, gentamicin, nalidixic acid and tetracyclines was also evaluated for *Campylobacter* isolates from poultry and poultry meat. In general, relatively high levels of resistance to ciprofloxacin, nalidixic acid and the tetracyclines was observed in *Campylobacter* isolates from poultry and poultry meat, whilst low levels were reported for erythromycin and gentamicin.

In 2005, the US Food and Drug Administration (FDA) banned the use of enrofloxacin in poultry production (FDA 2005). Studies were later carried out to evaluate the post-ban *Campylobacter* resistance to fluoroquinolones in poultry products. One such study was a year-long project carried out in Louisiana, which started in October 2006 and compared *Campylobacter* isolates from retail conventionally raised and organic chickens (Han et al. 2009). They looked at resistance to the four antibiotics tetracycline, erythromycin, ciprofloxacin and gentamicin. Tetracycline was the antibiotic most isolates were resistant to (31.5%), followed by erythromycin (20%). Significantly higher rates of resistance to erythromycin were observed in conventional chicken isolates compared to those from organic chickens. However, the results generally showed low resistance rates to ciprofloxacin, with all isolates from organic chickens being susceptible to this antibiotic compared to 8.5% of isolates from the conventional chickens being resistant. No isolates were found to be co-resistant to both ciprofloxacin and erythromycin. In comparison, another study in Maryland looked at the prevalence of fluoroquinolone-resistant campylobacters in poultry products from two conventional producers who announced they had stopped using fluoroquinolones from 2002 (Price et al. 2007). The results however showed no significant change in the amount of fluoroquinolone-resistant *Campylobacter* isolates from the two producers throughout the duration of the study. They also compared the rates of fluoroquinolone-resistance of *Campylobacter* spp. isolated from the conventional poultry products compared to those from antibiotic-free producers. They found that the isolates from the two conventional producers were significantly more likely to be resistant to

fluoroquinolones than the antibiotic-free isolates. Although these two US studies had different outcomes, they give an idea of fluoroquinolone-resistance rates of *Campylobacter* spp. immediately following the ban in 2005. However, the long-term effects of ceasing the use of fluoroquinolones in US poultry production will have to be determined over time.

1.4 PATHOGENESIS OF *C. JEJUNI*

The pathogenic mechanisms by which *C. jejuni* infections occur in humans remain poorly understood despite the availability of a wealth of genomic information from several different *Campylobacter* strains. A major reason for this is the lack of appropriate animal disease models which efficiently mimic infection in humans (Newell 2001). However, experimental *Campylobacter* infection of primates has been found to very closely simulate the disease and immune response of humans (Fitzgeorge et al. 1981; Russell et al. 1989; Russell et al. 1993; Islam et al. 2006), particularly the New World monkey *Aotus nancymae* (Jones et al. 2006). Despite this, the cost of such experimentation is quite excessive, making it unavailable to most research institutions and there have always been ethical concerns surrounding such types of research. Nevertheless, using the available animal models, such as Toll-like receptor (TLR)-deficient mice, ferrets and wax moth larvae, several putative virulence factors involved in pathogenesis of human campylobacteriosis have been previously described (Ketley 1997; van Vliet & Ketley 2001; Konkel et al. 2001; Young et al. 2007; Dasti et al. 2010). In addition, the complete genome sequence of *C. jejuni* NCTC 11168 (Parkhill et al. 2000) earlier revealed that many classical virulence factors, known to be

key for other enteropathogens, were not present. For instance, no virulence-associated pathogenicity island was found in NCTC 11168 (Parkhill et al. 2000). However several genes with homopolymeric G-tracts, which can be prone to phase variation through slipped-strand mispairing, were identified. In spite of this, the role that phase variation plays in pathogenesis of *Campylobacter* has yet to be determined.

Following exposure to *C. jejuni*, the lower intestinal tract of the gut (ileum, jejunum and colon) becomes colonised. Expression of a number of putative virulence determinants are predicted to be necessary for intestinal colonisation (see Figure 1-2), including those for chemotaxis and motility (Ketley 1997). Once colonisation occurs, other potential factors needed for virulence, including adhesion and invasion of host epithelial cells, toxin production, iron acquisition as well as oxidative, nitrosative and thermal stress defence mechanisms are necessary (Ketley 1997; van Vliet & Ketley 2001).

1.4.1 Chemotaxis and motility

Chemotaxis and flagellar motility are important for effective colonisation of the intestines as it is first necessary to penetrate the mucus layer of the intestinal cells. Chemotaxis is the ability of motile bacteria to sense and respond to specific chemical attractants. Several chemoattractants have been determined for *C. jejuni* including many constituents of mucin, which is a key component of mucus, such as L-fucose, L-glutamate, L-serine, pyruvate and succinate while many bile acids were found to act as chemorepellents (Hugdahl et al. 1988). The importance of chemotaxis in colonisation has been

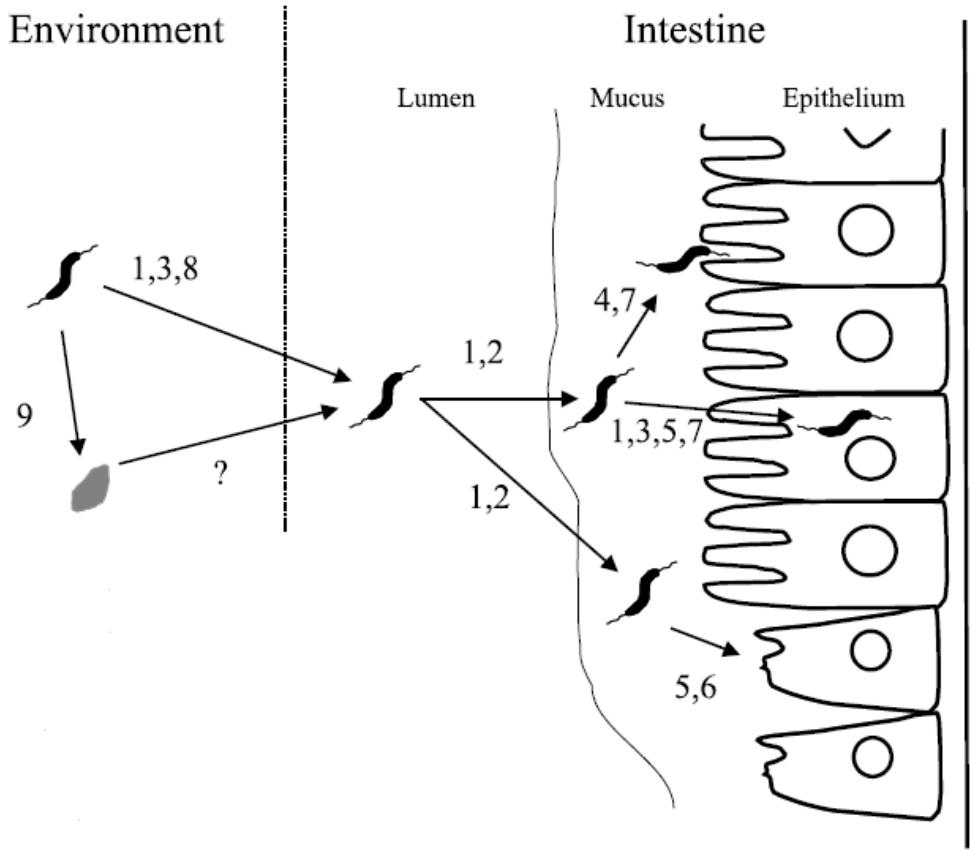


Figure 1-2. The different phases of *Campylobacter* intestinal colonisation.
The numbers represent putative *C. jejuni* virulence determinants and the different phase (s) where each is thought to be expressed; 1 – motility, 2 – chemotaxis, 3 – oxidative stress defence, 4 – adhesion, 5 – invasion, 6 – toxin production, 7 – iron acquisition, 8 – heat stress response and 9 – coccoid dormant stage.

Legend: - epithelial cell, - viable *Campylobacter* cell, - dormant coccoid *Campylobacter* cell. Reproduced from van Vliet & Ketley (2001).

Proven with non-chemotactic *C. jejuni* mutants isolated by chemical mutagenesis, which failed to colonise the intestinal tract of suckling mice (Takata et al. 1992).

Orthologues of the chemotaxis genes *cheA*, *cheW*, *cheV*, *cheY*, *cheB* and *cheR* have been identified in the genome sequence of *C. jejuni* (Parkhill et al. 2000) but their roles in chemotaxis, with the exception of *cheY*, *cheB* and *cheR*, have not been characterised. It has been reported that *C. jejuni* strains that lacked or over-expressed CheY, which is a response regulator that controls the direction of flagellar rotation when phosphorylated by CheA (Marchant et al. 2002), resulted in decreased virulence in a ferret disease model (Yao et al. 1997). The *cheA* and *cheV* genes also encode polypeptides that contain response regulator domains. The *cheB* and *cheR* genes, which respectively encode a methylesterase and methyltransferase, were determined to be arranged in an operon and have a putative role in the adaptation of *C. jejuni* to changes in the chemical gradient of their swimming environment (Kanungpean et al. 2011). Motility swarming assays were conducted to examine the chemotactic behaviour of deletion mutants of *cheB*, *cheR* and *cheBR*. The results showed significantly decreased swarm sizes compared to the wild-type, which suggested that the CheB and CheR proteins influence, but are not essential, for chemotaxis to occur in *C. jejuni* (Kanungpean et al. 2011). In addition, *in vitro* analyses using human intestinal INT-407 cell cultures revealed that a *cheR* deletion mutant was hyperadherent and hyperinvasive to these cells while a *cheB* mutation resulted in a non-adherent and non-invasive phenotype. *In vivo* experiments furthermore revealed that CheB and CheR are required for

colonisation of chickens as a *cheBR* mutant showed a reduced ability to colonise the caecum of 1-day old chicks compared to the wild-type strain.

C. jejuni also possesses ten putative chemoreceptors (designated Transducer-like proteins, Tlps), some of which show homology to the methyl-accepting chemotaxis proteins (MCPs) in *E. coli*. They are responsible for sensing chemoattractants in the environment and *C. jejuni* mutants lacking two of them, either *cj0019c* (DocB) or *cj0262c*, showed decreased colonisation of the gastrointestinal tract of 1-day old chickens, but the chemoattractants were unknown (Hendrixson & DiRita 2004). More recently, the *C. jejuni* group A Tlp receptor, Tlp3 (*cj1564*), was found to be a multi-ligand binding chemoreceptor that has several functions including recognition of the chemoattractants isoleucine, purine, malic acid and fumaric acid as well as the chemorepellants lysine, glucosamine, succinic acid, arginine and thiamine (Rahman et al. 2014). An isogenic mutant of *cj1564* showed altered morphology, reduced chemotactic motility but increased autoagglutination and biofilm formation (Rahman et al. 2014). In addition, the *tlp3* mutation reduced the ability of *C. jejuni* to adhere to and invade human intestinal Caco-2 cells but caused no change in its ability to colonise one day old chickens (Rahman et al. 2014).

Campylobacters are motile through the presence of a single flagellum, at one or both cellular poles, which provides their typical corkscrew-like morphology. The importance of the flagella in colonisation has been demonstrated using flagellar mutants which were unable to colonise the gut of chickens

(Nachamkin et al. 1993; Wassenaar et al. 1993), mice (Morooka et al. 1985; Newell & Mcbride 1985), rabbits (Caldwell et al. 1985; Pavlovskis et al. 1991) and hamsters (Aguero-Rosenfeld et al. 1990). Flagellar motility was also found to be important in causing disease in humans (Black et al. 1988). Furthermore, the importance of flagella in adhesion and invasion of host cells *in vitro* has been shown for flagellar mutants of *C. jejuni* 81176 that lost their ability to adhere to and invade human INT 407 cells (Wassenaar et al. 1991; Yao et al. 1994). The flagellum of *C. jejuni* is unsheathed and is composed mainly of flagellins, which are encoded by the genes *flaA* and *flaB* (Nuijten et al. 1990). The two flagellin genes show 95% sequence similarity but are expressed independently from their own promoters. The *flaA* gene is expressed at much greater levels than *flaB* and is regulated by σ^{28} (*fliA*), whilst σ^{54} (*rpoN*) controls expression of *flaB* (Guerry et al. 1991; Hendrixson et al. 2001; Jagannathan et al. 2001; Hendrixson & Dirita 2003; Carrillo et al. 2004). Inactivation of the flagellin genes has been found to affect motility in different ways. A *flaA*- *flaB*+ mutant resulted in formation of a truncated flagellum which led to a non-motile phenotype while a *flaA*+ *flaB*- mutant possessed full-length flagella and remained motile (Guerry et al. 1991; Wassenaar et al. 1991; Wassenaar et al. 1994).

1.4.2 Adhesion and invasion

Adherence and invasion of eukaryotic cells is an important feature of *C. jejuni* pathogenesis. In order to colonise hosts, microorganisms generally require adhesion and binding factors, including surface appendages such as pili which are found on the surfaces of many Gram-negative and Gram-positive bacteria.

However, the genome of *C. jejuni* does not appear to possess genes encoding homologues for obvious pilus-like structures and the organism has been shown not to produce such structures (Gaynor et al. 2001; Wiesner et al. 2003). Nevertheless, components of type II secretion systems which function in pilus biogenesis and assembly have been identified and are similar to systems used in assembly of type IV pili in organisms such as *Vibrio cholera* and *Neisseria gonorrhoeae* (Wiesner et al. 2003). Such components have been found to play a role in natural transformation of *C. jejuni* (Wiesner et al. 2003).

In order to characterise the interaction of *C. jejuni* with eukaryotic cells, a number of studies have used models with human cells; for example human laryngeal carcinoma (HEp-2), human cervical cancer (HeLa) and human epithelial kidney (HEK-293) cell lines. However, cell lines that mimic the conditions expected to be encountered *in vivo*, such as human intestinal epithelial (INT 407) and human colon (Caco2 or HCA-7) cells, have been more extensively used. As a result, a number of putative adhesion and binding factors have been found despite the absence of identifiable pili or pilus-like organelles in *C. jejuni*. These include CadF (Konkel et al. 1997), JlpA (Jin et al. 2001) and PEB1 (Pei & Blaser 1993). CadF is an outer membrane protein which facilitates binding of *Campylobacter* to fibronectin, a component of the extracellular matrix. Studies have shown that CadF is required for maximal adherence and invasion of INT 407 cells *in vitro* (Monteville et al. 2003) as well as for the efficient colonisation of infant leghorn chickens (Ziprin et al. 1999). JlpA is a surface-exposed liproprotein which has been found to be important in binding of *C. jejuni* to HEp-2 epithelial cells *in vitro* as adherence

of insertion and deletion *jlpA* mutants to HEp-2 cells was reduced compared to the wild-type strain (Jin et al. 2001). The periplasmic cell-binding protein PEB1 plays a role in *C. jejuni* adherence to HeLa cells *in vitro* (Kervella et al. 1993; Pei et al. 1998). A mouse challenge study also found that *peb1* mutants showed significantly reduced rates and duration of intestinal colonisation of mice compared to the wild-type (Pei et al. 1998).

The invasive ability of *C. jejuni* has been demonstrated *in vitro* using various human cell lines (Wassenaar et al. 1991; Grant et al. 1993; Szymanski et al. 1995; Pei et al. 1998) as well as *in vivo* using animal models of *Campylobacter* enteritis. Some of the experimentally challenged animals used were infant chickens (Ruiz-Palacios et al. 1981; Sanyal et al. 1984; Welkos 1984), infant mice (Newell & Pearson 1984; Yanagawa et al. 1994), newborn piglets (Babakhani et al. 1993) and infant monkeys (Russell et al. 1993). In addition, there is strong evidence to suggest that *Campylobacter* invades intestinal epithelial cells of humans as intracellular bacteria have been recovered from patients with colitis (van Spreeuwel et al. 1985). Another study found that there was a significant difference in the level of invasion between isolates from patients with *Campylobacter* colitis compared to those with non-inflammatory diarrhoea (Everest et al. 1992). Eighty-six percent of the colitis strains were also able to translocate across Caco-2 cell monolayers compared to 48% of isolates from patients with non-inflammatory diarrhoea.

A previous study found that during co-cultivation with INT 407 cells or in an INT 407 cell-conditioned medium, *C. jejuni* secreted at least eight proteins into

the culture medium (Konkel et al. 1999). These secreted proteins are collectively known as *Campylobacter* invasion antigens (Cia), and include the 73-kDa protein CiaB. Their precise role in the invasion process is still to be determined. However, mutation of the *ciaB* gene in *C. jejuni* significantly reduced the number of cells which were internalised, compared to the wild-type, and resulted in failure to secrete the other proteins (Konkel et al. 1999). It was earlier suggested that CiaB may belong to a type III secretion system (Konkel et al. 1999) but no type III system has been found in the *C. jejuni* genome. Nevertheless, it was later demonstrated that CiaB and other secreted proteins require a functional flagellar export apparatus for their secretion (Konkel et al. 2004). More recently, a functional type VI secretion system was identified in *C. jejuni* but the genes encoding this system were only found to be present in 10% of the *C. jejuni* isolates tested, all of which contained the CJIE3 integrative element (Bleumink-Pluym et al. 2013).

1.4.3 Toxin production

Toxins have been proposed as important virulence factors but their role in pathogenesis of *Campylobacter* enteritis remains unclear. Many cytotoxins and enterotoxins in campylobacters have been described (Wassenaar 1997) but to date cytolethal distending toxin (CDT) is the only toxin to be well characterised (Pickett et al. 1996; Whitehouse et al. 1998). CDT is produced by many *Campylobacter* species, including *C. jejuni*, *C. coli*, *C. lari*, *C fetus* and *C. upsaliensis* and was found to be cytolethal to CHO (Chinese hamster ovary), Vero, HeLa, HEp-2 and Caco2 cell lines (Johnson & Lior 1988; Whitehouse et al. 1998). The toxic effect on sensitive cells was characterised

by progressive cell elongation, swelling and eventual death. It was however found that not all cell types were sensitive to CDT since mouse Y-1 adrenal cells were unaffected (Johnson & Lior 1988). This toxin can cause a variety of mammalian cells to become irreversibly blocked in the G2 phase of the cell cycle due to failure to activate CDC2 kinase, which is required for transition into mitosis (Whitehouse et al. 1998). CDT is also produced by a number of other bacterial species including *E. coli* (Johnson & Lior 1987; Scott & Kaper 1994), *Shigella* spp. (Okuda et al. 1997), *Haemopilus ducreyi* (Gelfanova et al. 1999) and *Helicobacter hepaticus* (Young et al. 2000a). In *C. jejuni* and other Gram-negative bacteria, toxin delivery can be mediated by outer membrane vesicles (OMVs), which have been found to aid in the release of CDT into the surrounding environment by *C. jejuni* 81-176 (Lindmark et al. 2009).

The function of three structural genes, *cdtA*, *cdtB* and *cdtC*, is necessary for CDT cytotoxicity (Pickett et al. 1996; Pickett & Whitehouse 1999). It has been determined that CdtB is the enzymatically active subunit of the toxin (Pickett & Whitehouse 1999). The functions of CdtA and CdtC are still unclear but it has been demonstrated that when invidual Cdt components were purified and applied to cells, no toxic activity was observed (Lara-Tejero & Galan 2001). However when applied together they interacted to form a fully active tripartite complex (Lara-Tejero & Galan 2001). It was therefore proposed that CdtA and CdtC act as a heterodimeric B subunit and may be involved in the delivery of CdtB into eukaryotic cells (Lara-Tejero & Galan 2001). The role of CDT in *C. jejuni* pathogenesis was also tested using isogenic *cdtB* mutants (Purdy et al. 2000). HeLa cytoxicity assays showed that CDT activity was either greatly

reduced or not detected in the mutants whilst their colonisation ability during an intragastric challenge of adult severe combined immunodeficient (SCID) mice was unaffected by the mutation (Purdy et al. 2000). However, the results also showed that the *C. jejuni cdtB* mutants invaded blood, spleen and liver tissue of the SCID mice less readily than wild-type bacteria, therefore suggesting a potential role for CDT in invasion of host tissues.

1.4.4 Iron acquisition

The ability of campylobacters to acquire iron within their animal host is known to be a key step for infection. Free iron usually has very limited availability as it is complexed with iron-binding and transport proteins such as haemoglobin, transferrin (in serum) and lactoferrin (in mucosal secretions) (Andrews et al. 2003). This iron limited environment acts as a non-specific host defence mechanism against pathogenic bacteria, where a free iron concentration of around 10^{-18} M is maintained, whilst at least 10^{-7} to 10^{-5} M is generally required for optimal bacterial growth (Andrews et al. 2003). In order to overcome this limitation, many bacterial pathogens have evolved a number of mechanisms to enable them to scavenge iron from their extracellular environment, including the use of small iron-chelating compounds known as siderophores. However, siderophore biosynthesis genes have not yet been identified within genome sequences of *Campylobacter* strains (Parkhill et al. 2000; Chaudhuri & Pallen 2006). Although, Field et al. (1986) found that 7 of 26 *C. jejuni* human isolates tested produced detectable amounts of siderophores when grown under iron-limited conditions and that three of the strains were also able to utilize exogenously supplied enterochelin and

ferrichrome, which are known to be produced by *E. coli* and certain soil fungi respectively. *C. jejuni* was also found to be able to utilize haem-containing compounds (Pickett et al. 1992). Genes encoding enterochelin (*ceuBCDE*) (Richardson & Park 1995), ferrichrome (c*fhuABD*) (Galindo et al. 2001) and haemin/haemoglobin (*chuABCDZ*) (Rock et al. 1999) have been characterised in *Campylobacter* and were demonstrated to be involved in iron acquisition.

Iron can exist in either ferrous (Fe^{2+}) or ferric (Fe^{3+}) states, and depending on the pH and redox conditions of the surrounding environment, soluble ferrous iron can be rapidly oxidised to the insoluble ferric iron. Iron in the ferrous state can easily diffuse through outer-membrane porins (size limit ~600 Da) but Fe^{3+}-siderophore complexes are too large to pass through. Therefore the ferric iron acquisition system of Gram-negative bacteria is generally comprised of an outer-membrane (OM) receptor protein, which specifically binds to cognate ferri-siderophore or free iron and transports it through the OM to a periplasmic binding protein and subsequently to a cytoplasmic membrane (CM) ABC transporter, consisting of a permease and an ATP-binding protein. Transport of ferri-siderophores through OM receptors relies on energy transduced through the TonB-ExbB-ExbD protein complex, while ATP is hydrolysed to provide energy for transport across the IM into the cytoplasm (Braun et al. 1998).

C. jejuni typically acquires iron from ferri-enterochelin through the CfrA OM receptor, which shows high homology to a number of other ferric siderophore receptors (Palyada et al. 2004). The *cfrA* gene was previously found to be iron regulated (Guerry et al. 1997) and mutation of this gene in *C. jejuni* NCTC

11168 prevented growth when ferri-enterochelin was provided as the only source of iron (Palyada et al. 2004). Another ferri-enterochelin receptor, designated CfrB, was identified and characterised in *Campylobacter* (Xu et al. 2010). It is a homolog of the Cj0444 protein in *C. jejuni* NCTC 11168 (annotated as a pseudogene) and was found to be important in colonisation of chickens by both *C. jejuni* and *C. coli* (Xu et al. 2010). Most strains of *C. jejuni* that produced the CfrB receptor protein did not produce CfrA, whilst *C. coli* strains were found to widely produce both CfrA and CfrB but not CfrB alone (Xu et al. 2010).

The enterochelin transport system encoded by *ceuBCDE* is similar in *C. jejuni* and *C. coli*, with *ceuB* and *ceuC* encoding integral membrane proteins, *ceuD* an ATPase-binding protein and *ceuE* a periplasmic-binding lipoprotein (Parkhill et al. 2000; Palyada et al. 2004). The importance of enterochelin uptake in gut colonisation was demonstrated *in vivo* as *cfrA* and c*euE* mutants of *C. jejuni* NCTC 11168 were unable to colonise the gastrointestinal tract of chickens (Palyada et al. 2004).

A ferrichrome uptake operon was identified in *C. jejuni* strain M129, encoding proteins with homology to the ferrichrome uptake systems of *E. coli* and *Pseudomonas aeruginosa* (Galindo et al. 2001). The operon is comprised of the genes c*fhuABD* and has not been identified so far in any of the sequenced *Campylobacter* genomes (Chaudhuri & Pallen 2006). In *E. coli* the *fhuA* gene encodes an outer membrane receptor, *fhuB* a permease and *fhuD* a periplasmic binding protein. CfhuA was found not to be uniformly present in eleven *C.*

jejuni strains tested as Southern blot analysis showed that only six of them carried the *fhuA* homologue (Galindo et al. 2001). Under iron-limited conditions, *C. jejuni* strain M129 grew best when ferrichrome was used as an iron source compared to other sources (Galindo et al. 2001). However, the role of CfhuA in ferrichrome uptake is still unclear, along with the role of *cfhuABD* in *C. jejuni* pathogenesis.

The *cj1658-cj1663* locus of the *C. jejuni* NCTC 11168 genome is involved in iron uptake associated with rhodotorulic acid, which is a fungal hydroxamate siderophore that was previously isolated and characterised (Atkin & Neilands 1968). A 19-kDa periplasmic protein (*cj1659*) of *C. jejuni* designated *p19* was previously determined to be iron regulated (van Vliet et al. 1998). The gene directly upstream of *p19, cj1658,* encodes a putative membrane-associated protein but does not resemble a Ton-B dependent receptor. However, the genes *cj1661-cj1663* appear to encode an IM ABC transporter system and homologues of *cj1658* and *p19* can be found in a *Yersinia pestis* iron-uptake pathogenicity island (Carniel 2001). Mutation of *cj1658* and *p19* in *C. jejuni* NCTC 11168 affected the ability of this strain to utilize ferri-rhodotorulic acid for growth (Stintzi et al. 2008).

In the Enterobacteriaceae, uptake of ferrous iron is mediated by the Feo transport system and is quite different to siderophore-dependent systems. The system is comprised of the two proteins FeoA and FeoB, which lie in an operon with the putative [Fe-S] transcriptional repressor FeoC (Cartron et al. 2006). The function of FeoA is unknown but FeoB appears to encode an IM

protein. Homologues of the *E. coli feoB* and *feoA* genes have been identified in the *C. jejuni* NCTC 11168 genome sequence but a *feoC* homologue was not found (Parkhill et al. 2000). Raphael and Joens (2003) reported that FeoB was not required for uptake of ferrous iron in *C. jejuni* and site-directed mutations in *feoB* resulted in no change in $^{55}Fe^{2+}$ uptake. It was subsequently determined that the *feoB* and *feoA* genes form an operon in *C. jejuni* and the role of FeoB in iron acquisition, gut colonisation and intracellular survival in *C. jejuni* was examined (Naikare et al. 2006). It was found that *feoB C. jejuni* mutants were significantly affected in their ability to transport Fe^{2+} and that intracellular iron accumulated within the periplasm compared to the cytoplasm for the wild-type. The *feoB* mutant was also out-competed by the wild-type during colonisation and survival studies using the rabbit ileal loop model as well as during chicken colonisation studies. Infection of the intestinal tract of colostrum-deprived piglets was also significantly reduced in the mutant compared to the wild-type (Naikare et al. 2006). In contrast to the findings of Raphael and Joens (2003), these results therefore not only suggest a role for FeoB in iron acquisition but also in colonisation and pathogenesis.

1.4.5 Oxidative stress defence

Although bacteria need to secure iron for growth and survival within the host, it is necessary for them to ensure that the intracellular supply is safe and non-toxic. However, incomplete reduction of oxygen can generate a mixture of highly reactive intermediates of varying toxicity known as reactive oxygen species (ROS), which include super anions (O_2^-) and peroxides (RO_2). Increased exposure to these intermediates can cause oxidative stress, which

leads to lethal damage of cell membranes, proteins and nucleic acids. Super anions can destroy enzymes containing [4Fe-4S] clusters, causing the release of iron and subsequent inactivation of the enzyme (Fridovich 1995). Released iron can then react with ROS to generate hydroxyl radicals (OH•) through the Haber-Weiss and Fenton reactions (Miller & Britigan 1997) as shown in the equations below:

$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$	Iron reduction (eq. 1)
$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+}\ OH^- + HO\bullet$	Fenton reaction (eq. 2)
$O_2^- + H_2O_2 \longrightarrow HO\bullet + OH^- + O_2$	Haber-Weiss reaction (eq. 3)

Campylobacters possess oxidative stress defence systems mainly against stress from superoxides and peroxides (van Vliet & Ketley 2001). Superoxide dismutases (SODs) catalyse the breakdown of superoxide molecules to hydrogen peroxide and dioxygen (Fridovich 1995). A single iron cofactored SOD, encoded by the *sodB* gene, is expressed by *Campylobacter* and is the key component of stress defence against superoxides (Pesci et al. 1994; Purdy & Park 1994). It was found that *sodB C. jejuni* mutants were significantly less able to invade INT 407 cells *in vitro* (Pesci et al. 1994) while *C. coli sodB* mutants showed decreased colonisation ability during experimental infection of 1-day old chicks (Purdy et al. 1999). Absence of the *sodB* gene also previously affected the ability of *Campylobacter* to survive for prolonged periods in milk and on chicken skin (Purdy et al. 1999) and mutants were also more sensitive to freeze-thaw stress (Stead & Park 2000). It has also been shown that *sodB* mutants have increased sensitivity upon exposure to paraquat,

which is a strong oxidising agent (Garénaux et al. 2008). All of these results therefore suggest that the SodB protein plays an important role in infection, oxidative stress defence and survival of *Campylobacter* in different foods.

Mechanisms for peroxide stress defence mainly rely on the enzymes catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (Grant & Park 1995; Baillon et al. 1999). Catalase catalyses the conversion of hydrogen peroxide to hydrogen and water and can therefore assist in removal of the hydrogen peroxide produced by SOD and other metabolic reactions. Catalase-deficient mutants were found to be more sensitive to exposure to H_2O_2 than the parental strain and had a reduced ability to detoxifying this molecule, as more than 60% of the H_2O_2 remained for the duration of the experiment compared to wild-type cells which reduced the concentration to undetectable levels within 5 minutes (Grant & Park 1995). Interestingly, it was later found that the ability of *katA* mutants to grow and survive in aerobic conditions as well as to colonise an animal model were unaffected by the mutation (Purdy et al. 1999). This may therefore suggest that *katA* is not an important determinant in aerobic survival and optimal colonisation ability of *Campylobacter*. Catalase expression in *C. jejuni* is regulated by the PerR protein and is repressed by iron but induced by H_2O_2 exposure (Park 1999; van Vliet et al. 1999).

Alkyl hydroperoxide reductase, also referred to as thiol-specific antioxidant (Tsa or TsaA), reduces alkyl hydroperoxides to alcohols. *C. jejuni* expresses a homologue of the catalytic AhpC protein but does not contain the flavoprotein AhpF, found in *E. coli* and several other bacteria (Poole et al. 2000). The

importance of AhpC in aerobic survival of *C. jejuni* was demonstrated as an *ahpC* mutant showed significantly reduced aerotolerance compared to the wild-type and was more sensitive to an inducer of oxidative stress (Baillon et al. 1999). However, the *ahpC* mutant was not sensitive to H_2O_2 exposure (Baillon et al. 1999).

The genome of *C. jejuni* contains a number of other peroxidases that may have roles in oxidative stress defence. These include thiol peroxidase (Tpx) and the bacterioferritin comigratory protein (Bcp), both of which are represented by homologues in *C. jejuni* and are encoded in *C. jejuni* NCTC 11168 by *cj0779* and *cj0271* respectively. It was found that Tpx and Bcp are cytoplasmic enzymes, which together play important roles in oxidative and nitrosative stress defence as a *tpx/bcp* double mutant was hypersensitive to exposure to hydrogen peroxide, organic peroxides, superoxide and nitrosative stress agents compared to the wild-type and single mutants (Atack et al. 2008).

1.4.6 Nitrosative stress response

During human infection and pathogenesis, campylobacters encounter elevated levels of nitric oxide (NO) and other agents that cause nitrosative stress. This has previously been demonstrated in patients with infective gastroenteritis, including individuals suffering from campylobacteriosis (Forte et al. 1999; Enocksson et al. 2004). The main sources of NO in human hosts are NO synthases (particularly inducible NO synthase), dietary nitrate, which is reduced to nitrite and then to NO by bacteria, and salivary nitrite, which is reduced to NO through the action of stomach acid (Lundberg et al. 2004). In

response to this form of stress, *C. jejuni* expresses a single-domain haemoglobin (Cgb), which functions in the scavenging and detoxification of NO (Elvers et al. 2004; Lu et al. 2007b). A Cgb-deficient mutant showed increased sensitivity to NO and nitrosative stress as it was hypersensitive to the nitrosating agents *S*-nitrosoglutathione (GSNO) and sodium nitroprusside, and to the nitric oxide–releasing compound spermine NONOate (Elvers et al. 2004). The NO-sensing regulator NssR (nitrosative stress sensing regulator, *cj0466*), which is a member of the Crp-Fnr superfamily of transcription factors, was found to regulate NO-responsive expression of *cgb* (Elvers et al. 2005). *C. jejuni* also expresses a second globin (Ctb), which is a truncated haemoglobin that has also been found to be under the control of NssR (Elvers et al. 2005). The function of Ctb has not been fully established but it has an extremely high affinity for oxygen and has been implicated in oxygen metabolism (Wainwright et al. 2005; Wainwright et al. 2008) and in performing a role in cytochrome c peroxidase or cytochrome P450-like oxygen chemistry (Lu et al. 2007a).

1.4.7 Thermal stress response

Campylobacters are exposed to a range of temperatures including 37°C within human and animal hosts, 42°C within avian reservoirs and around 4°C within food products (water, milk or on poultry meat). The optimal temperature range for growth is between 37-42°C, where temperatures above this trigger a thermal stress response in *C. jejuni* and result in the synthesis of heat shock proteins (HSPs). HSPs are a group of highly conserved proteins that are co-regulated and play an important role in thermotolerance as well as in response

to other environmental stresses. They also participate in normal cell function by acting as chaperones to allow the correct folding of many cellular proteins and the proteolysis of misfolded proteins, which can be deleterious if allowed to accumulate. A number of HSPs have been identified in *C. jejuni* including the proteins GroEL, GroES, DnaJ, DnaK and ClpB (Konkel et al. 1998; Thies et al. 1999a; Thies et al. 1999b; Thies et al. 1999c). However, only DnaJ has been shown to play a role in *C. jejuni* pathogenesis as a *dnaJ* null mutant was unable to colonise newly hatched Leghorn chickens (Konkel et al. 1998). The *dnaJ* mutant also exhibited impaired growth at 46°C, implying the chaperone has a role in thermotolerance (Konkel et al. 1998). Konkel et al. (1998) demonstrated a heat shock response for *C. jejuni* immediately following exposure to temperatures above the optimal range (≥43°C), and found that the synthesis of at least 24 proteins increased in response to this thermal stress. Similarly, another study looked at the global transcriptional response of *C. jejuni* to a temperature increase from 37 to 42°C and found that approximately 20% of the genes in the genome were either significantly up- or down-regulated over a 50 minute period following the temperature increase (Stintzi 2003). Genes encoding chaperones, chaperonins and HSPs, including *groEL*, *groES*, *dnaK*, *dnaJ*, *hspR*, *hcrA* and *clpB*, showed increased levels of expression. The RacRS regulon, which is a two-component regulatory system, has been found to be involved in differential expression of proteins at 37 and 42°C in *C. jejuni* (Brás et al. 1999). Inactivation of the response regulator gene in *C. jejuni*, encoded by *racR*, also resulted in a reduced ability to colonise the intestinal tract of chickens (Brás et al. 1999).

1.5 REGULATION OF *CAMPYLOBACTER* VIRULENCE

1.5.1 Iron-responsive regulation

Iron is an absolute requirement for life but in the presence of oxygen, it is capable of generating toxic and potentially harmful metabolites (see section 1.4.5). Iron acquisition, metabolism and storage are therefore strictly regulated to allow iron homestasis to be carefully balanced. In *C. jejuni* and other Gram-negative bacteria, iron-regulated gene expression is under the control of the transcriptional regulator Fur (ferric uptake regulator). When the intracellular ferrous ion concentration exceeds a threshold level, Fur binds to Fe^{2+} ions and forms a dimeric Fe^{2+}-Fur complex (holo-Fur). This complex recognises specific consensus sequences (Fur box) in promoter regions upstream of iron-regulated genes and represses their transcription by blocking entry of RNA polymerase (Figure 1-3) (Andrews et al. 2003; Butcher et al. 2012). However, when the concentration of intracellular iron becomes low, the Fur complex dissociates (apo-Fur) and no longer binds to the recognition sequences, thus de-repressesing transcription of iron-regulated genes (Figure 1-3) (Andrews et al. 2003; Butcher et al. 2012). The structure of the Fur protein in *C. jejuni* was recently determined and differences were observed in the orientation of the apo-Fur DNA-binding domain compared to other structurally characterised Fur and Fur-like homologues (Butcher et al. 2012). It was found that the *C. jejuni* apo-Fur protein retains a canonical V-shape dimer conformation, and in doing so re-positions the DNA-binding domain to enable DNA interactions that can be modulated by the binding of iron (Butcher et al. 2012).

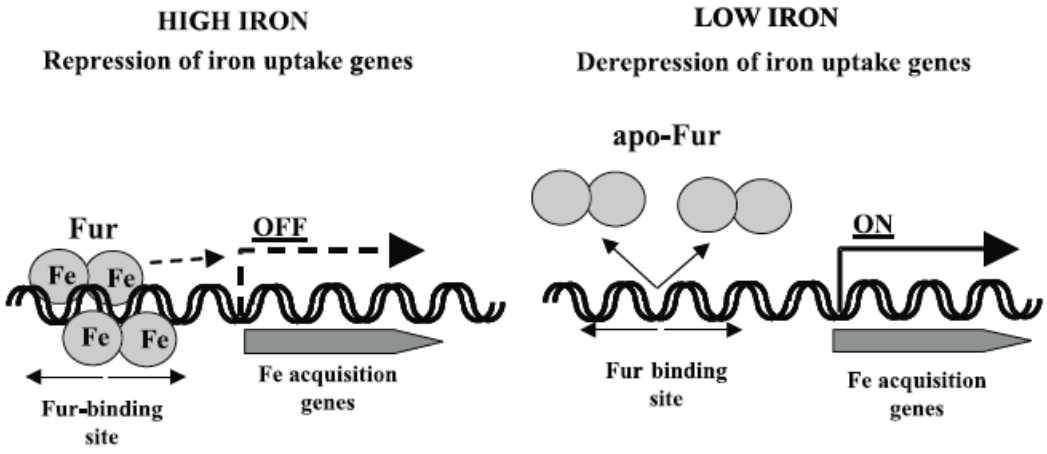


Figure 1-3. Schematic diagram of Fur regulation of iron-regulated genes. Fur represses transcription of iron-responsive genes once it forms a complex with its co-repressor Fe^{2+}, but causes de-repression in the absence of Fe^{2+}. (Reproduced from Andrews et al., 2003)

The *C. jejuni* Fur protein has been previously found to control transcription of at least 60 genes related to iron acquisition, oxidative stress defence, flagellar biogenesis and energy metabolism (Palyada et al. 2004; Holmes et al. 2005). Binding of purified Fur to the promoters upstream of *p19*, *cfrA*, *ceuBCDE*, *chuABCD* and c*j1613* (putative haem oxygenase) has also been demonstrated (Holmes et al. 2005; Ridley et al. 2006). More recently, a chromatin immunoprecipitation and microarray analysis assay (ChIP-chip) was used to define the role of Fur in *C. jejuni* and 95 genomic loci were identified that were bound by Fur regardless of the fold change (Butcher et al. 2012). These Fur binding sites were found to include some in the vicinity of genes encoding proteins involved in metal homeostasis, flagellar biogenesis, energy production and conversation, and stress response (Butcher et al. 2012).

It was previously found that a *C. jejuni fur* mutant was unable to regulate expression of iron acquisition genes, but there was still iron-responsive gene regulation (van Vliet et al. 1998). This suggested the presence of a second iron-response regulator, which was found to be the peroxide stress regulator PerR protein. Fur is known to regulate the genes *katA* and *ahpC*, which are also regulated by PerR (van Vliet et al. 1999). A *perR* mutation in *C. jejuni* caused hyper-resistance to peroxide stress as the *aphC* and *katA* promoters were de-repressed under these conditions, leading to high levels of expression of these genes (van Vliet et al. 1999). In addition, iron-responsive regulation of *aphC* and *katA* completely ceased in a *C. jejuni perR fur* double mutant (van Vliet et al. 1999).

1.5.2 Two-component regulatory systems

Two-component systems (TCSs) provide a stimulus-response mechanism that allows organisms to sense and respond to changes within their environment, mainly through changes in gene expression. TCSs are usually comprised of a membrane-located sensory histidine protein kinase and a cytoplasmic response regulator protein. The histidine kinase senses extracellular stimuli and transfers a phosphoryl group to a conserved aspartate residue on the N-terminal domain of the response regulator, which then interacts with the promoters of target genes and regulates their expression. The genome of *C. jejuni* is regulated by at least 37 putative transcription factors, including 10 response regulators (Wösten et al. 2008). Examples of two-component transduction systems in *C. jejuni* are: FlgS-FlgR which regulates the *fla* regulon and is essential for flagella biogenesis (Wösten et al. 2004); PhosS-PhosR which is activated by phosphate limitation and regulates the *pho* regulon (Wösten et al. 2006); RacR-RacS which is responsive to temperature and is important for colonisation of chickens (Brás et al. 1999); DccR-DccS which is activated in the late stationary phase of bacterial growth and is required for optimal *in vivo* colonisation (MacKichan et al. 2004) and CprR-CprS which is essential for expression of factors that are needed for biofilm formation, planktonic growth and optimal colonisation of chickens (Svensson et al. 2009).

1.6 GENOME SEQUENCE OF *C. JEJUNI* NCTC 11168

The *C. jejuni* NCTC 11168 genome has 1,641,481 base pairs (bp) arranged as a circular chromosome, with a G+C content of 30.6% and the presence of 1,643 putative coding sequences (CDS), including 19 probable pseudogenes

(Parkhill et al. 2000; Gundogdu et al. 2007). The genome generally lacks repetitive DNA sequences, with only four repeated sequences being identified throughout the entire genome, one of them being the ribosomal RNA operon. There are no inserted sequence (IS) elements, transposons, retrons or prophage-associated genes present. Shotgun assembly of the genome illustrated the presence of homopolymeric G:C tracts exhibiting length variations amongst identical clone (Parkhill et al. 2000). Variation in length of poly G:C tracts may be due to slipped-strand mispairing during chromosomal replication (Levinson & Gutman 1987) and is known to mediate phase variation of surface molecules in pathogenic bacteria such as *Haemophilus influenzae* (Hood et al. 1996). Hypervariable sequences were identified in gene clusters mainly responsible for lipooligosaccharide (LOS) biosynthesis (*cj1131c-cj1152*), capsular polysaccharide (CPS) biosynthesis (*cj1413c-cj1448c*) and flagellar modification (*cj1293-cj1342*).

Following publication of the genome sequence of *C. jejuni* NCTC 11168, the sequenced clone (11168-GS) was found to be non-motile, displaying a straight, rod-shaped morphology that invaded and translocated poorly through tissue culture cells, and was a poor coloniser of 1 day old chickens (Ahmed et al. 2002; Gaynor et al. 2004). However, variants exist with more typical phenotypes (Carrillo et al. 2004; Gaynor et al. 2004; Revez et al. 2012). Transcriptional analysis of the sequenced variant compared to the original isolate (11168-O) revealed differences in expression of genes relating to respiration and metabolism, suggesting that adaptation to different oxygen tensions may have contributed to the differences in phenotype (Gaynor et al.

2004). Another study with two variants of NCTC 11168 found differences in their virulence properties, including motility, and suggested these were largely due to changes in expression of σ^{28} and σ^{54}-regulated genes (Carrillo et al. 2004). More recently, *Campylobacter* isolates that were recovered from a diarrheic human and identified as strain NCTC 11168-GSv (American Type Culture Collection (ATCC) genome sequence reference strain) exhibited major genetic changes compared to NCTC 11168-GS (Thomas et al. 2014). These changes were found at 15 loci within the genome of NCTC 11168-GSv and included several insertion/deletion (indel) mutations in genes carrying homopolymeric regions as well as point mutations in two loci. On inoculation of interleukin-10 knockout mice with an isolate of NCTC 11168-GSv from the infected individual, the isolate was found to undergo further genetic variations, with specific mutations occurring at nine loci. These findings therefore illustrate that *C. jejuni* is capable of exhibiting host-specific genetic adaptation.

1.7 BACTERIOPHAGE

Bacteriophages (commonly referred to as phages) are bacterial viruses that infect, replicate and ultimately kill susceptible hosts. An estimated 10^{31} individual virus particles (mostly tailed phage) are thought to be in existence, making them the most abundant biological entities present on the planet (Hendrix et al. 1999). Bacteriophages are ubiquitous and can be found in most environments that support bacterial growth such as seawater, soil and within animal intestines; typically with a phage-to-bacterium ratio of 10:1 or greater. An early study reported on the high abundance of viruses present in marine environments (Bergh et al. 1989) where phage concentrations of up to 2.5 x

10^8 particles/ml have been detected in lake Plussee, Germany and approximately 10^7 phage particles/ml compared to 10^6 cells/ml of bacteria were present in marine samples. These concentrations were found to vary according to the season and geographical location.

1.7.1 Classification

Bacteriophages are classified by the International Committee on Taxonomy of Viruses (ICTV) primarily according to their morphology and nucleic acid content (whether they contain single- or double-stranded RNA or DNA), but host range and antigenic properties are also taken into account. For classification, the ICTV requires that capsid morphology be determined by visualising phage particles using an electron microscope. However, this procedure is not usually routinely performed by researchers, resulting in many of the completely sequenced bacteriophages in GenBank (Benson et al. 2002) remaining unclassified by the ICTV system. As of July 2013, bacteriophages were classified into the order *Caudovirales* (ICTV 2014), which consists of 3 families based on tail morphology (Figure 1-4): *Myoviridae* with long contractile tails, *Siphoviridae* with long non-contractile tails and *Podoviridae* with extremely short tails (Hendrix & Casjens 2005). A further 10 families also exist but are currently not assigned to an order (ICTV 2014). The viruses that typically represent bacteriophages are the tailed phages that contain linear double-stranded DNA and genome sizes ranging from 17 to 500 kb.

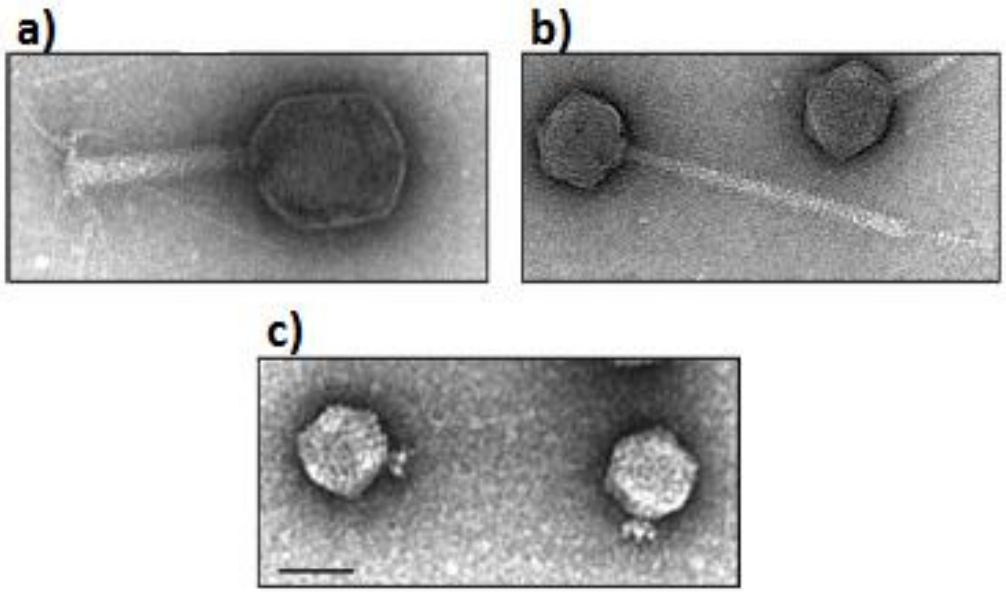


Figure 1-4. Viruses classified in the order *Caudovirales*.
Electron micrographs illustrating a T4-like myovirus (panel a), an *Escherichia coli* siphovirus HK97 (panel b) and a *Salmonella typhimurium* podovirus P22 (panel c). Each phage shows the characteristic tail morphology associated with its classification. The scale bar is 50 nm. Images reproduced from Krupovic et al. (2011).

1.7.2 Lifecycle

Apart from being classified on the basis of morphology, phages can also be grouped according to their lifecycle. Phages are generally either virulent (lytic) or lysogenic (Adams 1959) but alternative lifecycles also exist, including pseudolysogeny and the carrier state life cycle (Abedon 2009). An overview of the different lifecycles is discussed next.

1.7.2.1 Virulent bacteriophage

Virulent bacteriophages redirect host metabolism in order to produce new phage particles, which are finally released on lysis of the host cells (example bacteriophage T4). The mechanisms associated with the lifecycle of virulent phages were first investigated *in vitro* by Ellis and Delbrück (1939) with their one-step growth experiment (Figure 1-5). They were able to determine the minimum latent period needed for intra-cellular virus growth as well as the average burst size. The latent period represents the time which elapses between adsorption of phage to the host cell and lysis, while the burst size is the mean yield of phage particles per infected host cell. The main stages of the lytic lifecycle are (1) adsorption, (2) infection, (3) replication and (4) release of virions. Bacteriophages adsorb to bacterial host cell receptors such as flagella, pili or capsule. Some phages also require adsorption cofactors, for example coliphage T4 reversibly lost its ability to adsorb to its host cells in the absence of tryptophan (Anderson 1948).

Following adsorption the phage injects its DNA into the bacterial cytoplasm, signalling the beginning of the eclipse and latent periods. The DNA is then

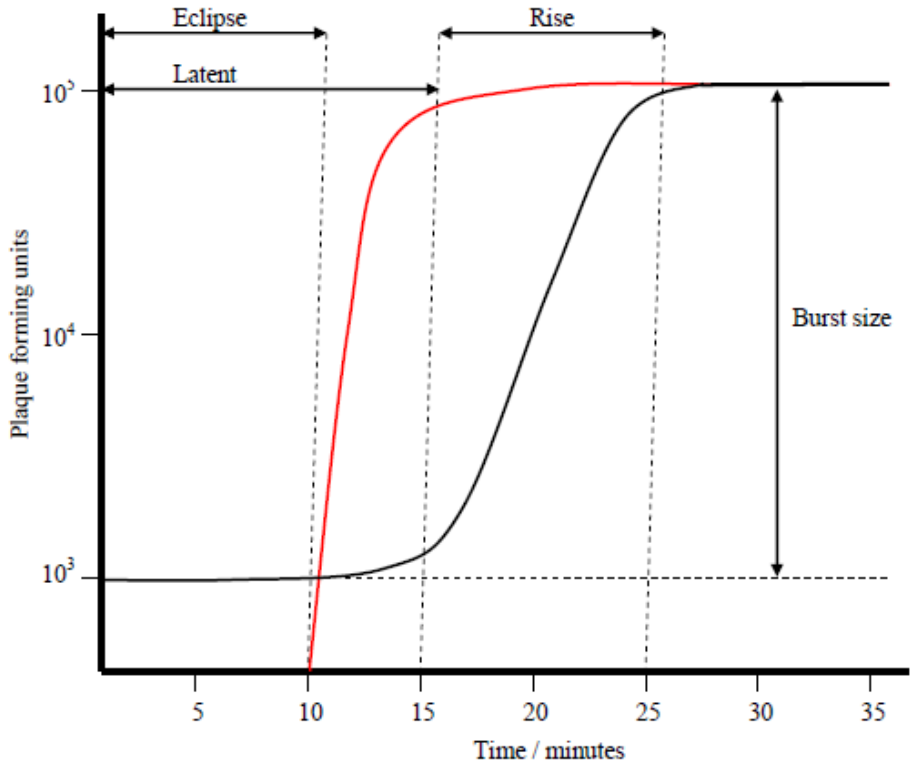


Figure 1-5. The bacteriophage one-step growth curve of Ellis and Delbrück (1939).

The bacteriophages are mixed with a specific bacterial host and incubated for a short period of time to allow phage adsorption. The mixture is diluted 1 in 1000 to prevent any further rounds of replication by reducing the number of available bacteria. The curve begins with the latent period, which represents the time that elapses between phage adsorption and bacterial cell lysis. The latent period also spans the eclipse phase where the bacteriophages are replicating and mature phage virions are accumulating within host cells (red line). As these mature virus particles are released into the extra-cellular environment, the curve enters the rise period. This can be used to calculate the burst size, which refers to the number of phage virions produced per infected host cell, for this single round of phage replication.

replicated to create multiple copies that are packaged into the capsid, which is constructed in the very late stages of phage infection. Assembly of the phage progeny is completed by attachment of tail fibres onto the DNA-filled heads and the eclipse phase comes to an end as the number of mature intra-cellular viruses accumulates. These mature phage virions are then released into the extra-cellular environment, which marks the end of the latent period.

1.7.2.2 Temperate bacteriophage

Temperate or lysogenic bacteriophages (lysogens) integrate phage DNA into the genome of the bacterial host cell, where replication occurs alongside the host DNA. The phage generally remains dormant, causing no cell lysis and producing no progeny (example bacteriophage λ). Lysogeny however means "production of lysis" and refers to the habit of some infected bacterial strains producing phage capable of lysing sensitive bacterial strains. This is possible because lysogenic phages can recommence the lytic life cycle either spontaneously or in response to environmental factors to produce infective phage progeny. In contrast, bacterial lysogens are generally resistant to super-infection by phages that are genetically related to their integrated prophage.

1.7.2.3 Pseudolysogeny and carrier state life cycle

Pseudolysogeny (false lysogeny) and the carrier state life cycle (CSLC) are two alternative bacteriophage lifecycles (Lwoff 1953; Adams 1959) that have previously been reported for either lytic or lysogenic phages (Miller & Ripp 2002). Pseudolysogeny describes an unstable condition where the viral DNA of the infecting phage fails to establish a long-term, stable relationship with the

host (lysogeny) or does not cause cell lysis, but is instead maintained within the cell in a non-active state (Ripp & Miller 1997). Such a situation may arise during nutrient limited conditions where the pseudoprophage can survive for extended periods. As nutrients are replenished, the unstable pseudolysogens can either be converted to true lysogens or elicit a lytic response (Miller & Ripp 2002). On the other hand, the CSLC refers to a mixture of bacteria and bacteriophages which exist in a stable equilibrium (Lwoff 1953). The majority of the bacteria are resistant but some sensitive variants are also present and these can be infected by the phage to allow multiplication. Bacteriophages are therefore constantly generated at the expense of the sensitive population (Siringan et al. 2014).

1.7.3 Bacteriophage therapy

1.7.3.1 History and early phage therapy trials

Bacteriophages were discovered independently in the early 1900's by Frederick Twort (1915) in England and Felix d'Herelle (1917) in France, who both described a phenomenon where bacterial cell lysis was taking place on solid media. Felix d'Herelle introduced the term bacteriophage, meaning 'bacteria eater', attributed to their bactericidal ability and was the first to formally propose the use of bacteriophages as therapeutic agents against bacterial infections. In 1919, he isolated phages from chicken faeces and used them to successfully control an epidemic of avian typhosis and subsequently went on to use this approach as a therapy to treat dysentery in human patients (Summers 2001). Bacteriophages were also used in 1921 by Richard Bruynoghe and Joseph Maisinto, who successfully treated staphylococcal skin

disease by injecting phages into and around surgically opened lesions, with regression of the disease occurring within 24-48 hours of administration (Bruynoghe & Maisin 1921). Dr. d'Herelle's laboratory in Paris commercially produced phage preparations against a number of bacterial infections, which included the products Bacté-coli-phage, Bacté-intesti-phage and Bacté-pyo-phage, which were marketed by what later became L'Oreal in France. In the 1940's, commercial phage products for human use were also produced by Eli Lilly Co. in the United States which targeted bacterial pathogens such as *Staphylococcus*, *Streptococcus* and *E. coli*. However, with the discovery and introduction of antibiotics, commercial production of bacteriophage preparations was discontinued in most of the Western world. On the other hand, the therapeutic use of bacteriophages still continued in Eastern Europe and the Russian Federation, in conjunction with or instead of antibiotics (Sulakvelidze et al. 2001).

1.7.3.2 Rediscovery and recent advances

Phage therapy trials ceased for some time after World War II, but the research of Smith and colleagues in the United Kingdom in the early 80's began a promising era of rediscovery (Smith & Huggins 1982). Their work focused on an *E. coli* strain which was a known pathogen of calves and they selected phages that utilised the K1 capsular antigen as a receptor for surface attachment. By doing this, they were able to successfully treat experimental *E. coli* infections in mice and found that a single-dose phage treatment was more effective than several doses of antibiotics including tetracycline, ampicillin and chloramphenicol (Smith & Huggins 1982). Anti-K1 phages were also used to

successfully treat experimentally induced *E.coli* diarrhoea in calves, lambs and piglets (Smith & Huggins 1983; Smith et al. 1987a; Smith et al 1987b). A decade later Barrow et al. (1998) extended these results by using anti-K1 phages for treatment of *E. coli* septicaemia and meningitis in chickens and calves.

Further experimental studies using animal models have been generally successful in treating bacterial pathogens including *Lactococcus garvieae* in yellowtail fish (Nakai et al. 1999), *Pseudomonas plecoglossicida* in Japanese sweetfish (Park et al. 2000), *Staphylococcus aureus* in mice and rabbits (Matsuzaki et al. 2003; Wills et al. 2005) and *P. aeruginosa* in mice (Watanabe et al. 2007). A number of studies have also evaluated the potential of using virulent phages to reduce bacterial loads on foods including *Listeria monocytogenes* on fresh-cut produce (Leverentz et al. 2003), *C. jejuni* on chicken skin (Atterbury et al. 2003b; Goode et al. 2003) and *L. monocytogenes* on soft cheeses (Carlton et al. 2005). In addition, some trials have used a mixture of two or more phages as a cocktail to cover a broader host range and reduce the bacterial load of contaminated food products (Pao et al. 2004; Abuladze et al. 2008). In recent times, commercial bacteriophage-derived products have become available for use in the food and agricultural industry. However, before such products are deemed safe for human consumption they have to meet certain safety standards. For instance, ListShield (Intralytix Inc, USA) and Listex P-100™ (Micreos, Netherlands) have been granted 'generally regarded as safe' (GRAS) status by the US Food and Drug Administration and are now available in the United States to treat *L. monocytogenes*

contamination. With the continued rise of multi-drug resistant bacterial pathogens, there has been re-newed interest in the use of bacteriophage therapy as an alternative to target infectious bacteria.

1.7.4 *Campylobacter* phages

1.7.4.1 Characteristics

The majority of *Campylobacter* bacteriophages are members of the *Myoviridae* family with long contractile tails, double-stranded DNA and icosahedral heads (Atterbury et al. 2003a; Loc Carrillo et al. 2005; Timms et al. 2010). Figure 1-6 shows an electron micrograph of *Campylobacter* phages. The sixteen virulent phages comprising the phage typing scheme of Frost et al. (1999) were characterised by Sails et al. (1998) and these could be subdivided into three groups based on their genome size and head diameter. Group I included two phages with head diameters of 140.6 and 143.8 nm and large genomes, 320 kb in size. Five phages with average head diameters of 99 nm and average genome sizes of 184 kb were classified into group II. Group III was comprised of nine phages with an average head size of 100 nm and average genome sizes of 138 kb. The sixteen phages were later classified into four groups according to their pattern of activity against spontaneous, transposon and defined mutants of *C. jejuni* (Coward et al. 2006). In addition, bacteriophage genomic DNA is usually resistant to restriction endonuclease digestion, however the DNA of group III phages is digestible with *Hha*I, making this a useful means of discriminating these *Campylobacter* phages (Sails et al. 1998). Several group III phages have been isolated from poultry (El-Shibiny et al. 2005; Loc Carrillo et al. 2005, 2007). The recent availability of the published genome

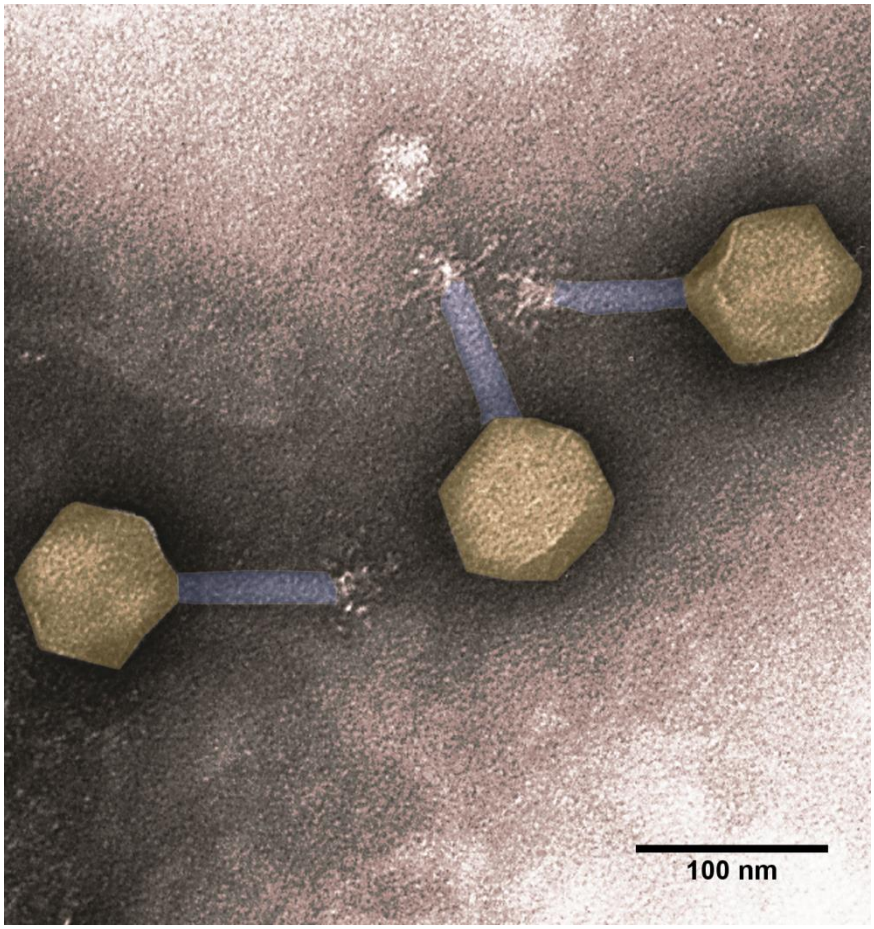


Figure 1-6. Transmission electron micrograph of *Campylobacter* phages. The phage particles have icosahedral heads and long contractile tails, making them members of the *Myoviridae* family. The phages have been artificially coloured. Image was produced courtesy of Dr. Catherine Loc Carrillo.

sequences of virulent *Campylobacter* phages has also made it possible for further classification (Javed et al. 2014). Based on whole genome homology and protein sequence similarities it was proposed to introduce two new genera, the "Cp220likevirus" and "Cp8unalikevirus" within the proposed subfamily "Eucampyvirinae" (Javed et al. 2014).

1.7.4.2 Sources of *Campylobacter* phages

Campylobacter bacteriophages have been isolated from multiple sources including the faeces of pigs, cattle and sheep (Bryner et al. 1970; Bryner et al. 1973; Firehammer & Border 1968) as well as abattoir effluent, sewage, manure and the faeces of chickens (Grajewski et al. 1985; Salama et al. 1989; Khakhria & Lior 1992; Sails et al. 1998; Connerton et al. 2004; Atterbury et al. 2005; El-Shibiny et al. 2005; Loc Carrillo et al. 2007). *Campylobacter*-specific phages have also been isolated from retail chicken samples (Atterbury et al. 2003a; Tsuei et al. 2007), therefore illustrating that these phages can still remain viable after processing and survive under commercial storage conditions.

1.7.4.3 *Campylobacter* prophages

As genome sequence data became available, the presence of lysogenic bacteriophages within *C. jejuni* was revealed in some strains. Notably, a Mu-like phage sequence (CJIE1/CLMP1) and other integrated prophages (CJIE2 and CJIE4) were identified within *C. jejuni* RM1221 (Fouts et al. 2005). A comparative genome analysis using PCR and a multistrain *C. jejuni* DNA microarray later demonstrated that these prophage sequences were also present in other *Campylobacter* strains (Parker et al. 2006). Similarly, a number of

studies have also reported the presence of Mu-like prophages integrated within the chromosomes of *Campylobacter* isolates (Barton et al. 2007; Scott et al. 2007b; Clark & Ng 2008). Furthermore, a *C. jejuni* strain originally isolated from the caecal contents of commercially reared broiler chickens, displayed genomic rearrangements that were found to be associated with intra-genomic recombination between multiple Mu-like phage sequences (Scott et al. 2007b). These variants showed resistance to infection by virulent phages, failed to colonise the intestine of broiler chickens and spontaneously produced infectious bacteriophage CampMu virions. However, reintroduction of these strains into chickens without bacteriophages present resulted in a reversion to a bacteriophage sensitive and colonisation proficient phenotype, by further recombination between the Mu-like phage sequences. These results illustrate a mechanism that enables the host to survive bacteriophage predation while influencing the *Campylobacter* population that colonises broiler chickens.

1.7.4.4 Application of *Campylobacter* bacteriophages

A quantitative risk assessment in Europe has predicted that a 3 $\log_{10}$ reduction in the number of *Campylobacter* in the chicken gut at slaughter would result in at least a 90% reduction in the risk to public health (EFSA 2011). It has also been predicted that a 1 $\log_{10}$ reduction in the number of *Campylobacter* contaminating chicken carcasses would be associated with a 50-90% reduced risk to public health, while reductions of more than 2 $\log_{10}$ units would reduce the risk to public health by more than 90% (EFSA 2011). To this end, targeting such *Campylobacter* reduction rates with phage therapy would be beneficial to the consumer. Initial bacteriophage therapy studies involving live birds used

group III phages only. Wagenaar et al. (2005) were the first to report on the use of bacteriophages to reduce *C. jejuni* colonisation of broiler chickens. They compared the effects of phage therapy as either a therapeutic or preventive measure of treating broiler chickens by using two of the phages (NCTC12669/phage 69 and NCTC 12671/phage 71) used in the phage typing scheme of Frost et al. (1999). A 3 $\log_{10}$ CFU/g decline in *C. jejuni* numbers was initially observed in the therapeutic group but after 5 days bacterial counts stabilised at a level 1 $\log_{10}$ CFU/g below the non-phage-treated controls. Preventative phage treatment resulted in a delay but not prevention of colonisation, with results reaching comparable levels to the therapeutic group after 1 week. In a similar investigation, Loc Carrillo et al. (2005) used broad lytic spectrum bacteriophages (CP8 and CP34) and *C. jejuni* host strains isolated from broiler chickens. This treatment successfully reduced *Campylobacter* levels by between 0.5 and 5 $\log_{10}$ CFU/g of caecal content as compared to the untreated chicks. Group II bacteriophages (CP220, phiCcolBB35, phiCcolBB37, phiCcolBB12) were also used in phage therapy trials and reduction levels of up to 2 $\log_{10}$ CFU/g were achieved (El-Shibiny et al. 2009; Carvalho et al. 2010) with these phages exhibiting an ability to infect both *C. jejuni* and *C. coli* strains.

Bacteriophages have also been used to reduce *Campylobacter* contamination levels on the surface of chicken carcasses artificially contaminated following slaughter (Goode et al. 2003; Atterbury et al. 2003b). The group III phages NCTC 12673 and NCTC 12674 were used in these studies and reduction levels of 1-2 $\log_{10}$ cm^{-2} were achieved. However, a greater reduction was observed

when bacteriophage application was combined with freezing (Atterbury et al. 2003b). Another study looked at the effects of using a lytic bacteriophage isolated from chicken faeces (phage Cj6) as a biocontrol agent against *Campylobacter* on cooked and raw beef (Bigwood et al. 2008). Phages were applied to the food at refrigerated and room temperatures (5 and 24°C) at high and low MOI (10 to 10^4) to either low or high host densities (<100 or 10^4 host cells cm^{-2}). The greatest reductions were observed at high host cell density and high MOI, with the degree of *Campylobacter* inactivation increasing with incubation time. After 24 hours incubation, reductions in excess of 2 $\log_{10}$ cm^{-2} were achieved on the raw and cooked beef at both temperatures.

1.7.4.5 Bacteriophage resistance

The development of bacteriophage resistant *Campylobacter* strains following phage therapy has always been perceived as a potential problem. However, experimentally these resistant types comprised a minor part of the overall population *in vivo* and appeared to be less virulent than the wild-type strains (Loc Carrillo et al. 2005; Scott et al. 2007a; Scott et al. 2007b). Loc Carrillo et al. (2005) found that the incidence of phage-resistant isolates recovered from the intestines of phage-treated bird was <4%. These resistant types were compromised in their ability to colonise new chickens and immediately reverted to a phage-sensitive phenotype *in vivo*. The phage-resistant phenotype therefore did not become the dominant population in these phage-treated chickens despite their continued presence and predation upon the sensitive population (Loc Carrillo et al. 2005).

Phage-resistance has been found to be overcome by using multiple bacteriophages in a 'cocktail' that are capable of targeting different cell surface receptors of the host. However, bacteria are capable of modifying their receptors as a means of avoiding bacteriophage infection. Coward et al. (2006) reported that resistance to some bacteriophages was associated with motility and capsular polysaccharide defects in *C. jejuni*. Similarly, Sørensen et al. (2011) found a novel phage receptor in *C. jejuni* and determined that a NCTC 11168-phage F336 resistant mutant, generated *in vitro*, had lost the phase variable O-methyl phosphoramidate (MeOPN) moiety attached to a Gal*f*NAc (N-acetylgalactosamine in furanose configuration) residue of the CPS. It was later determined that coinfection of chickens with *C. jejuni* NCTC 11168 and phage F336 resulted in the selection of resistant types *in vivo* as all isolates from phage-infected chickens were resistant to subsequent phage F336 infection (Sørensen et al. 2012).

1.7.4.6 Genome sequences

Highly purified, high titre suspensions are required for DNA sequencing of bacteriophages. However, due to the refractory nature of many *Campylobacter* bacteriophage genomes to restriction enzyme digestion as well as the difficulties associated with amplifying their DNA using *Taq* polymerase, sequencing phage genomes has proven problematic. To date, complete genome sequences are available for eight *Campylobacter* phages and a further incomplete sequence composed of 5 contigs has also been published (Table 1-1). The available genome sequences represent group II and III phages but no

Table 1-1. Published sequences of virulent *Campylobacter* phages to date

Phage	Accession number	Group	Length	Reference
CP220	FN667788	II	177,534	Timms et al. 2010
CPt10	FN667789	II	175,720	Timms et al. 2010
NCTC12673	GU296433	III	135,041	Kropinski et al. 2011
vB_CcoM-IBB_35 Contig 1	HM246720	II	53,237	Carvalho et al. 2012
vB_CcoM-IBB_35 Contig 2	HM246721	II	51,534	Carvalho et al. 2012
vB_CcoM-IBB_35 Contig 3	HM246722	II	27,985	Carvalho et al. 2012
vB_CcoM-IBB_35 Contig 4	HM246723	II	14,701	Carvalho et al. 2012
vB_CcoM-IBB_35 Contig 5	HM246724	II	24,608	Carvalho et al. 2012
CP81	FR823450	III	132,454	Hammerl et al. 2011
CP21	HE815464	II	182,833	Hammerl et al. 2012
CP30A	JX569801	III	133,572	-
CPX	JN132397	III	132,662	-
CP8	KF14816	III	132,667	-

group I sequence has yet been deposited. Group II phages CP220 and CPt10 were isolated at least fourteen years apart but interestingly showed very high levels of sequence conservation (Timms et al. 2010). Both genomes contained numerous copies of radical S-adenosylmethionine genes, which were suggested to be involved in enhancing host metabolism during bacteriophage infection. The other group II phages, CP21 and vB_CcoM-IBB_35, showed sequence homology with CP220 and CPt10, however the genomic organisation of CP21 is divergent (Hammerl et al. 2012). Group III phage CP81 was also found to display a relationship with phages CP220 and CPt10 as well as T4-like phages, with similarities being seen with proteins associated with capsid structure, DNA replication and recombination (Hammerl et al. 2011). However, some genes encoding membrane proteins, transposases and metabolic enzymes in CP220 and CPt10 were not present in CP81. Bacteriophage NCTC 12673 (Kropinski et al. 2011) was found to show closest homology to T4-related cyanophages, sharing very limited homology to CP220 and CPt10 at the nucleotide level and only 36.6% and 36.7% at the protein level respectively. What all of the phage genomes (groups II and III) appear to share in common however, is the presence of homing endonucleases related to Hef which are absent in the sequenced *Campylobacter* genomes.

1.8 OBJECTIVES OF STUDY

The main objective of this study was to examine phage-host interactions between *Campylobacter* and virulent phages. This was performed by observing the transcriptional response of *C. jejuni* to bacteriophage infection and determining the viral mechanisms necessary for redirection of normal host

metabolic functions to enable production of new phage progeny. The restriction-modification system of a phage-sensitive *C jejuni* strain and the role lack of phase variation plays in its restrictive ability were also examined.

Chapter 2 : Materials and Methods

2.1 PREPARATION OF GROWTH AND STORAGE MEDIA

Unless otherwise stated, all growth and storage media were prepared using reverse osmosis (RO) water and was sterilised by autoclaving at 121°C and 15 psi for 15 minutes on a liquid cycle. For agar media, molten agar was firstly tempered 45-50°C before adding any supplements or antibiotics. Plates were prepared inside a laminar flow cabinet. On setting, the agar plates were stored at 4°C for up to four weeks. All other chemicals were stored at room temperature after autoclaving and cooling, unless otherwise indicated.

2.1.1 Blood agar (BA)

Blood agar base No. 2 (Oxoid Ltd., Basingstoke, UK: CM0271; proteose peptone 15.0 g/l, liver digest 2.5 g/l, yeast extract 5.0 g/l, sodium chloride 5.0 g/l, agar 12.0 g/l) was prepared by suspending 40 g into 1 litre of RO water. After autoclaving, it was cooled to 45-50°C and defibrinated horse blood (TCS Biosciences, Buckingham, UK) was added to a final concentration of 5% (v/v).

2.1.2 *Campylobacter* blood-free selective agar (modified CCDA-Preston)

Campylobacter blood-free selective agar (Oxoid: CM0739; nutrient broth No.2 25.0 g/l, bacteriological charcoal 4.0 g/l, casein hydrolysate 3.0 g/l, sodium desoxycholate 1.0 g/l, ferrous sulphate 0.25 g/l, sodium pyruvate 0.25 g/l, agar 12.0 g/l) was prepared by suspending 45.5 g into 1 litre of RO water. However, an additional 2% (w/v) of bacteriological agar No. 1 (Oxoid) was occasionally added during preparation in order to prevent swarming of *Campylobacter*. To isolate *Campylobacter* from samples other than pure cultures, 1 vial of CCDA

selective supplement (SR 155E; 16 mg of cefoperazone and 5 mg of amphotericin B) was reconstituted in sterile RO water and was aseptically added to 500 ml of sterilised CCDA after tempering.

2.1.3 New Zealand Casamino Yeast Medium (NZCYM) basal agar

New Zealand Casamino Yeast Medium broth (Difco, Oxford, UK: 240410; pancreatic digest of casein 10.0 g/l, casamino acids 1.0 g/l, yeast extract 5.0 g/l, sodium chloride 5.0 g/l, magnesium sulphate, anhydrous 0.98 g/l) was prepared by adding 22 g of NZCYM broth to 1 litre of RO water. NZCYM basal agar was then made by adding 15 g of bacteriological agar No. 1 (Oxoid) to the broth to a final agar concentration of 1.5% (w/v).

2.1.4. NZCYM overlay agar

NZCYM broth (Difco) was prepared according to the manufacturer's instructions. NZCYM overlay agar was then made by adding bacteriological agar No. 1 (Oxoid) to the broth to a final agar concentration of 0.6% (w/v). Following sterilisation, 5 ml volumes were aseptically aliquoted into sterile glass universal bottles and were allowed to temper in a 50-55°C water bath until ready for use.

2.1.5 Nutrient broth No.2

Nutrient broth No. 2 (Oxoid: CM0067; 'Lab-Lemco' powder 10.0 g/l, peptone 10.0 g/l, sodium chloride 5.0 g/l) was prepared by suspending 25 g into 1 litre

of RO water. After sterilisation, it was stored at room temperature for up to eight weeks.

2.1.6 Mueller-Hinton (MH) broth

Mueller-Hinton broth (Oxoid: CM0405; dehydrated beef infusion 300 g/l, casein hydrolysate 17.5 g/l, starch 1.5 g/l, Ca^{2+} 3.854 mg/l, Mg^{2} 5.072 mg/l) was prepared by suspending 21 g into 1 litre of RO water. After sterilisation, it was stored at room temperature for up to eight weeks.

2.1.7 Mueller-Hinton (MH) agar

Mueller-Hinton agar was prepared by adding bacteriological agar No. 1 (Oxoid) to MH broth (2.1.6) to a final agar concentration of 1.5% (w/v).

2.1.8 *Campylobacter* MH motility agar

MH motility agar was prepared as previously described for MH agar (2.1.7) but instead the bacteriological agar was added to a final concentration of 0.4% (w/v).

2.1.9 Modified Semi-solid Rappaport Vassiliadis (MSRV) agar (ISO)

Modified Semi-solid Rappaport Vassiliadis agar (Oxoid: CM1112; enzymatic digest of animal and plant tissue 4.6 g/l, acid hydrolyate of casein 4.6 g/l, sodium chloride 7.3 g/l, potassium dihydrogen phosphate 1.5 g/l, magnesium chloride anhydrous 10.9 g/l, malachite green oxalate 0.04 g/l, agar 2.7 g/l) was

prepared by suspending 31.6 g of MSRV (ISO) agar base into 1 litre of RO water. After boiling and without autoclaving, the medium was cooled to 50°C. One vial of novobiocin supplement (SR0181; novobiocin 10 mg) was reconstituted in RO water and was then aseptically added. The medium was mixed well and poured into sterile petri dishes. Due to the semi-solid nature of the medium care was taken after the agar was set and the petri dishes were also not inverted.

2.1.10 Xylose Lysine Desoxycholate (XLD) agar

Xylose Lysine Desoxycholate agar (Oxoid: CM0469; yeast extract 3.0 g/l, L-Lysine HCl 5.0 g/l, xylose 3.75 g/l, lactose 7.5 g/l, sucrose 7.5 g/l, sodium desoxycholate 1.0 g/l, sodium chloride 5.0 g/l, sodium thiosulphate 6.8 g/l, ferric ammonium citrate 0.8 g/l, phenol red 0.08 g/l, agar 12.5 g/l) was prepared by suspending 53 g into 1 litre of RO water. The medium was boiled with frequent agitation but was not autoclaved.

2.1.11 Buffered Peptone Water (BPW)

Buffered Peptone Water (Oxoid: CM0590; peptone 10 g/l, sodium chloride 5.0 g/l, disodium phosphate 3.5 g/l, potassium dihydrogen phosphate 1.5 g/l) was prepared by suspending 20 g into 1 litre of RO water. It was aliquoted into 10 ml volumes prior to autoclaving and was stored at room temperature until ready for use.

2.1.12 Phosphate Buffered Saline (PBS)

Phosphate buffered saline (Oxoid: BR0014) was prepared according to the manufacturer's instructions to a pH of 7.3±0.2 at 25°C. The final composition was 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na_2HPO_4 and 0.02% (w/v) KH_2PO_4. Following sterilisation, the PBS was stored at room temperature until ready for use.

2.1.13 Maximum Recovery Diluent (MRD)

Maximum recovery diluent (Oxoid: CM0733; peptone 1.0 g/l, sodium chloride 8.5 g/l) was prepared by suspending 9.5 g into 1 litre of RO water. It was stored at room temperature following sterilisation until ready for use.

2.1.14 Salt Magnesium (SM) buffer

Salt Magnesium buffer was prepared by combining NaCl (Fisher Scientific, Loughborough, UK) to a final concentration of 0.1 M, $MgSO_4.7H_2O$ (Fisher Scientific) to a final concentration of 10 mM, gelatine (Sigma-Aldrich, Dorset, UK) to a final concentration of 0.01% and Tris (Sigma-Aldrich) to a final concentration of 50 mM. The solution was adjusted to pH 7.5 with concentrated HCl (Acros Organics, Loughborough, UK) and was then made up to a final volume of 1 litre with RO water. After sterilising, the buffer was stored at room temperature until ready for use.

2.1.15 Bacteriological storage medium

Nutrient broth No.2 (2.1.5) was prepared according to the manufacturer's instructions but was supplemented with 20% (v/v) glycerol (Fisher Scientific). After sterilisation, it was stored at room temperature until ready for use.

2.1.16 Antibiotics

Stock solutions were prepared by dissolving the desiccated antibiotic in their appropriate solvents and then filter sterilising through a Minisart 0.2 µm filter (Sartorius Stedim Biotech, Goettingen, Germany). These were stored at 4°C until ready for use. Table 2-1 shows the stock and working concentrations for the antibiotics used in this thesis.

Table 2-1. Antibiotics used within this thesis.

Antibiotic	Solvent	Stock Concentration	Working Concentration
Kanamycin	RO water	50 mg ml^{-1}	50 µg ml^{-1}
Chloramphenicol	Ethanol	50 mg ml^{-1}	30 µg ml^{-1}

2.1.17 Iron chelator

2', 2'-dipyridyl (DIP) (Fisher Scientific), was dissolved in ethanol to give a 10 mM stock solution. This was then added to the appropriate nutrient media to give a final concentration of 20 µM.

2.2 CAMPYLOBACTER

2.2.1 Strains

The *Campylobacter* strains used in this thesis are detailed in Table 2-2.

Table 2-2. *Campylobacter* strains used within this thesis.

C. jejuni strain or genotype	Description	Source	Reference
PT14	Clinical isolate previously used to propagate bacteriophages in the UK phage typing scheme.	Dr. J. Richardson, HPA, Colindale, UK	Brathwaite et al. 2013
NCTC 11168	Wild-type human isolate.	NCTC, PHLS, Colindale, UK	Parkhill et al. 2000
NCTC 11168H	Hypermotile variant of NCTC 11168.	Dr. M. Jones, University of Nottingham, UK	Jones et al. 2004
HPC5	Isolated from broiler chickens within the UK.	C. Loc Carrillo PhD thesis, University of Nottingham	
PT14CP30ACS	Stable carrier strain life cycle (CSLC) isolate obtained from phage treatment of biofilms.	P. Siringan PhD thesis, University of Nottingham	
PT14CP8CS	Stable CSLC isolate obtained from phage treatment of biofilms.	P. Siringan PhD thesis	
NCTC 11168Δ0031::kan	Transformed by electroporation with plasmid DNA from pUC-11168Δcj0031::kan. This plasmid contained a 700-bp region of the 5' end of *cj0031* (flanked by *Pst*I and *Bam*HI sites) which was ligated to a 600-bp region of *cj0032* (flanked by *Eco*RI and *Bam*HI sites). A kanamycin resistance cassette derived from pJMK30 was inserted into the *Bam*HI site.	Dr. C. Bayliss, University of Leicester, UK	
NCTC 11168ON	Variant of NCTC 11168 with *cj0031* gene phased on.	Dr. C. Bayliss	
CH11168ON	Chicken isolate with *cj0031* gene phased on.	Dr. C. Bayliss	

Table 2-2 continued

C. jejuni strain or genotype	Description	Source	Reference
CH11168Δcj0031::kan	Chicken isolate naturally transformed with chromosomal DNA from NCTC 11168Δcj0031::kan.	Dr. C. Bayliss,	
PT14Δ00150::kan	Naturally transformed with chromosomal DNA from NCTC 11168Δcj0031::kan.	This thesis	
PT14Δ00150::kan, 00230::00150cam	The complementation vector PCfdxA:PerR was used to replace the ferredoxin promoter and *perR* gene with the cj0031-ON promoter and gene fragment of NCTC 11168. The vector was then amplified in *E. coli* and was used to transform and complement the PT14Δ00150::kan mutant.	This thesis	
11168 *cgb*::Kan^r	The gene encoding *cgb* (*cj1586*) in *C. jejuni* NCTC 11168 was disrupted by insertion of a kanamycin resistance cassette using the suicide plasmid pKE18.	Prof. R. Poole, University of Sheffield	Elvers et al. 2004
11168 *ctb*::Kan^r	The gene encoding *ctb* (*cj0465c*) in *C. jejuni* NCTC 11168 was disrupted by insertion of a kanamycin resistance cassette using the suicide plasmid pKE29.	Prof. R Poole	Wainwright et al. 2005
PT14 *cgb*::Kan^r	Naturally transformed with chromosomal DNA from11168 *cgb*::Kan^r	This thesis	
PT14 *ctb*::Kan^r	Naturally transformed with chromosomal DNA from 11168 *ctb*::Kan^r	This thesis	

2.2.2 Growth conditions

Campylobacter strains were subcultured on BA (2.1.1) and incubated in 3.5L anerobic gas jars (Oxoid) without catalyst unless otherwise stated. Prior to incubation, the internal atmospheric pressure of the jar was reduced to -22 psi and was restored using a gas mixture containing 85% (v/v) N_2, 10% (v/v) CO_2 and 5% (v/v) H_2 (BOC Limited, Surrey, UK). This resulted in microaerobic growth conditions of approximately 83% (v/v) N_2, 7.3% (v/v) CO_2, 3.6% (v/v) H_2 and 5.6% (v/v) O_2. Alternatively, *Campylobacter* was grown in a Modular Atmospheric Controlled System (MACS) cabinet (Don Whitley Scientific, Shipley, UK) containing a gas mixture of 88% (v/v) N_2, 5% (v/v) CO_2, 2% (v/v) H_2 and 5% (v/v) O_2 (BOC Limited). Incubation in either the gas jars or MACS cabinet was for 18-24 hours at 42°C unless otherwise specified.

2.2.3 Storage

2.2.3.1 Long-term storage

For long-term storage, *Campylobacter* strains were firstly grown overnight on BA (2.1.1). A sterile cotton swab was then used to harvest growth from the plates and dispersed into 1 ml aliquots of bacteriological storage medium (2.1.15). These were stored at -80°C until ready for use.

2.2.3.2 Short-term storage

Long-term stocks were revived monthly by quickly thawing an aliquot, spreading 40µl onto a BA plate (2.1.1) and incubating at 42°C for 18-24 hours under microaerobic conditions (2.2.2). Plates with overnight growth were then stored microaerobically (2.2.2) at 4°C for up to one month. This provided an

immediate source of cells without having to continuously thaw and re-freeze the long-term stocks. The plates were used to sub-culture growth by placing an inoculum onto a fresh BA plate (2.1.1) and incubating under microaerobic conditions (2.2.2) at 42°C for 18-24 hours.

2.2.4 Enumeration

Enumeration of *Campylobacter* was performed using a modification of the Miles and Misra (1938) technique. Serial ten-fold dilutions of the cell suspension to be enumerated were firstly prepared in PBS (2.1.12). Five 10 µl aliquots of each dilution were then spotted onto pre-dried modified CCDA-Preston plates (2.1.2). The spots were allowed to dry with the help of a Bunsen flame and the plates were incubated in a MACS cabinet (Don Whitley Scientific) at 42°C for 48 hours. Dilutions with counts of 3-30 colonies were counted and the average of the five spots was determined. The number of colony forming units per ml (CFU ml^{-1}) was then calculated by multiplying the average number of colonies by the inverse of the dilution factor and by 100.

2.2.5 Confirmatory tests

2.2.5.1 Gram stain

Gram stain reagents were firstly prepared according to the manufacturer's instructions immediately prior to use. Smears of colonies to be tested were then prepared aseptically on a glass microscope slide (BDH Lab Supplies, Leicestershire, UK) in PBS (2.1.12) using a sterile 1 µl plastic loop and sterile water. The Gram staining procedure was followed after air drying and heat fixing of the smears. Slides were placed in crystal violet solution (Pro-lab

Diagnostics, Bromborough, Wirral, UK) for 1 minute, with the excess rinsed off with RO water. Next, the slides were immersed in Lugol's iodine (Pro-lab Diagnostics) for 30 seconds, again rinsing the excess with RO water. The stained smears were then decolourised by placing the slides into ethanol (Fisher Scientific) for 1 minute, followed by counterstaining with carbol fuschin solution (Pro-lab Diagnostics) for 30 seconds followed by rinsing with RO water. Slides were air dried and viewed under oil immersion at 100 x magnification using a light microscope.

2.2.5.2 Oxidase test

N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Acros Organics) was dissolved in RO water to give a final concentration of 0.01 g ml^{-1} immediately before use. A sterile swab, moistened with the solution, was then used to harvest cells from overnight growth from the surface of BA plate (2.1.1). The appearance of a deep blue colour within 10 seconds indicated a positive result while a light blue colour or no colour change was considered to be negative.

2.2.5.3 Hippurate test

Sodium hippurate (Sigma-Aldrich) was dissolved in RO water to give a final concentration of 5% (w/v). The solution was filter sterilised and dispensed into 500 µl aliquots. These were stored at -20°C until ready for use. Hippurate activity was tested by inoculating overnight growth from a BA plate (2.1.1) into the thawed solution. This was then incubated at 37°C for 2 hours under aerobic conditions followed by addition of 100 µl of freshly prepared 7% (w/v)

1,2,3-triketohydrindene monohydrate (ninhydrin reagent) and a further incubation period of 10 minutes. A positive result was seen by a deep violet colour while a light purple colour or no colour change was considered a negative result.

2.3 MOTILITY ASSAY

Using a sterile 1 µl loop, an inoculum of overnight *Campylobacter* growth was stabbed into the centre of a MH motility agar plate (2.1.8), avoiding stabbing through the agar onto the base of the petri dish. The plates were incubated upright at 42°C for 24-48 hours under microaerobic conditions (2.2.2). The diameter of the motility zone was subsequently measured.

2.4 NATURAL TRANSFORMATION OF *CAMPYLOBACTER*

Cells from an overnight BA plate (2.1.1) were resuspended in 10 ml of MH broth (2.1.6.) and the OD_{600} was adjusted to 0.5. Next, 0.5 ml of the *Campylobacter* suspension was added to a 15 ml Falcon tube (Fisher Scientific) containing 1 ml of MH agar (2.1.7). This was incubated for 5 hours under microaerobic conditions in the MACS (2.2.2). Five micrograms of DNA containing the marker to be transformed was then added to the cells followed by a further overnight incubation in the MACS (2.2.2). The culture was collected from the Falcon tube and was centrifuged at 13,000 g for 15 minutes in order to concentrate the cells. The resulting pellet was resuspended in 100 µl of MH broth (2.1.6) and spread onto the appropriate selective medium and incubated for 2-3 days in the MACS. Seven to eight transformed colonies were individually collected and spread onto selective agar to produce confluent

growth. The growth was harvested with a sterile cotton swab and was dispersed into 1 ml aliquots of bacteriological storage medium (2.1.15) and stored at -80°C until ready for use.

2.5 BACTERIOPHAGE

2.5.1 Strains

The bacteriophages used within this thesis, along with their propagating strains are listed in Table 2-3. Propagation of the phages is described in section 2.5.2.

2.5.2 Propagation, harvesting and storage of bacteriophage

The propagating strains of *Campylobacter* were cultured on BA (2.1.1) under microaerobic conditions (2.2.2) at 42°C for 18-24 hours. The overnight growth was then harvested into 10 ml of 10 mM $MgSO_4$ using a sterile cotton swab to give an approximate final cell density of 8 $\log_{10}$ CFU ml^{-1}. One hundred microlitres of bacteriophage stock at 8 $\log_{10}$ PFU ml^{-1} was then added to the cell suspension and this was incubated for 20-30 minutes in the MACS cabinet (Don Whitley Scientific) at 42°C. During this time, 5 ml aliquots of NZCYM overlay agar (2.1.4) were heated in a microwave and were allowed to temper in a 55°C water bath. Five hundred microlitres of the *Campylobacter*-bacteriophage suspension was then transferred to the cooled overlay agar and mixed. This was poured onto pre-dried NZCYM plates (2.1.3) which were allowed to set prior to incubation under microaerobic conditions (2.2.2) at 42°C for 18-24 hours. The bacteriophage was harvested from the plates by dispensing 5 ml of SM buffer (2.1.14) onto the surface of the agar followed by

Table 2-3. Bacteriophages used within this thesis and their propagating strains.

Bacteriophage	Propagating strains	Source
CP8	*C. jejuni* PT14 *C. jejuni* HPC5 *C. jejuni* NCTC 11168 *C. jejuni* NCTC 11168H	C. Loc Carrillo PhD thesis, University of Nottingham
CP30	*C. jejuni* PT14 *C. jejuni* HPC5 *C. jejuni* NCTC 11168 *C. jejuni* NCTC 11168H	Dr. P. Connerton, University of Nottingham
CP220	*C. jejun*i PT14 *C. jejuni* HPC5	A. Scott PhD thesis, University of Nottingham
A14	*C. jejuni* PT14	Dr. P. Connerton
A1B	*C. jejuni* PT14	Dr. P. Connerton
CP20	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CP25	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CP28	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CP32	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CP34	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CP51	*C. jejuni* PT14	C. Loc Carrillo PhD thesis
CPX	*C. jejuni* PT14	S. Al-Kandari PhD thesis, University of Nottingham
E5	*C. jejuni* PT14	Dr. P. Connerton
G1	*C. jejuni* PT14	Dr. P. Connerton
G3	*C. jejuni* PT14	Dr. P. Connerton
G4	*C. jejuni* PT14	Dr. P. Connerton
G5	*C. jejuni* PT14	Dr. P. Connerton
G6	*C. jejuni* PT14	Dr. P. Connerton
G7	*C. jejuni* PT14	Dr. P. Connerton
G8	*C. jejuni* PT14	Dr. P. Connerton
mc1a	*C. jejuni* PT14	Dr. P. Connerton
X3	*C. jejuni* PT14	Dr. P. Connerton
NQ1	*C. jejuni* PT14	Dr. P. Connerton
4B	*C. jejuni* PT14	Dr. P. Connerton
7	*C. jejuni* PT14	Dr. P. Connerton
9	*C. jejuni* PT14	Dr. P. Connerton
10b	*C. jejuni* PT14	Dr. P. Connerton
11	*C. jejuni* PT14	Dr. P. Connerton
18b	*C. jejuni* PT14	Dr. P. Connerton
19	*C. jejuni* PT14	Dr. P. Connerton

Table 2-3 continued

Bacteriophage	Propagating strain	Source
19b	*C. jejuni* PT14	Dr. P. Connerton
NCTC 12669	*C. jejuni* PT14	NCTC, PHLS, Colindale, UK
NCTC 12670	*C. jejuni* PT14	NCTC
NCTC 12671	*C. jejuni* PT14	NCTC
NCTC 12672	*C. jejuni* PT14	NCTC
NCTC 12673	*C. jejuni* PT14	NCTC
NCTC 12674	*C. jejuni* PT14	NCTC
NCTC 12675	*C. jejuni* PT14	NCTC
NCTC 12676	*C. jejuni* PT14	NCTC
NCTC 12677	*C. jejuni* PT14	NCTC
NCTC 12678	*C. jejuni* PT14	NCTC
NCTC 12679	*C. jejuni* PT14	NCTC
NCTC 12680	*C. jejuni* PT14	NCTC
NCTC 12681	*C. jejuni* PT14	NCTC
NCTC 12682	*C. jejuni* PT14	NCTC
NCTC 12683	*C. jejuni* PT14	NCTC
NCTC 12684	*C. jejuni* PT14	NCTC
PT14CP30ACS	*C. jejuni* PT14	P. Siringan PhD thesis, University of Nottingham
PT14CP8CS	*C. jejuni* PT14	P. Siringan PhD thesis
HPC5CP30ACS	*C. jejuni* HPC5	P. Siringan PhD thesis
HPC5CP8CS	*C. jejuni* HPC5	P. Siringan PhD thesis

shaking at 60 rpm aerobically overnight at 4°C. The SM buffer was recovered with a syringe and was filtered through a 0.2 µm filter to remove the remaining *Campylobacter* cells and any cell debris present. The recovered phage was stored indefinitely at 4°C in glass McCartney bottles. The stocks were routinely filter-sterilised if there was any sign of contamination.

2.5.3 Titration of bacteriophage

Bacteriophage titration was performed using the appropriate propagating host strain. Briefly, an overnight culture of the desired host strain was prepared on a BA plate (2.1.1) and the cells were harvested into 10 ml of 10 mM $MgSO_4$ using a sterile cotton swab to give an approximate final cell density of 8 $\log_{10}$ CFU ml^{-1}. This suspension was incubated in the MACS cabinet (Don Whitley Scientific) for 15-20 minutes to allow recovery of the bacterial cells. During this time, 5 ml aliquots of NZCYM overlay agar (2.1.4) were heated in a microwave and cooled in a 55°C water bath. Five hundred microlitres of the *Campylobacter* suspension was then transferred to the cooled overlay agar, mixed and poured onto pre-dried NZCYM basal plates (2.1.3) with gentle swirling to form an even lawn. The plates were allowed to dry prior to incubation in the MACS cabinet (Don Whitley Scientific) for 10-15 minutes. Serial ten-fold dilutions of the bacteriophage to be enumerated were prepared in PBS (2.1.7). Five 10 µl aliquots of each dilution were then spotted onto the bacterial lawns and the spots allowed to dry. The plates were incubated for 18-24 hours under microaerobic conditions (2.2.2) at 42°C. Dilutions resulting in the appearance of 3-30 plaques were counted and the average of the five spots was determined. The number of plaque forming units per ml (PFU ml^{-1}) was

then calculated by multiplying the average number of plaques by the inverse of the dilution factor and by 100.

2.5.4 Bacteriophage lytic spectra

Bacterial lawns for each *Campylobacter* strain were prepared as previously described (2.5.3). The bacteriophages of interest were diluted to the routine test dilution of approximately 6 $\log_{10}$ PFU ml^{-1} and 10 µl aliquots were spotted onto the surface of the lawns, with a maximum of 16 spots per plate. The spots were allowed to dry with the help of a Bunsen flame prior to microaerobic incubation (2.2.2) at 42°C for 18-24 hours. The lytic ability of each phage was scored according to the nomenclature used by Frost et al. (1999) in the UK phage typing scheme.

2.5.5 Efficiency of plating

In order to determine the susceptibility of various test strains of *Campylobacter* to phage infection, the efficiency of plating (EOP) was calculated. This was done by determining plaque formation on one strain and comparing it to that on another strain. For instance, in the case of wild-type and mutant strains, EOP values were calculated by dividing the PFU ml^{-1} of the phage grown on the mutant by the PFU ml^{-1} of the phage grown on the wild-type.

2.6 *IN VITRO* GROWTH EXPERIMENTS

2.6.1 Bacteriophage infection of *C. jejuni*

Three independent cultures of the appropriate *Campylobacter* strain were grown overnight on BA plates (2.1.1) under microaerobic conditions (2.2.2) at 42°C. *Campylobacter* from each plate was suspended into individual 10 ml volumes of PBS (2.1.12) using sterile cotton swabs. OD_{600} readings were taken using the Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer with PBS used as the control. The estimated bacterial concentration was then calculated using an empirical formula based on optical density (eq. 4) which was derived from a standard *Campylobacter* dilution series, previously carried out by Dr. A. Scott.

Campylobacter titre (CFU ml^{-1}) = (OD_{600} x 2 x 10^9) – 6 x 10^6 (eq. 4)

Three biological replicates were prepared by adding the suspensions to 250 ml conical flasks containing 150 ml of nutrient broth No. 2 (2.1.5) to give final estimated concentrations of 7 $\log_{10}$ CFU ml^{-1} of culture. Three biological replicates were also prepared for non-bacteriophage infected cultures. One millilitre aliquots were taken at 0 h for actual enumeration of the initial concentration of *Campylobacter* cells (2.2.4). The flasks were then placed into anaerobic gas jars (Oxoid) and were incubated under microaerobic conditions at 42°C (2.2.2) with shaking at 100 rpm in an IS-971R Lab Companion shaking incubator (Fisher Scientific). After 2 hours, the appropriate bacteriophage was added to the broth to generate an effective multiplicity of infection (MOI) of >1. Aliquots were again taken for enumeration of

Campylobacter (2.2.4) and for the bacteriophages (2.5.3). The latter samples were filtered through a Minisart 0.2 µm filter (Sartorius Stedim Biotech) in order to remove bacterial cells. Microaerobic incubation at 42°C (2.2.2) with shaking was then resumed. Aliquots for enumeration of *C. jejuni* and to titre bacteriophages were taken at hourly intervals.

2.6.2 Bacteriophage infection of *C. jejuni* for transcriptome analysis experiments

The desired *Campylobacter* strain was grown and infected with the appropriate bacteriophage as previously described (2.6.1) until the phage reached the eclipse phase of growth. Cells were harvested from three biological replicates at the time point confirmed by enumerations to have occurred directly before cell lysis (population crash) in order to capture *Campylobacter* cells that were under phage infection.

2.6.3 Determination of bacteriophage resistance

A 50 µl aliquot of the bacteriophage of interest at a concentration of 7 $\log_{10}$ PFU ml^{-1} was pipetted evenly down the centre of a MH agar plate (2.1.7) in a single line and allowed to dry. Twenty individual *Campylobacter* colonies from the *in vitro* growth experiment (2.6.1) were chosen at random to represent the time points of interest. Each of these colonies was streaked perpendicularly across the phage line using a single streak. Phage resistance was determined by the presence of growth across the phage line.

2.7 IRON-DEPLETION GROWTH EXPERIMENT

Independent cultures of *C. jejuni* PT14 were grown overnight on MH agar (2.1.7) under microaerobic conditions (2.2.2) at 42°C. Cells were harvested and cultures were grown and infected with bacteriophages as previously described (2.6.1), however MH broth (2.1.6) was used instead of NB No. 2 (2.1.5). All glassware was previously treated with 8 M HCl and then rinsed three times with RO water to remove residual iron from their surfaces. In order to induce iron-limited conditions, 2', 2'-dipyridyl (DIP) (2.1.17) was added to the broths at a final concentration of 20 µM prior to their inoculation.

2.8 ISOLATION OF GENOMIC DNA

Campylobacter genomic DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions. Cells were harvested from a quarter of an overnight BA plate (2.1.1) into 1.5 ml of PBS (2.1.9). The suspension was then centrifuged at 12,000 x g for 2 minutes at room temperature using a Heraeus Pico 17 bench-top centrifuge. The pellet was then resuspended in 180 µl of Lysis Solution T (BB6678: composition confidential) and 20 µl of RNase A solution (R6148: composition confidential) was added followed by incubation at room temperature for 2 minutes. 20 µl of 20 mg ml^{-1} Proteinase K (Sigma-Aldrich) was then added and the mixture was incubated for 30 minutes at 55°C. Afterwards, 200 µl of Lysis Solution C (B8803: composition confidential) was added and the mixture was thoroughly vortexed for 15 seconds to achieve a homogenised mixture followed by a further incubation at 55°C for 10 minutes. During this time, a column was prepared by adding 500 µl of Column Preparation Solution

(C2112: composition confidential) and centrifuging at 12,000 x g for 1 minute using a Heraeus Pico 17 bench-top centrifuge. The eluate was then discarded. After incubation, 200 µl of absolute ethanol (Fisher Scientific) was added to the lysate and this was homogenised thoroughly by vortexing for 5-10 seconds. This mixture was then loaded onto the prepared column and was centrifuged at 6,500 x g for 1 minute using a Heraeus Pico 17 bench-top centrifuge. The eluate and collection tube were both discarded and the column was placed into a new 2 ml collection tube. 500 µl of Wash Solution 1 (W0263: composition confidential) was added to the column and this was again centrifuged for 1 minute at 6,500 g using a Heraeus Pico 17 bench-top centrifuge. Next the collection tube was discarded and replaced and 500 µl of Wash Solution 1 Concentrate (B6553: composition confidential) was added to the column for a second wash. The column was centrifuged in a Heraeus Pico 17 bench-top centrifuge at maximum speed for 3 minutes to dry the column. The collection tube was discarded and replaced. 200 µl of Elution Solution (B6803: 10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) was added directly to the centre of the column. The tube was then centrifuged at 6,500 g for 1 minute in a Heraeus Pico 17 bench-top centrifuge in order for the DNA to elute. The genomic DNA was stored at 4°C for short-term storage and more immediate use and at -20°C for long-term storage.

2.9 POLYMERASE CHAIN REACTION (PCR)

2.9.1 Primer design

The primers used within this thesis are listed in Table 2-4. The Cgb and Ctb forward and reverse primers were designed based on the gene homologues in

Table 2-4. Primers used within this thesis.

Primer name	Primer sequence (5'→3')	Tm /°C	Source	Amplicon size (bp)
Cgb-F	GGAGAGGATTTAACCAATGAG	60	This thesis	1711
Cgb-R	CCATAAGCAACTTCCCAAGC	60	This thesis	
Ctb-F	ACAATTAATCAAGAAAGCATAGC	60	This thesis	1797
Ctb-R	GCTATCATTTGTGCACGCTG	60	This thesis	
PT14-00150F	GTTTAACCCCAACGATGC	54	This thesis	2856
PT14-00150R	GCTTTTAAAATTTCATCGAC	52	This thesis	

Note: Primer sequences are given in the 5' to 3' direction.

Tm - melting temperature

F and R represent the forward and reverse gene directions respectively.

C. jejuni PT14 to *cj1586* (*cgb*) and *cj0465c* (*ctb*) in NCTC11168. The PT14-00150 primers were designed to amplify a fragment of the A911_00150 gene in PT14. This was done with the help of the RNA-seq mapping data for the PT14Δ00150::kan mutant using PT14 as the reference genome. Sequence reads for *C. jejuni* NCTC11168 and PT14 were both available, which made it possible to design primers that could amplify DNA from either source.

Primers were designed in such a way that the difference in melting temperature between the two primers did not exceed 5°C. An optimal GC content of 40-60% was strived for and where possible, the primer was terminated with a guanine or cytosine residue at the 3'-end. Primers were designed so that they were shorter than 25 nucleotides and their melting temperatures were estimated using the equation below (eq. 5):

$$T_m = 4\,(G+C) + 2\,(A+T) \qquad \text{(eq. 5)}$$

All primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

2.9.2 PCR conditions

PCR was carried out using Fermentas DreamTaq Green PCR Master Mix (2x) (Fisher Scientific) containing 2 U DreamTaq DNA polymerase, 2x DreamTaq Green buffer, 0.4mM of each dNTP and 4mM of $MgCl_2$, along with two dyes (blue/yellow) to monitor electrophoresis progress. Reactions (1x) were carried out in 0.5 ml thin-walled PCR tubes (Fisher Scientific) in 50 µl volumes with

100 ng of template DNA. Amplification of PCR products was conducted using the BIOER XP thermal cycler (Alpha Laboratories, Hampshire, UK). Initial denaturation of the template DNA and enzyme activation was done at 95°C for 3 minutes. This was followed by 35 cycles each of denaturation at 95°C for 30 seconds, annealing of the primers at 45-55°C for 30 seconds and extension at 72°C for 1-3 minutes. The length of the extension step was based on the size of the expected PCR product, with 1 minute extension per kb for products up to 2 kb being deemed sufficient. A final extension step was also done at 72°C for 5-15 minutes.

2.10 AGAROSE GEL ELECTROPHORESIS

Routine analysis of DNA was performed using agarose gel electrophoresis. Molecular Biology grade agarose (Melford Laboratories, Suffolk, UK) was melted in a microwave in 1x TAE buffer (40 mM tris-acetate adjusted with glacial acetic acid to pH 8.3, 1 mM disodium ethylenediaminetetraacetic acid) to give a 1% (w/v) solution. After cooling, ethidium bromide was added to the solution to a final concentration of 0.4 µg ml^{-1} prior to pouring into a gel casting tray with an appropriately sized comb. The gel was allowed to set at room temperature followed by removal of the comb. DNA markers used were 100-bp DNA ladders (New England Biolabs, Ipswich,UK) and 1 kb ladders (Promega). Samples were run for 1-2 hours at 5-7 V/cm in 1 x TAE buffer. Gels were subsequently visualised under UV using the Gel Doc XR system (BIO-RAD) with the Quantity One basic software, version 4.6.5 (BIO-RAD).

2.11 GENOME ANALYSIS AND SEQUENCING

2.11.1 Genome sequencing of *C. jejuni* PT14 and phages CP30 and CPX

Genomic DNAs from *C. jejuni* PT14 and bacteriophage CP30 were previously isolated by Dr. P. Siringan and genomic DNA from bacteriophage CPX was isolated by Dr. S. Alkandari. Sequencing was performed by Deep Seq (University of Nottingham) using the 454 GS FLX platform (Roche Diagnostics, Indiana, USA). Raw data consisting of a total of 257,645, 26,922 and 37,429 reads for *C. jejuni* PT14, CP30 and CPX respectively was generated, with an average read length of 352, 324 and 321 bases respectively. Initial processing of the raw data was done by Deep Seq using Newbler Assembly software (454 Life Science, Connecticut, USA) and a single contig was produced for CPX. This was however not the case for the other genomes.

2.11.2 Phage sequence accession numbers

No further processing needed to be done for CPX, therefore the complete genome was annotated and deposited into GenBank by Dr. A. Timms under the accession number JN132397. The CP30 genome was *de novo* assembled by Prof. I. Connerton using CLC Genomics Workbench software (CLC Bio, Swansea, UK). The genome was found to have multiple sequence configurations, which were separated and annotated. One of the configurations, designated CP30A, was annotated in Artemis and was deposited in GenBank under accession number JX569801. CP30A is the only configuration that will be referred to in this thesis.

2.11.3 Genome assembly of *C. jejuni* PT14

The *C. jejuni* PT14 454 reads (2.11.1) were de novo assembled using CLC Genomics Workbench v6.0 software. The sequence was subsequently compared and confirmed with 5 million 50-bp reads generated using MiSeq technology operating in paired-end mode (Illumina).

2.11.4 Annotation of *C. jejuni* PT14

The complete genome of *C. jejuni* PT14 was sent to NCBI for annotation using their Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (Angiuoli et al. 2008). Frameshifts or any errors in the sequence assembly were confirmed using a combination of translated Basic Local Alignment Search Tool (BLAST) (Camacho et al. 2009), to observe protein translations for each nucleotide sequence and Artemis (Rutherford et al. 2000), to examine the six reading frames and determine which bases were responsible for the frameshifts. Once all potential frameshifts were confirmed the complete genome of *C. jejuni* PT14 was deposited in GenBank under the accession number CP003871.

2.11.5 Genome analysis

The complete annotated genome of *C. jejuni* PT14 was analysed using CLC Genomics Workbench software to identify the major genetic features. Genome comparisons were also made between *C. jejuni* PT14 and other *Campylobacter* strains using the genome database Biocyc (Caspi et al. 2014) as well as the online version of Artemis Comparison Tool (WebACT) (Abbott et al. 2005).

2.12 TRANSCRIPTOME ANALYSIS

2.12.1 RNA isolation and purification

C. jejuni PT14 cultures were grown and infected with phage as previously described (2.6.2). Cells were harvested by centrifuging the samples for 15 minutes at 10,000 x g with a J2-21 centrifuge (Beckman Coulter, High Wycombe, UK) using a JA14 rotor. Cell pellets were transferred to 1.5 ml microcentrifuge tubes and were washed three times in 1 ml of PBS (2.1.12) to get rid of the growth medium. This was done in an Eppendorf 5415R benchtop centrifuge (Fisher Scientific) at 10,000 x g for 15 minutes each at 4°C. Total RNA was then extracted from the cell pellets using the Trizol Max Bacterial Isolation kit with Max Bacterial Enhancement Reagent (Invitrogen, Paisley, UK). The RNA was ethanol-precipitated and an on-column DNase treatment was performed using an RNase-free DNase set (Qiagen). The RNA was subsequently purified using an RNeasy Mini kit (Qiagen, Crawley, UK). Briefly, the sample volume was adjusted to 200 µl with RNase-free water and 700 µl of Buffer RLT (cat. no. 79216: composition confidential but contains high concentration of guanidine isothiocycanate) was added. Five hundred microlitres of absolute ethanol was added to the diluted sample and this was mixed well by pipetting action. The sample was transferred to an RNeasy Mini spin column placed in a collection tube and was centrifuged for 15 seconds at 8,000 x g in a Heraeus Pico 17 bench-top centrifuge. The flow-through was discarded and 500 µl of Buffer RPE (cat. no. 101813: ethanol added but composition confidential) was added and the sample centrifuged for 15 seconds at 8,000 x g in a Heraeus Pico 17 bench-top centrifuge to wash the spin column. A second wash was carried out using Buffer RPE but with a spin

of 2 minutes at 8,000 x g. The column was then placed into a new collection tube and was centrifuged at full speed for 1 minute. The spin column was transferred to a new 1.5 ml collection tube and 30-50 µl of RNase-free water was added directly to the column membrane. The RNA was then eluted by centrifuging the column for 1 minute at 8,000 x g. The total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the integrity was assessed with a bioanalyzer 2100 (Agilent Technologies Inc., South Queensferry, UK) using the Prokaryote Total RNA Nano assay. Total RNA samples with RIN values of at least 7.0 were selected for cDNA library preparation.

2.12.2 Depletion of rRNA and library construction

Depletion of 23S, 16S and 5S rRNA from samples was achieved using the Ribo-Zero rRNA removal kit for Gram-negative bacteria (Epicentre Biotechnologies, Madison, Wisconsin) according to the manufacturer's instructions. Depletion of the rRNA was confirmed using a bioanalyzer 2100 (Agilent). Library preparation and sequencing were carried out at either Source BioScience (Nottingham, UK) or Ark Genomics (University of Edinburgh, Scotland, UK). To prepare the cDNA libraries, the ScriptSeq mRNA-Seq library preparation kit (Epicentre Biotechnologies) and the ScriptSeq v2 RNA-Seq library preparation kit (Epicentre Biotechnologies) were used respectively according to the manufacturer's instructions. Briefly, 2 µg of rRNA-depleted RNA was chemically fragmented by using the RNA fragmentation component of each kit (composition confidential) and incubating for 5 minutes at either 85 or 94°C. The fragmentation reaction was stopped by placing the mixture on

ice. Fragments of approximately 300-350 bp were reverse transcribed to cDNA using random-sequence primers containing a tagging sequence at their 5'ends. The RNA was then digested and the 3' ends of the cDNA were randomly tagged using the Terminal Tagging Oligo (TTO), which contained a 3'-blocking group on the 3'-terminal nucleotide to prevent extension of the TTO. The TTO was hydrolysed and the cDNA produced was tagged at both ends. The libraries were then individually indexed with a unique identification adapter sequence (TruSeq adapter index sequences) and the second strand of the cDNA was generated during a PCR amplification step of at least 15 cycles. At Source BioScience, the libraries were validated using the Shimadzu MultiNA and were normalised to approximately 10 nM while Ark Genomics used qPCR to quantify the libraries prior to sequencing and checked their quality using a bioanalyser 2100 (Agilent).

2.12.3 RNA-seq analysis

Paired-end, directional sequencing was carried out using the Illumina HiSeq 2000 sequencer. The indexed libraries were pooled and loaded onto a single lane of a HiSeq 2000 flowcell at a concentration of 8 pM. Cluster formation, primer hybridisation as well as 50-bp, 36 cycle sequencing at Source BioScience and 100-bp, 36 cycle sequencing at Ark Genomics were performed according to the Illumina standard protocol. On completion of the run, the raw sequence data were extracted, sorted according to index and converted to the FASTQ format using the sequencing analysis software Real Time Analysis (RTA) v1.10 and CASAVA v1.8.

2.12.4 Processing of the RNA-seq data

All processing of the RNA-seq data was carried out on a 64 bit desktop computer with 24 GB RAM and an Intel core i7 3.20 GHz processor. Three main steps were involved in the processing. The first was a quality control step where filtering of the raw reads was done, this was followed by mapping all reads against a reference genome and finally analysis of differential gene expression was carried out.

2.12.4.1 Filtering of the raw reads

The raw sequence reads were imported into the CLC Genomics Workbench v6.0 package and were filtered to remove low quality reads and the TruSeq adapter indexes. A duplicate read removal tool was also downloaded into the Workbench in order to filter any duplicated reads generated during PCR amplification.

2.12.4.2 Mapping filtered reads to reference genomes

The filtered reads were analysed using the RNA-Seq feature of CLC Genomics Workbench. Ignoring non-specific matches, reads were mapped to the *C. jejuni* PT14 genome sequence (GenBank accession number CP003871), and bacteriophages CP30A (GenBank accession number JX569801) and CPX (GenBank accession number JN132397).

2.12.4.3 Analysis of differential gene expression

The filtered reads were analysed using the RNA-seq Analysis tool of the CLC Genomics Workbench v6.0 package in order to identify differential gene

expression. This was achieved using the prokaryote setting under the exon identification and discovery section. Expression values were determined using reads per kilobase of transcript per million mapped reads (RPKM) (Mortazavi et al. 2008). Significant differential gene expression was assessed using the Baggerly's statistical test on expression proportions, which assumes a Beta-Binomial distribution and tests for RPKM differences between replicate samples (Baggerly et al. 2003). To correct for multiple hypothesis testing, p-values were calculated from the original p-values for the test statistic with a confidence rate of 0.05 by applying the false discovery rate controlling method of Benjamini and Hochberg (1995).

2.13 *CAMPYLOBACTER* COLONISATION OF BROILER CHICKENS

2.13.1 Pre-trial rearing of *Campylobacter*-free broilers

Commercial male Ross 308 broiler chickens were obtained from PD Hook (Oxfordshire, UK) as hatchlings. The *Campylobacter*-free day old chicks were group housed in a Home Office designated chicken house at Sutton Bonington Campus (Loughborough, UK) in accordance with the UK Home Office Regulations: Animal (Scientific Procedures) Act, 1986. The initial temperature of the house was 35°C but this was reduced on a daily basis by 1°C until it reached 21°C. All broilers were kept in full sight of other cohort members and in keeping with normal commercial rearing practices, they were provided with 23 hours of daylight and 1 hour of darkness every day. They were given food and water *ad libitum* and were fed a 'chicken starter' mixture containing 18% protein and supplemented with 105 mg/kg lasalocid sodium to

prevent coccidiosis. The birds were also checked twice a day for any changes in their appearance or behaviour. On reaching 16 days of age, they were weighed and randomly assigned to 12 caged groups of 7 birds. To ensure that they were still free of naturally occurring infection, the chicks were tested for the presence of *Campylobacter* by direct plating of cloacal swabs onto CCDA agar (2.1.2); and for *Salmonella* by pre-enrichment in BPW (2.1.11), selective isolation on MSRV agar (2.1.9) and confirmation of presence on XLD agar (2.1.10).

2.13.2 Preparation of bacterial suspensions

Two of the strains that were administered during the trials were *C. jejuni* PT14 and PT14Δ00150::kan mutant. The strains were grown on blood agar (2.1.1) incubated at 42°C for 24 hours under microaerobic conditions (2.2.2) and was supplemented with kanamycin (2.1.16) for the mutant strains. Three suspensions of each strain were prepared from the overnight growth to give approximate concentrations of 7 $\log_{10}$ CFU ml^{-1}, 5 $\log_{10}$ CFU ml^{-1} and 3 $\log_{10}$ CFU ml^{-1}. These concentrations were determined by OD_{600} measurements but the actual dose administered was confirmed by enumeration on CCDA (2.1.2).

2.13.3 Administration of *C. jejuni* strains

On day 22, three groups of 7 birds were dosed with 1 ml of *C. jejuni* PT14 and another three groups were dosed with PT14Δ00150::kan. For both strains, each individual group of 7 birds was dosed with either 7 $\log_{10}$ CFU ml^{-1}, 5 $\log_{10}$ CFU ml^{-1} or 3 $\log_{10}$ CFU ml^{-1} of *Campylobacter* (Table 2-5), which was administered by oral gavage.

Table 2-5. Dosing schedule for chicken colonisation trial

Bird ID	Strain	Dose (CFU ml^{-1})	Bird ID	Strain	Dose (CFU ml^{-1})
10-1	*C. jejuni* PT14 WT	10^{-3}	7-1	PT14Δ00150::kan	10^{-3}
10-2	*C. jejuni* PT14 WT	10^{-3}	7-2	PT14Δ00150::kan	10^{-3}
10-3	*C. jejuni* PT14 WT	10^{-3}	7-3	PT14Δ00150::kan	10^{-3}
10-4	*C. jejuni* PT14 WT	10^{-3}	7-4	PT14Δ00150::kan	10^{-3}
10-5	*C. jejuni* PT14 WT	10^{-3}	7-5	PT14Δ00150::kan	10^{-3}
10-6	*C. jejuni* PT14 WT	10^{-3}	7-6	PT14Δ00150::kan	10^{-3}
10-7	*C. jejuni* PT14 WT	10^{-3}	7-7	PT14Δ00150::kan	10^{-3}
11-1	*C. jejuni* PT14 WT	10^{-5}	8-1	PT14Δ00150::kan	10^{-5}
11-2	*C. jejuni* PT14 WT	10^{-5}	8-2	PT14Δ00150::kan	10^{-5}
11-3	*C. jejuni* PT14 WT	10^{-5}	8-3	PT14Δ00150::kan	10^{-5}
11-4	*C. jejuni* PT14 WT	10^{-5}	8-4	PT14Δ00150::kan	10^{-5}
11-5	*C. jejuni* PT14 WT	10^{-5}	8-5	PT14Δ00150::kan	10^{-5}
11-6	*C. jejuni* PT14 WT	10^{-5}	8-6	PT14Δ00150::kan	10^{-5}
11-7	*C. jejuni* PT14 WT	10^{-5}	8-7	PT14Δ00150::kan	10^{-5}
12-1	*C. jejuni* PT14 WT	10^{-7}	9-1	PT14Δ00150::kan	10^{-7}
12-2	*C. jejuni* PT14 WT	10^{-7}	9-2	PT14Δ00150::kan	10^{-7}
12-3	*C. jejuni* PT14 WT	10^{-7}	9-3	PT14Δ00150::kan	10^{-7}
12-4	*C. jejuni* PT14 WT	10^{-7}	9-4	PT14Δ00150::kan	10^{-7}
12-5	*C. jejuni* PT14 WT	10^{-7}	9-5	PT14Δ00150::kan	10^{-7}
12-6	*C. jejuni* PT14 WT	10^{-7}	9-6	PT14Δ00150::kan	10^{-7}
12-7	*C. jejuni* PT14 WT	10^{-7}	9-7	PT14Δ00150::kan	10^{-7}

2.13.4 Enumeration of caecal contents

The broiler chickens were sacrificed on day 23 according to the Home Office Humane Killing of Animals Schedule 1 code of practice. The birds were rendered unconscious via CO_2 asphyxiation and their necks were dislocated to confirm death. The birds were dissected using aseptic technique. With the breast facing upward, an incision was carefully made with a sterilised scalpel from the middle of the bird towards the vent. The breast muscle was then exposed by pulling the skin back, followed by carefully exposing the intestines by cutting and removing all surrounding tissue. The intestines were pulled away to expose the two caeca, which were cut using scissors and their contents squeezed into a pre-weighed Falcon tube. The weight of the caecal contents was then determined and a 1 in 10 (w/v) dilution was prepared using sterile MRD (2.1.13). This was vortexed vigorously to mix the sample as thoroughly as possible and further ten-fold dilutions were performed. *Campylobacter* enumerations were carried out on CCDA (2.1.2).

2.14 CONSTRUCTION OF CJ0031 COMPLEMENTATION CONSTRUCT

2.14.1 Complementation vector

The PCfdxA:PerR complementation shuttle vector was kindly provided by Bruce Pearson (Institute of Food Research, Norwich, UK). It contains the ferredoxin promoter *fdxA* (van Vliet et al. 2001), a *perR* (van Vliet et al. 1999) gene fragment, a chloramphenicol resistance cassette and two fragments of the *cj0046* pseudogene, which allow recombination into this gene locus in the NCTC 11168 genome following transformation.

2.14.2 Amplification of target gene

Primers were designed that could amplify the *cj0031* gene in NCTC 11168 and the upstream promoter region. (Primers-: Cj0031-fw:5'-ATCGTCTCCAGGAAGCATATATAAAGGTATT-3'; Cj0031-rv:5'-TATCGGCCGTTTCCCTTCAATGATTTTTATTTC-3'). The forward primer contained a *Bsm*BI (*Esp*3I) restriction site while the reverse primer contained *Bsi*EI, *Eae*I and *Eco*52I restriction sites. The desired fragment (3700-bp) was amplified using Q5 polymerase (New England Biolabs) and the PCR product was cleaned up with the Qiaquick PCR purification kit (Qiagen). The fragment was then subcloned into a pCR2.1 TOPO vector (Invitrogen).

2.14.3 Ligation of vector and amplified *cj0031* fragment

The amplified *cj0031* product was digested with *Bsm*BI and *Eco*52I and the PCfdxA:PerR complementation vector was linearised by digesting it with *Bsm*BI. The fragment was then ligated into the vector, where the *fdxA* promoter and *perR* gene were replaced by the *cj0031* promoter and gene fragment.

2.14.4 Recombination of the complementation construct into the *C. jejuni* PT14 genome

The vector was amplified in *E. coli*. Genomic DNA was then used to naturally transform and complement the PT14Δ00150::kan deletion mutant.

2.15 *C. JEJUNI* INFECTION OF CULTURED HUMAN EPITHELIAL CELL LINES

2.15.1 Maintenance of cell cultures

Human colonic epithelial HCA-7 cells (Kirkland 1985) were grown as monolayer cultures in 24-well plates in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with foetal calf serum (FCS) at a final concentration of 10% (v/v) (Invitrogen) at 37°C in 5% (v/v) CO_2. Cells were stained with 0.4% (v/v) Trypan Blue (Invitrogen) and their viability was monitored by microscopic examination.

2.15.2 Adhesion and invasion assay

Adhesion and invasion assays were performed as previously described by Al-Sayeqh et al. (2010). Duplicate cell monolayers at 70% confluence were covered with cells of the *Campylobacter* strain of interest. This was done at an approximate multiplicity of infection of 100 in D-MEM supplemented with 10% FCS and incubated at 37°C for 3 h in 5% CO_2. Both monolayer plates were washed three times with PBS (2.1.12) but one was also treated with 1 ml of fresh D-MEM supplemented with 250 μg gentamicin ml^{-1} and incubated for a further 2 hours in order to kill extracellular bacteria. The cell monolayers were lysed by adding 0.1% (vol/vol) Triton X-100 in PBS to release internalized bacteria and cells were enumerated on CCDA plates (2.1.2). The number of adherent bacteria was calculated by subtracting the number of internalized bacteria from the total number of adherent and invaded cells and then expressing this value as a percentage of the inoculum. The invasion

efficiency was expressed as the percentage of the inoculum that survived gentamicin treatment.

Chapter 3 : Genomic characterisation of *Campylobacter jejuni* PT14 and examination of phage-susceptibility

3.1 INTRODUCTION

Campylobacter jejuni strain PT14 is a clinical isolate which was previously used to propagate bacteriophages in the United Kingdom phage typing scheme (Frost et al. 1999). It has proven useful in the isolation of *Campylobacter* bacteriophages from several sources and has successfully colonised chickens during experimental infection trials (Atterbury et al. 2003a; Atterbury et al. 2003b; Atterbury et al. 2005; Connerton et al. 2004; El-Shibiny et al. 2005; Loc Carrillo et al. 2005). The strain is available from the Public Health England Board as NCTC 12662 (www.phe-culturecollections.org.uk).

Over the last decade, a large number of *Campylobacter* genomes have either been partially or completely sequenced. Publication of the first complete *Campylobacter* genome sequence, that of *C. jejuni* NCTC 11168 (Parkhill et al. 2000; Gundogdu et al. 2007), provided an ideal reference genome for a number of comparative genome studies which examined genetic variability within the genus (Pearson et al. 2003; Taboada et al. 2004; Parker et al. 2006). Prophage-related DNA sequences were notably absent from the genome of *C. jejuni* NCTC 11168. In constrast, the chicken isolates *C. jejuni* RM1221 (Fouts et al. 2005) and *C. jejuni* S3 (Cooper et al. 2011) were found to possess *Campylobacter jejuni* integrated elements (CJIEs), one of which was identified as a Mu-like prophage. It has been reported that the Mu-like prophage sequences are substrates for recombination adding to the diversity of *Campylobacter*. Genomic rearrangements observed in *C. jejuni* HPC5 occurred as a result of intra-genomic inversions between Mu-like prophage DNA

sequences provoked by exposure to virulent bacteriophages, which resulted in the activation of dormant Mu-like prophages (Scott et al. 2007a).

Bacteria have evolved a number of defence mechanisms to help protect them from invading foreign nucleic acids, such as those of bacteriophages and plasmids. One such system in many bacterial species, including *C. jejuni*, is the presence of the clustered regularly interspaced short palindromic repeats (CRISPR) and associated (*cas*) genes. CRISPR-*cas* loci are comprised of an array of multiple DNA repeats that generally vary between 21 and 48 bp in length, separated by non-repetitive sequences called spacers that are approximately 26-72 bp in length (Grissa et al. 2007). Cas proteins, ranging from 4 to 20 in number (Haft et al. 2005), are usually present in the vicinity of the CRISPR array and an AT-rich sequence known as the leader is also located directly upstream of this locus.

The CRISPR-*cas* system was firstly identified in 1987 in *E. coli* K12 (Ishino et al. 1987), followed by identification of CRIPSR arrays in a number of other bacterial and archeal species in the early 2000s, including *Streptococcus pyogenes* (Hoe et al. 1999), *Haloferax mediterranei* (Mojica et al. 1995), *Anabaena* sp. PCC 7120 (Masepohl et al. 1996) and *Mycobacterium tuberculosis* (Groenen et al. 1993). It was later determined that the spacer sequences of the CRISPR-*cas* system were actually capable of providing resistance against bacteriophages and plasmids (Barrangou et al. 2007; Marraffini & Sontheimer 2008; Horvath & Barrangou 2010). Barrangou and colleagues (2007) looked at the CRISPR sequences of numerous

bacteriophage-sensitive *Streptococcus thermophilus* strains and found that after a viral challenge, the bacteria had integrated new spacers derived from the DNA of the infecting phage (protospacer). Acquisition of this new protospacer resulted in resistance to subsequent infection by phages carrying the same sequence in their genome. The protospacer sequence was found to be integrated adjacent to the leader sequence of the CRISPR array.

The CRISPR locus is generally transcribed as a large single RNA transcript (precursor CRISPR RNA or pre-crRNA), which is processed by Cas proteins to generate small antisense RNA molecules (CRISPR RNA or crRNA). These cRNAs contain a single spacer that is flanked by direct repeat fragments, which then target and cleave the invading DNA during subsequent reinfection (Brouns et al. 2008). Spacer acquisition as well as target recognition and cleavage are usually mediated by recognition of a short protospacer adjacent motif (PAM), which is located adjacent to either end of the protospacer (Shah et al. 2013). There are three major types of CRISPR-*cas* systems (Type I, II and III), all of which contain the highly conserved Cas proteins, Cas1 (DNA endonuclease) and Cas2 (RNA endonuclease). Each system is characterised by the specific Cas proteins involved in maturation of crRNAs, nucleic acid targeting and cleavage of the invading DNA element (Makarova et al. 2011). Type II CRISPR-*cas* is considered to be the minimal CRISPR-*cas* system and typically contains genes encoding various configurations of Cas1, Cas2, Cas4 or Csn2/Csn2-like and Cas9 (Makarova et al. 2011; Chylinski et al. 2014). It is predominately associated with pathogenic bacteria such as *C. jejuni*, which possesses a type II-C system that lacks a Cas4 or Csn2/Csn2-like protein

(Figure 3-1). The crRNA maturation step that takes place within type II systems is characterised by a requirement for a trans-activating CRISPR RNA molecule (tracrRNA), host RNase III and Cas9 (Deltcheva et al. 2011; Jinek et al. 2012). It has previously been demonstrated that the mature crRNA can be complementary base-paired to the tracrRNA in the presence of Cas9 to form an RNA duplex, which subsequently guides the Cas9 protein to cleave the target DNA at sites that are complementary to the crRNA (Jinek et al. 2012). This step is referred to as CRISPR interference.

Another important bacterial defence system that utilises a self/non-self DNA recognition mechanism is the use of restriction-modification (R-M) systems that degrade unmethylated or unmodified incoming DNA. R-M systems typically consist of a methyltransferase that catalyses methylation of a specific host DNA sequence and a restriction enzyme which cleaves or restricts foreign DNA lacking that specific methylation. Approximately 90% of bacterial genomes were found to contain at least one R-M system and approximately 80% contain multiple R-M systems (Vasu & Nagaraja 2013). In general, a relationship exists between the number of R-M systems present in a genome and the genome size of a bacterial strain, (Vasu & Nagaraja 2013). However the reason for the presence of multiple R-M systems in a single host is not clearly understood. R-M systems are classified into four main groups (Types I, II, III and IV) based primarily on enzyme subunit composition, cofactor requirements, symmetry of the recognition sequence and positioning of the DNA cleavage site (Boyer 1971; Roberts et al. 2003; Yuan 1981). Each group can be divided into sub-categories, especially the Type II.

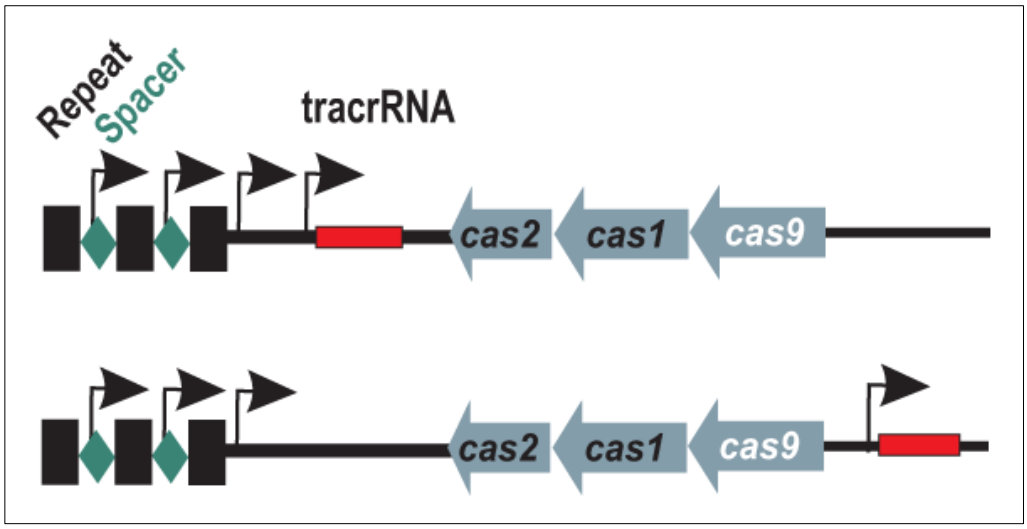


Figure 3-1. Typical type II-C CRISPR-*cas* systems.

The CRISPR repeats are represented by black boxes and the spacers by green diamonds. Each spacer is capable of independent expression due to the presence of transcriptional start sites within their sequences (black arrows). The tracrRNA sequence is illustrated by a red box and it can either be located upstream or downstream of the genes encoding the CRISPR-associated Cas proteins *cas2*, *cas1* and *cas9* (blue arrows). Adapted from Dugar et al. 2013.

Type I R-M systems are comprised of a multisubunit complex which functions as both a methyltransferase and a restriction endonuclease. It usually contains two R subunits (restriction), two M subunits (modification) and one S subunit (specificity) (Dryden et al. 2001). Sequence recognition is determined by two target recognition domains (TRDs) in the S subunit. The genes present in Type I systems are *hsdR, hsdM* and *hsdS* and the proteins encoded by the latter two are sufficient for methyltransferase activity, but all three are required for restriction activity. Type I restriction enzymes require ATP, S-adenosylmethionine (AdoMet) and Mg^{2+} during cleavage of unmethylated substrates, which occurs at a variable distance from the recognition sequence following DNA translocation. However, if the enzyme complex targets a hemimethylated substrate, where only one strand is methylated, it can act as a methyltranferase to methylate the other strand and requires AdoMet to donate the methyl group. Methylation with a Type I system produces N6-methyladenine (m6A) and the recognition sequence is asymmetrical and bipartite. Type I systems are currently divided into five sub-categories (A, B, C, D and E) based on genetic complementation, where subunits from different enzymes associate to produce a single functional enzyme; DNA sequence similarity and immunological cross-reactivity (Titheradge et al. 2001; Chin et al. 2004).

Type II R-M systems are the simplest and most commonly known. They are comprised of separate subunits for restriction and modification but some classes are known to produce a single bifunctional protein due to fusion of the R and M genes. The endonuclease component of the system requires Mg^{2+} ions

as a cofactor and the methyltransferase requires AdoMet as a methyl group donor to produce N4-methylcytosine (m4C), 5-methylcytosine (m5C) or m6A. The Type II enzymes generally recognise a 4-8 bp palindromic sequence and DNA cleavage occurs symmetrically on both strands at a defined position at or close to the recognition site. Type II restriction enzymes have been subdivided into 11 different classes according to their enzymatic behaviour and sequence cleaving nature (A, B, C, E, F, G, H, M, P, S and T) and some enzymes can belong to more than one subcategory (Roberts et al. 2003).

The Type III R-M systems comprise of two genes, *mod* and *res*, which encode protein subunits M (recognition and modification) and R (restriction). The M subunit can act independently as a methyltransferase but both M and R subunits are required for restriction activity (Janscak et al. 2001; Mucke et al. 2001). Methylation requires AdoMet while cleavage requires ATP in the presence of AdoMet. For successful cleavage, Type III enzymes recognise two copies of a non-palindromic 5-6 bp sequence which are oppositely positioned within the DNA substrate. Cleavage occurs in a similar fashion to Type I enzymes where the DNA is cleaved at a distinct location from one copy of the recognition sequence following ATP-dependent DNA translocation. Methylation takes place primarily on one strand but cleavage will only occur if both strands are unmodified. The product of methylation is hemimethylated m6A.

Type IV R-M systems have been re-classified to include modification-dependent restriction enzymes that specifically recognise methylated DNA

sites (Roberts et al. 2003). These systems possess no associated methyltransferase activity. Foreign DNA is therefore targeted if it contains methylated bases in a particular recognition sequence. One well defined Type IV system is that of McrBC in *E. coli* (Raleigh & Wilson 1986; Stewart et al. 2000), which generally targets two variably spaced dinucleotides in the form RmC (m4C or m5C), and cleaves approximately 30-bp away from one of these recognition elements with a GTP requirement for translocation of the DNA. The RmC recognition site can be found on both strands or may only be present on one.

C. jejuni strains are known to possess many hypervariable homopolymeric repeats within genes related to the assembly of their capsule, lipooligosaccharides (LOS) and flagella structures (Parkhill et al. 2000). The presence of these repeats makes the genes prone to slipped-strand mispairing during chromosomal replication leading to phase variation (Parkhill et al. 2000). Surface capsular polysaccharide (CPS) structures and flagella of *C. jejuni* have been recognised as important factors in the binding of bacteriophages (Coward et al. 2006). However, phase variation of such genes can alter cell surfaces, which in turn can affect phage propagation by restricting access to cell surface recognition sites. The *cps* locus of *C. jejuni* is known to contain conserved as well as variable genes (Karlyshev et al. 2005). The gene cluster responsible for the biosynthesis of heptosyl residues (Hep), including *hddC, gmhA2* and *hddA,* is highly variable while the transport related *kps* genes flanking the Hep residues are conserved among strains (Karlyshev et al. 2005).

In this chapter the genome sequence of *C. jejuni* PT14 was characterised and an attempt was made to determine what genetic factors may be contributing to the phage-sensitive nature of this strain.

3.2 RESULTS

3.2.1 Genome analysis

The circular genome of *C. jejuni* PT14 was found to be 1,635,252-bp in length with 1607 putative open reading frames (ORFs) and an average G+C content of 30.5%. The genome sequence contains 3 copies of the ribosomal RNA operon and 41 tRNA genes covering all amino acids, with arginine being represented most frequently followed by alanine and leucine. The genome was automatically annotated using the NCBI programme PGAAP (Angiuoli et al. 2008) (2.11.4), from which it was possible to assign functional information to 80.9% of the 1607 ORFs on the basis of what was available in the database. Figure 3-1 shows a genome map of *C. jejuni* PT14 with annotation related to as CDS, pseudogenes and conserved hypothetical proteins illustrated. There is no evidence for the presence of any prophage-associated genes or plasmids within this genome.

C. jejuni PT14 contains 26 probable pseudogenes, with many in common with those in other *C. jejuni* genomes. Table 3-1 shows a comparison of the pseudogenes present in the PT14 genome with their gene homologues in the NCTC 11168 genome. The majority of these are also probable pseudogenes in NCTC 11168 and have been highlighted in yellow. However, twelve pseudogenes present in PT14 remain intact within NCTC 11168. Similarly, on examination of the NCTC 11168 genome, there are three annotated pseudogenes that are intact within the PT14 genome. These are *cj0501* (A911_02450), *cj1064* (A911_05150) and *cj1470c* (A911_07080).

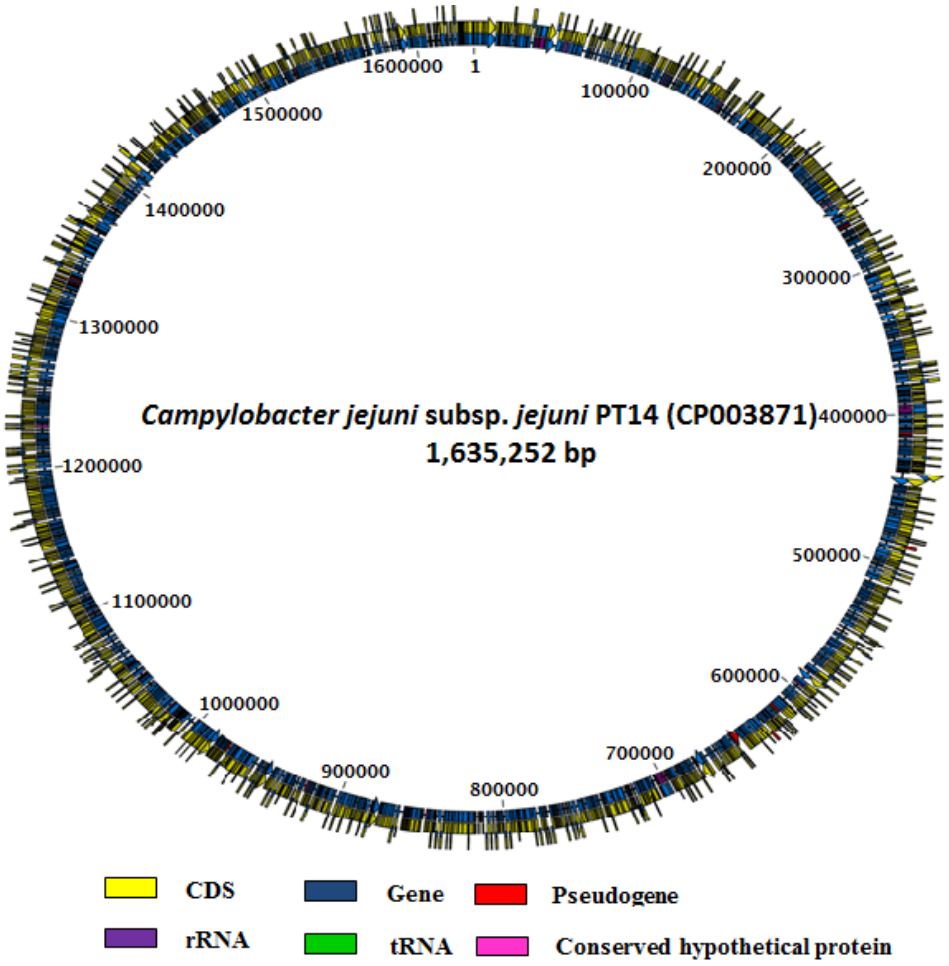


Figure 3-2. Circular representation of the *C. jejuni* PT14 genome.

The annotation types are colour coded according to the key. Colour coded are 1607 CDS, 1657 genes, 26 pseudogenes, 42 conserved hypotheticals, 9 rRNAs and 41 tRNAs.

Table 3-1. Probable pseudogenes within the *C. jejuni* PT14 genome.

No.	PT14		NCTC 11168	
	Gene ID	Product	Gene ID	Product
1	A911_00225 A911_00230 A911_00235 A911_00240 A911_00245	C4-dicarboxylate transporter	Cj0046	Putative sodium sulfate transmembrane transport protein
2	A911_00335 A911_00340	Hemerythrin family non-heme iron protein	Cj0072c	Putative iron-binding protein
3	A911_00820	Methyl transferase	Cj0170	Methyl transferase
4	A911_01045 A911_01050 A911_01055 A911_01060	Serine protease eatA precursor	Cj0223	Putative IgA protease family protein
5	A911_01405	Glycerol-3-phosphate transporter	Cj0292c	Putative glycerol-3-phosphate transporter
6	A911_02170 A911_02175	TonB-dependent receptor	Cj0444	Putative TonB-dependent outer membrane receptor
7	A911_02370	MFS transport protein	Cj0484	Putative MFS transport protein
8	A911_02550	Na+/Pi cotransporter protein	Cj0522	Putative Na+/Pi cotransporter protein
9	A911_02750 A911_02755	Hypothetical protein	Cj0565	Hypothetical protein
10	A911_03205	Di-/tripeptide transporter	Cj0654	Putative transmembrane transport protein

Table 3-1 continued

No.	PT14		NCTC 11168	
	Gene ID	Product	Gene ID	Product
11	A911_03280 A911_03285	Potassium-transporting ATPase, subunit A	Cj0676	Potassium-transporting ATPase A chain
12	A911_03300	Potassium-transporting ATPase, subunit C	Cj0678	Potassium-transporting ATPase C chain
13	A911_03365	Restriction/modification enzyme	Cj0690c	Putative restriction/modification enzyme
14	A911_03610	Flamentous hemagglutinin domain protein	Cj0738-Cj0740	Hypothetical proteins
15	A911_03630 A911_03635	Putative outer membrane protein	Cj0742	Putative outer membrane protein
16	A911_03645 A911_03650	ISCco1, transposase orfB	Cj0752	IS element transposase
17	A911_04205 A911_04210 A911_04215 A911_04220 A911_04225	Arylsulfotransferase	Cj0866	Arylsulfatase
18	A911_04680 A911_04685	Putative periplasmic protein	Cj0969	Putative periplasmic protein
19	A911_04765	MFS transport protein	Cj0987	Putative MFS transport protein
20	A911_05030	Putative transmembrane transport protein	Cj1040	Putative MFS transport protein
21	A911_06755 A911_06760	Cryptic C4-dicarboxylate transporter, DcuD	Cj1389	Putative C4-dicarboxylate anaerobic carrier

Table 3-1 continued

No.	PT14		NCTC 11168	
	Gene ID	Product	Gene ID	Product
22	A911_06780	Adenylosuccinate lyase	Cj1395	Putative MmgE/PrpD family protein
23	A911_06985	Integral membrane protein	Cj1452	Putative integral membrane protein
24	A911_07015	Periplasmic protein	Cj1456	Putative periplasmic protein
25	A911_07345	C4-dicarboxylate anaerobic carrier, putative	Cj1528	Putative C4-dicarboxylate anaerobic carrier
	A911_07350	C4-dicarboxylate anaerobic carrier, putative		
	A911_07355	Cryptic C4-dicarboxylate transporter, DcuD		
26	A911_07485	Putative permease	Cj1560	Putative permease
	A911_07490			

Note: Predicted pseudogenes shared between the *C. jejuni* NCTC 11168 and PT14 genomes have been highlighted in yellow in the NCTC 11168 genome. Genes that remain intact within *C. jejuni* NCTC 11168 compared to those in the PT14 genome have not been highlighted.

C. jejuni PT14 was found to contain 27 homopolymeric G:C tracts (defined as containing ≥7 consecutive G:C residues) which bears comparison to 29 reported for NCTC 11168 (Parkhill et al. 2000; Gundogdu et al. 2007), 25 for RM1221 (Parker et al. 2006) and 23 for CG8486 (Poly et al. 2007). The locations of homopolymeric tracts are generally conserved between *C. jejuni* strains. Within the *C. jejuni* PT14 genome, there are five tracts residing in intergenic regions, two in probable pseudogenes and nine within genes of unknown function (Table 3-2). Variation in the length of the G:C tracts within genes results in phase variable expression. Five of the tracts identified in *C. jejuni* PT14 show phase variation at the sequence level. These are genes encoding two putative methyltransferases, one of unknown function, an aminoglycosidase N3'-acetyltransferase and the invasion protein CipA. *C. jejuni* PT14 also contains a phase variable A:T region within the gene A911_06060 encoding a GMP synthase.

3.2.2 Nucleotide sequence statistics

The nucleotide distribution displayed in Figure 3-3 is typical of an AT-rich *Campylobacter* genome. The average G+C content of the *C. jejuni* PT14 genome is 30.5% and this percentage is similar to that observed for other strains including NCTC 11168 (30.6%) (Parkhill et al. 2000; Gundogdu et al. 2011), CG8486 (30.4%) (Poly et al. 2007) and 81-176 (30.6%) (Hofreuter et al. 2006). Table 3-3 shows the nucleotide frequency in codon positions. "A" residues are observed most frequently in the first and second position whilst "T" residues are most frequent in the third position. "C" residues were the least frequently occurring nucleotide in the first and third position while "G"

Table 3-2. Listing and location of homopolymeric repeats within the C. jejuni PT14 genome

No	Position	Homopolymeric G/C tract	Gene ID/Region affected	Putative function	Note
1	37029-37038	C (10)	Intergenic	N/A	N/A
2	66340-66349	C (10)	A911_00220	Hemerythrin-like iron -binding protein	N/A
3	167095-167103	G (9)	A911_00820	Methyl transferase	Pseudogene
4	527148-527157	G (10)	Intergenic	N/A	N/A
5	577352-577361	G (10)	A911_03015	Unknown	N/A
6	588134-588143	G (10)	A911_03065	Lipoprotein	N/A
7	629763-629772	G (10)	A911_03285	Potassium transporting ATPase, A-subunit	Pseudogene
8	640595-640603	G (9)	A911_03335	Invasion protein CipA	Phase variable
9	697531-697541	C (11)	Intergenic	N/A	N/A
10	1074514-1074524	C (11)	A911_05520	Beta-1,3 galactosyltransferase	N/A
11	1080064-1080070	C (7)	A911_05550	Unknown	N/A
12	1227262-1227270	G (9)	A911_06290	Unknown	Phase variable
13	1228732-1228741	G (10)	A911_06295	Aminoglycosidase N3'-acetyltransferase	Phase variable
14	1235061-1235069	C (9)	A911_06340	Unknown	N/A
15	1236300-1236308	C (9)	A911_06345	Unknown	N/A
16	1240439-1240447	C (9)	A911_06365	Unknown	N/A
17	1241666-1241674	C (9)	A911_06370	Unknown	N/A
18	1250228-1250237	G (10)	Intergenic	N/A	N/A

Table 3-2 continued

No	Position	Homopolymeric G/C tract	Gene ID/Region affected	Putative function	Note
19	1253090-1253098	G (9)	A911_06440	Methyltransferase	Phase variable
20	1263087-1263095	G (9)	A911_06490	Motility accessory factor	N/A
21	1274781-1274789	C (9)	A911_06515	Unknown	N/A
22	1353164-1353172	C (9)	A911_06906	SAM-dependent methyltransferase	Phase variable
23	1355346-1355354	C (9)	A911_06907	Sugar transferase	N/A
24	1364471-1364479	C (9)	Intergenic	N/A	N/A
25	1366633-1366640	C (9)	A911_06918	Unknown	N/A
26	1385824-1385832	G (9)	A911_07000	Sugar transferase	N/A
27	1591849-1591858	G (10)	A911_08080	Lipoprotein	N/A

Note: Homopolymeric G:C tracts are defined as containing ≥7 consecutive G:C residues. The relative chromosomal position within the PT14 genome is indicated with respect to the nucleotide sequence beginning at dnaA, along with the gene or intergenic regions where the G:C tracts occur. Genes that are observed to be phase variable at sequence level are noted.

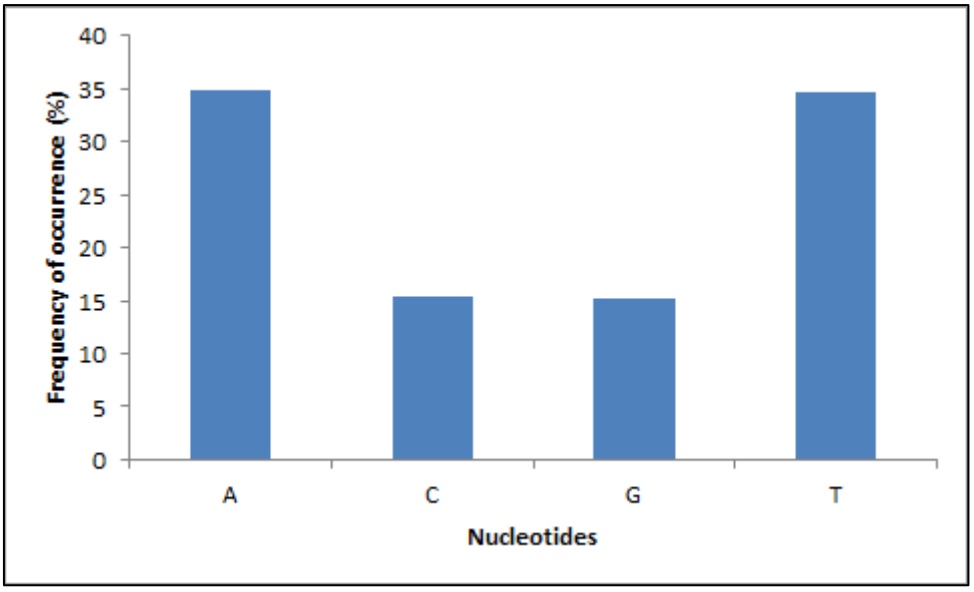


Figure 3-3. Nucleotide distribution histogram for the *C. jejuni* PT14 genome.

The occurrence of each nucleotide within the genome is illustrated. The average G+C content of the genome is shown to be 30.5%.

Table 3-3. Frequency of nucleotide codon positioning for *C. jejuni* PT14 genome.

Codon position	Nucleotide frequency in codon position (%)			
	A	C	G	T
1st position	36	13	30	21
2nd position	37	17	14	33
3rd position	36	9	10	45

residues were the least in the second position. The reduced frequency of G and C residues is consistent with their low distribution within the genome. The codon "AAA", encoding the amino acid lysine, occurred 41,343 times and was the most abundant codon. However, "CGG" encoding arginine was the least frequent, occurring only 58 times.

3.2.3 Phylogenetic relation to other *C. jejuni* strains

Figure 3-4 illustrates the relationship between *C. jejuni* PT14 and its nearest neighbours based on genomic BLAST comparisons. The dendogram shows that although there is strong synteny with NCTC 11168, the genome with the most genetic similarity is that of *C. jejuni* IA3902. This is quite interesting because PT14 and NCTC 11168 are both human *C. jejuni* isolates while IA3902 was originally isolated from an aborted ovine fetus and is a highly abortifacient strain (Burrough et al. 2009). It is also notable that the two strains known to contain prophage-associated elements, *C. jejuni* RM1221 and *C. jejuni* S3, are clustered furthest away from PT14 since this genome does not appear to contain any such integrated elements.

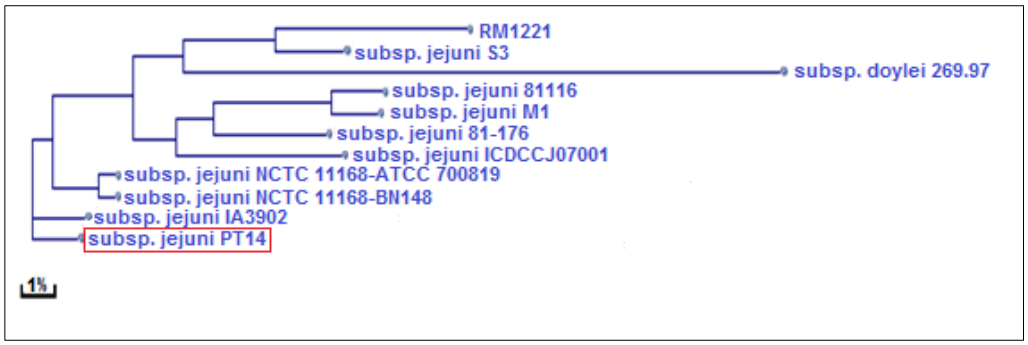


Figure 3-4. Phylogenetic comparison of *C.jejuni* PT14 with its closest relatives.

The comparisons are based on genomic BLAST searches. The PT14 genome is clustered furthest away from the two genomes known to contain *Campylobacter jejuni* integrated elements (CJIEs), RM1221 and *C. jejuni* S3. The dendogram was calculated using the Genome feature of the NCBI website.

3.2.4 CRISPR-*cas* system of *C. jejuni* PT14

The CRISPR-*cas* system within the genome of *C. jejuni* PT14 was identified and found to be intact. PT14 contains a minimal type II-C CRISPR array, with four 36-bp direct repeats (start position of first repeat: 1448528, end position of last repeat: 1448761) flanking three 30-bp spacer sequences (start position of first spacer: 1448564, end position of last spacer: 1448725). Interestingly, the first direct repeat sequence is missing the initial "G" residue which is present in the other three repeats. A putative transcriptional -10 start site (5'-TAAAAT-3') was identified 123-bp upstream of the initial direct repeat. The genes encoding three CRISPR-associated Cas proteins, *cas2* (A911_07325) (position: 1448906-1449337), *cas1* (A911_07330) (position: 1449330-1450220) and *cas9* (A911_07335) (position: 1450217-1453180) were also identified, beginning 145-bp downstream of the terminal direct repeat sequence. A tracrRNA sequence was also determined that could potentially base-pair with the repeat sequence. The CRISPR array sequences can be found in Table 3-4.

BLAST searches revealed that for each spacer sequence of the *C. jejuni* PT14 CRISPR array, there were no obvious matches to phage or plasmid DNA but instead nucleotide sequences were found that mapped to the PT14 chromosome. The first spacer sequence mapped to the gene encoding the peptidoglycan-associated lipoprotein Omp18 (A911_00540) while the second spacer mapped to the genes encoding apolipoprotein N-acyltransferase (A911_05300) and isoleucyl-tRNA ligase (A911_05135). BLAST searches also found matches to other *C. jejuni* strains, with the first and third spacer

Table 3-4. CRISPR array sequences for *C. jejuni* PT14

CRISPR feature	Sequence (5'→3')
Direct repeat	GTTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAAT
Spacer 1	ATAATTTCTAATTTCATTTATAACCTTTCA
Spacer 2	TAGTAGCTAAGAATAAAATAAGAAACACTG
Spacer 3	GTTGGAATGCTTAAGCAGGGGTGGAGTGAA
tracrRNA	AAGAAAUUUAAAAAGGGACUAAAA

sequences displaying 100% sequence identity to spacer sequences of three other *C. jejuni* strains for the full length of the sequence. The second spacer showed 100% identity to spacer sequences of five other strains, with two of them matching 100% for the full sequence length. A BLAST search of the direct repeat sequence displayed 100% nucleotide sequence identity to those of at least 99 other *C. jejuni* strains. The CRISPR element of *C. jejuni* PT14 has a G+C content of 28.4%, which is lower than the average G+C content of the rest of the genome.

3.2.5 The *cps* gene locus of *C. jejuni* PT14

The *C. jejuni* PT14 *cps* gene locus extends from A911_06875 to A911_06960 and has a G+C content of 27%. On comparing the genomes of PT14 to NCTC 11168, the capsular polysaccharide loci were found to have variable regions. However, both genomes contained *kps* orthologs with seven genes that make up two clusters and flank the polysaccharide biosynthesis genes. The first cluster contains the genes *kpsS* and *kpsC* (A911_06875 and A911_06880) while *kpsF*, *kpsD*, *kpsE*, *kpsT* and *kpsM* (A911_06940, A911_06945, A911_06950, A911_06955 and A911_06960) make up the second cluster (Figure 3-5). The Kps proteins allow translocation of the capsular polysaccharide to the bacterial surface. Within the PT14 genome, twenty-two polysaccharide biosynthetic genes were identified but none appear to encode proteins that are responsible for biosynthesis of Hep residues as observed within NCTC 11168 (Parkhill et al. 2000). However, the capsular polysaccharide locus in *C. jejuni* PT14 was found to be identical to that of *C. jejuni* IA3902, which also contained no genes for biosynthesis of Hep residues.

An insertion of six genes as well as a gene substitution of fourteen genes has occurred within NCTC 11168 compared to PT14 (A911_06908 to A911_06935, excluding A911_06918) and IA3902 (CJSA_1353 to CJSA_1367 excluding CJSA_1363). This occurs between *cj1422* and *cj1442c* within the NCTC 11168 genome. In addition, within the *cps* locus of PT14 there are three genes containing homopolymeric G:C tract repeats and two of them appear phase variable at sequence level. These are A911_06906 and A911_06907, which encode a SAM-dependent methyltransferase and a putative sugar transferase respectively while the hypothetical gene represented by A911_06918 does not appear to be phase variable. The nearest homologues to A911_06906, A911_06907 and A911_06918 within *C. jejuni* NCTC 11168 are *cj1420c*, *cj1421c* and *cj1422c* respectively, which are all phase variable within that genome. Figure 3-5 shows an alignment of the capsular polysaccharide loci between *C. jejuni* PT14, NCTC 11168 and IA3902. These two genomes were chosen for the alignment because of their close phylogenetic relationship with PT14 based on genomic BLAST searches (3.2.3). The gene arrangement within this locus is identical between PT14 and IA3902. The gene substitutions in the NCTC 11168 genome are also clearly illustrated in Figure 3-5.

3.2.6 The lipooligosaccharide biosynthesis locus of *C. jejuni* PT14

The *C. jejuni* PT14 LOS locus extends from A911_05480 (*galE*) to A911_05580 (*gmhB*) (Figure 3-6) and has a G+C content of 27.8%. Genes

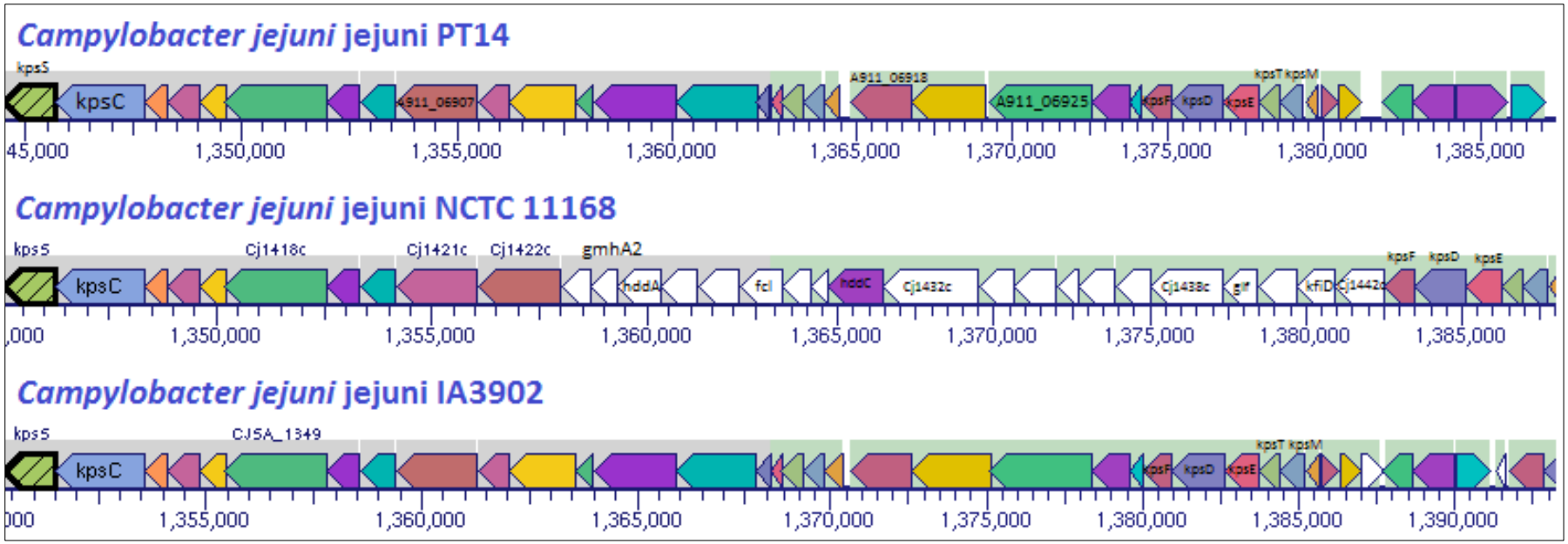


Figure 3-5. Alignment of the capsular polysaccharide loci of *C. jejuni* PT14, NCTC 11168 and IA3902.

Gene names are shown on or above each ORF and the direction of transcription is indicated by an arrow. Corresponding homologous genes are assigned the same colour and the *kps* genes clusters are indicated for each genome. In PT14, this locus extends from the first gene shown, *kpsS*, until the *kpsM* gene (A911_06875 to A911_06960). Gene substitutions occur in NCTC 11168 compared to the other two genomes directly after *cj1422c* until *cj1442c*. In NCTC 11168 this region includes genes responsible for heptosyl biosynthesis but not in *C. jejuni* PT14 and *C. jejuni* IA390. The alignment was performed using the Biocyc databases (Caspi et al. 2014).

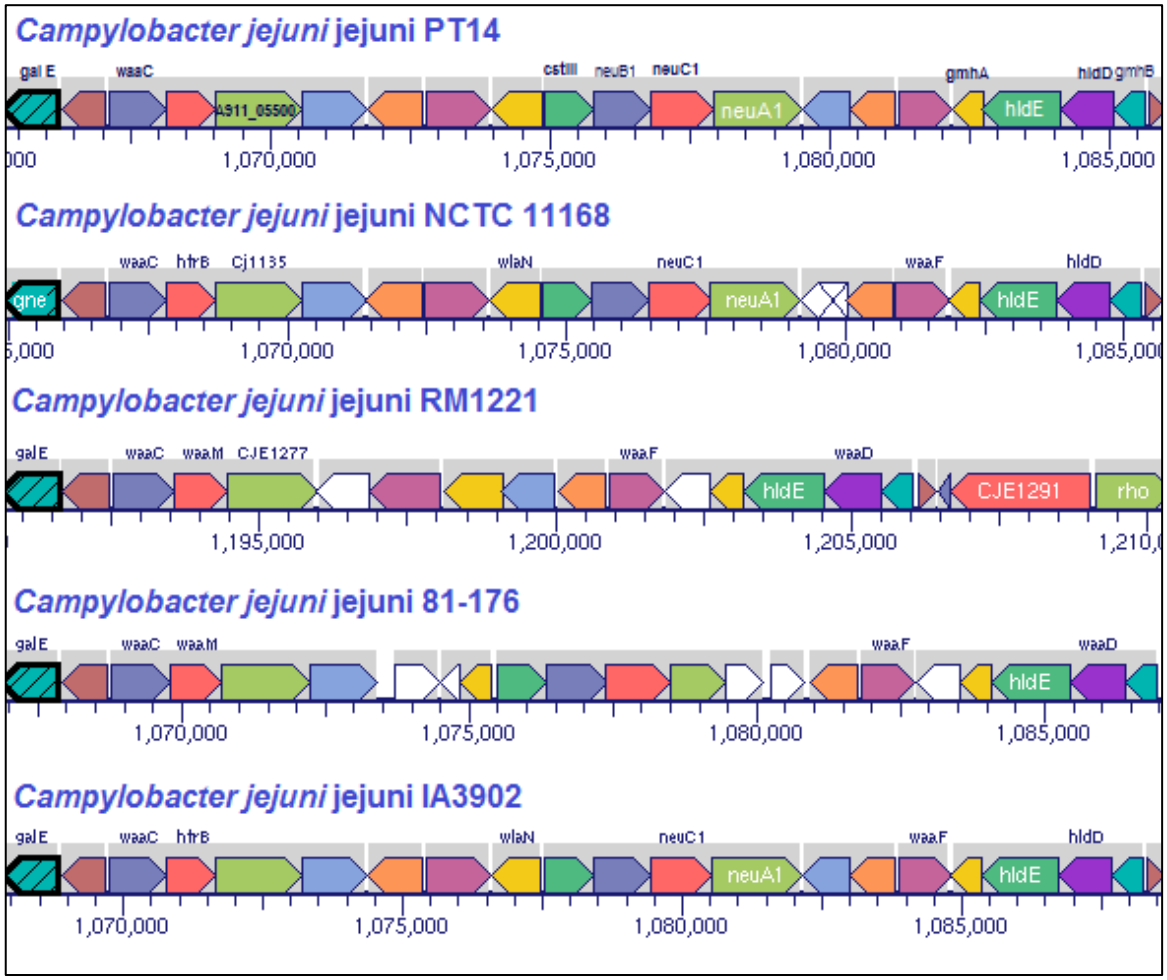

Campylobacter jejuni jejuni PT14
galE
waaC
A911_05500
cstIII
neuB1
neuC1
neuA1
gmhA
hldE
hldD
gmhB
1,070,000
1,075,000
1,080,000
1,085,000
Campylobacter jejuni jejuni NCTC 11168
waaC
hfrB
Cj1135
wlaN
neuC1
waaF
hldD
gne
neuA1
hldE
1,070,000
1,075,000
1,080,000
Campylobacter jejuni jejuni RM1221
galE
waaC
waaM
CJE1277
waaF
waaD
hldE
CJE1291
rho
1,195,000
1,200,000
1,205,000
Campylobacter jejuni jejuni 81-176
galE
waaC
waaM
waaF
waaD
hldE
1,070,000
1,075,000
1,080,000
1,085,000
Campylobacter jejuni jejuni IA3902
galE
waaC
hfrB
wlaN
neuC1
waaF
hldD
neuA1
hldE
1,070,000
1,075,000
1,080,000
1,085,000

Figure 3-6. Alignment of the LOS biosynthesis loci of *C. jejuni* PT14, NCTC 11168, RM1221, 81-176 and IA3902.

Gene names are shown on or above each ORF and the direction of transcription is indicated by an arrow. Corresponding homologous genes are assigned the same colour and white shading accompanied by an X represents a pseudogene. The LOS locus in PT14 extends from *galE* (A911_05480) to *gmhB* (A911_05580) and genes involved in LOS heptose biosynthesis pathway are present. The *neuBCA* gene operon and *cstIII* gene, which are involved in sialic acid biosynthesis are present in all of the aligned genomes, with the exception of RM1221. The alignment was performed using the Biocyc databases (Caspi et al. 2014).

involved in the LOS heptose biosynthesis pathway are present such as *waaC* (A911_05490) encoding heptosyltransferase I, *gmhA* (A911_05565) encoding phosphoheptose isomerase and *hldD* (A911_05575) encoding ADP-L-glycero-D-manno-heptose-6-epimerase. Three glycosyltransferases (A911_05505, A911_05510 and A911_05515) were identified as well as the *neuBCA* gene operon (A911_05530, A911_05535 and A911_05540), which is required for sialic acid biosynthesis. The *cstIII* gene (A911_05525) encoding α-2,3 sialyltransferase is also present. These genes are known to be involved in biosynthesis of sialylated LOS structures with the potential for human ganglioside mimicry (Gilbert et al. 2002). Figure 3-6 shows an alignment of the LOS biosynthesis loci of *C. jejuni* PT14, NCTC 11168, RM1221, 81-176 and IA3902. As seen with other loci, the gene arrangement is identical for PT14 and IA3902.

3.2.7 The flagellar modification locus in *C. jejuni* PT14

In *C. jejuni* PT14, the flagellar modification locus extends from A911_6280 (*pseB*) to A911_06515 (hypothetical protein) and has a G+C content of 30.2%. Within this locus, nine genes and an integenic region contain homopolymeric G:C tracts. These genes encode a number of hypothetical proteins as well as known motility accessory factors. Two of the genes, an aminoglycosidase N3'-acetyltransferase (A911_06295) and a methyltransferase (A911_06440), are observed to be phase variable at sequence level.

3.2.8 Restriction and modification systems in *C. jejuni* PT14

C. jejuni PT14 possesses multiple R-M systems. A type I R-M system was identified containing the genes *hsdR*, *hsdS* and *hsdM* (A911_07460, A911_07470 and A911_07480). The genome also contains a type IIs system (A911_00150), with the nearest homologue in NCTC 11168 being *cj0031*/*cj0032*. Adenine-specific (A911_01010), site-specific (A911_07040) and methionine-dependent (A911_03525) DNA methyltransferases were also identified as well as two putative R-M enzymes (A911_03365 (pseudogene) and A911_05085).

3.2.9 Lytic profiles

Bacteriophage-sensitivity of *C. jejuni* PT14 was tested against the activity of 60 selected lytic phages (Figure 3-7), including the 16 UK typing phages (Frost et al. 1999). All phage were initially propagated using *C. jejuni* PT14, with the exception of those on plate 4 where the propagating strain is indicated. For example, CP8/11168 refers to initial propagation of phage CP8 with *C. jejuni* NCTC 11168 as the propagating host strain. The bacteriophages of interest were diluted to the routine test dilution of approximately 6 $\log_{10}$ PFU ml^{-1} and 10 µl aliquots were spotted onto the surface of the PT14 lawns (2.5.4). The plaque forming ability of each phage was observed and 35 phages displayed either confluent or semi-confluent lysis, while only 9 phages (E5, 10b, 9, NCTC 12671, NCTC 12677, NCTC 12672, NCTC 12675, NCTC 12683 and CP220/PT14) showed no lytic ability.

3.2.10 *In vitro* growth curves

After examining the activity of lytic bacteriophages against *C. jejuni* PT14 (3.2.9), two were selected to perform *in vitro* growth experiments. *Campylobacter* phages CP30A and CPX were selected on the basis of their ability to produce confluent lysis on *C. jejuni* PT14 and that genome sequence data were available, identifying them as group III *Campylobacter* bacteriophages and representatives of the CP8unalikevirus genera (Javed et al. 2014). A decision not to use the group II *Campylobacter* phage CP220 for the *in vitro* experiments was made, despite also being a sequenced strain, because of its inability to lyse PT14 cells as noted by the lack of plaques on the PT14 lawns (Figure 3-7).

It was necessary to conduct *in vitro* growth experiments in order to evaluate growth and infection conditions during phage infection of PT14 as well as to develop appropriate modelling parameters to allow a measurable output of phage-infected cells to be generated for subsequent transcriptomic analysis (see Chapter 4). The experiment was conducted in 50 ml volumes of nutrient broth no.2 over 24 hours using the procedure described in section 2.6.2. Figure 3-8 shows the two growth curves for infection of *C. jejuni* PT14 with CP30A (A) and CPX (B). An initial density of 7.45 $\log_{10} \pm 0.09$ CFU ml^{-1} and 7.20 $\log_{10} \pm 0.06$ CFU ml^{-1} of *C. jejuni* PT14 was grown for two hours and was then infected with 5.91 $\log_{10} \pm 0.09$ PFU ml^{-1} of phage CP30A and 6.02 $\log_{10} \pm 0.05$ PFU ml^{-1} of phage CPX respectively. These concentrations and the ratio of bacteria to phage were chosen to take into consideration the results of *in vitro* growth experiments previously conducted by Cairns et al. (2009). Figure 3-8

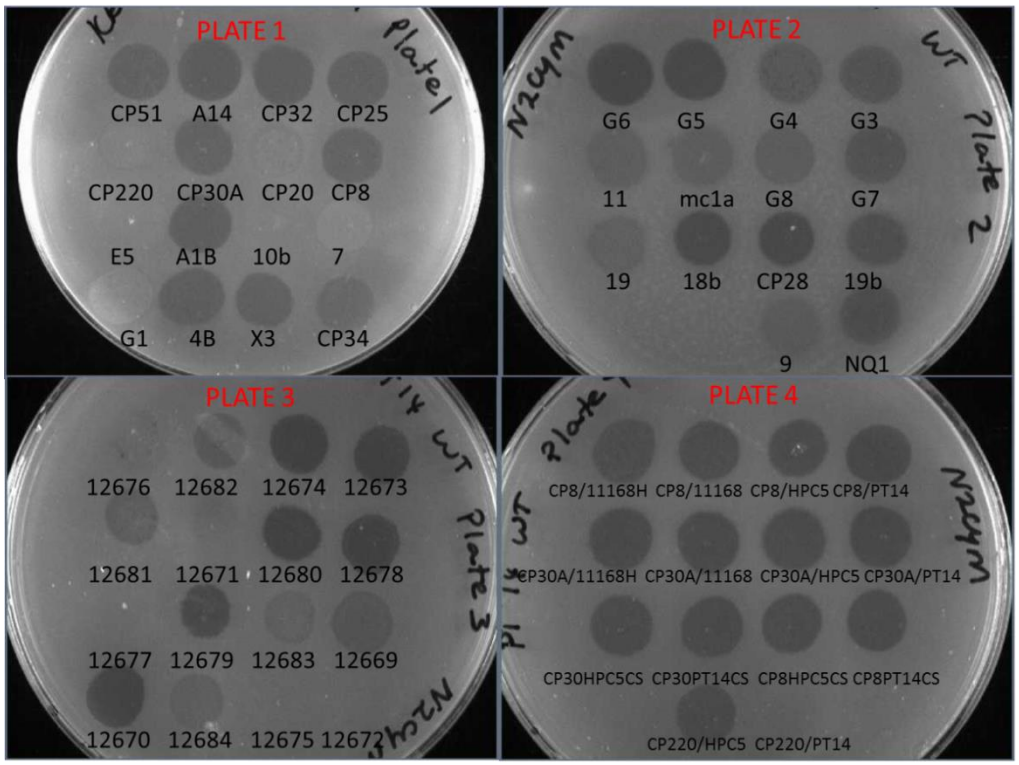


Figure 3-7. Sensitivity of *C. jejuni* PT14 to selected bacteriophages.

The phages were diluted to the routine test dilution of 6 $\log_{10}$ PFU ml^{-1} and were spotted onto PT14 lawns The phages tested include the 16 typing phages (Frost et al. 1999), the sequenced group II phage CP220 (Timms et al. 2010) and the sequenced group III phages CP30A and CP8. The plaque forming ability of each phage was observed. Confluent lysis was exhibited by phages A14, A32, CP8, A1B, G6, 18b, CP28, NQ1, NCTC 12670, NCTC 12673, NCTC 12674, NCTC 12678, CP8/11168, CP8/HPC5, CP8/PT14, CP30A/11168H, CP30A/11168, CP30A/HPC5, CP30A/PT14, CP30A/PT14PL and CP8HPC5CS, semi-confluent lysis by phages CP51, CP25, CP30A, 4b, X3, CP34, G5, 19b, NCTC 12679, NCTC 12680, CP8/11168H, CP30AHPC5CS, CP8PT14CS and CP220/HPC5 and opaque lysis by phages CP220, CP20, 7, G1, G4, G3, 11, mc1a, G8, G7, NCTC 12669, NCTC 12681, NCTC 12682, NCTC 12684. No lysis was exhibited by phages E5, 10b, 9, NCTC 12671, NCTC 12677, NCTC 12672, NCTC 12675, NCTC 12683 and CP220/PT14. Lytic activity was scored according to the nomenclature used during the UK phage typing scheme (Frost et al. 1999).

shows uninfected *C. jejuni* PT14 in blue, phage-infected *C. jejuni* PT14 in brown and bacteriophage growth in green.

Infection with CP30A resulted in an immediate drop in bacterial numbers by at least 3 logs on addition of the phage at the second hour. However, a similar drop in the host population in response to CPX infection was not observed until two hours post-infection. Following the fall in the host viable count, the CP30A-infected and CPX-infected *Campylobacter* populations began to increase in growth from the 6^{th} and 8^{th} hour respectively until the final sampling point at the 24^{th} hour. Although growth of the phage-infected host appeared to be relatively constant in both cases, a slight drop was observed for the CP30A-infected population at the 18^{th} hour which occurred with a simultaneous rise in CP30A. This is indicative of further lysis of phage-sensitive *C. jejuni* cells. Statistical analysis showed that there was no significant difference (Student's t-Test, >0.05, n=3) between the post-crash growth rate of the CP30A-infected population compared to the CPX-infected population. There was also no significant difference (Student's t-Test, >0.05, n=3) in the phage yields generated from cell lysis of these two populations. Mean burst sizes of 5.0 and 5.2 (infectious virions per cell) could be estimated from the phage yields with respect to the synchronous fall in the bacterial populations for CP30A and CPX respectively.

In order to establish whether the host population during the resurgence in growth contained a mixed population of phage-sensitive and resistant cells, that were both capable of growing following the crash, samples were collected

at the 6th, 8th, 18th and 24th hour and phage resistance was determined with the assistance of Dr. A. Timms as described in section 2.6.3. The results confirm that subsequent to the bacterial crash at the 8th hour, a high percentage of the isolates were indeed resistant to CP30A (65-85%) and CPX (84-90%) infection (Table 3-5). However, there was a decline in the frequency of phage resistance by the 24th hour. The results also illustrate that the percentage of isolates that were sensitive to CP30A infection at the 18th hour was quite low, suggesting a reason for the small drop in host numbers observed at that time point compared to the large drop previously encountered at the 2nd hour.

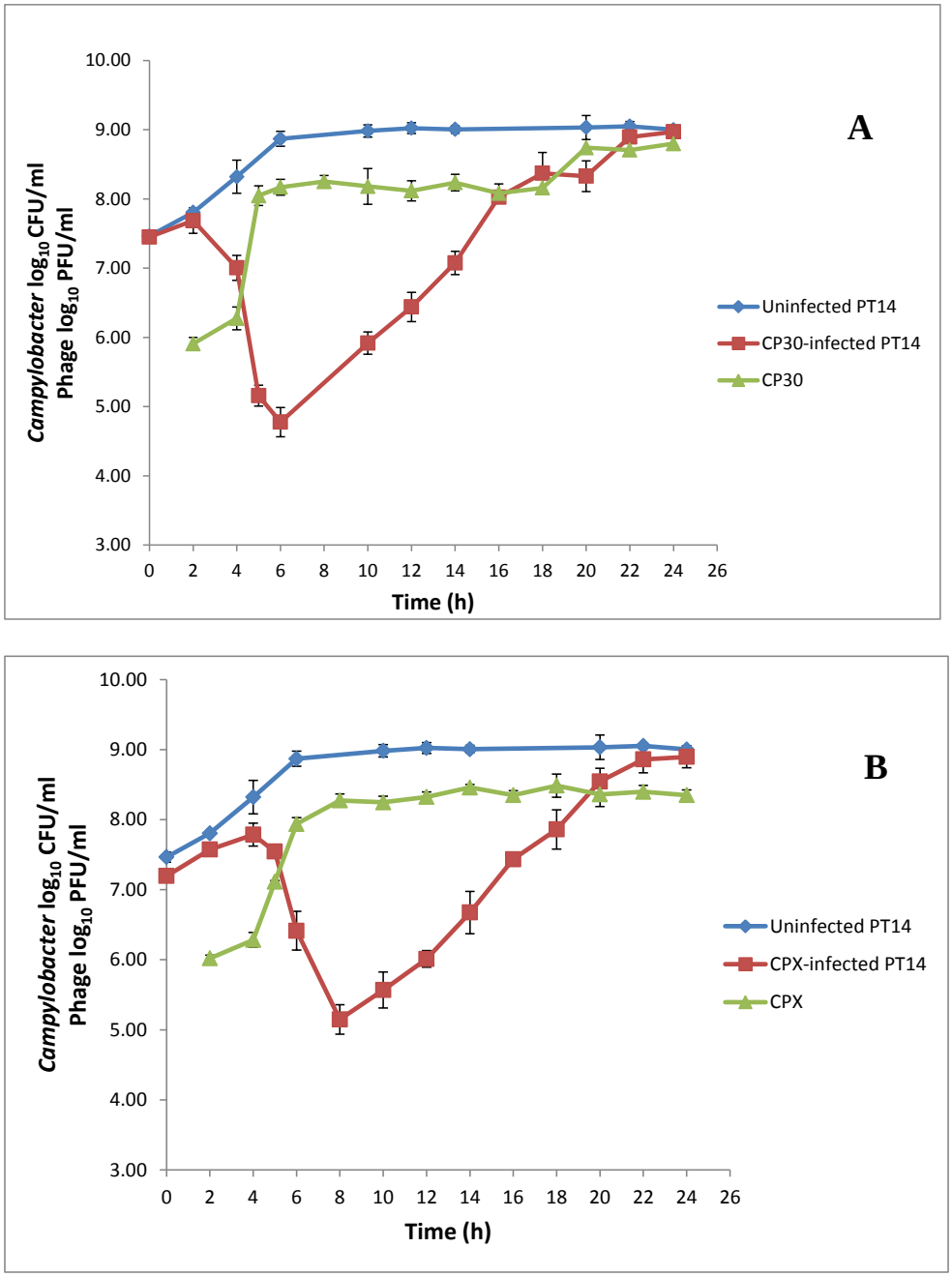


Figure 3-8. *In vitro* growth curves of *C. jejuni* PT14 infected with bacteriophages CP30A (A) and CPX (B).

Uninfected *C. jejuni* PT14 are indicated by the blue lines, phage-infected by the brown and bacteriophage growth by the green lines. The phages were added at the second hour of growth, at which point the host population infected with CP30A crashed, while a reduction in the CPX-infected cell numbers occurred 2 hours post-infection. A simultaneous rise in phage titre occurred at these times. Following the crash, the *C. jejuni* PT14 population steadily increased in growth until the final sampling point.

Table 3-5. Frequency of phage-resistant *C. jejuni* PT14 isolates during infection with phages CP30A and CPX

Time/hours	Phage resistance to CP30A									Phage resistance to CPX								
	Culture									Culture								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
6	ND	ND	ND	0.10	0.05	0.15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	0.70	0.65	0.85	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.84	0.90	0.85
18	ND	ND	ND	0.73	0.75	0.82	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.60	0.67	0.73
24	0.00	0.00	0.05	0.60	0.53	0.50	ND	ND	ND	0.05	0.10	0.10	ND	ND	ND	0.44	0.29	0.67

Key -

Cultures 1-3: Uninfected *C. jejuni* PT14 replicates 1-3

Cutures 4-6: CP30A-infected *C. jejuni* PT14 replicates 4-6

Cultures 7-9: CPX-infected *C. jejuni* PT14 replicates 7-9

ND: Not determined

3.3 DISCUSSION

Analysis of the *C. jejuni* PT14 genome has identified features such as an intact CRISPR-*cas* system (3.2.4), multiple R-M systems (3.2.8) as well as the presence of homopolymeric tract repeats with phase variable regions within genes that have the potential to impact on bacteriophage replication (Table 3-2). These features are all related to bacterial defence and therefore suggest that although this strain is highly phage-sensitive, mechanisms are still in place to enable potential escape from bacteriophage predation.

3.3.1 Phage-derived protospacers not detected in PT14 CRISPR array

The CRISPR direct repeat sequence of *C. jejuni* PT14 is highly conserved with other members of the species. BLAST searches for each spacer repeat sequence of the *C. jejuni* PT14 CRISPR array did not reveal any phage-associated or plasmid DNA sequence matches but instead identified nucleotide matches to the PT14 chromosome and other *C. jejuni* strains (3.2.4). This finding was not surprising as PT14 was not resistant to many of the bacteriophages examined in this study, which may be a consequence of the strain not previously integrating phage-derived proto-spacers into its CRISPR region. However, the BLAST searches also showed that none of the spacer sequences were unique to PT14, with other *C. jejuni* strains carrying identical spacers.

CRISPR spacers that target chromosomal genes have previously been examined by Stern et al. (2010), who found that amongst 23,550 spacers from

330 CRISPR-encoding prokaryotic organisms, one in every 250 spacers were self-targeting. As a result, they initially hypothesised that the CRISPR system may be involved in gene regulation and therefore sought to determine conservation amongst self-targeting spacers. However, no conserved sequences were identified and this hypothesis was dismissed. It was noted that 37% of the self-targeting spacer sequences were found to be located close to the leader sequence, either in the first or second position of the array. Generally, spacers are integrated into the array towards the leader end and this finding may suggest that acquisition of self-targeting spacers is a recent event that could possibly have deleterious effects. A similar finding was observed for PT14 with both the first and second spacers containing nucleotide sequences that can be aligned to chromosomal genes. Stern et al. (2010) also found that the two direct repeats flanking the self-targeting spacer sequence were twice as likely to contain mutations compared to those flanking the other spacers. This could therefore affect crRNA maturation of the self-targeting spacer whilst allowing the rest of the CRISPR array to remain functional. It was finally suggested that targeting of chromosomal genes by the CRISPR system may have been accidental and this may therefore be a form of autoimmunity which could eventually lead to a negative fitness cost to the organism (Stern et al. 2010).

3.3.2 Gene encoding specific phage target not phase variable in PT14

Three genes within the CPS locus of *C. jejuni* PT14 were found to contain homopolymeric tract repeats. However, one of these genes, A911_06918, does not appear to be phase variable at sequence level. Its nearest homologue within

the NCTC 11168 genome is *cj1421* and this gene is phase variable. It encodes a MeOPN transferase, which allows attachment of MeOPN to a GalfNAc (N-acetylgalactosamine in furanose configuration) residue of the CPS. It has been identified in other *C. jejuni* strains (Karlyshev et al. 2005) and has also been determined to be a specific target for bacteriophage F336 (Sørensen et al. 2011). It was found that a phage F336 resistant NCTC 11168 variant selected *in vitro* had in fact lost the MeOPN as a result of having an additional G residue in *cj1421*, thus causing the gene to be in a non-functional phase (Sørensen et al. 2011). Later, Sørensen and colleagues (2012) tested whether *C. jejuni* NCTC 11168 could become resistant to bacteriophage F336 *in vivo* during chicken colonisation whilst observing the MeOPN transferase status of any resistant variants. They found that the phage-sensitive isolates expressed 9 G residues in the *cj1421* gene whereas phage-resistant isolates contained either 8 or 10 G residues, which would cause the gene to switch to the "off" phase. Phase variable disruption of *cj1421* of *C. jejuni* NCTC 11168 therefore prevents MeOPN attachment to Gal*f*Nac of capsular polysaccharide, which leads to non-infection by bacteriophage F336 (Sørensen et al. 2011) and allows bacteriophage evasion during chicken colonisation (Sørensen et al. 2012). In contrast, the *cj1421* homologue identified in *C. jejuni* PT14 (A911_06918) does not appear in a syntenic region and as it is not phase variable, bacteriophage targeting the MeOPN capsular modification are unlikely to be hindered by such a mechanism in *C. jejuni* PT14.

3.3.3 *C. jejuni* PT14 appears to possess a class C LOS locus

Comparative genome analyses have shown that the LOS biosynthesis locus of *C. jejuni* is a highly variable region within the genome (Gilbert et al. 2002; Leonard II et al. 2003; Leonard II et al. 2004; Parker et al. 2005). These variations are responsible for the differences observed in LOS structures amongst *C. jejuni* strains. Five classes (A-E) of the LOS locus have been identified within *C. jejuni* based on the general organisation of the LOS biosynthetic genes (Gilbert et al. 2002; Godschalk et al. 2004). Godschalk et al (2004) examined the correlation between expression of ganglioside-like structures and the class of LOS locus assigned as well as the prevalence of the different classes amongst GBS-associated *C. jejuni* strains. They found that classes A, B and C contained genes involved in biosynthesis and transfer of sialic acid while classes D and E lacked such genes. Classes A and B loci were also previously found to contain the *cstII* gene encoding the bi-functional α2,3/α2,8-sialyltransferase which catalyses transfer of sialic acid molecules to sugar structures at the cell envelope (Gilbert et al. 2002; Chiu et al. 2004). However, class C was reported to carry the *cstIII* variant encoding the mono-functional α2,3-sialyltransferase (Gilbert et al. 2002). Likewise, the LOS locus of *C. jejuni* PT14 contains the sialic acid biosynthesis genes as well as α2,3-sialyltransferase (*cstIII*), which suggests that this strain has a class C LOS locus. In addition, Godschalk et al. (2004) further reported that class A was specifically associated with GBS and the expression of GM1 ganglioside-like structures whereas class B was linked to MFS and the production of GQ1b-like structures. No significant results were achieved for classes C, D or E but some class C isolates were found to produce GM1 structures in their LOS as well.

3.3.4 Phage-sensitivity determined by ganglioside-like LOS structures

Louwen et al. (2013) examined the link between bacteriophage resistance in *C. jejuni* as a consequence of the expression of ganglioside-like LOS structures and the association with preservation of the CRISPR-*cas* system. Their findings suggest possible reasons for the phage-sensitivity of *C. jejuni* PT14 to many phages. Firstly, Louwen et al. (2013) found that isolates producing GM1 and GD1 ganglioside-like structures (classes A and B) were significantly more phage-resistant compared to isolates that did not express these structures. They also tested the involvement of the sialyltransferase gene *cstII* and the *cas* gene *csn1* in bacteriophage-resistance. They found that *cstII* mutants were more susceptible to phage infection than wild-type isolates and that *cstII*-positive *csn1* mutants were less susceptible to bacteriophage infection than *cstII*-negative csn1 mutant. This therefore suggests that *cstIII* isolates require the CRISPR-*cas* system for defence against bacteriophages. Further analysis also showed that strains containing the *cstII* gene and therefore expressing GM1 or GD1 ganglioside-like structures, possessed mutations or frameshifts within their *cas* genes or contained short repeats (1-3) or no CRISPR element at all. There was no mention of integration of proto-spacer sequences into the CRISPR elements of the strains tested. All of these findings therefore suggest that *C. jejuni* PT14, which possesses a *cstIII* sialyltransferase gene, would not be as resistant to bacteriophage infection as the *cstII*-positive isolates used within their study. Based on these results, an assumption can also be made that *C. jejuni* PT14 retains a functional CRISPR-cas system as a bacterial defence mechanism against bacteriophage infection.

3.3.5 Genome analysis does not provide clear rational for the phage-sensitivity of *C. jejuni* PT14

The genome of *C. jejuni* PT14 has considerable synteny with the genomes of *C. jejuni* IA3902 and NCTC 11168, containing no prophage-associated elements or genomic islands. No obvious features were identified via the genome analysis that could explain the bacteriophage-sensitive nature of *C. jejuni* PT14 over any other strain. Nevertheless, continued exposure to bacteriophages may cause *C. jejuni* PT14 to evolve over time or may even force this strain to make better use of its host defence mechanisms in order to overcome phage predation.

Chapter 4 : Transcriptome analysis of *Campylobacter* in response to bacteriophage infection

4.1 INTRODUCTION

Bacteriophages rely on their bacterial host during reproduction as a means of providing the appropriate substrates and metabolic machinery to allow efficient production of progeny. Usually this involves redirecting the host cellular metabolism for viral production and thus allows the phages to regulate host macromolecular synthesis. Earlier studies have shown that in some instances, such as with the T-even bacteriophages (T2, T4 and T6) of *E. coli*, there is extensive shutdown of host DNA synthesis within a few minutes of infection (reviewed by Koerner & Snustad 1979). On the other hand, transcription of host DNA has been found to continue late into the phage lytic cycle during infection with phages like Φ29 of *Bacillus subtilis* (Schachtele et al. 1972).

Active bacteriophage replication is a density-dependent process whereby a threshold density of host bacteria must be present in order for phage numbers to increase (Wiggins & Alexander 1985; Payne et al. 2000; Payne & Jansen 2001). Wiggins and Alexander (1985) investigated the existence of critical thresholds that may be required for phage replication to take place. Using a number of different hosts they found that a minimum concentration of about 4 $\log_{10}$ CFU ml^{-1} was required for phage numbers to increase. This host density threshold value was later referred to by Payne and Jansen (2001) as the proliferation threshold, which refers to the minimum concentration of bacteria required for there to be an increase in the phage population. Payne and Jansen (2001) also introduced another term, the viral inundation threshold, which refers to the concentration of bacteriophage required to cause a decrease in the host population.

Cairns et al. (2009) investigated phage-bacteria interactions using the *Campylobacter* bacteriophage CP8 and the minimally passaged broiler chicken isolate *C. jejuni* strain GIIC8 (Loc Carrillo et al. 2005). Their models took into account the proliferation and inundations thresholds hypothesised by Payne and Jansen (2001). The results revealed a proliferation threshold of around 5-6 $\log_{10}$ CFU ml^{-1} for their chosen strain of phage and bacterium. The minimum concentration of bacteriophage before a decrease in *Campylobacter* occurred, or the inundation threshold, was around 7 $\log_{10}$ PFU ml^{-1}. These interaction experiments therefore illustrated that there was no net increase in the bacteriophage population until the host concentration reached the proliferation threshold.

To date, only a few studies have examined global changes in host gene expression during infection by lytic bacteriophages (Karlsson et al. 2005; Poranen et al. 2006; Ravantti et al. 2008; Fallico et al. 2011; Ainsworth et al. 2013). These studies have all used microarray technology validated by quantitative real-time PCR to profile the bacterial transcriptome under phage infection. Cells were generally harvested from samples with the aim to span the entire phage replication cycle. A consistent feature arising from these studies, was that the majority of changes in host gene expression only occurred during the later stages of phage infection, after virion components had already been synthesised (Poranen et al. 2006; Ravantti et al. 2008; Ainsworth et al. 2013). However, the results thus far do not suggest a universal host transcriptional response to phage infection. For instance, changes in *E. coli* gene expression in response to bacteriophage PRD1 infection showed that

some of the most highly up-regulated genes included chaperonins, proteases and other stress-related genes of the heat shock regulon as well as components of the phage shock regulon and several genes related to synthesis of exopolysaccharides of the cell surface (Poranen et al. 2006). However, investigating the transcriptional response of *Pseudomonas aeruginosa* to infection with phage PRR1, which uses a similar cell surface receptor complex to that of phage PRD1 and as a result shares the same broad host range, resulted in. differential expression of genes encoding products whose functions were mostly linked to transport, energy production and protein synthesis (Ravantti et al. 2008). In addition, host gene changes during phage c2 infection of *Lactococcus lactis* subspecies *lactis* IL1403 were mostly associated with cell envelope processes, regulatory functions and carbohydrate metabolism, with up-regulation of genes encoding proteins involved in membrane stress, D-alanylation of cell wall lipoteichoic acids, maintenance of the proton motive force and energy conservation (Fallico et al. 2011). However, infection of *L. lactis* UC509.9 with phages c2 and Tuc 2009 resulted in more phage-specific host responses, although there were only a few shared differentially transcribed genes between cells infected by the two phages (Ainsworth et al. 2013). The shared host response, which showed similarities to that observed by Fallico et al. (2011), involved differential expression of genes related to catabolic flux and energy production, cell wall modification and the conversion of ribonucleotides to deoxyribonucleotides.

A number of studies have opted to monitor expression of phage-specific transcripts over an infection cycle instead of characterising host transcription

using either solely quantitative real-time PCR (Clokie et al. 2006) or microarray technology validated by quantitative real-time PCR (Luke et al. 2002; Duplessis et al. 2005; Liu & Zhang 2008; Berdygulova et al. 2011; Pavlova et al. 2012). Phage gene expression was generally regulated in a temporal manner by identification of early, middle and late transcriptional gene classes. However, temporal assignments varied amongst the different studies, which were either based on the initial appearance of a transcript (Luke et al. 2002), the time interval during which the greatest change in transcript abundance occurred (Duplessis et al. 2005; Sevostyanova et al. 2007) or the time interval during which transcript abundance peaked (Clokie et al. 2006; Berdygulova et al. 2011; Pavlova et al. 2012). Many of the early genes encoded products of no known function as well as proteins involved in transcriptional regulation. The middle genes encoded proteins predicted to be involved in DNA replication, recombination and nucleotide metabolism, whereas the late genes mostly encoded proteins related to capsid and tail morphogenesis, DNA packaging and host cell lysis.

Using bioinformatic and biochemical approaches, it was also possible to identify early, middle and late phage promoters (Sevostyanova et al. 2007; Berdygulova et al. 2011; Pavlova et al. 2012). These promoters were recognised by the host RNA polymerase enzyme, which typically catalyses bacterial transcription. RNA polymerase is composed of four catalytic subunits (α, β, β' and ω) and a regulatory subunit (sigma factor). Bacterial transcription is initiated when the catalytic core of the RNA polymerase binds to a sigma factor and recognises a specific consensus sequence of the promoter, which is

located around positions -35 and -10 relative to the transcriptional start site at +1. Some bacteriophages, such as phage T4, do not encode their own RNA polymerase or associated sigma factors but instead utilise those of the host in order to transcribe their genes. Previously, two phage-encoded proteins were identified that bind to RNA polymerase *in vitro* and prevented recognition of the consensus sequence (Berdygulova et al. 2011; Pavlova et al. 2012). This therefore inhibited transcription from host promoters but however allowed transcription from the middle or late phage promoters (Berdygulova et al. 2011; Pavlova et al. 2012). These proteins may therefore be involved in shutting off host transcription and causing the shift from host to viral transcription. Such studies have provided a better understanding of the temporal regulation of gene expression in bacteriophages.

With the introduction of direct cDNA sequencing (RNA-seq) for whole-transcriptome analysis, some of the limitations associated with previous high-throughput approaches, such as microarray technology, were clearly highlighted. For instance, microarrays are usually designed based on known transcripts and may therefore cause gene expression to be viewed in a biased manner. In fact, this technique lacks the ability to detect novel transcripts and also has the potential for non-specific or cross-hybridisations to occur. Microarrays also rely on indirect measurements of the hybridisation intensity of labelled cDNA to oligonucleotide probes whilst the RNA-seq approach allows direct mapping of sequence data to a sequenced reference genome. In addition, RNA-seq has provided many advantages over hybridisation-based techniques including greater sensitivity, a larger dynamic range, less

background noise and the ability to detect transcripts in an annotation-independent manner. Thus, early studies revealed RNA-seq as a revolutionary tool for investigating the transcriptome of prokaryotic organisms as these studies were able to identify rare or novel transcripts, accurately define operons, discover expression of putative noncoding RNAs (ncRNAs) and correct existing gene annotations (Yoder-Himes et al. 2009; Passalacqua et al. 2009; Perkins et al. 2009; Parkhomchuk et al. 2009; Sharma et al. 2010; Filiatrault et al. 2010). However, it has been more challenging to examine the bacterial transcriptome compared to that of eukaryotes. This has mainly been due to the fact that total RNA of bacterial cells is typically comprised of less than 5% of messenger RNA (mRNA). In addition, the lack of polyadenylation at the 3'-end, which occurs with mature mRNA in eukaryotes, prevents specific targeting of bacterial mRNA. To overcome this, mRNA enrichment protocols have been used to increase mRNA recovery, including the removal of ribosomal RNA (rRNA) by hybridisation to complementary oligonucleotide sequences that target specific regions of the 5S, 16S and 23S rRNAs (Chen & Duan 2011; Giannoukos et al. 2012; Peano et al. 2013).

Two key factors to be considered when carrying out a transcriptome analysis using sequencing are read lengths and the number of reads obtainable in a sequencing reaction. It has previously been reported that short read lengths may cause reads to align to multiple locations of the reference genome (Bloom et al. 2009; Passalacqua et al. 2009). As a result, such ambiguously mapped reads may influence subsequent analyses to determine gene expression levels, and may be better excluded (Passalacqua et al. 2009). In addition, the bacterial

transcriptome generally consists of coding RNA (mRNA) and small ncRNAs (rRNA, tRNA and regulatory RNAs). However, if transcripts of ncRNAs are highly abundant and therefore more frequently sequenced, this can reduce the number of reads available for low abundance mRNA transcripts (Bloom et al. 2009). Bloom et al. (2009) separated their reads into unique sequences which mapped to either an ORF, a rRNA gene or a tRNA gene and compared them to reads which mapped to multiple locations. They found that most unique reads originated from ORFs but that a large number of the multiple-mapping reads were derived from highly abundant rRNA genes despite including a rRNA depletion step prior to their cDNA library preparation.

A potential shortcoming of RNA-seq is the inability of standard cDNA libraries to preserve information on the direction of transcription. Directionality is especially crucial in cases where transcripts may be generated from both strands and would therefore influence determination of gene expression levels. Several protocols have been developed to allow strand-specific RNA-seq, including modification of the RNA molecule prior to reverse transcription by ligation of a 5' RNA linker (Passalacqua et al. 2012), or chemical modification during second strand cDNA synthesis by adding specific residues to the 3'-end, followed by subsequent degradation of the unmarked strand (Parkhomchuk et al. 2009; Levin et al. 2010). Another very useful feature of RNA-seq is the ability to discriminate between primary and processed transcripts (van Vliet 2010). Primary transcripts, including mRNAs and ncRNAs, carry a 5' triphosphate end (5'PPP) while processed transcripts, including rRNAs and tRNAs, carry a 5' monophosphate (5'P). A sequencing

approach known as differential RNA-seq (dRNA-seq) allows selective sequencing of primary transcripts without prior rRNA removal (reviewed by Sharma & Vogel 2014) and can be used for annotation of transcriptional start sites (TSSs). The dRNA-seq libraries are prepared by splitting the RNA sample into two and leaving one untreated to capture both 5'PPP and 5'P RNAs. For the second sample, processed RNAs are depleted by treatment with 5'P-dependent terminator exonuclease (TEX), which degrades the 5'P RNAs. Directionality is also maintained with this method. The dRNA-seq method was first used to describe the primary transcriptome of *H. pylori* under five different growth conditions and was successful in identification of at least 1,900 TSSs and assignment of antisense TSSs to 46% of the genes (Sharma et al. 2010).

A number of studies have examined the transcriptome of *C. jejuni* using RNA-seq technology (Chaudhuri et al. 2011; Dugar et al. 2013; Taveirne et al. 2013; Porcelli et al. 2013; Butcher & Stintzi 2013). In some instances, dRNA-seq was used to enable selection of unprocessed RNA species and allowed the primary transcriptome of *C. jejuni* to be determined (Dugar et al. 2013; Porcelli et al. 2013), along with identification of a novel type-II CRISPR/Cas system amongst multiple *Campylobacter* strains (Dugar et al. 2013). Variations of strand-specific sequencing were utilised in other cases to determine ncRNAs and antisense transcription (Chaudhuri et al. 2011; Taveirne et al. 2013; Butcher & Stintzi 2013). The earliest RNA-seq study of the *C. jejuni* transcriptome characterised the regulon of the sigma factor RpoN (σ^{54}), which regulates expression of genes involved in flagellar biosynthesis,

and expression data was compared to previous microarray data (Chaudhuri et al. 2011). A proteome study was also conducted to supplement the transcriptome data (Chaudhuri et al. 2011). RNA-seq technology has also been used to examine the transcriptome of *Campylobacter* during colonisation of the chicken caecum and 250 genes were identified that were differentially expressed *in vivo* (Taveirne et al. 2013). More recently, the transcriptional response of *C. jejuni* to iron limitation was reported (Butcher & Stintzi 2013) and a more extensive examination of the transcriptome under these conditions was provided compared to previous work utilising microarray technology (Palyada et al. 2004; Holmes et al. 2005).

In this chapter, the transcriptome of *C. jejuni* PT14 was examined during phage infection with group III phages CP30A and CPX in order to characterise differential expression patterns. Thus far, there are no published studies reporting host or phage transcription during bacteriophage infection of *Campylobacter* with virulent phages.

4.2 RESULTS

4.2.1 *In vitro* growth curves for RNA isolation and protein extraction

Growth experiments were conducted to harvest cells for RNA isolation and protein extraction as described in section 2.6.1. Briefly, three independent cultures of *C. jejuni* PT14 were grown for 6 hours in 150 ml of nutrient broth No. 2 (2.1.5) under microaerobic conditions (2.2.2) with shaking at 100 rpm. In order to determine the response of *C. jejuni* to bacteriophage infection, samples for RNA isolation were taken during the eclipse phase of bacteriophage growth (2.6.2). This would therefore allow *Campylobacter* cells that were most likely under bacteriophage infection to be captured. This approach was adopted taking into account the findings of previous studies where major changes in the host transcriptional response to phage infection occurred only during the later stages of infection (Poranen et al. 2006; Ravantti et al. 2008; Ainsworth et al. 2013).

In order to generate an infectable host population that could achieve a synchronous fall and provide sufficient cells to be harvested for RNA isolation purposes, appropriate infection conditions were sought. A number of growth experiments were performed with the aim of determining suitable host and viral concentrations that would encourage phage to potentially infect at a rate of 1 phage per 1 bacterial cell. An effective MOI of 1 was found to achieve a synchronous infection and fall in the *Campylobacter* population. The initial *Campylobacter* concentration was chosen to take into account the finding of Cairns et al. (2009), who observed that an increase in bacteriophage growth

was observed after their *Campylobacter* population had reached 5-6 $\log_{10}$ CFU ml^{-1}. Therefore initial *C. jejuni* densities of at least 6.0 $\log_{10}$ CFU ml^{-1} were selected. However, it was determined that a bacterial density of 7.0 $\log_{10}$ CFU ml^{-1} for CP30A-infected cells was required to enable the harvesting of sufficient phage-infected cells for RNA isolation over a 6 hour period. Viable counts of triplicate cultures were performed and the RNA preparations selected *post-hoc* for RNA-seq analysis represented infected cell populations directly before synchronous lysis. For CPX-infected *C. jejuni* PT14 populations, synchronous lysis occurred when the bacterial density reached close to 7.0 $\log_{10}$ CFU ml^{-1} (Figure 4-1). Therefore to achieve exponentially growing *C. jejuni* PT14 cultures at 7.0 $\log_{10}$ CFU ml^{-1}, initial inoculums of 6.0 $\log_{10}$ CFU ml^{-1} were prepared in order that similar eclipse phase phage-infected cell populations to that of the CP30-infected cells could be harvested prior to lysis.

Figure 4-1 illustrates growth of *C. jejuni* PT14 in the absence and presence of CP30A (A) and CPX (B) following optimisation of the infection conditions. Bacteriophages were added at the second hour of bacterial growth and an approximate 1-log decrease in the CP30A- and CPX-infected *C. jejuni* PT14 population was observed at 3 and 4 hours post-infection respectively. This population decline occurred when the concentration of *Campylobacter* cells infected with CP30A and CPX had reached approximately 7.7 $\log_{10}$ CFU ml^{-1} and 7.1 $\log_{10}$ CFU ml^{-1} respectively. These concentrations are in excess of the bacteriophage proliferation threshold. Although bacteriophage growth is not illustrated in Figure 4-1, the phages were titrated at intervals throughout the experiment and the phage progeny produced following the decrease in

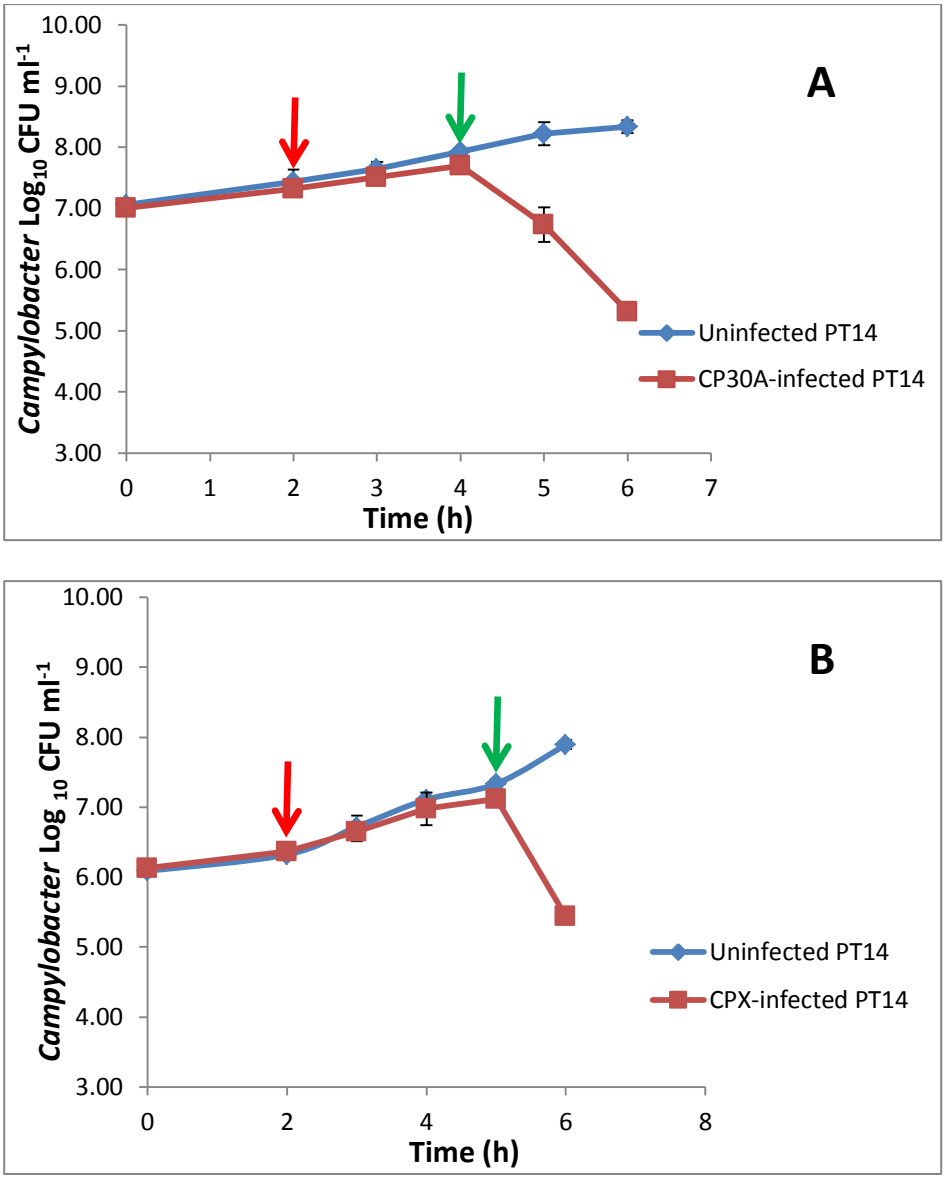


Figure 4-1. *In vitro* growth curves of *C. jejuni* PT14 in the presence and absence of bacteriophages CP30A (A) and CPX (B).

Cultures were grown in 150 ml of NB no. 2 and initial *C. jejuni* densities were chosen to enable harvesting of sufficient phage-infected cells for RNA isolation. The red arrows indicate the time point where CP30A and CPX were added. Samples were collected for total RNA isolation at the time points prior to host cell lysis (2 and 3 hours post-infection respectively) and at the equivalent time points for phage-uninfected cultures (green arrows). Values for bacterial growth (CFU ml^{-1}) are generated from 3 independent cultures ± SD and plotted as a function of time. Bacteriophages were titrated at intervals throughout the experiment to confirm their replication and effective MOI (data not shown).

C. jejuni population was verified (2.5.3). Cells were harvested from the three biological replicates for RNA isolation at 2 and 3 hours post-infection for the CP30A and CPX-infected cultures repectively. These time points represent the points directly before the decrease in *C. jejuni* PT14 numbers was observed and should also correspond to the esclipse period of phage growth. In Figure 4-1, these are illustrated by the green arrows and bacterial concentrations were verified by *Campylobacter* enumerations (2.2.4).

4.2.2 Transcriptome analysis of *C. jejuni* during phage infection

Total RNA was isolated from the uninfected and phage-infected cells using the Trizol Max Bacterial Isolation kit (Invitrogen) according to section 2.12.1 and was purified using an RNeasy Mini kit (Qiagen). The RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the integrity was measured using a Bioanalyser 2100 (Agilent Technologies Inc.). All samples selected for cDNA library preparations had RIN numbers of at least 7.0. Ribosomal RNA was depleted from the total RNA according to section 12.2.2 using the Ribo-Zero rRNA removal kit for Gram negative bacteria (Epicentre Biotechnologies). Two µg of the rRNA depleted RNA was then used to prepare cDNA libraries using the ScriptSeq mRNA-Seq library preparation kit (Epicentre Biotechnologies). The libraries were individually indexed, pooled and then sequenced using an Illumina HiSeq 2000 sequencing system.

The RNA-sequencing generated an average of approximately 28 million reads per sample (Table 4-1), each of which was 50 nucleotides in length and from

paired-ends. CLC Genomics Workbench version 6.0 software was used to remove the adapter indexes and filter the raw reads (section 2.12.4.1). Principle component analysis of the datasets demonstrated the replicates clustered according to their origin (Figure 4-2). The filtered reads were mapped against the *C. jejuni* PT14 reference genome (Brathwaite et al. 2013) according to section 2.12.4.2. Table 4-1 provides a summary of the number of reads attained after filtering as well as illustrates the number of reads that aligned uniquely to the reference genome. Figures 4-3 to 4-6 illustrate how the mapped reads were distributed over specific genes and displayed within the CLC Genomics Workbench. All unmapped reads were collected and BLAST searches confirmed some of them were of bacteriophage origin.

4.2.3 Differential host gene expression during bacteriophage infection of *C. jejuni*

In order to identify genes that were regulated in response to bacteriophage infection, reads that mapped uniquely to the *C. jejuni* PT14 genome were firstly normalized for transcript length and the total number of reads by calculating the reads per kilobase of transcript per million mapped reads (RPKM) (Mortazavi et al. 2008). This was done using the CLC Genomics Workbench 6.0 package and generated expression values that allowed for comparisons between genes and infection conditions. Significant differential gene expression was assessed using the Baggerly's statistical test on expression proportions (Baggerly et al. 2003). To correct for multiple hypothesis testing, p-values were adjusted by applying the false discovery rate controlling method of Benjamini and Hochberg (1995).

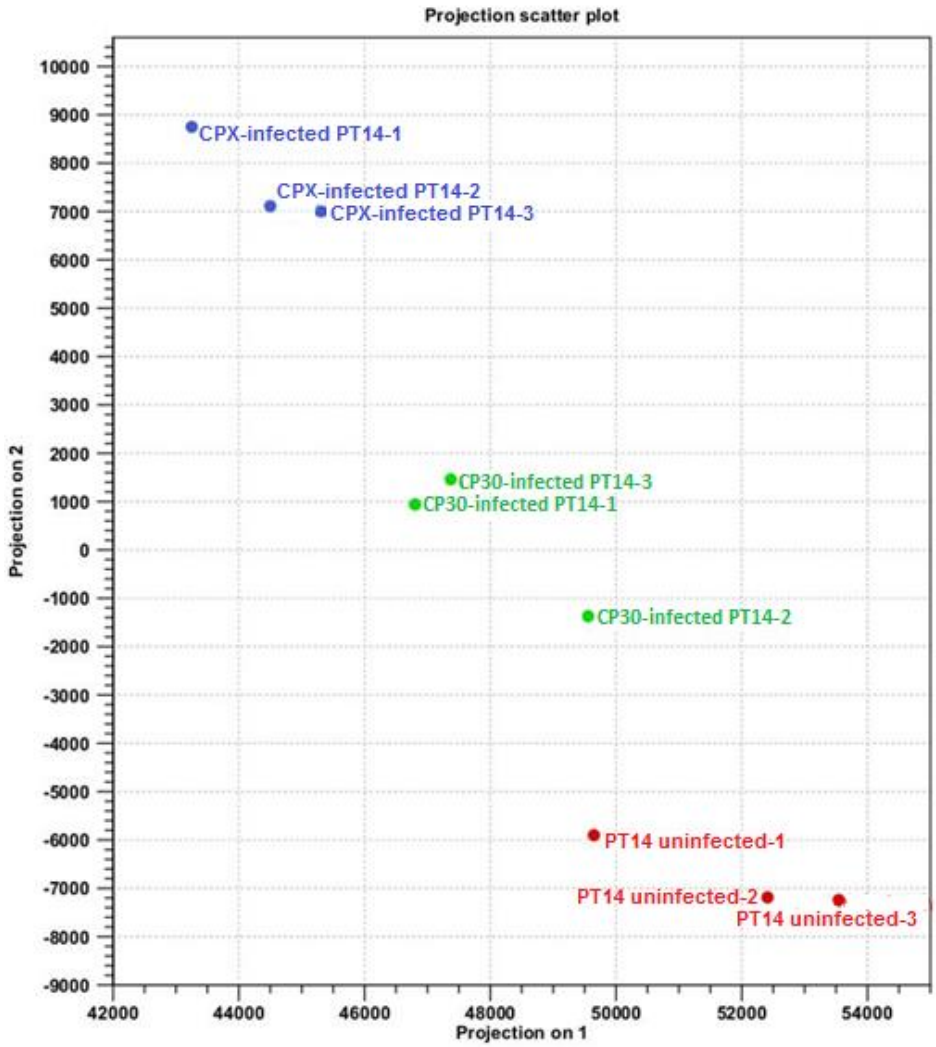


Figure 4-2. Principle component analysis of RNA-seq reads for *C. jejuni* under bacteriophage infection.

The uninfected *C. jejuni* PT14 replicates are illustrated in red while the CP30A-infected and CPX-infected replicates are shown in green and blue respectively. Each condition is displayed as a distinct group, with patterns of similarity being exhibited in each case.

Table 4-1. Analysis of RNA-seq data for reads mapped against the *C. jejuni* PT14 genome.

Condition	Original read count	Number of reads after trimming	Number of uniquely mapped reads
Uninfected *C. jejuni* PT14 replicate 1	47,972,332	22,793,829	18,326,585
Uninfected *C. jejuni* PT14 replicate 2	37,012,236	17,115,514	12,893,941
Uninfected *C. jejuni* PT14 replicate 3	24,676,752	13,051,011	10,660,977
CP30A-infected *C. jejuni* replicate 1	25,744,022	11,585,083	6,603,483
CP30A-infected *C. jejuni* replicate 2	25,935,970	15,119,809	10,237,483
CP30A-infected *C. jejuni* replicate 3	23,851,478	14,041,695	10,596,450
CPX-infected *C. jejuni* replicate 1	22,349,820	14,512,973	10,790,373
CPX-infected *C. jejuni* replicate 2	20,865,460	13,067,709	9,149,500
CPX-infected *C. jejuni* replicate 3	23,071,418	15,172,122	10,545,329

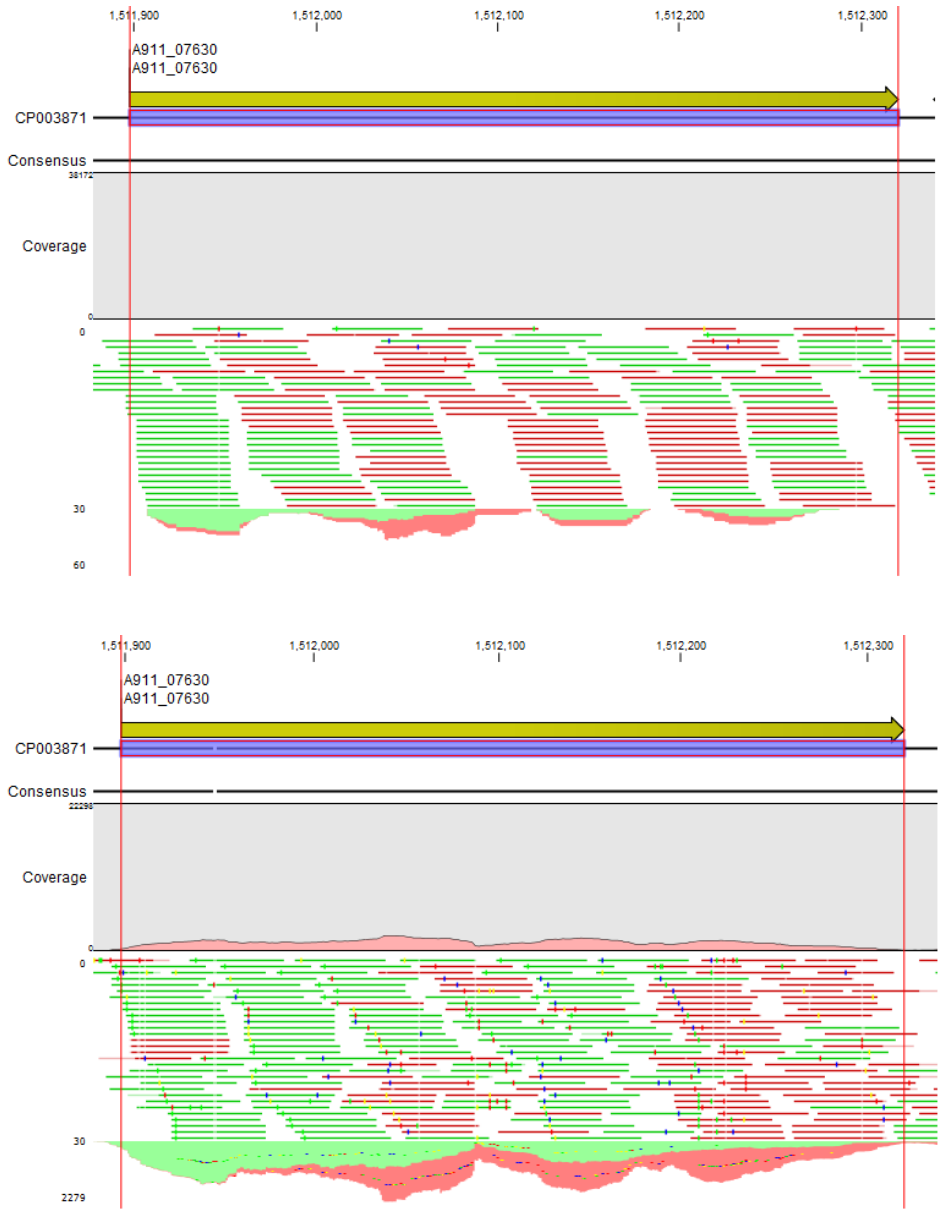


Figure 4-3. RNA-seq data mapped against the *C. jejuni* PT14 genome, showing read distribution for the *cgb* gene (A911_07630).

Data are shown for uninfected (top panel) and CP30-infected PT14 (bottom panel). Reads are illustrated by horizontal green (forward orientation) and red (reverse orientation) lines. After 30 rows of reads, an overflow graph following the same colour scheme is displayed below the reads. Mismatches between the reference sequence and the mapping data are highlighted in red (A), blue (C), green (T) and yellow (G). Coverage is displayed relative to the total number of reads and is shown above the reads. The *cgb* gene is up-regulated in the CP30-infected PT14 sample. The RNA-seq data have been visualised using CLC Genomics Workbench.

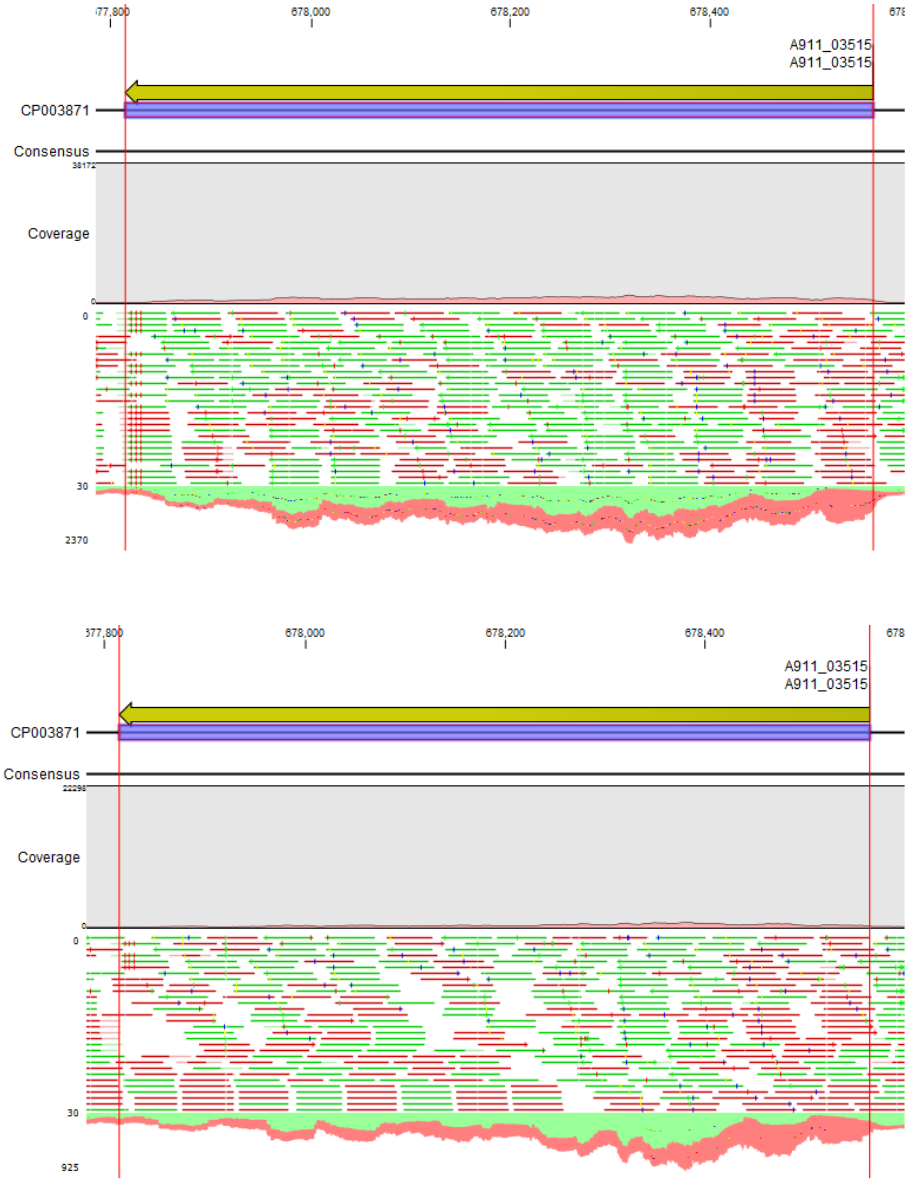
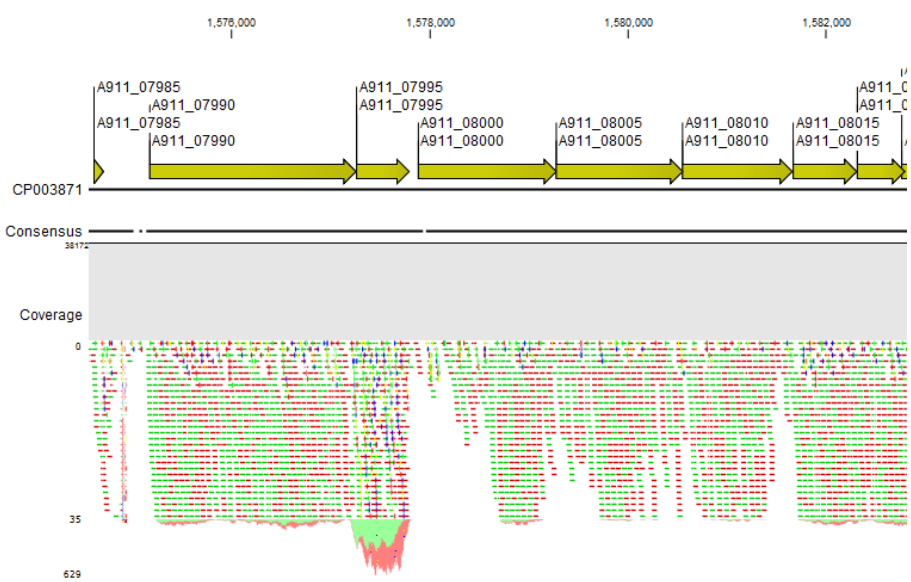


Figure 4-4. RNA-seq data mapped against the *C. jejuni* PT14 genome, showing read distribution for the *flaC* gene (A911_03515).

Data are shown for uninfected (top panel) and CP30-infected PT14 (bottom panel). Reads are illustrated by horizontal green (forward orientation) and red (reverse orientation) lines. After 30 rows of reads, an overflow graph following the same colour scheme is displayed below the reads. Mismatches between the reference sequence and the mapping data are highlighted in red (A), blue (C), green (T) and yellow (G). Coverage is displayed relative to the total number of reads and is shown above the reads. The *flaC* gene is down-regulated in the CP30-infected PT14 sample. The RNA-seq data have been visualised using CLC Genomics Workbench.


1,576,000
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A911_07985
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A911_08005
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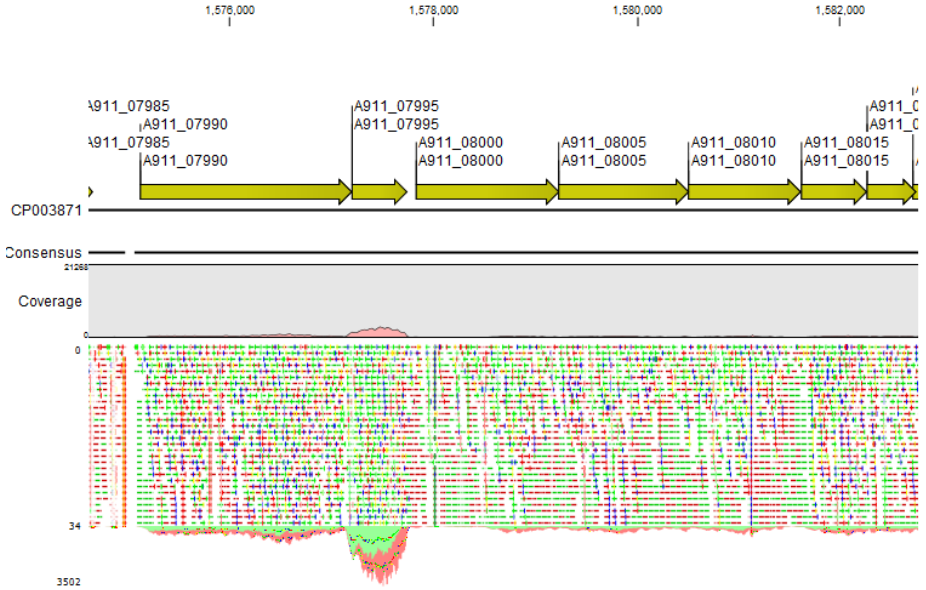
A911_08015
CP003871
Consensus
Coverage
0
35
629


1,576,000
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1,582,000
A911_07985
A911_07990
A911_07995
A911_08000
A911_08005
A911_08010
A911_08015
CP003871
Consensus
Coverage
0
34
3502

Figure 4-5. RNA-seq data mapped against the *C. jejuni* PT14 genome, showing read distribution for the iron uptake genes A911_07990-A911_08015 (*cj1658-cj1663*).

Data are shown for uninfected (top panel) and CPX-infected PT14 (bottom panel). Reads are illustrated by horizontal green (forward orientation) and red (reverse orientation) lines. After 34-35 rows of reads, an overflow graph following the same colour scheme is displayed below the reads. Mismatches between the reference sequence and the mapping data are highlighted in red (A), blue (C), green (T) and yellow (G). Coverage is displayed relative to the total number of reads and is shown above the reads. Genes A911_07990, A911_07995, A911_08000, A911_08005, A911_08010 and A911_08015 are all up-regulated in the CPX-infected PT14 sample. The RNA-seq data have been visualised using CLC Genomics Workbench.

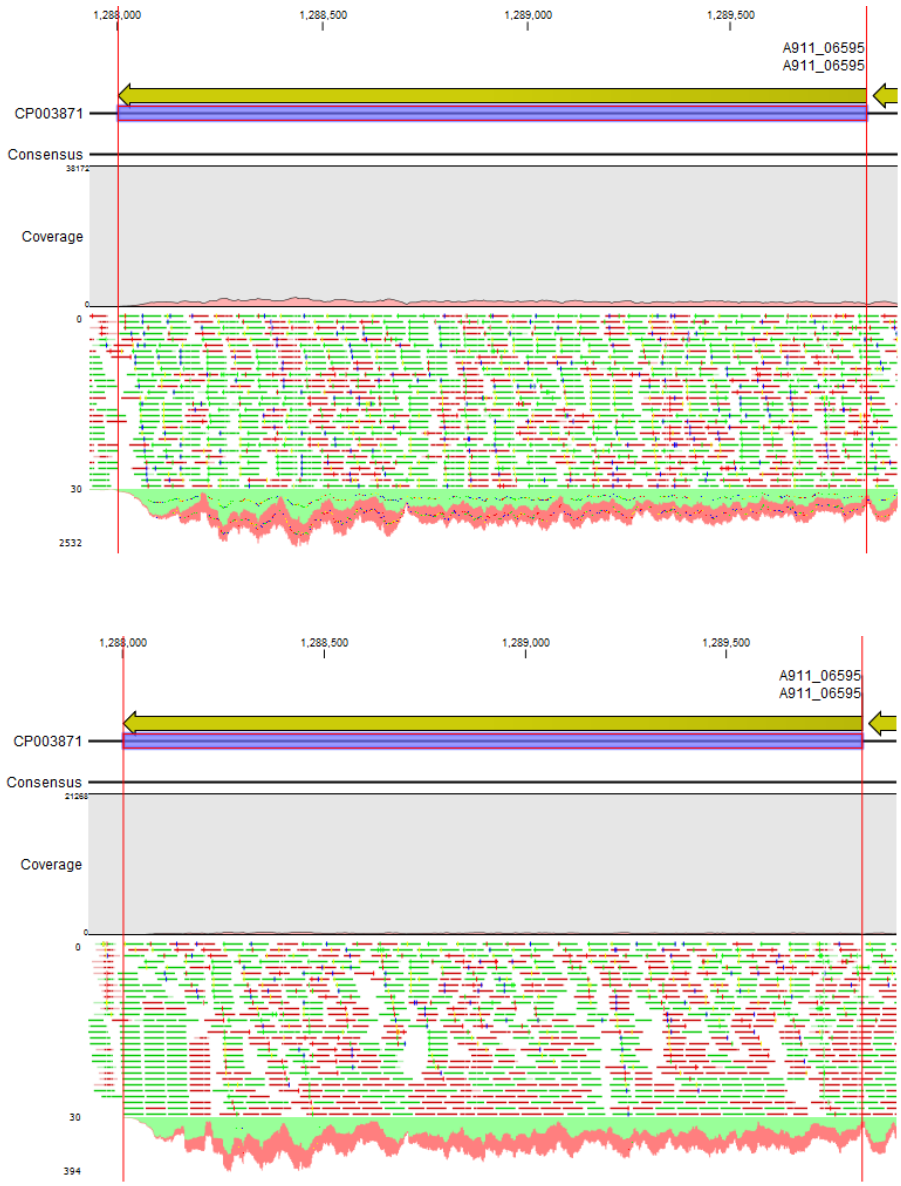


Figure 4-6. RNA-seq data mapped against the *C. jejuni* PT14 genome, showing read distribution for the *nrfA* gene (A911_06595).

Data are shown for uninfected (top panel) and CPX-infected PT14 (bottom panel). Reads are illustrated by horizontal green (forward orientation) and red (reverse orientation) lines. After 30 rows of reads, an overflow graph following the same colour scheme is displayed below the reads. Mismatches between the reference sequence and the mapping data are highlighted in red (A), blue (C), green (T) and yellow (G). Coverage is displayed relative to the total number of reads and is shown above the reads. The *nrfA* gene is down-regulated in the CPX-infected PT14 sample. The RNA-seq data have been visualised using CLC Genomics Workbench.

Infection of *C. jejuni* PT14 with CP30A produced differential regulated expression (≥ 1.5-fold, p_{adj}<0.05) of 100 genes compared to uninfected control cultures, which may be compared to differential regulation of expression of 466 genes in response to CPX infection. These values represent 6.2% and 28.7% of the overall host genetic makeup, suggesting that CPX had more of a measurable effect on the host genes than CP30A. A total of 68 genes were identified that showed common transcriptional responses to infection by bacteriophages CP30A and CPX. In addition, there was quite an even distribution between up- and down-regulated genes following CP30 infection as just under half of the genes showed increased abundance (49 out of 100). However, the distribution of regulated genes in response to CPX infection was less evenly spread with more than three quarters of the CPX-regulated genes (360 out of 466) showing up-regulation. Figure 4-7 provides a summary of the distribution of the regulated genes in response to infection with either phage and the number of common genes exhibiting similar behaviour in response to infection by either phage. In addition, Figure 4-8 illustrates the $\log_2$(RPKM+1) values obtained for uninfected PT14 plotted against the equivalent values for CP30A and CPX-infected PT14 respectively. Up- and down-regulated genes are colour-coded in yellow and red respectively.

Genes displaying significant differential expression were classified into functional categories, according to the scheme originally used by the Sanger Centre for the *C. jejuni* genome database (Parkhill et al. 2000). Figure 4-9 shows Circos diagrams (Krzywinski et al. 2009) depicting these classifications. Genes from the two datasets show similarities in the functional profiles of the

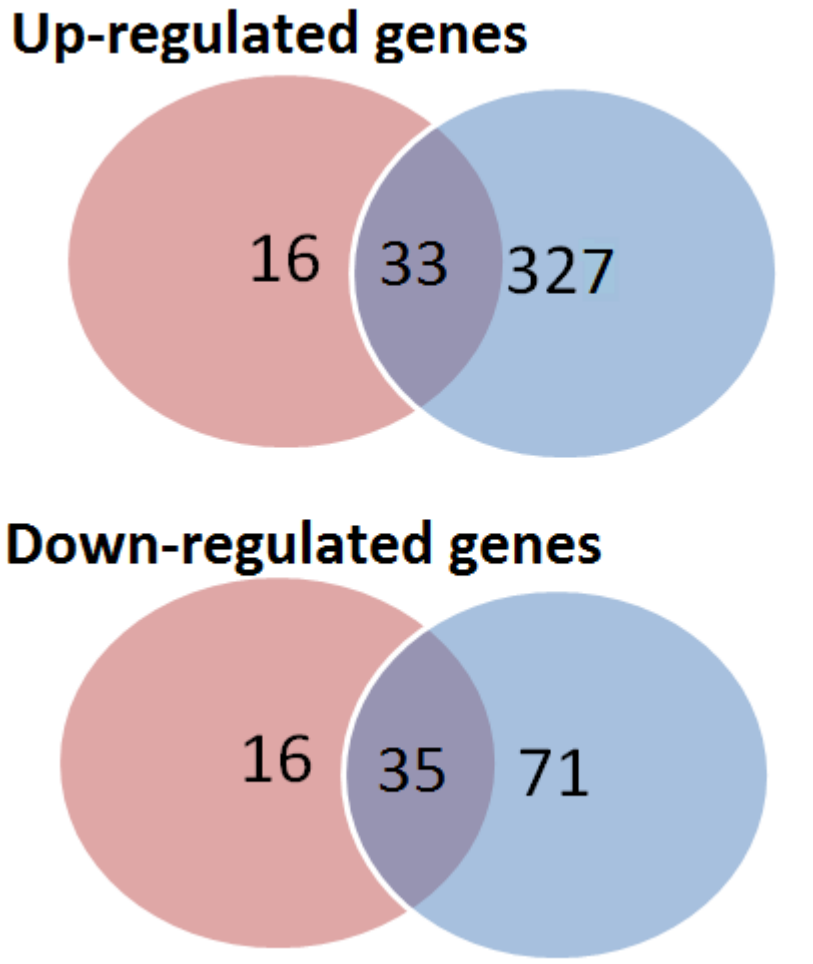


Figure 4-7. Distribution of differentially expressed genes in response to bacteriophage infection.

The distribution of genes significantly differentially expressed in response to phage CP30A infection is represented by the red circle, while the blue circle shows those for the CPX infection. Between the two conditions, there were 32 genes in common that were activated in response to bacteriophage infection while 35 common genes were found to be repressed.

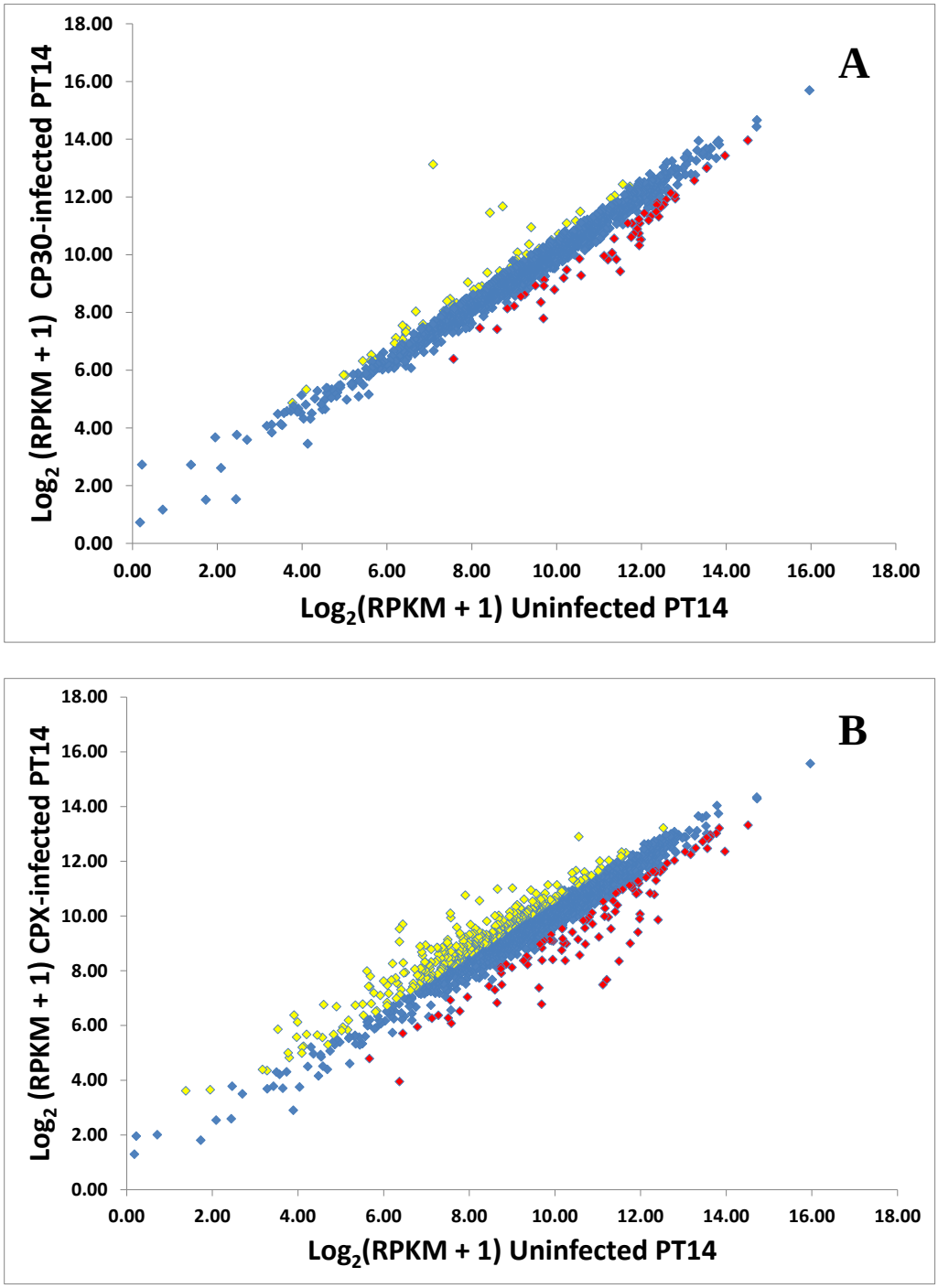


Figure 4-8. Differential gene expression in *C. jejuni* PT14 in response to infection with bacteriophages CP30A(A) and CPX(B).

The average $\log_2$(RPKM+1) value for each gene in the PT14 genome was calculated and plotted for the uninfected and phage-infected PT14 isolates using RPKM values for 3 biological replicates. Significantly differentially expressed genes (≥1.5-fold, *p_{adj}<0.05) are highlighted in yellow (up-regulated) and red (down-regulated).

*p_{adj}<0.05 – corrected p-value representing a false discovery rate of <5%

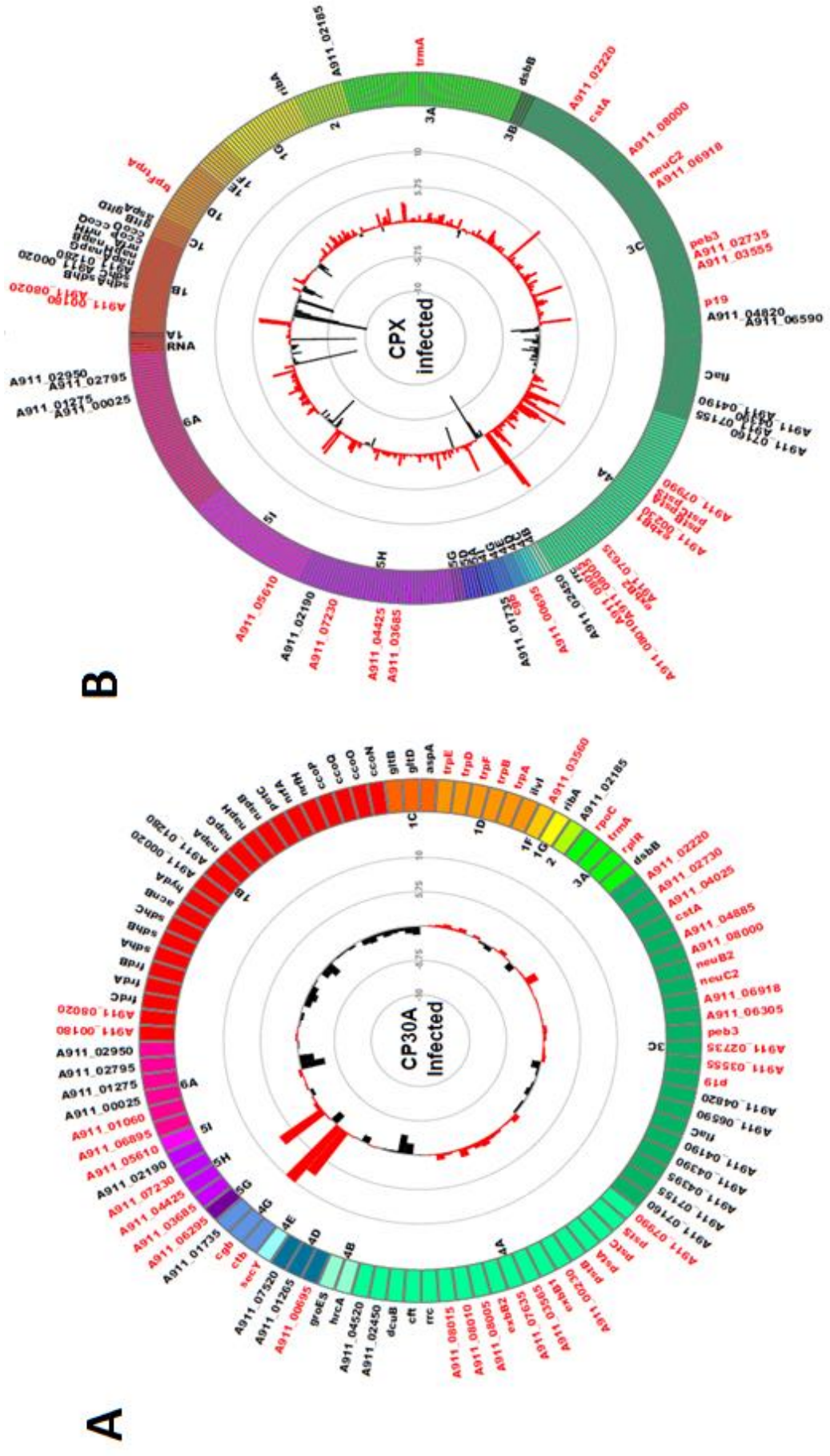

B
CPX
infected
A
CP30A
Infected

Figure 4-9. *C. jejuni* PT14 genes differentially expressed in response to phage infection ordered by functional category.

Individual genes that were found to be greater than 1.5 fold differentially expressed in response to CP30 phage infection (A) or CPX phage infection (B), compared to uninfected bacteria, are represented using Circos diagrams (Krzywinski et al. 2009) with each gene represented and colour-coded according to functional class (Parkhill et al. 2000; Gundogdu et al. 2007) as follows: 1A Degradation; 1B Energy metabolism; 1C Central intermediary metabolism; 1D Amino acid biosynthesis; 1E Polyamine synthesis; 1F Purines, pyrimidines, nucleosides and nucleotides; 1G Biosynthesis of cofactors, prosthetic groups and carriers; 2 Broad regulatory functions and signal transduction; 3A Synthesis and modification of macromolecules; 3B Degradation of macromolecules; 3C Cell envelope; 4A Transport/binding proteins; 4B Chaperones, chaperonins, heat shock; 4C Cell division; 4D Chemotaxis and mobility; 4E Protein and peptide secretion; 4G Detoxification; 4I Pathogenicity; 5A IS elements; 5D Drug/analogue sensitivity; 5G Antibiotic resistance; 5H Conserved hypothetical proteins; 5I Unknown; 6A Miscellaneous. Histograms in the central rings represent the fold change with increased expression on infection represented by red bars and reduced expression represented by black bars. Asterisk in A indicates a greater than 5 fold change in expression. Each gene is labelled individually in A whilst only genes that are differentially expressed in both data sets, are labelled in B to avoid congestion.

differentially regulated genes, with the CP30A-infected regulated genes being assigned to 17 categories while the CPX-infected regulated genes were classified into 24 categories. A high number of up-regulated genes in response to both infection conditions were related to cell envelope and transport or binding protein functions, while those regulated in response to CPX infection were also involved in synthesis of macromolecules. Several down-regulated genes for both infection conditions were associated with energy metabolism and cell envelope functions.

Among the genes that were up-regulated in response to infection of *C. jejuni* PT14 by phages CP30A or CPX, were a substantial number classified in transport or binding protein functions that encode products involved in iron acquisition. For infection with either phage, these include the gene loci associated with the transport of rhodotorulic acid (A911_07990/*cj1658*, A911_07995/*p19*, A911_08000, A911_08005, A911_08010 and A911_08015/*cj1660-cj1663*). With the exception of the *p19* gene, this operon represents the top five most highly expressed genes in response to CPX infection of *C. jejuni* PT14, with A911_08005 being the most up-regulated at 9.5-fold higher than the uninfected samples. There were also several other iron acquisition genes that showed increased abundance in the CPX-infected samples, including most of those involved in the ferric enterochelin uptake system (*cfrA*, *ceuBDE*), haemin uptake system (*chuABC*) and the ferri-transferrin uptake system (*cfbpBC*). Many components of the three TonB energy transduction systems (*exbB1* (both phages), *exbB2* (both phages)-*exbD2-tonB2* and *exbD3-tonB3*) also showed elevated levels of transcription in

response to CPX infection. In addition, some genes known to be associated with oxidative stress defence in *C. jejuni* were also up-regulated in response to infection with CPX, including *katA*, *sodB*, *perR*, A911_06735/*cj1386*, A911_08020/*cj1664* (CP30A and CPX), A911_08025/*cj1665* and A911_02725/*cj0559*.

The genes exhibiting the greatest differential expression in response to CP30A infection of *C. jejuni* PT14 were *cgb* (+63.97-fold) and *ctb* (+7.92-fold), which encode a single domain haemoglobin and a truncated haemoglobin respectively, and are known to be involved in nitrosative stress defence. The *cgb* gene was also up-regulated (+1.98-fold) in response to CPX-infection, but although *ctb* was also up-regulated by this phage, it fell below the 1.5 fold cut-off (+1.18-fold). Two other nitrosative stress responsive genes of unknown function were also up-regulated in response to CP30A infection, these are A911_03685/*cj0761* (CPX also) and A911_04025/*cj0830*. In addition, CP30A increased expression of genes encoding a ribosomal protein (*rplR*), a subunit of RNA polymerase (*rpoC*) and the tryptophan biosynthesis gene locus (*trpABFDE*). The *trpA* and *trpF* genes were also up-regulated by CPX.

Genes coding for proteins involved in cell envelope functions related to surface polysaccharides, lipopolysaccharide and antigens (*kpsS*, *neuB2*, *neuC2*, A911_06918/*cj1421c*) also showed increased expression in response to phage infection. Interestingly, *cj1421c* was previously reported to encode a product that was identified as a specific target for bacteriophage F336 (Sørensen *et* al., 2011). A considerable number of genes co-regulated by CPX were also related

to synthesis and modification of macromolecules including several that encoded proteins involved in DNA replication, restriction/modification and repair (*dnaA*, *dnaX*, *nth*, *mutY*, *polA*, *ruvB*, *ruvC*, *topA*, *ung*, *uvrC* and *xerD*).

Four genes encoding products related to the host defence system were up-regulated in response to CPX infection. These were the genes encoding a CRISPR-associated Cas protein, *cas*2 (A911_07325), the gene encoding a member of the Type I R-M system of PT14, *hsdS* (A911_07470) as well as genes encoding an adenine specific (A911_01010) and site-specific DNA methyltransferase.

Genes encoding products involved in respiration (*hypD* and *hypF*) and in particular, aerobic respiration (*nuoA* and *nuoB*), were found to be up-regulated by CPX. Other notable genes that showed elevated levels of transcription for the phage-infected samples included those predicted to encode proteins associated with aerobic transport of C4-dicarboxylates (A911_00230/*cj0046*), transport of phosphates (*pstSCAB*) and phosphate-containing molecules (*peb3*) as well as transport [*cstA*, A911_00990/*cj0204* (CPX only), A911_07600/*cj1580c* (CPX only)] and secretion of peptides [*secY* (CP30 only)].

The RNA-seq data indicated that in response to infection by either bacteriophage, there was an increase in expression of the gene A911_00180/*cj0037c* which encodes a cytochrome c. This was an interesting finding because other similar genes encoding products related to electron

transport during respiration were found to be down-regulated. The complete dataset of genes up-regulated in PT14 in response to infection with CP30A and CPX, along with their fold changes and adjusted p-values, is listed in appendices 1 and 3 respectively.

The down-regulated genes in response to phage infection encoding products related to energy metabolism including some involved in glycolysis [*fba*, *gapA* and *pyk* (all CPX only)], the tricarboxylic acid cycle [*sdhABC*, *frdABC* (CP30A only) and *acnB* (CP30A only)] and electron transport during respiration [A911_01280/*cj0265c*, *nrfA*, *nrfH*, *ccoN* (CP30A only), *ccoOPQ*, *napAGHB*, *petC* (CP30A only) and *fdxA* (CPX only)]. Two notable genes that were down-regulated in response to infection with either phage were those encoding a molybdopterin oxidoreductase (A911_00025/*cj0005c*), which exhibited the greatest differential fall in response to CP30A infection (-4.33-fold), and a hypothetical protein in *C. jejuni* PT14 that encodes a monohaem cytochrome c in NCTC 11168 (A911_00020/*cj0004c*). These two genes have been found to represent a sulphite respiration system in *C. jejuni*. There was also a reduction in transcription levels of the genes *dcuA* (CPX only) and *dcuB*, which encode two anaerobic C4-dicarboxylate transporters. In addition, the down-regulated genes encoding products related to amino acid biosynthesis were *ilvI* (CP30A only), *leuA*, *leuB*, *leuC*, *metC'*, *dapA* and *aroC* (all CPX only) while some of those related to central intermediary metabolism functions include *gltB*, *gltD*, *aspA* and *glmU* (CPX only).

The majority of the genes down-regulated by CPX that were related to cell envelope functions encode products associated with flagellar biogenesis, including those involved in formation of the flagellar filament (*flaB* and *flaG*), hook (*flaD*, *flgE*, *flgE2* and *flgK*) and rod (*flgB*, *flgG*, *flgG2*, *flgH* and *flgI*) as well as flagellar chaperones (*flgD* and *fliK*). The third flagellin gene (*flaC*) was also found to be down-regulated in response to infection by either CP30A or CPX. Genes related to chemotaxis and mobility (A911_01265/*cj0262c* and A911_07520/*cj1564*) as well as heat shock (*hrcA* and *groES*) were also down-regulated in response to infection of PT14 with CP30A only.

A striking observation was the down-regulation of the *cft* gene in response to CP30A infection and of the *fdxA* gene in response to CPX-infection, which are suggested to have roles in oxidative stress defence in *Campylobacter*. These genes encode a non-haem iron-containing ferritin and a ferredoxin protein respectively. This was particularly interesting because genes with similar function were found to be up-regulated in response to phage infection. The complete dataset of genes down-regulated in PT14 in response to infection with bacteriophages CP30A and CPX is listed in appendices 2 and 4 respectively.

4.2.4 Examination of bacteriophage transcription

In order to have an idea of bacteriophage transcription during infection of *C. jejuni* PT14, all reads from the CP30A- and CPX-infected samples were mapped against the reference genomes for CP30A (GenBank accession number JX569801) and CPX (GenBank accession number JN132397)

according to section 2.12.4.2. The reads were normalized for gene length and the total number of reads by calculating the RPKM (Mortazavi et al. 2008). The average RPKM was calculated for the triplicate phage-infected samples and this was used as a measure of gene expression. The complete datasets showing the average RPKM values for the CP30A- and CPX-infected PT14 samples during bacteriophage infection are listed in appendices 5 and 6 respectively. The gene encoding the major capsid protein was the most highly expressed transcript by both bacteriophages and other genes encoding products that are known to be expressed late in transcription were also abundant such as the tail sheath protein, tail tube protein, neck protein, and putative major head protein II. The genes encoding the co-chaperone GroES and the prohead core scaffold and protease, which function in phage capsid morphogenesis, were also highly expressed. Genes encoding products involved in DNA synthesis also showed high levels of expression by both phages, including the putative sliding clamp DNA polymerase accessory protein, putative sliding clamp loader subunit, the single-stranded DNA binding protein and the ribonucleotide reductase subunits.

4.2.5 *In vitro* growth of CP30A- and CPX-infected *C. jejuni* PT14 under iron-limited conditions

Based on the RNA-seq results, it was decided to examine the requirement for iron during bacteriophage infection of *C. jejuni* PT14. This was done by carrying out *in vitro* growth experiments under iron-depleted conditions (2.7) to determine whether phage infection would be affected. Iron depletion was mediated by adding 2', 2'-dipyridyl (DIP) (2.1.17) to MH broth (2.1.6) to give

a final concentration of 20 µM. Uninfected and phage-infected cultures were grown in triplicate for 5 hours under these conditions according to section 2.6.1. *Campylobacter* cells were enumerated (2.2.4) every two hours and at the final hour. This revealed a decrease in viable bacterial density from 7.0 $\log_{10}$ CFU ml^{-1} to 4.0 $\log_{10}$ CFU ml^{-1} for both the uninfected and phage-infected cultures over the 5 hours. This therefore suggested that the concentration of available iron was too low to support growth of the *Campylobacter* and negatively impacted its survival under these conditions, which would confound investigations of bacteriophage infection.

4.2.6 Designing the Cgb-FR and Ctb-FR primers

In order to further investigate the role of the haemoglobins Cgb and Ctb during bacteriophage infection of *C. jejuni* PT14, the corresponding knockout mutations were constructed. Prof. R. Poole (University of Sheffield, UK) kindly provided knockout mutant strains in which the *cgb* (*cj1586*) and *ctb* (*cj0465c*) genes had been disrupted in the *C. jejuni* NCTC 11168 genome (Elvers et al. 2004; Wainwright et al. 2005). The *cgb* mutant, 11168 *cgb*::Kan^r (known as RKP1336 or CJCGB01), was constructed by introducing plasmid DNA from the suicide plasmid pKE18 into the NCTC 11168 genome by electroporation. The plasmid contained a cloning vector comprised of a 1,187-bp DNA fragment of the *cgb* gene, where a *Bgl*II restriction site was introduced. The vector was digested with *Bgl*II and a kanamycin resistance (Kan^r) cassette derived from pJMK30 (van Vliet et al. 1998) was inserted as a *Bam*HI fragment. Similarly, the *ctb* mutant, 11168 ctb::Kan^r (known as RKP1386) was constructed by introducing plasmid DNA from the suicide

plasmid pKE18 into the NCTC 11168 genome by electroporation. The plasmid contained a disruption of the *ctb* gene to create 11168 cgb::Kan^r.

The *cgb* and *ctb* genes of *C. jejuni* PT14 are sufficiently conserved to enable homologous recombination. Therefore genomic DNAs from the knockout mutants 11168 cgb::Kan^r and 11168 ctb::Kan^r, were used to introduce the disruption into the PT14 genome by natural transformation to generate PT14 cgb::Kan^r and PT14 ctb::Kan^r (section 2.4). To determine whether the natural transformation had been successful, primers Cgb-F and Cgb-R as well as Ctb-F and Ctb-R were designed as previously described (2.9.1) to amplify 1,711-bp and 1,797-bp DNA fragments of *cgb* and *ctb* respectively.

4.2.7 PCR confirmation of *C. jejuni* PT14 *cgb* and *ctb* knockout mutation

Following natural transformation of *C. jejuni* PT14 with genomic DNAs from 11168 *cgb*::Kan^r and 11168 *ctb*::Kan^r, genomic DNA was isolated from candidate PT14 *cgb*::Kan^r and PT14 *ctb*::Kan^r transformants (2.8). The DNAs were PCR amplified using the Cgb-FR and Ctb-FR primers (Table 2-4). Genomic DNA was also isolated from 11168 *cgb*::Kan^r and 11168 *ctb*::Kan^r to provide positive PCR controls, and from the parental strains, PT14 and NCTC 11168, to provide negative controls. Figure 4-10 illustrates the agarose gel electrophoresis image showing successful amplification of these DNA isolates, generating fragments of expected amplicon size for the wild-type and mutant strains.

4.2.8 *In vitro* growth of Cgb- and Ctb-deficient *C. jejuni* PT14 mutants under bacteriophage infection

Due to the elevated transcription levels of the PT14 haemoglobin genes *cgb* (A911_07630) and *ctb* (A911_02270) during bacteriophage infection, their importance was further investigated by conducting *in vitro* growth experiments. The knockout mutants, PT14 *cgb*::Kan^r and PT14 *ctb*::Kan^r, were grown in triplicate in 50 ml volumes of Nutrient broth No. 2 (2.1.5) over 24 hours according to section 2.6.1. Bacteriophages CP30A and CPX were added to the cultures after 2 hours of initial growth and samples were taken hourly up to 4 hours post infection and then every 2 hours until the 24th hour in order to monitor the viable count of the host and the phage titre. An initial host concentration of 7.0 $\log_{10}$ CFU ml^{-1} was used for the Cgb- and Ctb-deficient mutants. Infection with CP30A resulted in an immediate 3 log drop in bacterial numbers, which yielded an increased phage titre of around 2 logs (Figure 4-11B and C). This was characteristic of what occurred with the wild-type strain (Figure 4-11A) and is indicative of synchronous infection, with approximately 99.9% of the bacterial population under phage infection. The crash in host population following CPX infection occurred 2 hours post-infection with either mutant, after the bacterial numbers had reached close to 7.5 $\log_{10}$ CFU ml^{-1}. This again exhibited an infection pattern similar to that of the wild-type strain (Figure 4-12A). The viable counts of the host bacteria were reduced by approximately 2.5 logs, which yielded increases in phage titre of around 1.4 logs for CP30A and 2 logs for CPX (Figures 4-12B and C). However, the mean burst sizes calculated from triplicate globin mutant cultures were significantly less than those observed for wild type *C. jejuni* PT14 (ANOVA,

$p<0.05$, n=3). CP30A infection produced the following burst sizes: wild type=5.0±0.1, Δ*cgb*=3.7±0.3, Δ*ctb*=2.5±0.5 pfu per cell; whilst CPX infection produced the burst sizes: wild type=5.2±0.2, Δ*cgb*=3.0±0.3, Δ*ctb*=2.1±0.7 pfu per cell.

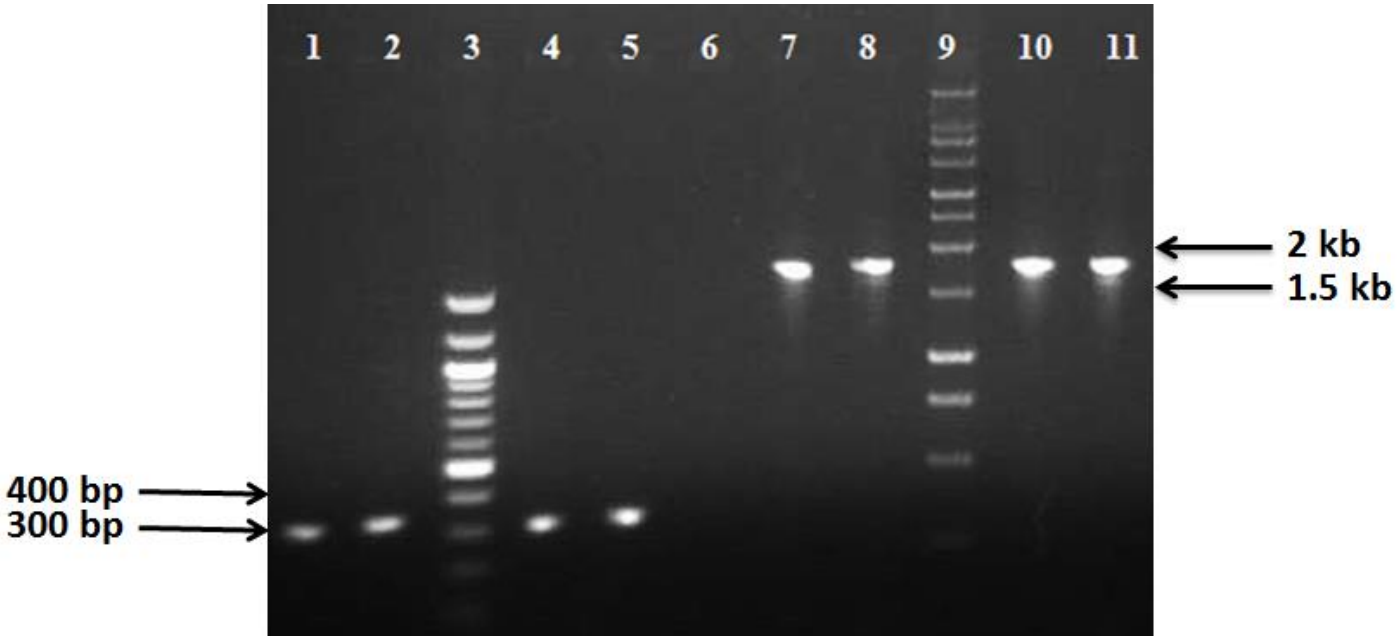
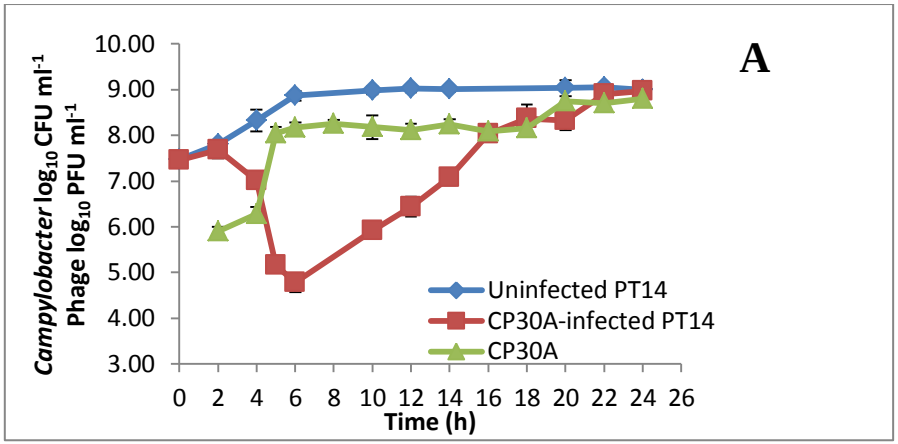
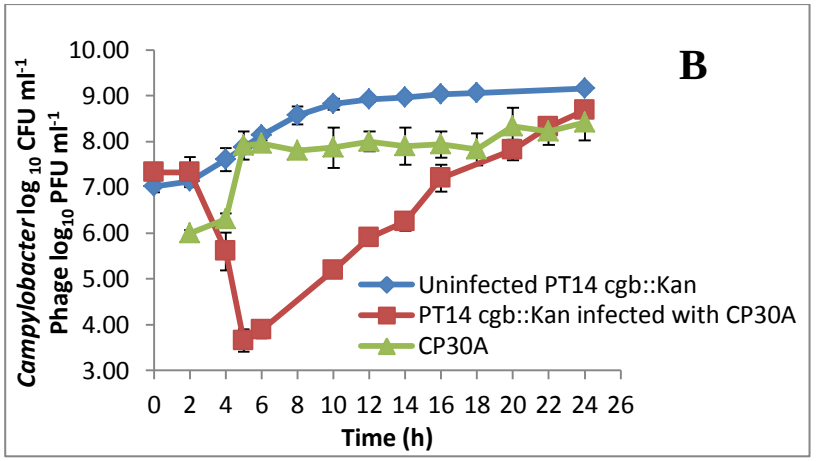


Figure 4-10. 1% Agarose gel of PCR products obtained using Cgb-FR and Ctb-FR primers.

Lane 1: PT14 WT/Cgb-FR, Lane 2: PT14 WT/Ctb-FR, Lane 3: NEB 100-bp ladder, Lane 4: NCTC 11168 WT/Cgb-FR, Lane 5: NCTC 11168 WT/Ctb-FR, Lane 6: empty, Lane 7: PT14 *cgb*::Kan^r/Cgb-FR, Lane 8: PT14 *ctb*::Kan^r/Ctb-FR, Lane 9: Promega 1 kb ladder, Lane 10: 11168 *cgb*::Kan^r/Cgb-FR, Lane 11: 11168 *ctb*::Kan^r/Ctb-FR. The expected amplicon sizes for the fragments generated from the wildtype strains using Cgb-FR and Ctb-FR were approximately 311-bp and 397-bp respectively while for the mutants they were 1,711-bp and 1,797-bp respectively.


A
Campylobacter log10 CFU ml-1
Phage log10 PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14
CP30A-infected PT14
CP30A


B
Campylobacter log 10 CFU ml-1
Phage log10 PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14 cgb::Kan
PT14 cgb::Kan infected with CP30A
CP30A

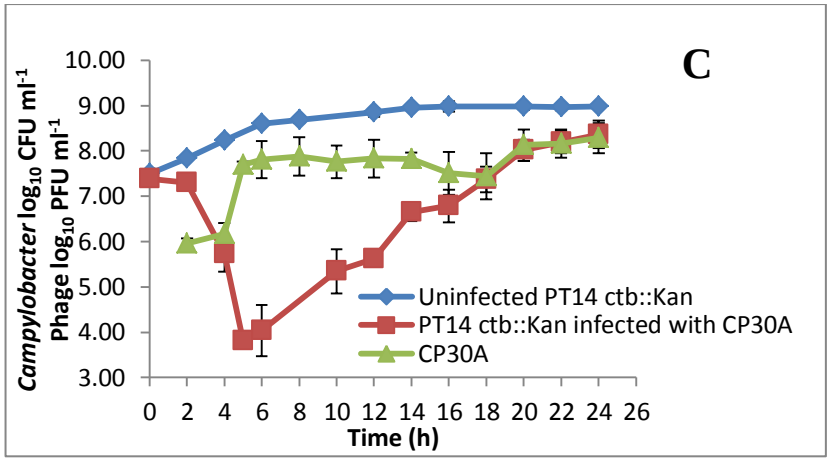
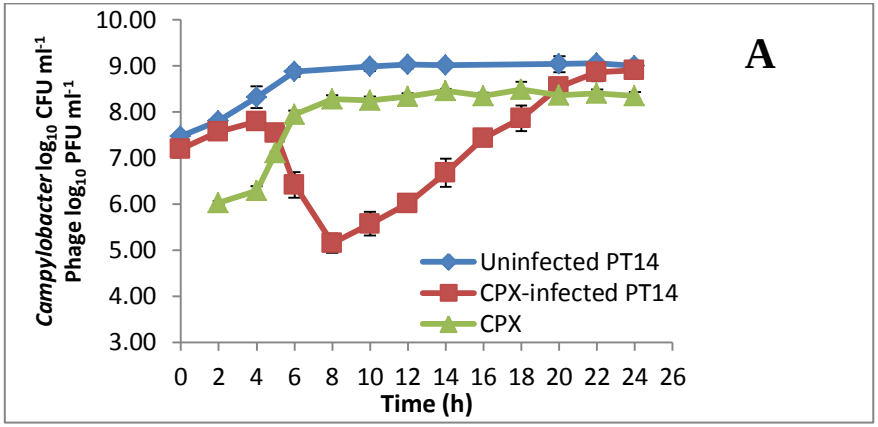
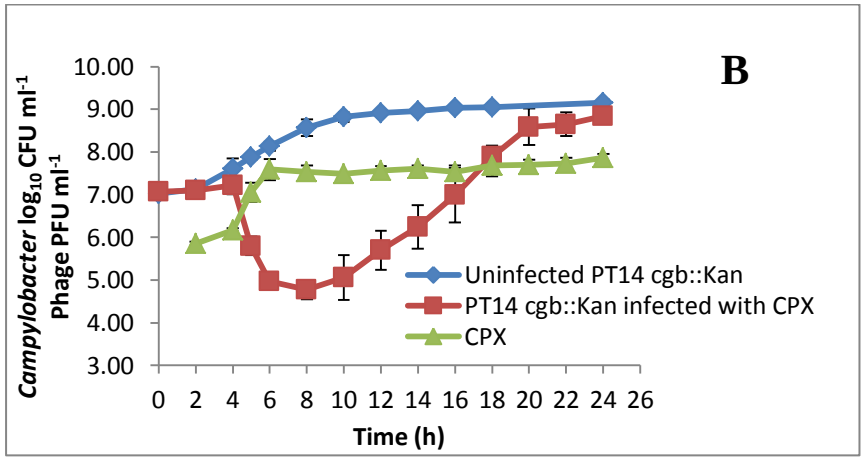

C
Campylobacter log10 CFU ml-1
Phage log10 PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14 ctb::Kan
PT14 ctb::Kan infected with CP30A
CP30A

Figure 4-11. *In vitro* growth of wild-type *C. jejuni* PT14 (A), and Cgb- (B) and Ctb-deficient (C) *C. jejuni* PT14 mutants in the absence and presence of bacteriophage CP30A.

Uninfected bacteria are represented by blue lines, phage-infected by brown lines and phage growth by green lines. On infection with CP30A at the second hour, there was an immediate approximate 3-log reduction in bacterial numbers. Following this, there was resurgence in growth of the host population, which continued until the final sampling point. The phage titre also remained relatively constant after the initial rise, however a second rise occurred between the 18-20th hour despite no clear drop in the host population being observed. Values for bacterial growth (CFU ml^{-1}) and phage titre (PFU ml^{-1}) are generated from 3 independent cultures ± SD and plotted as a function of time. The pattern of growth for the wild-type and mutants is generally identical, however the mean burst sizes that were calculated from the triplicate cultures were significantly less that that observed for the wild-type (ANOVA, $p<0.05$, $n=3$).


A
Campylobacter log10 CFU ml-1
Phage log10 PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14
CPX-infected PT14
CPX


B
Campylobacter log10 CFU ml-1
Phage PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14 cgb::Kan
PT14 cgb::Kan infected with CPX
CPX

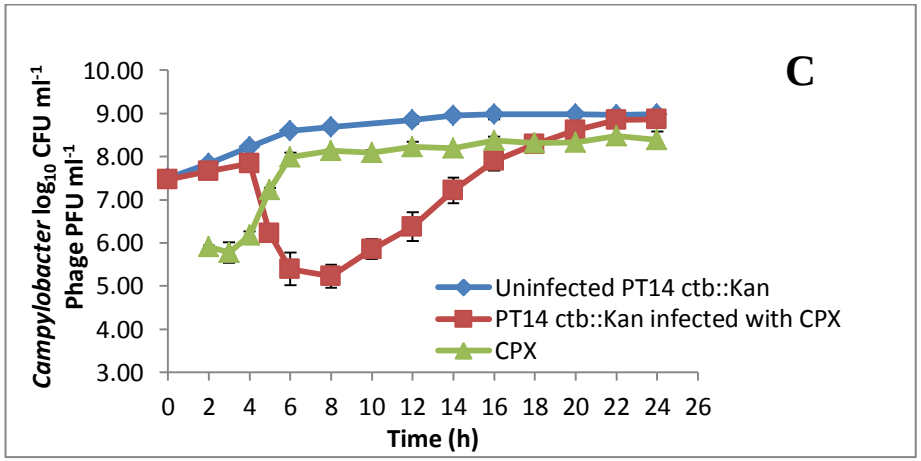

C
Campylobacter log10 CFU ml-1
Phage PFU ml-1
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (h)
Uninfected PT14 ctb::Kan
PT14 ctb::Kan infected with CPX
CPX

Figure 4-12. *In vitro* growth of wild-type *C. jejuni* PT14 (A), and Cgb- (B) and Ctb-deficient (C) *C. jejuni* PT14 mutants in the absence and presence of bacteriophage CPX.

Uninfected mutants are represented by blue lines, phage-infected by brown lines and phage growth by green lines. Phage CPX was added at the second hour of bacterial growth and two hours later an approximate 2.5 log reduction in host numbers was observed. Following this, a resurgence in growth of the host population could be observed, which continued until the final sampling point. The phage titre remained relatively constant between the 6-24th hour following the burst at the 4th hour. Values for bacterial growth (CFU ml^{-1}) and phage titre (PFU ml^{-1}) are generated from 3 independent cultures ± SD and plotted as a function of time. The pattern of growth for the wild-type and mutants is generally identical, however the mean burst sizes that were calculated from the triplicate cultures were significantly less that that observed for the wild-type (ANOVA, $p<0.05$, $n=3$).

4.3 DISCUSSION

The transcriptional profile of *C. jejuni* PT14 under infection by bacteriophages CP30A and CPX was characterised using RNA-seq technology. RPKM values were calculated and provided a measure of gene expression levels between uninfected and phage-infected samples. It was also possible to determine measurements of gene expression for phage-specific transcripts. These results confirmed that RNA was isolated during the eclipse phase of phage replication as several genes known to be expressed late in transcription exhibited high expression levels, with the major capsid protein being the most abundant transcript expressed by both phages.

4.3.1 The phage-infected host transcriptome mimics an iron-limited environment

The transcriptome of *C. jejuni* PT14 under bacteriophage infection is consistent with that of an iron-restricted environment since very similar genes were previously found to be regulated during iron limitation (Palyada et al. 2004; Holmes et al. 2005; Butcher & Stintzi 2013). Previous studies have characterised gene expression of *C. jejuni* in response to iron limitation using microarray analysis (Palyada et al. 2004; Holmes et al. 2005) and RNA-seq technology (Butcher & Stintzi 2013). Regardless of the growth conditions used, a number of common genes were activated in response to the lack of iron. The gene products relate to different iron-acquisition systems, which include ferri-enterochelin (*cfrA*, *ceuE*) (Palyada et al. 2004), haem (*chuABCD*, *cj1613c*) (Ridley et al. 2006), transferrin (*cfbpABC*) (Miller et al. 2008) and rhodotorulic acid (*cj1658/p19*, *cj1660-cj1663*). The majority of these genes

were also found to be activated in response to phage infection, with the exception of *chuD* and *cfbpA*. These both encode periplasmic binding proteins and although they were both up-regulated in response to infection by CP30A and CPX, they fell below the 1.5 fold cut-off which was selected as a measurable and potentially meaningful change. In addition, in response to iron-limited conditions (Palyada et al. 2004; Holmes et al. 2005; Butcher & Stintzi 2013), several members of the putative TonB energy transduction systems were also up-regulated including *exbB1-exbD1-tonB1*, *exbB2-exbD2-tonB2* and *tonB3*. In comparison to the genes up-regulated in response to phage infection, all of these genes were also activated with the exception of *exbD1* and *tonB1*.

Iron-uptake systems of *C. jejuni* typically comprise of an outer-membrane (OM) receptor protein, which specifically binds to and transports ferric iron sources through the OM into the periplasm using energy derived from the ExbB-ExbD-TonB energy transduction system. The iron is then transported to a periplasmic iron-binding protein (highlighted in red in Figure 4-13 (A), which subsequently delivers it to the cytoplasmic membrane (CM) ABC transporter system, which consists of a permease and an ATP-binding protein. Figure 4-13 (A) provides a schematic of a typical iron-uptake system of *C. jejuni* and Figure 4-13 (B) illustrates the role each gene plays in the uptake of enterochelin, rhodotorulic acid, haemin and transferrin. The complete array of iron uptake

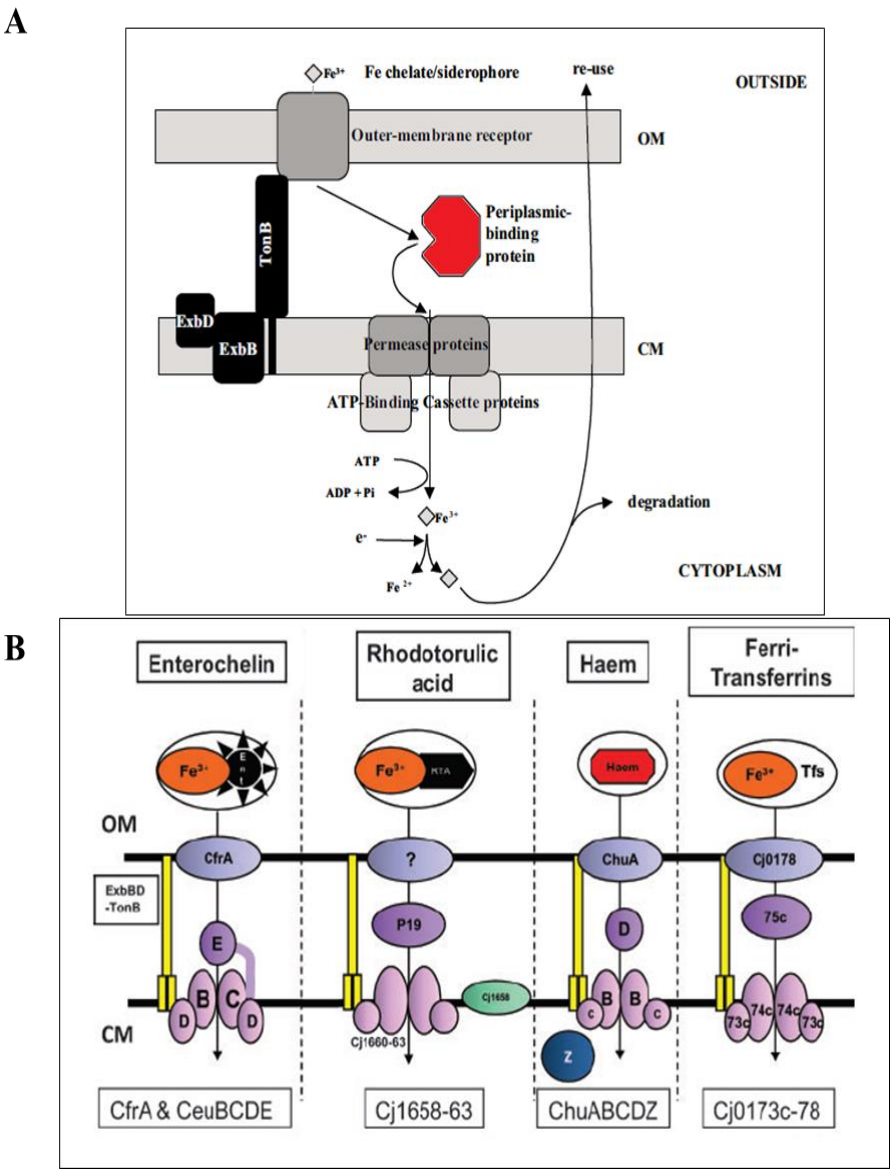


Figure 4-13. Schematic representation of siderophore-based iron acquisition systems in *C. jejuni*.

(A) Ferri-siderophore complexes transport iron via outer-membrane (OM) receptors to the periplasmic binding protein (red), which then delivers it to the cytoplasmic membrane (CM) ABC transporter (comprised of a permease and an ATP-binding protein). Iron transport is mediated into the periplasm via the outer-membrane by energy derived from the ExbB-ExbD-TonB energy transduction system. (B) Several components that allow uptake of enterochelin, rhodotorulic acid, haem and ferri-transferrin in *C. jejuni* have been determined. Adapted from Andrews et al. 2003 (A) and Miller et al. 2009 (B).

genes that were up-regulated in response to phage infection, suggests an increased need for siderophore-based iron acquisition as most genes required for uptake of enterochelin and rhodotorulic acid were activated. On the other hand, since the genes encoding the periplasmic binding proteins for transport of haem and transferrin proteins failed to be activated, this may suggest that there was no increased need to transport haem and transferrin into the cytoplasm via the ABC transporter system, despite the genes encoding the permeases (*chuB* and *cfbpB* (*cj0174*)) and ATP-binding proteins (*chuC* and *cfbpC*/*cj0173c*) being up-regulated. Although it should be noted that the gene encoding the OM receptor protein for transport of transferrin (A911_00860/*cj0178c*) was up-regulated in response to infection by either phage, it was not significantly differentially expressed compared to the uninfected control. Failure of the phages to up-regulate all of the genes related to haem and transferrin uptake is most likely due to the lack of available sources to allow their acquisition under the experimental conditions.

4.3.2 Regulation of iron acquisition genes in response to phage infection

Bacteria generally regulate iron acquisition, metabolism and storage in response to iron availability and to avoid iron toxicity when excess iron interacts with oxygen. In *C. jejuni* and other Gram-negative bacteria, iron-regulated gene expression is mediated by the Fur protein (1.5.1). Under iron-rich conditions, the Fur protein binds to ferrous ions and forms a dimeric Fe^{2+}-Fur complex (holo-Fur), which recognises a specific consensus sequence within the promoter region of iron-regulated genes known as the Fur box and

represses their transcription by blocking entry of RNA polymerase. Under iron-limited conditions, the complex dissociates (apo-Fur) and transcription of iron-regulated genes is de-repressed. The expression profile of the iron acquisition genes regulated in response to bacteriophage infection of *C. jejuni* PT14 is therefore consistent with the model of Fur as a transcriptional repressor of such genes when sufficient iron is available. The findings therefore demonstrate that Fur appears to have derepressed transcription of these genes in what it perceives to be iron-limited conditions.

The Fur regulon of *C. jejuni* was previously defined by examining the transcriptome of a wild-type and *fur* deletion mutant in the absence and presence of iron (Palyada et al. 2004; Holmes et al. 2005). It was demonstrated that that apo- and holo-Fur were both capable of activating and repressing gene expression (Palyada et al. 2004), with more than 60 genes encoding products related to iron acquisition, oxidative stress defence, flagellar biogenesis and energy metabolism being regulated (Palyada et al. 2004; Holmes et al. 2005). More recently, using a chromatin immunoprecipitation and microarray analysis approach (ChIP-chip), it was reported that Fur regulates 95 transcriptional units in *C. jejuni* (Butcher et al. 2012). However, the Fur regulon identified using this technique was significantly different to those that had previously been reported (Palyada et al. 2004; Holmes et al. 2005), possibly because the ChIP-chip identified all direct targets that were bound by Fur regardless of the fold change. Nevertheless, apo- and holo-Fur gene repression and activation was also observed (Butcher et al. 2012). Butcher et al. (2012) also determined

the structure of apo-Fur and suggested possible mechanisms for its regulatory role (discussed in section 1.5.1).

4.3.3 Regulation of oxidative stress defence genes in response to phage infection

A relationship exists between oxidative stress and iron metabolism as exposure of cellular ferrous iron to oxygen can generate the formation of reactive oxygen species (ROS), such as superoxide anions (O_2^-), peroxides (RO_2) and hydroxyl radicals (OH•), which are strong oxidants. *C. jejuni* has developed a number of defence mechanisms for protection against this type of stress (discussed in section 1.4.5). Genes encoding proteins with functions related to oxidative stress defence include *ahpC*, *katA*, *perR*, *sodB*, *tpx*, *cj0559*/A911_02725, *cj1386*/A911_06735, *cj1664*/A911_08020 and *cj1665*/A911_08025, which overlap with some of the genes that respond to iron limitation. (Palyada et al. 2004; Holmes et al. 2005; Butcher & Stintzi 2013). All of these genes were also found to be activated in response to CPX infection of *C. jejuni* PT14, with the exception of *ahpC* and *tpx*. Although the peroxide stress regulator PerR has been found to control transcription of a number of genes encoding proteins relating to oxidative stress defence in *C. jejuni* (van Vliet et al. 1999; Holmes et al. 2005; Palyada et al. 2009), some of these genes are also co-regulated by Fur, including the peroxide stress genes *aphC* and *katA* (van Vliet et al. 1999). The genes *cj1386*, *cj1664* and *cj1665* were also previously found to be Fur regulated (Holmes et al. 2005). In addition, in response to phage infection, *perR* was up-regulated in response to

CPX infection whereas expression of the *fur* gene remained unchanged between uninfected and phage-infected samples.

There are other genes which have been found to contribute to oxidative stress defence in *C. jejuni*, either directly or indirectly. For instance, the ferritin protein, Cft, stores iron inside the ferritin cavity in an inactive but reusable state, and is therefore able to avoid generation of ROS. A *cft*-deficient mutant was previously found to be more sensitive to exposure to H_2O_2 than the parental strain (Wai et al. 1996). This was probably due to the inability of the mutant to collect and store iron from the cytoplasm and therefore resulting in the formation of ROS. In addition, ferritin can provide intracellular iron reserves during iron limitation and as would be expected, the *cft* gene was found to be up-regulated in response to iron-limited conditions (Palyada et al. 2004; Holmes et al. 2005). However, in response to phage infection, there was no change in expression of this gene in response to CPX infection but interestingly, it was found to be 1.5-fold down-regulated in response to CP30A infection. Nonetheless, a similar finding was previously reported for *H. pylori*, with expression of the non-haem containing ferritin gene *pfr* being repressed by apo-Fur (Delany et al. 2001). This suggests that the cft gene may have been regulated by apo-Fur during CP30A infection of PT14.

Another noteworthy protein is the ferredoxin FdxA, which does not appear to show resistance to particular peroxide stress but an *fdxA*-deficient mutant exhibited significantly reduced aerotolerance (van Vliet et al. 2001). The *fdxA* gene was also previously found to show iron-induced gene expression (van

Vliet et al. 2001; Palyada et al. 2004). In response to CPX infection of *C. jejuni* PT14, *fdxA* expression was down-regulated. However, as with the *cft* gene it has been reported that expression of *fdxA* was repressed by apo-Fur in *C. jejuni* (Palyada et al. 2004). This therefore suggests that the *fdxA* gene was also likely under the control of apo-Fur during CPX infection of PT14.

4.3.4 Regulation of flagellar biogenesis genes in response to phage infection

Transcription of flagellar genes in *C. jejuni* is primarily predicted to be controlled by the sigma factors *rpoN* (σ^{54}) and *fliA* (σ^{28}) (Guerry et al. 1991; Hendrixson et al. 2001; Jagannathan et al. 2001; Hendrixson & Dirita 2003; Carrillo et al. 2004). Nevertheless, several genes related to flagellar biogenesis in *C. jejuni* have also previously been found to be controlled either directly or indirectly by Fur, including *flaA*, *flaB*, *flaD*, *flgE2*, *flgG2*, *flgH* and *flgI* (Palyada et al. 2004; Butcher et al. 2012). With the exception of *flaA* and *flaB*, all other genes were found to be apo-Fur activated and repressed on addition of iron to a previously iron-limited medium, but were unusually overexpressed at mid-log phase under iron-rich conditions (Palyada et al. 2004). Moreover, a recent study reported that a large number of flagellar chaperones, regulatory proteins and genes involved in flagellar biogenesis were iron activated (Butcher & Stintzi 2013). In response to infection by CPX, several genes related to flagellar biogenesis were found to be down-regulated in *C. jejuni* PT14. If such genes are indeed iron-activated and PT14 is being perceived as experiencing iron-limited conditions during phage infection, these findings therefore imply that the flagellar biogenesis genes were apo-Fur repressed.

4.3.5 Regulation of nitrosative stress responsive genes

The RNA-seq data indicated that in response to phage infection of *C. jejuni* PT14 there was strong up-regulation of the nitrosative stress responsive genes. These genes were *cgb*, *ctb*, A911_03685/*cj0761* and A911_04025/*cj0830*, whose gene expression have been found to be regulated by *cj0466*, designated as NssR (Nitrosative stress sensing Regulator) (Elvers et al. 2005). The haemoglobin gene *cgb*, which was the most significantly differentially expressed gene in response to CP30A infection, was previously strongly and specifically induced following exposure to nitrosative stress, but not superoxide or peroxide (Elvers et al. 2004). However, the transcriptomic and proteomic response of *C. jejuni* to nitrosative stress revealed up-regulation of a number of genes encoding heat shock proteins as well as products related to oxidative stress defence and iron acquisition and transport (Monk et al. 2008). In addition, it was also demonstrated that Cgb and Ctb were regulated by oxygen availability and in particular when *C. jejuni* cultures were grown at higher rates of oxygen diffusion and then exposed to nitrosative stress. Under these circumstances there was increased transcription of the NssR-regulated genes *cgb, ctb, cj0761* and *cj0830* (Monk et al. 2008). These findings provide a rationale for the pattern of up-regulated gene expression with regards to phage infection of PT14 since NssR-regulated genes respond to the presence of oxygen prior to exposure to nitrosative stress. It is implied that there was a relatively strong source of nitrosative stress particularly during CP30A infection. Cultures of Cgb- and Ctb-deficient *C. jejuni* PT14 mutants could be infected with CP30A or CPX but infection led to reduced burst sizes compared to infected wild type cultures. (Figures 4-11 and 4-12).

4.3.6 Host response to phage infection includes a demand for oxygen

The pattern of down-regulated gene expression was consistent with what would be expected to occur within an aerobic environment. This was evident by the strong repression of genes predicted to be involved in a number of anaerobic electron transport pathways. *C. jejuni* is an obligate microaerophile, which has an aerobic respiratory metabolism that utilises oxygen as the terminal electron acceptor. However, a number of reductases are present in the genome that have previously been shown to allow *C. jejuni* to carry out respiration with alternative electron acceptors to oxygen (Kelly 2001; Sellars et al. 2002; Myers & Kelly 2005b). *In vitro* studies have shown that *C. jejuni* is unable to grow under strictly anaerobic conditions in the presence of these alternative electron acceptors. However, addition of fumarate, nitrate, nitrite, trimethylamine-N-oxide or dimethyl sulfoxide to microaerobic cultures that were under severely oxygen-limited conditions resulted in increased growth (Sellars et al. 2002). In response to phage infection of *C. jejuni* PT14, genes encoding several of these reductases showed reduced expression levels, including subunits of fumarate reductase (*frdCAB*), nitrate reductase (*napAGHB*), nitrite reductase (*nrfA* and *nrfH*) and trimethylamine-N-oxide reductase (A911_01280/*cj0265c*). This therefore suggests that under phage infection, the host generates energy in an electron acceptor-dependent manner, with a demand for oxygen as the terminal electron acceptor. In addition, genes encoding anaerobic C4-dicarboxylate transporters (*dcuA* and *dcuB*) were also down-regulated in response to phage infection while elevated levels of

transcription was observed for a gene encoding a product that allows aerobic transport of C4-dicarboxylates (A911_00230/*cj0046*).

C. jejuni possesses two terminal oxidases: a cytochrome bd-type quinol oxidase (Cyd homologues) and a cytochrome c oxidase, cb (ccb3)-type (Cco homologues). The latter accepts electrons from cytochrome c via a bacterial ubiquinol:cytochrome c oxidoreductase or bc_1 complex encoded by genes from the *petABC* operon, while the previous accepts electrons directly from ubiquinol. The RNA-seq data show that in response to phage infection of *C. jejuni* PT14, genes encoding the cb-type cytochrome c (*ccoNOPQ*) and a subunit of the cytochrome bc_1 complex (*petC*) were repressed, while there was no change in expression for the bd-type quinol oxidase. Figure 4-14 provides a schematic of the pathways of electron transport to oxygen and alternative electron acceptors in *C. jejuni*. All major genes that were down-regulated in response to bacteriophage infection of PT14 are denoted by boxes in reverse fields.

In addition to being able to use a wide range of alternative electron acceptors to oxygen, *C. jejuni* is also capable of utilising a number of electron donors, including formate, hydrogen, D-lactate and succinate. Genes encoding products that have been predicted to allow sulphite to be used as an electron donor (A911_00020/*cj0004c* and A911_00025/*cj0005c*) were also down-regulated in response to phage infection (Myers & Kelly 2005a). In *C. jejuni* NCTC 11168, *cj0004c* and *cj0005c* encode a monohaem cytochrome c and molydopterin oxidoreductase respectively. It was previously demonstrated that

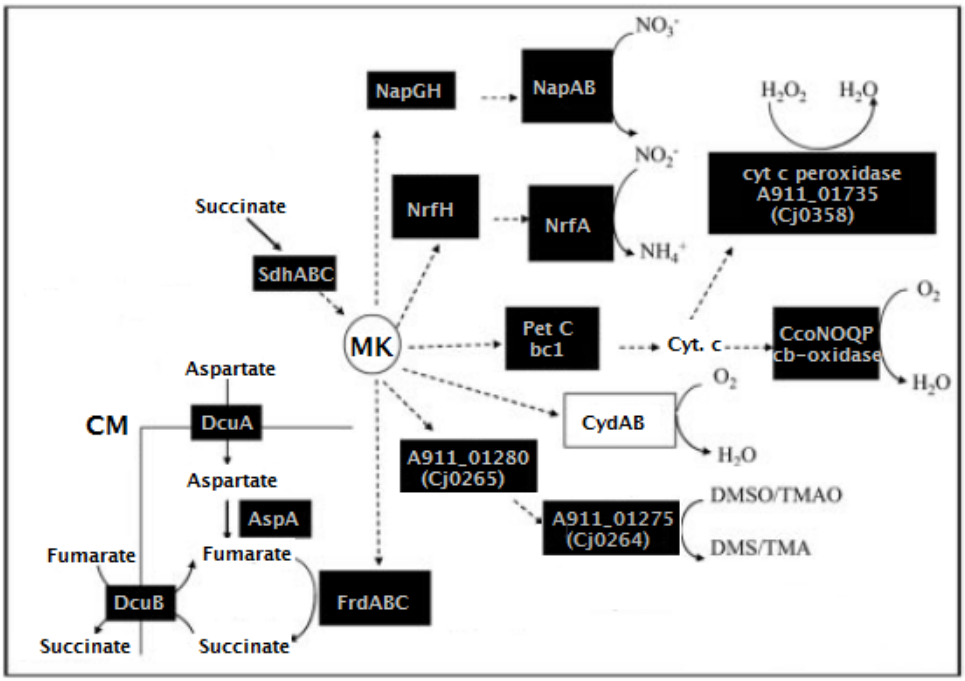


Figure 4-14. Predicted electron transport pathways in *C. jejuni.*

Illustrated are pathways of electron transport to oxygen and alternative electron acceptors, such as fumarate (*frdABC*), nitrate (*napABGH*), nitrite (*nrfAH*) and trimethylamine N-oxide reductase (A911_01280/*cj0265c*). The dotted lines represent electron transfer pathways via the menaquinone pool (MK) and the solid lines indicate possible electron donors for respiration or electron acceptor transformations. Black boxes in reverse fields show products whose gene expression were down-regulated at least -1.5-fold or more in response to phage infection of *C. jejuni* PT14, while the white box shows a product with no change in gene expression. Anaerobic transport of C4-dicarboxylate substrates through the cytoplasmic membrane (CM) occurs via the C4-dicarboxylate transporters DcuA and DcuB. The diagram is based on the findings of Woodall et al. (2005) and also takes into account the electron transport pathways described by Sellars et al. (2002) and Myers and Kelly (2005b). Adapted from Woodall et al. (2005).

a *cj0004c* deletion mutant was unable to utilise sulphite or metabisulphite as electron donors but could respire with formate (Myers & Kelly 2005a). In addition, formate respiration which is cytochrome bc_1 complex-dependent, was inhibited by 50% in the presence of the bc_1 inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide, while sulphite respiration was unaffected. This suggests that electrons donated through sulphite respiration do not rely on the bc_1 complex to enter the respiratory chain and therefore enter at the level of cytochrome c (Myers & Kelly 2005a).

In contrast to the transcriptional repression observed for the respiratory cytochromes, the gene A911_00180/*cj0037c*, encoding a cytochrome c, was up-regulated in response to phage infection of PT14. However, this gene was previously found to be down-regulated in response to the limited oxygen conditions experienced during colonisation of the chicken gut (Woodall et al. 2005). Moreover, the vast majority of the genes associated with energy metabolism (TCA cycle and electron transport) that were activated at the low oxygen tensions of the chicken caecum, were down-regulated in response to phage infection of PT14. This further supports the prospect that the transcriptome of *C. jejuni* PT14 under phage infection is an aerobic environment and also justifies up-regulation of genes involved in oxidative stress defence (4.3.3). It also raises the question as to where bacteriophages replicate in the chicken intestine.

4.3.7 Possible iron- and oxygen-dependent mechanisms during phage infection

Based on the changes observed in host gene expression during phage infection of *C. jejuni* PT14, there is a clear demand for iron and oxygen. A likely explanation for such a requirement is the existence of an iron- and oxygen-dependent metabolic process that is important for phage replication. This process is the reduction of ribonucleotides to form 2'-deoxyribonucleotides for DNA synthesis and repair, and is solely catalysed by the iron-dependent enzyme ribonucleotide reductase (RNR). Following a single reduction, RNR receives electrons from the dithiol group of thioredoxin, which is regenerated through reduction of its disulphide groups by NADPH.

RNRs can be classified into three distinct classes (Ia/Ib, II, III) based on their interaction with oxygen and the mechanism used to generate a stable tyrosyl radical (Nordlund & Reichard 2006). Phages CP30A and CPX both possess class Ia RNRs, which are comprised of two subunits: α and β. The subunits of class Ia enzymes of *E. coli* are known to associate to form a kinetically stable α2:β2 complex (Minnihan et al. 2013) (Figure 4-15B). The active site of the α2 subunit contains a conserved cysteine residue at position 439, which is necessary for catalysis; as well as multiple binding sites for deoxynucleotides and ATP (Figure 4-15A). The β2 subunit contains a di-iron tyrosyl radical cofactor (Y_{122}•) that requires oxygen for its formation and is needed to initiate the nucleotide reduction process in the α2 subunit (Figure 4-15A). The di-iron centre of the tyrosyl radical is flanked by a number of ligands that act as iron-binding sites. These are aspartate (D_{84}), three glutamates (E_{115}, E_{204} and E_{238})

and two histidines (H_{118} and H_{241}) (Figure 4-15A). During catalysis, the tyrosyl radical is continuously shuttled to the α2 subunit to allow oxidation of the cysteine residue and has been shown to involve the residues Y_{356}, Y_{730} and Y_{731} (Seyedsayamdost et al. 2007; Minnihan et al. 2011). The pathway for electron transfer in *E. coli* has been proposed as: $Y_{122}\bullet\leftrightarrow W_{48}\leftrightarrow Y_{356}$ in the β2 subunit to $Y_{731}\leftrightarrow Y_{730}\leftrightarrow C_{439}$ in the α2 subunit (Stubbe et al. 2003; Reece et al. 2006).

Examination of the protein sequences deduced from the genomes of CP30A and CPX confirmed the presence of the RNR α2 and β2 subunits as well as the location of the conserved cysteine (C_{440}) and tyrosine residues (Y_{289}/Y_{290}) in α2 (Figure 4-16). However, the C-terminus of α2 is flanked beween two Hef-like homing endonucleases and surprisingly is separated from the N-terminus and the β2 subunit by more than 60-kbp. The tyrosine residues were found to be present in the C-terminus while the cysteine was located in the N-terminus of the α2 subunit. Nevertheless, once these subunits are able to associate, it is possible that a functional and stable α2:β2 complex could form (Minnihan et al. 2013). The findings obtained from the transcriptome analysis of *C. jejuni* PT14 in response to infection by CP30A and CPX are therefore consistent with what would be expected during the functioning of RNR. In addition, affinity of the di-iron centre of β2 would be sufficient to displace iron from other iron-containing proteins such as Fur. Moreover, loss of holo-Fur has been demonstrated to de-repress transcription of iron-regulated genes (Butcher et al. 2012).

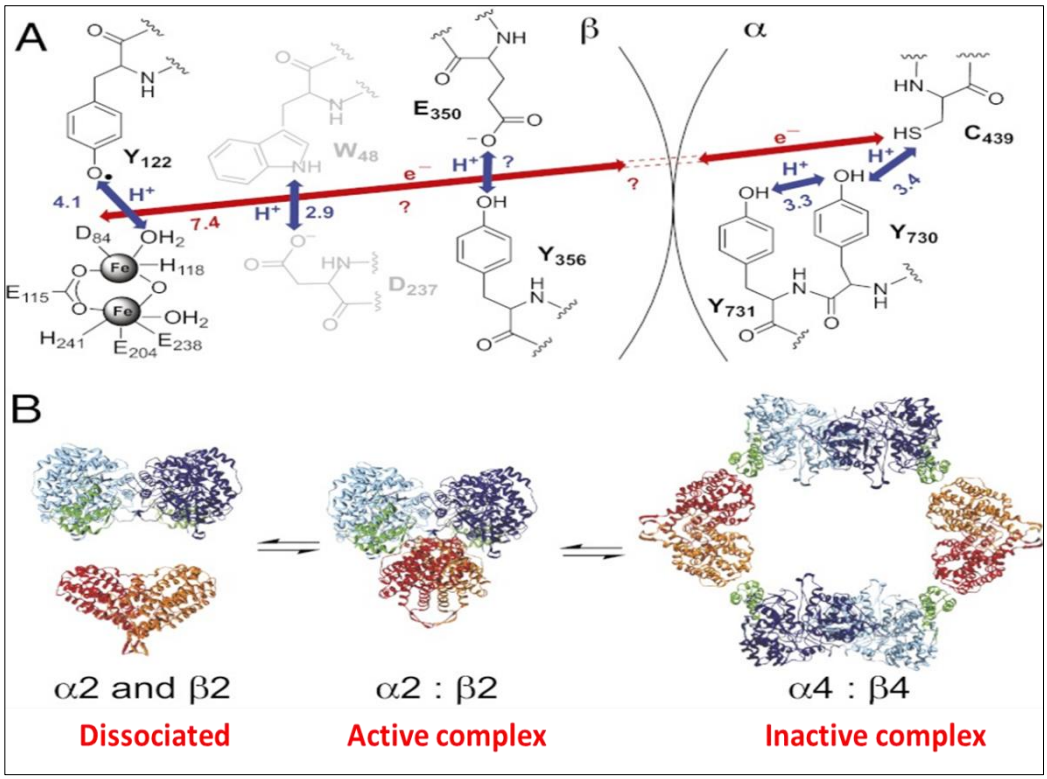


Figure 4-15. Mechanisms of class Ia ribonucleotide reductase (RNR) in *E. coli*.

(A) Class Ia RNRs are composed of an α and β subunit, which associate to form an α2β2 complex. The α2 subunit contains an active site cysteine residue (C_{439}) which is needed for reduction of ribonucleotides to deoxyribonucleotides, while β2 contains a stable di-iron tyrosyl radical cofactor (Y_{122}•). Reversible transfer of the radical electron from Y_{122}• to C_{439} takes place over a long distance and involves the residues Y_{356} in β2 andY_{730} and Y_{731} in α2. (B) A solution equilibrium exists for class Ia RNRs in *E. coli* across three inter-converting subunit states: α2 + β, α2β2 and α4β4. The relative distribution of these states is dependent on protein concentration and the identity and concentration of the nucleotides present. Taken from Minnihan et al. 2013.

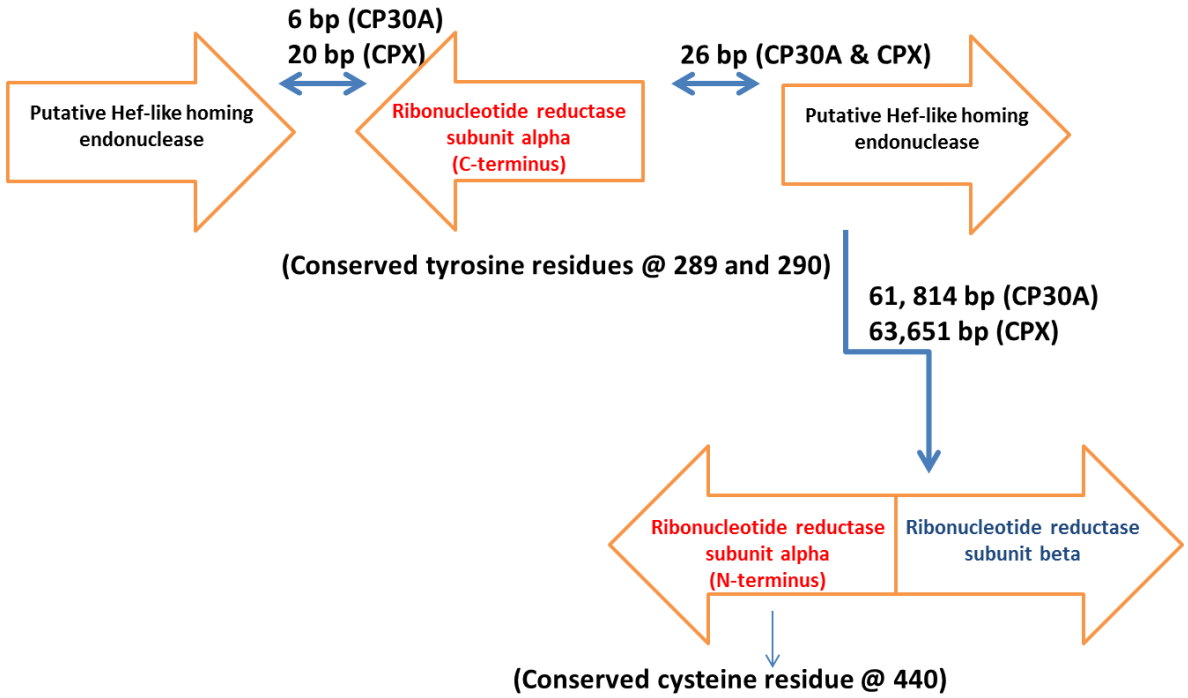


Figure 4-16. Arrangement of the ribonucleotide reductase (RNR) subunits in phages CP30A and CPX.

The C-terminal domain of the α2 subunit contains the two conserved tyrosine residues at positions 289 and 290. It is located between two Hef-like homing endonucleases and is at least 60-kbp from the N-terminal domain and the β2 subunit, which contains the conserved cysteine residue at position 440. The arrangement and positioning of the subunits appear to be different to that of the prototypical *E. coli* RNR.

Chapter 5 : Examination of a type II restriction-modification system in *Campylobacter jejuni* PT14

5.1 INTRODUCTION

Bacterial restriction-modification (R-M) systems generally protect the host cell against invading foreign DNA including those of viral or plasmid origin as well as from DNA fragments being transferred during natural transformation (reviewed by Bickle & Krüger 1993; Wilson & Murray 1991). Classical R-M systems comprise of a modification methyltransferase, which catalyses methylation of a specific DNA sequence in the host genome, and a restriction enzyme which cleaves or restricts foreign DNA lacking that specific methylation. Methyltransferases and restriction endonucleases from the same system are able to recognise the same sequence of nucleotides. Cognate methylation can therefore prevent restriction of host DNA. On the other hand, some restriction enzymes exist that cleave DNA only when the target sequence has been modified and as such, these restriction enzymes do not coexist with a cognate methylation enzyme.

The methyltransferase component of the R-M system catalyses transfer of a methyl group from S-adenosylmethionine (AdoMet) to an adenine or cytosine residue within each strand of the recognition sequence. In general, the same base is methylated on both DNA strands but it is also possible for cognate methylation of only one strand (hemimethylation). Methylation in bacterial genomes has been found to produce either N6-methyladenine (m6A) (Dunn & Smith 1958), 5-methylcytosine (m5C) (Doskocil & Sormova 1965) or N4-methylcytosine (m4C) (Ehrlich et al. 1987; Janulaitis et al. 1983; Janulaitis et al. 1984). Using single molecule real-time (SMRT) sequencing, the complete methylation pattern of a number of bacterial genomes has been determined including *B. cereus*, *H. pylori* and *C. jejuni* (Murray et al. 2012; Krebes et al. 2013). Active methyltransferase genes and their recognition sequences were identified

including those that are part of an R-M system. For instance, *C. jejuni* NCTC 11168 possesses a Type II system assigned RM.CjeNII (*cj1051c*), which recognises the sequence 5'-GAGN_5GT-3' and has been shown to methylate the A residues on both strands (Murray et al. 2012).

Preliminary studies in the early 1950's (Anderson & Felix 1952; Bertani & Weigle 1953; Luria & Human 1952; Ralston & Krueger 1952) revealed that some bacterial strains could inhibit the propagation of bacteriophages that were previously grown on different host strains. This mechanism is known as host-controlled modification and allows the new host to restrict the phage DNA and prevent infection. In order to test the susceptibility of the bacterial strains to bacteriophage infection, the ability of the phages to form plaques was measured. This principle is known as the efficiency of plating (EOP) and it was found that propagation on a non-restricting host resulted in an EOP of 1 while for bacteria capable of restriction it was between 10^{-3} and 10^{-5} fold lower. A decade later, Arber and Dussoix (1962) looked at host-controlled modification of bacteriophage λ when grown on a pair of *E.coli* K-12 strains. Based on the EOP results, they concluded that the phage DNA became strain-specifically marked by the host during viral propagation and that subsequent host strains could only become infected by phage carrying that particular modification. Using ^{32}P-labelled phage, they observed the effects of infecting a restrictive host with unmodified phage DNA. Extensive breakdown of the phage DNA was observed. However, it had previously been observed that unmodified phages could escape restriction and complete the lytic stage of their life cycle, therefore passing on the strain-specific modification that they possess to their progeny (Bertani & Weigle 1953; Luria & Human 1952). The resulting EOP values were close to 1 on both

restrictive and non-restrictive host strains. In addition, after a single passage on a non-restrictive host, the modified progeny phage lost their ability to re-infect the restrictive host therefore confirming that they had not inherited the strain-specific modification of that host. These early experiments highlighted the use of bacterial R-M systems as a defence mechanism against bacteriophages.

There are four major groups of R-M systems (Types I, II, III and IV) and they are classified primarily according to enzyme subunit composition, cofactor requirements, symmetry of the recognition sequence and positioning of the DNA cleavage site (Boyer 1971; Yuan 1981; Roberts et al. 2003) (described in detail in Chapter 3). Each group can be divided into sub-categories, especially the Type II, which is divided into 11 groups (A, B, C, E, F, G, H, M, P, S and T). Of these, the Type IIS subcategory was the earliest to be recognised and was retained by Roberts et al. (2003) to describe enzymes that are capable of cleavage of at least one DNA strand outside of the recognition sequence, thereby suggesting shifted cleavage away from the recognition site (e.g. *Fok*I and *Alw*I). Two further classes, Types IIP and IIA, were also introduced (Roberts et al. 2003). Type IIP describes enzymes, such as *Eco*RI and *Hind*III, which recognise palindromic sequences and cleave DNA at a defined position at or close to the recognition site while Type IIA refers to those that recognise asymmetrical sequences and can cleave DNA either close to or away from the recognition sequence. The Type II enzymes also include those with atypical features such as enzymes that cleave on both sides of the recognition sequence (e.g. *Bcg*I [Type IIB]), which may be stimulated or inhibited by AdoMet (e.g. *Eco*57I and *Bsg*I [Type IIG]), or can interact with two copies of the recognition sequence and cleave one (e.g *Eco*RII [Type IIE]) or both of them (e.g *Bsp*MI [Type IIF]) as well as may possess unusual subunit structures

(e.g. *Bbv*CI [Type IIT]). The remaining enzymes are the Type IIC, which contain cleavage and modification functions within a single polypeptide; the Type IIH, which possess similar genetic structures to Type I enzymes but biochemically behave as Type II; and the Type IIM enzymes, which recognise a specific methylated target sequence and cleave at a fixed site.

There is a wide distribution of R-M systems among prokaryotes, with more than 90% of the analysed genomes containing at least one R-M system (Roberts et al. 2010). It has also been found that within sequenced genomes there is generally an increase in the number of R-M systems with an increase in genome size (Vasu & Nagaraja 2013). For instance, organisms with a genome 2 to 3-Mbp in size possess a median number of 3 R-M systems per genome, those 3 to 4-Mbp in size have 4 R-M systems per genome and organisms with a genome size of 4 to 5-Mbp possess 5 R-M systems per genome (Vasu & Nagaraja 2013). However, the distribution of R-M systems with organism possessing smaller genome sizes (1 to 2-Mbp) is not as clear cut. For example, many *Brucella* species contain only 1 R-M system per genome while multiple R-M systems have been found amongst species of *Helicobacter* and *Campylobacter*. Such organisms can switch between different R-M systems when necessary and are therefore equipped to be naturally more competent than organisms with fewer systems. Miller et al. (2005) characterised the Type I R-M systems of 73 *C. jejuni* strains and assigned them to Type IC as well as two additional families, Types IAB and IF. They found no evidence that these strains contained multiple Type I systems observed in *H. pylori*.

In some instances, methyltransferase genes are known to play a role in physiological processes, such as with the DNA adenine methyltransferase (Dam) of *E. coli*, which produces a m6A residue and functions in DNA mismatch repair, initiation of DNA replication and the regulation of gene expression (Boye & Løbner-Olesen 1990; Marinus & Casadesus 2009). In many Gram-negative bacteria such as *E. coli*, *Salmonella* spp., and *Yersinia* spp., gene regulation by a Dam methyltransferase is known to affect expression of a number of genes including those related to virulence phenotypes such as motility, adherence to and invasion of human epithelial cells and colonisation of the host (Low et al. 2001; Julio et al. 2001; Fälker et al. 2005; Balbontín et al. 2006). Transcriptional regulation can occur when genes are reversibly switched on-and-off (phase variation) through methylation of promoter sequences (Blyn et al. 1990) or via the presence of homopolymeric tract repeats in the promoter region of these genes (van der Ende et al. 1995). Phase variable methyltransferase *mod* genes exist in a number of bacterial Type III R-M systems, including *Haemophilus influenzae* (De Bolle et al. 2000; Srikhanta et al. 2005), *H. pylori* (Vries et al. 2002), *Pasteurella haemolytica* (Ryan & Lo 1999), *Neisseria meningitidis* (Srikhanta et al. 2009) and *Neisseria gonorrhoeae* (Srikhanta et al. 2009). Altered expression of such methyltransferases has been reported to cause coordinated phase variation of multiple genes (Srikhanta et al. 2005). This novel system is referred to as the 'phasevarion' (phase variable regulon) and is known to regulate gene expression in *H. influenzae* (Srikhanta et al. 2005), *N. meningitidis* (Srikhanta et al. 2009) and *N. gonorrhoeae* (Srikhanta et al. 2009). Differential regulation of genes related to pathogenesis and virulence were observed within these studies and functional confirmations revealed the presence of phenotypically distinct populations in regards to antimicrobial resistance, invasion and adhesion to human cells as well as biofilm formation.

In this chapter, *C. jejuni* PT14 (Brathwaite et al. 2013) was naturally transformed using DNA from *C. jejuni* NCTC 11168Δcj0031, which is a non-restrictive *cj0031* deletion mutant (*cj0031* gene homologue: A911_00150). The restriction ability of the wild-type and mutant against bacteriophages previously propagated on PT14 and other strains was then tested. The gene structure of the mutant was examined and functional tests were carried out to determine whether distinct phenotypes exist between the wild-type and mutant. Finally, to investigate the biological role of the A911_00150 gene in the PT14 R-M system, the effect of the A911_00150 deletion mutant on the transcriptional profile of PT14 was also examined.

5.2 RESULTS

5.2.1 A *C. jejuni* PT14 putative Type II R-M system

In *C. jejuni* NCTC 11168, the gene *cj0031* encodes a putative Type IIS R-M system which is phase variable and capable of reversibly switching the expression of the reading frame "on/off" by virtue of the sequence content of a variable polyG tract. The polyG tract repeat length ranges from nine to ten guanine bases in the reading frame of *cj0031* and the adjacent gene *cj0032* (Bayliss et al. 2012). A G9 tract length results in the fusion of *cj0031* and *cj0032* which places this fused gene in frame and therefore in the "on" configuration, whilst G10 separates the reading frames and produces an "off" variant. It has previously been found through SMRT sequencing that the gene is predominately in the "off" configuration and produces no active methyltransferase (Murray et al. 2012). This nearest homologue to this gene in *C. jejuni* PT14 was found to be A911_00150 and although it is not phase variable, a nucleotide BLAST search showed that there is 98% sequence identity to the N-terminal end of *cj0031* in NCTC 11168 and 90% identity to the C-terminal end. Figure 5-1 shows a comparison of these two genes using the Artemis Comparison Tool web-based version (WebACT) (Abbott et al. 2007). Regions of sequence similarity are aligned using red (same orientation) or blue (reverse orientation) blocks, while differences in sequences are illustrated with white gaps. A pairwise comparison of A911_00150 and *cj0031* shows that the N-terminal ends of the genes display sequence homology. However, the C-terminal ends show differences, which coincide with the position of the polymorphic poly-G tract within *cj0031*. The reading frames corresponding to *cj0031* and *cj0032* are in-frame in PT14, revealing a relationship where *cj0032* is replaced in all but the extreme C-terminal coding sequence, which is conserved along with the subsequent gene (A911_00155/*cj0033*).

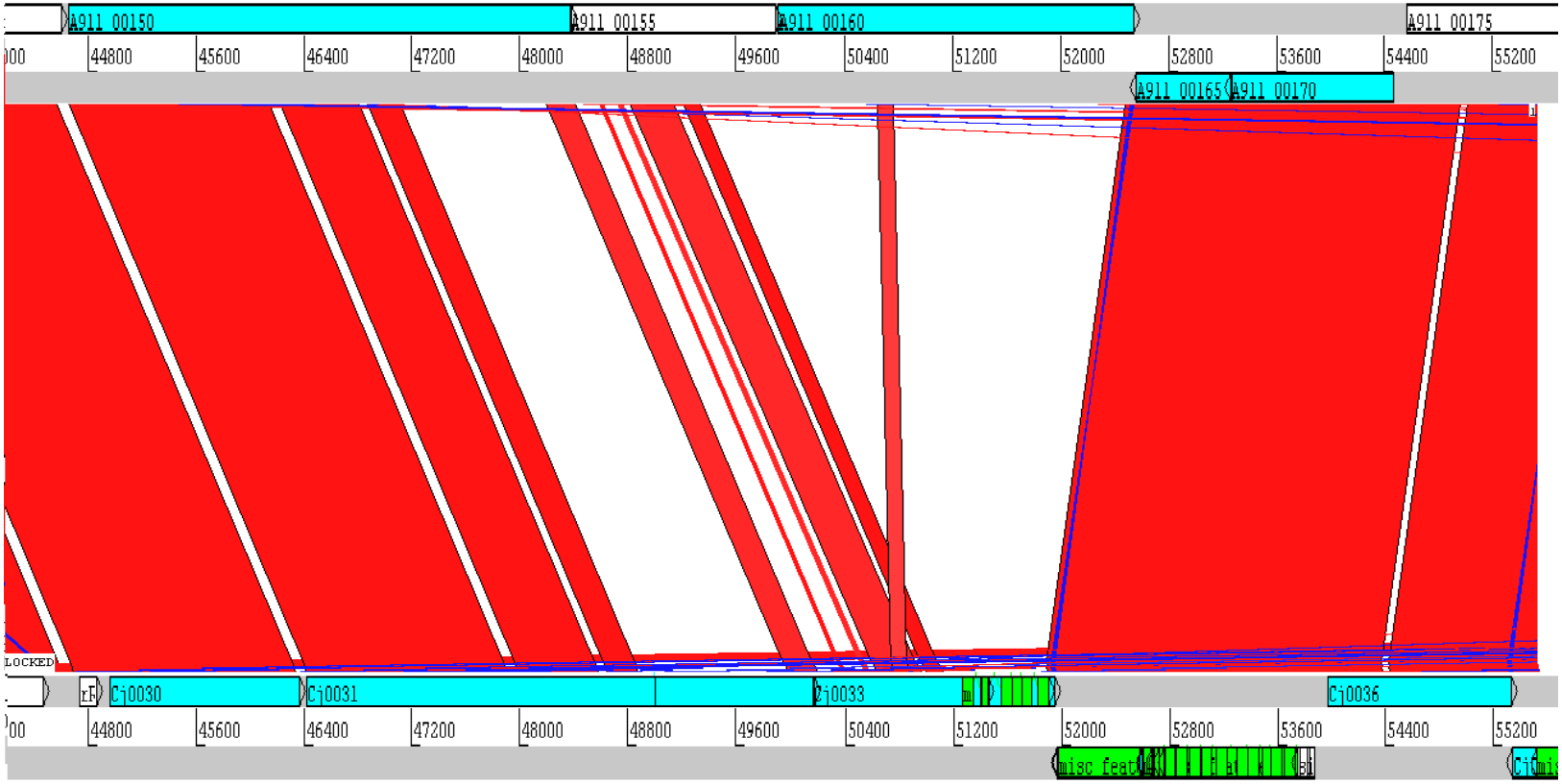


Figure 5-1. An Artemis Comparison Tool (ACT) analysis of the *cj0031* gene in NCTC 11168 and its homologue in PT14.

The genes corresponding to PT14 are represented on the top bar (A911_00150) of the image whilst those for NCTC 11168 are on the bottom (*cj0031*). The red blocks show regions of homology and the differences are represented by white gaps. It can be observed that the N-terminal end of A911_00150 and *cj0031* show homology but their C-terminal ends appear to be different. The sequence homology returns at the extreme end of *cj0032*.

5.2.2 Biofilm-derived *cj0031* mutants in PT14

Biofilms were previously prepared by Dr. P. Siringan according to the method of Siringan et al. (2011) by dispensing 10 µl aliquots of diluted *C. jejuni* PT14 culture into a petri dish containing glass coverslips. Once formed, the biofilms were treated with bacteriophages and significant ($p<0.05$) reductions were observed for all phages used. However, some viable cells recovered from the biofilm had become resistant to subsequent infection with the bacteriophages previously used to infect the same biofilm. EOP values for these isolates were lower than those of the wild-type (data not shown). Phase variation was one possible explanation for the frequent recovery of these mutants, and in particular phase variation within a restriction-modification system. Using the *C. jejuni* NCTC 11168 genome as a guide, since at this time the PT14 genome sequence had not been determined, PCR primers were designed to enable the amplification of candidate phase variable genes. DNA sequencing of *cj0031*/*cj0032* amplicons revealed differences in the genome sequences of NCTC 11168 and PT14 located in the 3'-end of the reading frame. DNA sequences of the genes amplified from the biofilm-derived mutants revealed the presence of point mutations towards the C-terminal coding sequence of *cj0031*/*cj0032*, where the sequences of NCTC 11168 and PT14 differ. Figure 5-2 shows a sequence alignment for a section of the cj0031 gene in NCTC 11168 and its homologue in PT14 for eight biofilm-derived bacteriophage escape mutants (P1-cp30, R1-30, R2-30, R4-30, R1-8, R2-8, R3-8 and R4-8). Some mutants occurred in locations where the sequences of NCTC 11168 and PT14 were identical, including mutants P1-cp30 and R4-30 where deletion of an adenine base has taken place at position 2418, with the adenine base of the start codon in *cj0031* representing position 0. Other mutants have arisen at

```
PT14A911_00150   TTTGATGAAAAAGAAGCCACAAAAGAATTTGCAAATCTTAAAAAAGAATACGACAATATC 2340
11168cj0031      TTTGATGAAAAAGAAGCCATAAAAGAATTTGCAAATCTTAAAAAAGAATACGACAATATC 2340
****************** ****************************************

PT14A911_00150   TTCAACCTAGAAAGCAATCATCCTTTTGAATGGCGTTTTGAATTCCCTGAAATTTTGGAC 2400
11168cj0031      TTCAACCTAGAAAGCAATCATCCTTTTGAATGGCGTTTTGAATTCCCTGAAATTTTGGAC 2400
************************************************************

PT14A911_00150   GATGATGGAAATTTCAAAGGCTTTGATCTCATCATTGGCAATCCGCCTTATATAAAAGAA 2460
11168cj0031      GATGATGGAAATTTCAAAGGCTTTGATCTCATCATCGGCAATCCACCTTATATAAGACAA 2460
********************************** ******** ********** * **
P1-cp30                       TTGCAA-GGC
R1-30                                      TCAT-TCG
R2-30
R3-30
R4-30                         TTGCAA-GGC
R1-8                                                  TCC-CCTT
R2-8                                       TCATC-TT
R3-8
R4-8

PT14A911_00150   AATGATAATAAAGATCTTTTTACCAATACAA--AAAAGTTA-AGAACTTATCAAGGAAAA 2517
11168cj0031      GAAGAGCTTAAAGAACTCAAGCCCCATTTAGCTAAAAATTATAAGGTTTATAAAGGCACG 2520
* **   ****** **     ** **  *   **** *** *    **** **** *
P1-cp30
R1-30
R2-30             AA-GATA
R3-30
R4-30
R1-8
R2-8
R3-8                                                        AGG--AA
R4-8                                       CA-AAAA
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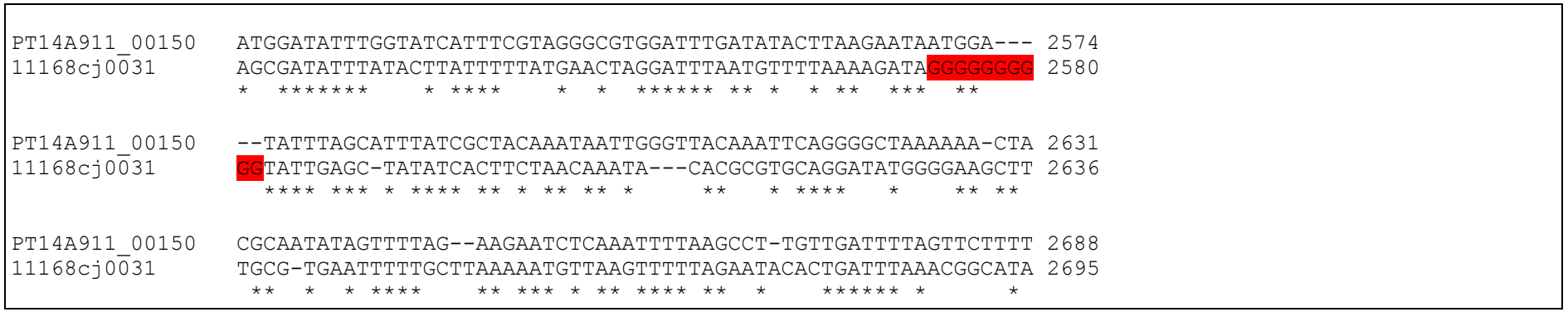


Figure 5-2. DNA sequence alignment of a section of the *cj0031* gene in *C. jejuni* NCTC 11168 with its homologue in PT14.

The alignment revealed eight bacteriophage-resistant biofilm-derived PT14 isolates (P1-cp30, R1-30, R2-30, R4-30, R1-8, R2-8, R3-8 and R4-8) that carry point mutations corresponding to different locations within the *cj0031* gene. Some of the mutations occurred in a region where there is a break in sequence homology between NCTC 11168 and PT14 and close to the homopolymeric G-tract (shown in red) in NCTC 11168. These mutations are highlighted in yellow, while sequence identity is represented by an asterisk.

locations where base substitutions are visible in the PT14 sequence. For instance, deletion of a thymine base at position 2463 produces the R2-30 mutant in a region of the PT14 sequence that is different to NCTC 11168.

5.2.3 Designing the PT14-00150 primers

To further investigate the role of *cj0031* in PT14, a knockout mutant was constructed and designated PT14Δ00150::kan. Dr. C. Bayliss (University of Leicester, UK) kindly provided a knockout mutant in which the *cj0031/32* locus had been disrupted in *C. jejuni* NCTC 11168 by introducing plasmid DNA into the genome from pUC-11168Δcj0031::kan by electroporation. This plasmid contained a 700-bp region from upstream of *cj0031* (flanked by *Pst*I and *Bam*HI sites) which was ligated to a 600-bp fragment downstream of *cj0032* (flanked by *Eco*RI and *Bam*HI sites) and the kanamycin resistance (Kan^r) gene *aphA-3*, derived from pJMK30 (van Vliet et al. 1998), was inserted into the *Bam*HI site. Genomic DNA from the knockout mutant NCTC 11168Δcj0031::kan was used to introduce this disruption into the PT14 genome by natural transformation (section 2.4).

In order to confirm whether the natural transformation of *C. jejuni* PT14 had been successful, primers PT14-00150F and PT14-00150R were designed as previously described in section 2.9.1 to amplify a 2.9 kb fragment within A911_00150. Figure 5-7 shows the RNA-seq mapping data which was used to confirm the appropriate sequence for designing the primers so they could amplify DNA from either the PT14 or NCTC 11168 genome.

5.2.4 PCR confirmation of *C. jejuni* PT14Δ00150 transformation

After naturally transforming *C. jejuni* PT14 with DNA from NCTC 11168Δcj0031::kan (2.4), nine transformants were selectively grown on BA agar (2.1.1) supplemented with kanamycin (50µg/µl) under microaerobic conditions (2.2.2). Confluent growth was harvested from these plates and stored in bacteriological storage medium (2.1.15) at -80°C until ready for used. Genomic DNA was extracted (2.8) from the PT14Δ00150::kan transformants as well as NCTC 11168Δcj0031::kan, PT14 wild-type, NCTC 11168 wild-type and NCTC 11168WT-ON (strains described in Table 2-2). The DNA was then PCR amplified using the PT14-00150FR primers (Table 2-4) and the gel electrophoresis image is presented in Figure 5-3. The primers successfully amplified DNA from all isolates, with the exception of transformants 4 and 8. Expected band sizes were achieved for all of the bands visualised as indicated by Figure 5-3. The band sizes suggest successful disruption of A911_00150 in the mutant by the *aphA-3* kanamycin resistance gene because bands representing the mutant isolates were 1400-bp larger than those of the wild-type strains, which corresponds to the approximate size of that gene.

5.2.5 Bacteriophage lytic spectra

Bacteriophage lytic reactions were scored according to the nomenclature used in phage typing scheme of Frost et al. (1999) (2.5.4). *Campylobacter* lawns were firstly prepared as described in section 2.5.3 and 10 µl aliquots of the bacteriophages of interest, diluted to the routine test dilution of approximate concentration of 6 $\log_{10}$ PFU ml^{-1} (the routine test dilution or RTD), were then spotted onto the lawns. A maximum of 16 spots were pipetted onto each petri dish in duplicate. The lytic ability of each phage was then recorded following overnight incubation. Table 5-1 presents

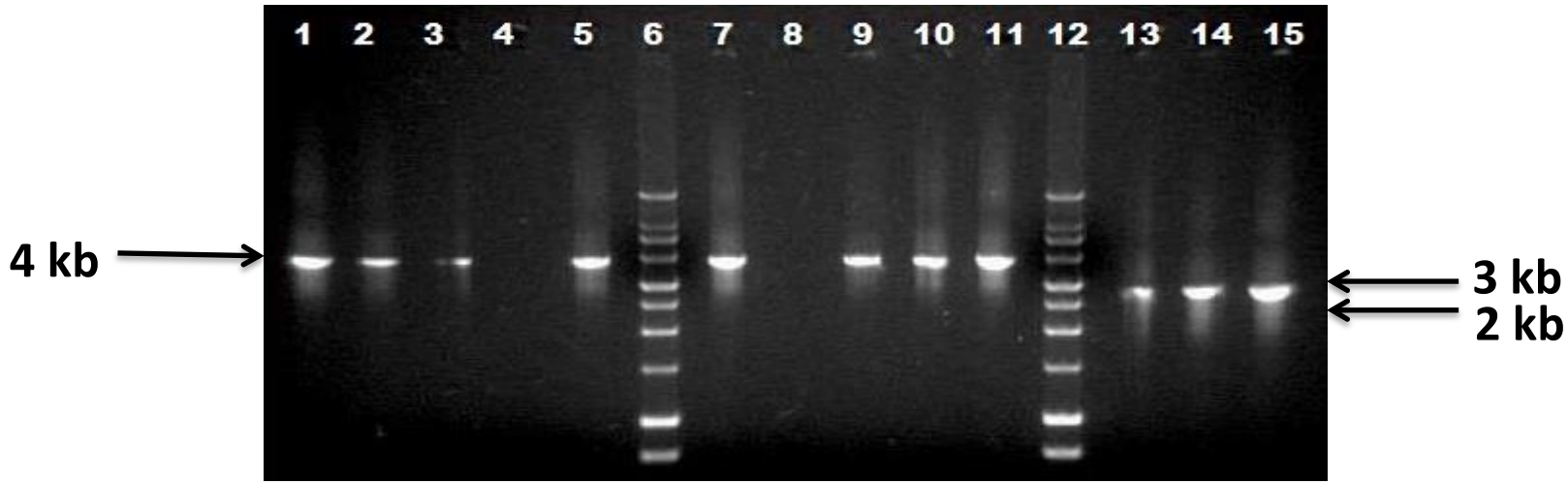


Figure 5-3. 1% Agarose gel of PCR products obtained using PT14-00150 primers.

Lane 1: PT14Δ00150::kan transformant 1, Lane 2: PT14Δ00150::kan transformant 2, Lane 3: PT14Δ00150::kan transformant 3, Lane 4: PT14Δ00150::kan transformant 4, Lane 5: PT14Δ00150::kan transformant 5, Lane 6: Promega 1 kb ladder, Lane 7: PT14Δ00150::kan transformant 6, Lane 8: PT14Δ00150::kan transformant 7, Lane 9: PT14Δ00150::kan transformant 8, Lane 10: PT14Δ00150::kan transformant 9, Lane 11: NCTC 11168Δ00150::kan, Lane 12: Promega 1 kb ladder, Lane 13: PT14WT, Lane 14: NCTC 11168WT, Lane 15: NCTC 11168 WT-ON. The approximate amplicon size for each A911_00150 mutant and wild-type strain was expected to be 4256-bp and 2856-bp respectively.

the scores for the propagation ability of phages from spot tests on PT14 wild-type and PT14Δ00150::kan lawns. Phages that displayed differences in their plaque forming ability are indicated in red. Twenty bacteriophages fit this criterion but efficiency of plating (EOP) values were only determined for those that formed plaques on both the wild-type and mutant strains (referred to as original phages in Table 5-2). EOP values were calculated by dividing the PFU ml^{-1} of each bacteriophage by the PFU ml^{-1} of the phages propagated on the wild-type strain (2.5.5). This therefore gave an EOP value of 1 for all bacteriophages that were propagated on the wild-type. The results indicate that all bacteriophages formed plaques more efficiently on the wild-type strain than on the PT14Δ00150::kan mutant, and in many cases no phage could be recovered from the mutant.

Bacteriophages that could be recovered as plaques from the mutant strain (referred to as recovered phages in Table 5-2) were titred against wild-type and PT14Δ00150::kan mutant strains to re-examine their plaque forming ability. The EOPs of the recovered phages are presented in Table 5-2, and with the exception of bacteriophage CP20, all bacteriophages continued to infect the wild-type strain more efficiently than on the PT14Δ00150::kan mutant. Bacteriophage that replicated in the absence of the PT14Δ00150::kan DNA methyltransferase appeared no less infectious to wild type PT14 and remained impaired in their ability to infect PT14Δ00150::kan.

Table 5-1. Bacteriophage lytic spectra on *C. jejuni* PT14

Phage	Previous Host	PT14WT	PT14ΔA911_00150	Phage	Previous Host	PT14WT	PT14ΔA911_00150
4b	PT14	SCL	SCL	CP8	11168H	SCL	SCL
7	PT14	OL	-	CP8	HPC5	CL	CL
9	PT14	-	-	CP8	PT14	CL	CL
10b	PT14	-	-	CP30	11168H	CL	CL
11	PT14	OL	-	CP30	11168	CL	CL
18b	PT14	CL	CL	CP30	HPC5	CL	CL
19	PT14	<OL	-	CP30	PT14	CL	CL
A1B	PT14	CL	CL	NCTC 12669	PT14	OL	±
A32	PT14	CL	CL	NCTC 12670	PT14	CL	CL
CP8	PT14	CL	CL	NCTC 12671	PT14	-	-
CP20	PT14	OL	++	NCTC 12672	PT14	-	-
CP51	PT14	SCL	SCL	NCTC 12673	PT14	CL	++
CP220	PT14	OL	-	NCTC 12674	PT14	CL	CL
E5	PT14	-	OL	NCTC 12675	PT14	-	-
G1	PT14	OL	+++	NCTC 12676	PT14	±	±
G3	PT14	OL	SCL	NCTC 12677	PT14	-	-
G4	PT14	OL	SCL	NCTC 12678	PT14	CL	SCL
G5	PT14	SCL	SCL	NCTC 12679	PT14	SCL	<SCL
G8	PT14	OL	-	NCTC 12680	PT14	CL	<SCL
mc1a	PT14	OL	-	NCTC 12681	PT14	OL	-
NQ1	PT14	CL	CL	NCTC 12682	PT14	OL	-
X3	PT14	CL	SCL	NCTC 12683	PT14	-	-
				NCTC 12684	PT14	OL	-

Abbreviations used for scoring are as follows: CL or OL = confluent clear or opaque lysis; < CL or < OL = less than confluent lysis; SCL or SOL = semi-confluent; < SCL or < SOL = less than semi-confluent but no discrete plaques; '+++' = > 100 discrete plaques; '++' = 50-99 discrete plaques; '+' = 20-49 discrete plaques; '±' = 1-19 discrete plaques; '-' = no plaques.

Table 5-2. Relative efficiency of plating (EOP) for phages propagated on PT14 wild-type and PT14Δ00150::kan mutant

Original phages[a]		
		EOP
Phage	PT14WT	PT14Δ00150
CP20	1	3.78E-04
E5	1	3.10E-03
G1	1	2.03E-05
G3	1	2.68E-03
G4	1	3.87E-03
NCTC12669	1	2.37E-04
NCTC 12680	1	7.47E-04
X3	1	6.57E-04
Recovered phages[b]		
Phage		EOP
	PT14WT	PT14Δ00150
CP20	1	2.7
E5	1	7.51E-02
G1	1	5.20E-04
G3	1	3.42E-03
G4	1	2.20E-03
NCTC12669	1	2.13E-03
NCTC 12680	1	6.47E-04
X3	1	5.00E-03

a – EOP values determined for the original phages propagated on *C. jejuni* PT14 highlighted in Table 5-1 that showed differences in their plaque forming ability but formed plaques after propagation on both the wild-type and mutant strains.

b – EOP values determined for phages recovered after infection of the mutant strain.

5.2.6 Motility assay

Swarming motility assays were performed as previously described in section 2.3 in order to determine the functional effect of the A911_00150 deletion mutation on the motility phenotype of *C. jejuni* PT14. In Figure 5-4, there is a visible difference in the size of the motility zones of the two strains, illustrating that PT14Δ00150::kan exhibited reduced motility compared to the wild-type strain. Zones of motility for the wild-type and all PT14Δ00150::kan transformants were measured in triplicate and the average diameter of growth in cm for each strain is shown in Table 5-3. The transformants (T1-T9) showed a reduced spread of growth compared to the wild-type PT14 and a Student's t-Test ($p<0.05$) confirmed that all PT14Δ00150::kan mutants were significantly less motile than the wild-type.

5.2.7 Complementation of the PT14Δ00150::kan mutant

The PT14Δ00150::kan 11168cj0031 complement was constructed by PhD. student L. Lis (University of Nottingham) as described in section 2.14. This complementation strain allowed the *cj0031* gene to be expressed from the *C. jejuni fdxA* promoter via the *cj0046* pseudogene-based complementation vector, pCfdxA:PerR no7 (Bruce Pearson, IFR, Norwich). Briefly, on PCR-amplification and pCR2.1 TOPO vector cloning of the *cj0031* gene in NCTC 11168 and its upstream promoter region, the PCR fragment was integrated into the complementation vector. The *perR* gene was replaced with the *cj0031* promoter and gene fragment. The vector was amplified in *E. coli* and genomic DNA was afterwards used to naturally transform and complement the deletion mutant to create PT14Δ00150::kan, 00230::00150cam.

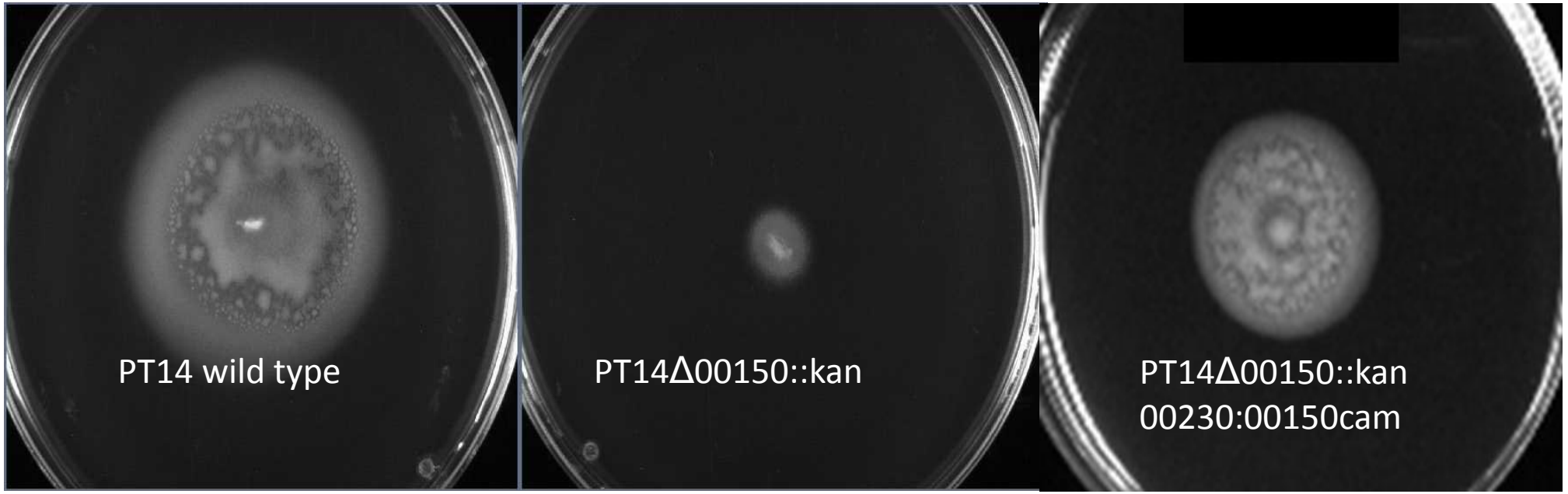


Figure 5-4. Motility assays for *C. jejuni* PT14 wild-type, mutant PT14Δ00150::kan and complement.

Motility was determined by measuring the diameter of the spread of growth in cm after 24 hours. There was a significant reduction in motility ($p<0.05$) of the PT14Δ00150::kan mutant compared to the wild-type. However, the wild-type phenotype was restored with the PT14Δ00150::kan 00230::00150cam complement.

Table 5-3. Average diameter of motility zones for PT14 wild-type and PT14Δ00150::kan mutant strains

Strain	Average growth (cm)[a]
PT14WT	4.33 ± 0.12
PT14Δ00150 T1	2.13 ± 0.05
PT14Δ00150 T2	2.07 ± 0.05
PT14Δ00150 T3	3.10 ± 0.42
PT14Δ00150 T4	2.87 ± 0.19
PT14Δ00150 T5	1.53 ± 0.39
PT14Δ00150 T6	1.30 ± 0.14
PT14Δ00150 T7	2.60 ± 0.29
PT14Δ00150 T8	1.60 ± 0.14
PT14Δ00150 T9	0.93 ± 0.17

Note: [a]Averages of triplicate measurements are given ± standard deviation.

The complemented mutant strain which was found to be motile, was then used along with strains PT14 wild-type and PT14Δ00150::kan, to determine the effect of the A911_00150::kan deletion mutation of adhesion and invasion phenotypes.

5.2.8 Adhesion and Invasion

Adhesion and invasion assays were performed with the assistance of Prof. I. Connerton (University of Nottingham) as previously described by Al-Sayeqh et al. (2010) and as detailed in section 2.15.2. Briefly, HCA-7 cells (Kirkland 1985) were grown as monolayer cultures in 24-well plates in D-MEM supplemented with FCS at a final concentration of 10% (v/v) (Invitrogen). Duplicate cell monolayers were individually covered with *C. jejuni* PT14 wild-type, PT14Δ00150::kan and PT14Δ00150::kan, 00230::00150cam complement cells at an approximate multiplicity of infection of 100. Both sets of monolayer plates were washed three times with PBS (2.1.12) and one of them was treated with gentamicin. The cell monolayers were lysed to release internalized bacteria and cells were enumerated on CCDA plates (2.1.2). The adherent bacterial count was derived by subtracting the number of internalized bacteria from the total number and expressing this as a percentage of the inoculum. The invasion efficiency was expressed as the percentage of the inoculum that was gentamicin protected.

For each experiment, means and standard deviations were derived from triplicate readings. Figure 5-5 illustrates that the number of bacteria that adhered to the HCA-7 cell monolayer was significantly reduced with

PT14Δ00150::kan as compared to the wild-type by almost 3-fold (Student's t-Test, $p<0.05$). Complementation of the mutant restored the wild-type adhesion phenotype. Similarly, cell invasion was significantly reduced with the mutant compared to the wild-type by 7.5-fold (Student's t-Test, $p < 0.05$) and the ability to invade the monolayer was also restored in the complemented mutant (Figure 5-6).

5.2.9 *In vivo* colonisation study

During the study, a batch of 21 chickens was divided into 3 groups of 7 and administered doses of either 7 $\log_{10}$ CFU ml^{-1}, 5 $\log_{10}$ CFU ml^{-1} or 3 $\log_{10}$ CFU ml^{-1} of PT14Δ00150::kan culture according to section 2.13.3 (Table 2-5). As a control a second batch of 21 chickens was infected with equivalent doses of the wild-type strain. For birds administered PT14Δ00150::kan by oral gavage, no *Campylobacter* colonies were recovered on CCDA enumeration media (2.1.2) from their caeca at any dose. The limit of detection was ≤2 $\log_{10}$ CFU g^{-1} caecal content. On the other hand, all chickens administered the PT14 wild-type strain were successfully colonised at a rate of >6 $\log_{10}$ CFU g^{-1} caecal content with any of the three doses administered. Table 5-4 presents the colonisation results.

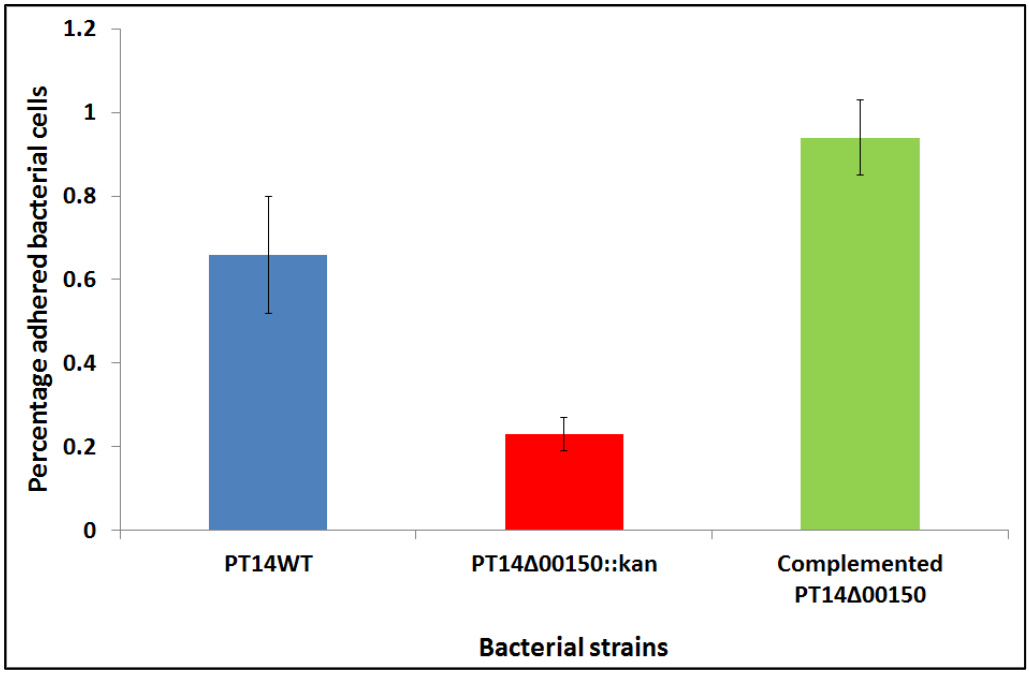


Figure 5-5. Adherence of *C. jejuni* PT14 strains to HCA-7 human colonic cells in response to the A911_00150 knockout mutation.

The ability of the PT14Δ00150::kan mutant to adhere to the HCA-7 cells was significantly ($p<0.05$) reduced compared to the wild-type and complementation of the mutant restored the wild-type phenotype. Results are derived from the mean of triplicate experiments and error bars represent the standard deviation.

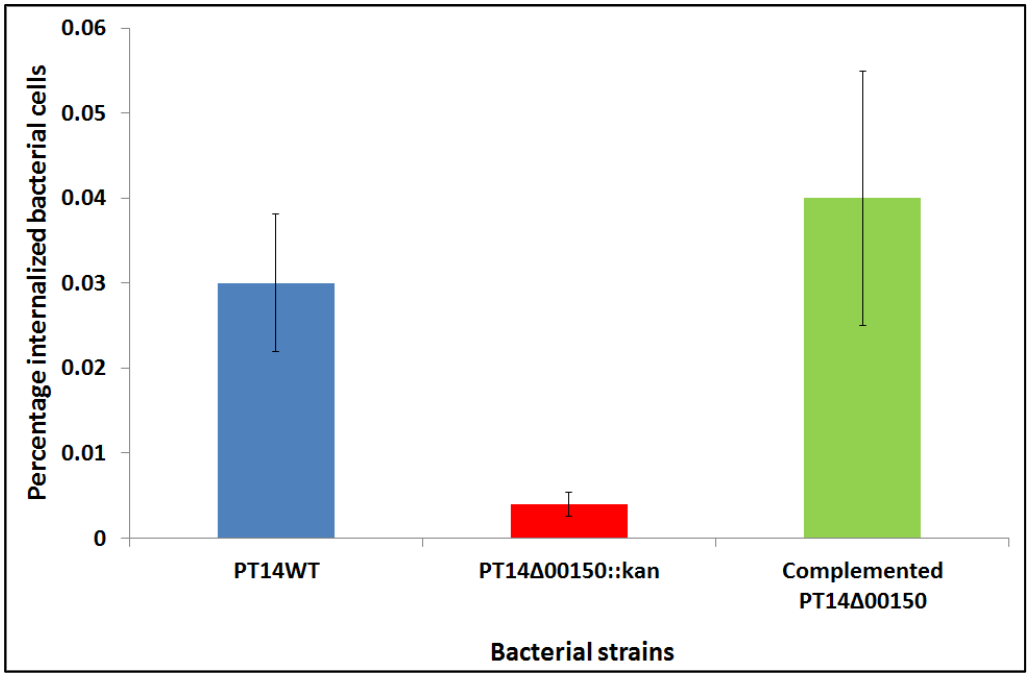


Figure 5-6. Invasion of HCA-7 human colonic cells by *C. jejuni* PT14 strains in response to the A911_00150 knockout mutation.

Invasion of the HCA-7 cells by the PT14Δ00150::kan mutant was significantly reduced ($p<0.05$) compared to the wild-type and complementation restored the wild-type phenotype. Results are derived from the mean triplicate experiments and error bars represent the standard deviation.

Table 5-4. Chicken colonisation levels after 24 hours post-administration of PT14 wild-type and PT14Δ00150::kan mutant strains

Strain	Designated group	Average quantity of recovered bacteria (CFU g^{-1})[a]
PT14Δ00150::kan	group 7 (3 $\log_{10}$ CFU ml^{-1})	≤2
	group 8 (5 $\log_{10}$ CFU ml^{-1})	≤2
	group 9 (7 $\log_{10}$ CFU ml^{-1})	≤2
PT14 wild-type	group 10 (3 $\log_{10}$ CFU ml^{-1})	6.27 $\log_{10}$ ± 0.39
	group 11 (5 $\log_{10}$ CFU ml^{-1})	6.83 $\log_{10}$ ± 0.40
	group 12 (7 $\log_{10}$ CFU ml^{-1})	6.57 $\log_{10}$ ± 0.96

Note: [a] Mean counts for 7 birds ± SD.

5.2.10 Transcriptome analysis of *C. jejuni* PT14 in response to A911_00150 mutation

For RNA-seq analysis of *C. jejuni* PT14 wild-type and the PT14Δ00150::kan mutant, triplicate cultures were grown and the RNAs harvested according to the method in section 2.12.1. Briefly, three biological replicates were grown in 150 ml of MH broth (2.1.6) with shaking at 100 rpm under microaerobic conditions at 42°C (2.2.2). This was done until the cultures reached mid-log phase of growth, after which RNA was isolated using the Trizol Max Bacterial Isolation kit (Invitrogen). The RNA was purified using an RNeasy Mini kit (Qiagen) and only samples with a RIN number of at least 7.0 were used for sequencing. Ribosomal RNA was removed using the Ribo-Zero rRNA removal kit for Gram-negative bacteria (Epicentre Biotechnologies) and directional cDNA libraries were then prepared using the ScriptSeq v2 RNA-seq library preparation kit (Epicentre Biotechnologies) according to section 12.2.2. The libraries were individually indexed and their quality was verified using a bioanalyser 2100 (Agilent). They were quantified by qPCR and pooled prior to loading onto an Illumina HiSeq 2000 sequencer. Sequencing was carried out using the Illumina standard protocol.

The RNA-sequencing generated an average of approximately 28 million reads per sample, each of which was 100 nucleotides in length and from paired-ends. CLC Genomics Workbench version 6.0 software was used to remove the adapter indexes and filter the raw reads (2.12.4.1). Table 5-5 provides a summary of the number of reads attained after filtering and also illustrates the number of reads that aligned uniquely to the reference genome for each

replicate sample. A principle components analysis was performed to validate the replicates (Figure 5-7) and the filtered reads were then mapped against the *C jejuni* PT14 reference genome (Brathwaite et al. 2013) according to section 2.12.4.2. Figure 5-8 shows RNA-seq data at the A911_00150 locus for the PT14Δ00150::kan mutant mapped against the PT14 genome. This data was also used in the design of the PT14-00150FR primers (5.2.3). Panels (a) and (b) show reads with faded ends, which represent the beginning and end of where the kanamycin resistance cassette was inserted within the PT14Δ00150::kan genome respectively. This mapping also provided unequivocal confirmation that A911_00150 had been successfully disrupted by the aphA-3 gene.

Table 5-5. Analysis of the RNA-seq data

Condition	Original count	Number of reads after trimming	Number of uniquely mapped reads
C. jejuni PT14 wild-type replicate 1	27,046,844	12,343,573	10,139,255
C. jejuni PT14 wild-type replicate 2	28,227,850	13,127,628	10,552,880
C. jejuni PT14 wild-type replicate 3	23,251,450	6,837,881	3,177,087
PT14Δ00150::kan replicate 1	34,455,968	14,792,032	10,336,677
PT14Δ00150::kan replicate 2	27,031,598	12,630,072	9,354,104
PT14Δ00150::kan replicate 3	29,370,616	12,769,998	8,674,015

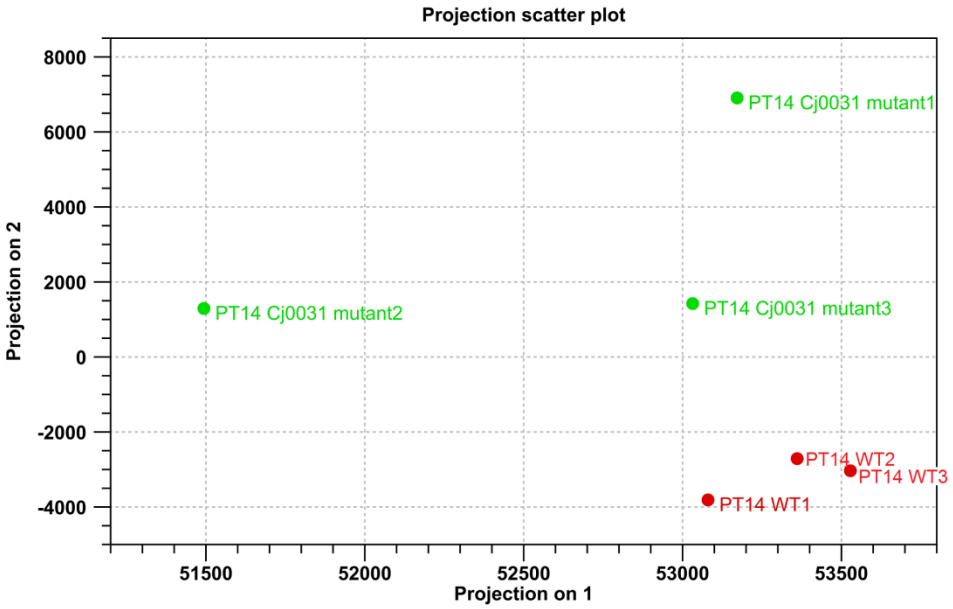
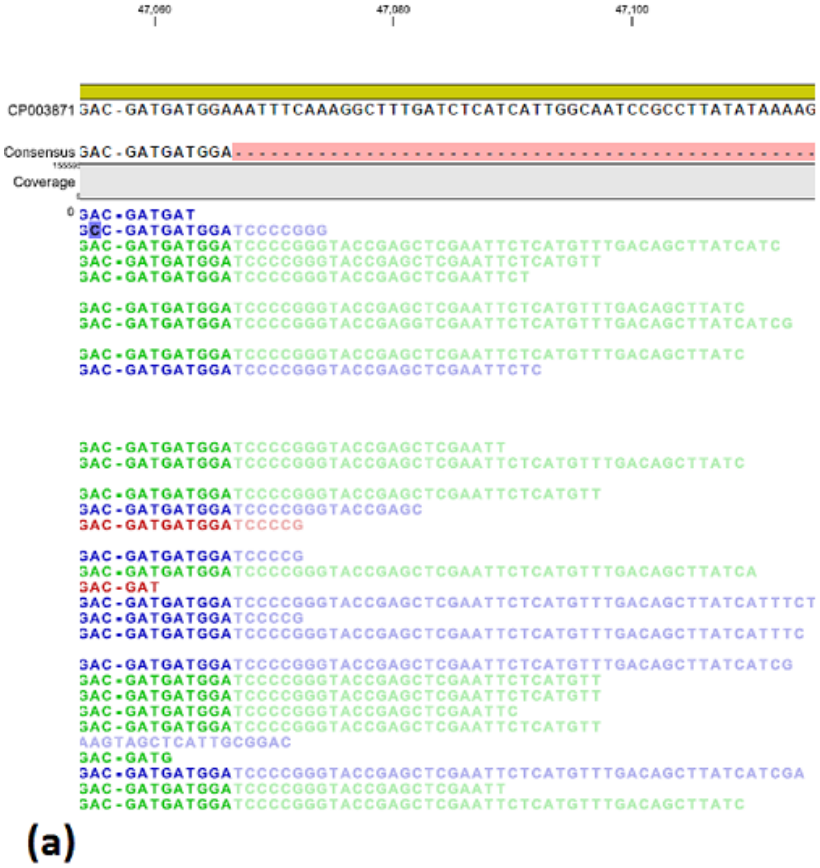


Figure 5-7. Principle component analysis of RNA-seq reads.

Each *C. jejuni* PT14 wild-type replicate and PT14Δ00150::kan mutant replicate are plotted on the PCA projection coordinates. The PCA successfully validates the reads as two distinct groups (wild-type vs mutant) regardless of the variation observed for the mutant reads compared to those of the wild-type on these two projections.


47,060
47,080
47,100
CP003871
Consensus
Coverage
(a)

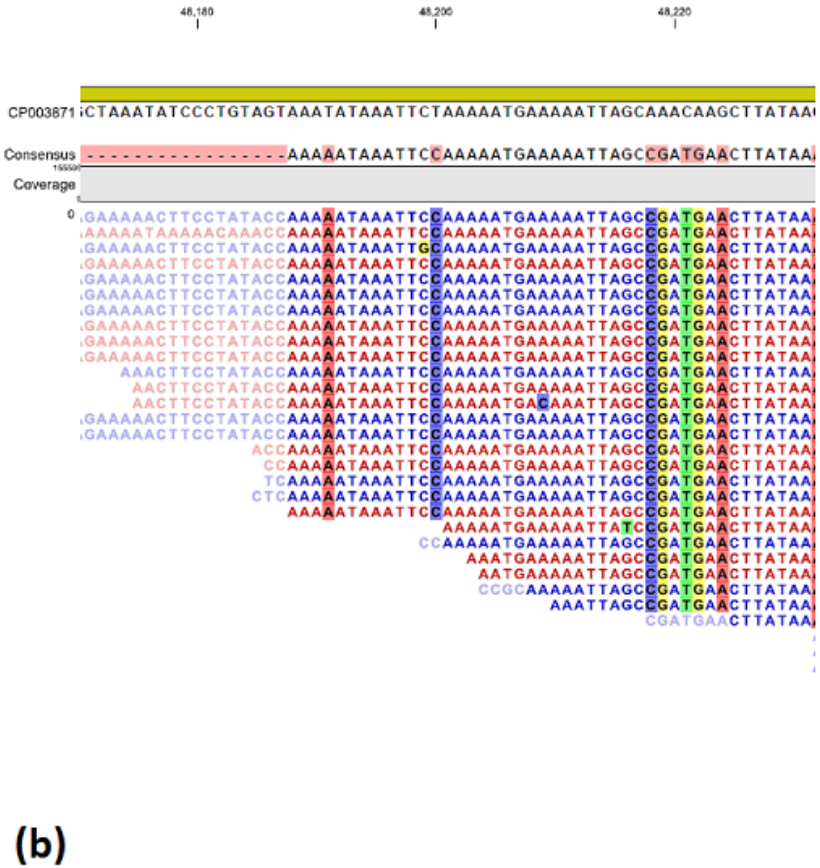

48,180
48,200
48,220
CP003871
Consensus
Coverage
(b)

Figure 5-8. *C. jejuni* PT14Δ00150::kan reads mapped against the PT14 genome.

The PT14 reference sequence is seen directly below the yellow bar to the top of the image and all Illumina reads mapped to this sequence are coloured in blue (paired reads), green (broken pairs, forward orientation) and red (broken pairs, reverse orientation). For all reads shown, their ends are faded because they do not match the reference sequence and were therefore not aligned. Mismatches between the reference sequence and the mapping data are highlighted in red (A), blue (C), green (T) and yellow (G). In this case, the faded read ends represent the sequence containing the A911_00150/*cj0031* knockout mutation and the nucleotide changes seen in (b) originated from the *C. jejuni* NCTC 11168 genome. Primers were designed in two regions where base changes did not occur and the sequence was the same for both *C. jejuni* PT14 and NCTC 11168, which would allow DNA amplification from either strain. The figure illustrates where the insertion of the kanamycin resistance gene *aphA-3* begins and ends, represented by panels (a) and (b) respectively, to create the disruption of A911_00150.

5.2.11 PT14-A911_00150 regulated gene expression

The RNA-seq data was processed using the CLC Genomics Workbench package (2.12.4.3). Reads that mapped uniquely to the *C. jejuni* PT14 genome were normalized by calculating the reads per kilobase of transcript per million mapped reads (RPKM) (Mortazavi et al. 2008) as a means of determining the expression level of the genes. The Baggerley's test on expression proportions (Baggerly et al. 2003) was used to determine genes with differential expression between the wild-type and mutant. To account for multiple testing, p-values were adjusted using the false discovery rate controlling method of Benjamini and Hochberg (1995), A total of 65 genes displayed significant changes in their expression levels ($\geq$ 1.5-fold, $p_{adj}<0.05$) in response to the A911_00150 knockout mutation in *C. jejuni* PT14. Figure 5-9 shows the $\log_2$ (RPKM+1) values obtained for PT14WT plotted against the equivalent values for PT14Δ00150::kan. Up- and down-regulated genes are colour-coded in yellow and red respectively.

Twenty-nine genes were activated in response to the A911_00150 deletion mutation in PT14. A substantial number of these were found to be associated with bacterial stress responses, including the transcriptional regulator *hrcA* as well as chaperones and chaperonins *grpE*, *dnaK*, *clpB* and *cpbA*. Although the heat shock proteins GroES and GroEL were found to be significantly regulated, they fell below the fold change cut-off of 1.5, which was selected as significant. A large number of genes encoding ribosomal proteins (*rplCDFNORWX*, *rpsE*) were also co-regulated. In addition, genes involved in central intermediary metabolism (*gltB*, *gltD*), amino acid biosynthesis (*hisF*)

and protein and peptide secretion (*secY*) were found to be up-regulated by *C. jejuni* PT14 as well. The most activated gene in response to the mutation was the heat shock protein GrpE, which was just over two-fold higher in the mutant than in the wild-type. The complete list of up-regulated genes in response to the A911_00150 knockout mutation can be found in Appendix 7.

Thirty-six genes were down-regulated in PT14 as a result of the mutation. Many were found to be associated with flagellar biogenesis, including a number of putative hook and basal body structural components. The gene encoding one of the minor flagellins, *flaB*, was also repressed in response to inactivation of A911_00150 in PT14. Genes involved in tryptophan biosynthesis (*trpD*, *trpE*), degradation of amino acids (*putA*) and fatty acid biosynthesis (*acs*) were down-regulated as well, along with a large number of hypothetical proteins. Three genes (A911_00155, A911_00160, A911_00170) which are located directly downstream of the disrupted A911_00150 gene in the PT14 mutant were found to be down-regulated. It is possible that the disruption of A911_00150 has impacted on the the expression of the following genes. Genes A911_00155 and A911_00160 encode hypothetical membrane associated proteins that contain penta peptide repeats. Gene A911_00170 encodes amembers of the major facilitator superfamily thought to be involved in secondary transport functions. The most highly repressed gene was a hypothetical protein (A911_00195) with a fold change of 20.6 followed by the flagellar hook protein FlgE which was 18-fold lower in the mutant than wild-type. The complete list of down-regulated genes in response to the A911_00150 knockout mutation can be found in Appendix 8.

Differentially expressed genes were functionally assigned (Figure 5-10) according to the Sanger Centre *C. jejuni* genome database. Up-regulated genes were placed into 11 functional categories while the down-regulated genes represented 8 groups. The highest number of activated genes related to synthesis and modification of macromolecules whilst the greatest numbers of down-regulated genes were involved in cell envelope functions.

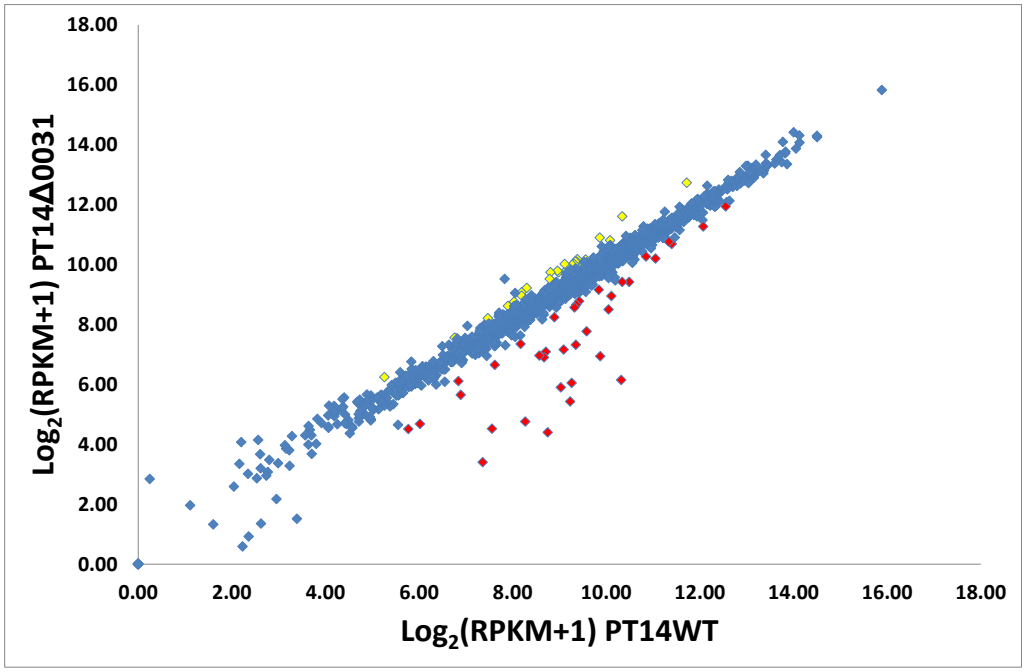


Figure 5-9. Differential gene expression in *C. jejuni* PT14 in response to the A911_00150 mutation.

The average $\log_2$(RPKM+1) value for each gene in the PT14 genome was calculated and plotted in the graph above for the PT14WT and PT14Δ00150::kan isolates using RPKM values for 3 biological replicates. Significantly differentially expressed genes (≥1.5-fold, $*p_{adj}<0.05$) are highlighted in yellow (up-regulated) and red (down-regulated).

$*p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

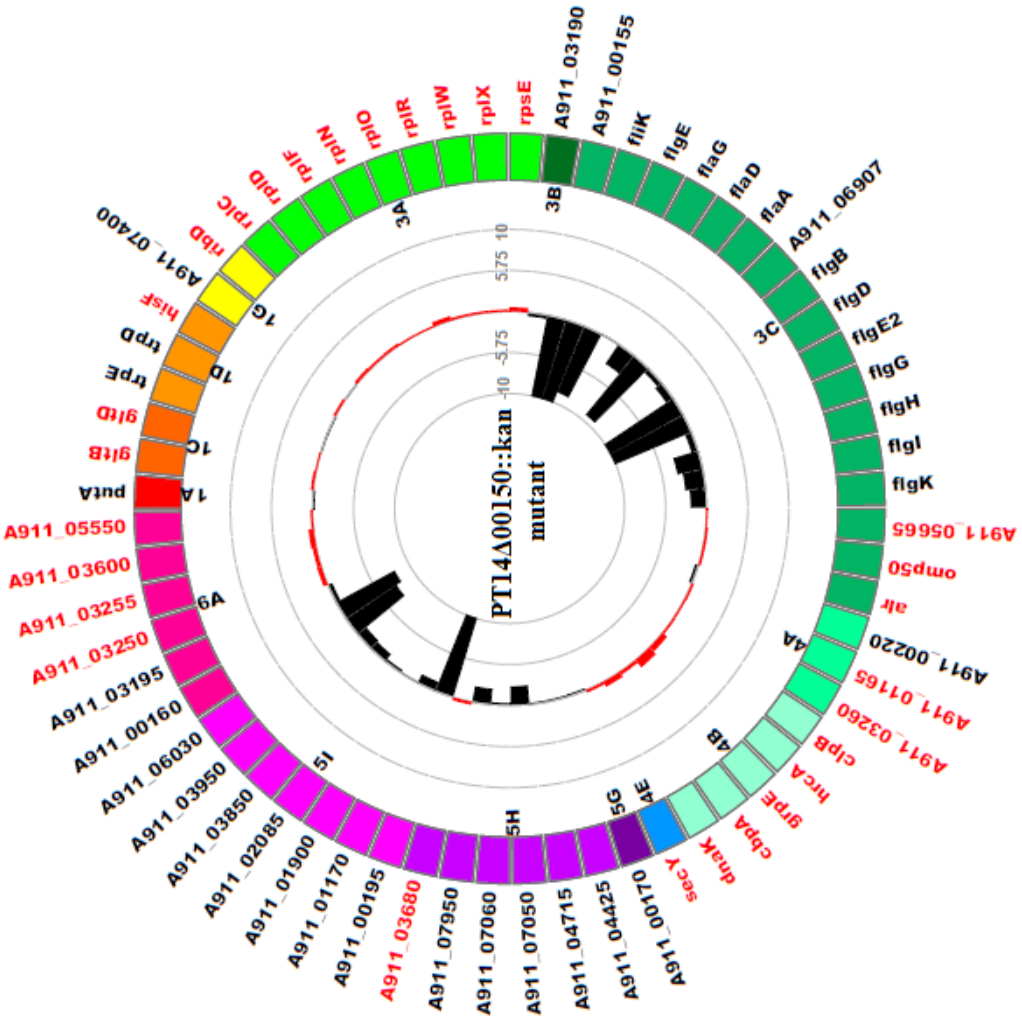

PT14Δ00150::kan
mutant
10
5.75
-5.75
-10
A911_03190
A911_00155
fliK
flgE
flaG
flaD
flaA
A911_06907
flgB
flgD
flgE2
flgG
flgH
flgI
flgK
A911_05665
ompS0
alr
A911_00220
A911_01165
A911_03260
clpB
hrcA
grpE
cbpA
dnaK
secY
A911_00170
A911_04425
A911_04715
A911_07050
A911_07060
A911_07950
A911_03680
A911_00195
A911_01170
A911_01900
A911_02085
A911_03850
A911_03950
A911_06030
A911_00160
A911_03195
A911_03250
A911_03255
A911_03600
A911_05550
putA
glnB
glnD
trpE
trpD
hlpF
A911_07400
rnbD
rplC
rplD
rplF
rplN
rplO
rplR
rplW
rplX
rpsE
3B
3C
4A
4B
4E
5G
5H
5I
6A
7C
7D
7G
3A

Figure 5-10. *C. jejuni* PT14 genes differentially expressed in the PT14Δ00150::kan mutant compared to wild type, ordered by functional category.

Individual genes that were found to be greater than 1.5 fold differentially expressed, are represented using a Circos diagram (Krzywinski et al. 2009) with each gene represented and colour-coded according to functional class (Parkhill et al. 2000; Gundogdu et al. 2007) as follows: 1A, Degradation; 1C, Central intermediary metabolism; 1D, Amino acid biosynthesis; 1G, Biosynthesis of cofactors, prosthetic groups and carriers; 3A, Synthesis and modification of macromolecules; 3B, Degradation of macromolecules; 3C, Cell envelope; 4A, Transport/binding proteins; 4B, Chaperones; chaperonins, heat shock; 4E, Protein and peptide secretion; 5G, Antibiotic resistance; 5H, Conserved hypothetical proteins; 5I, Unknown; 6A, Miscellaneous;. Differentially expressed genes are labelled by locus-tag on the outer circle in red type for increased expression in the PT14Δ00150::kan mutant compared to wild type PT14 or black type for decreased expression. Histograms in the central rings represent the fold change with increased expression in the PT14Δ00150::kan mutant compared to wild type represented by red bars and reduced expression represented by black bars. Scale indicated by grey circles with values greater than 10 fold truncated so bars touching the inner ring represent a fold change of greater than or equal to -10.

5.3 DISCUSSION

In *C. jejuni* NCTC 11168, the phase variable gene *cj0031* encodes a Type IIS R-M system. The homologue of this gene in *C. jejuni* PT14, A911_00150, is not phase variable but displays an overall 76% amino acid identity to Cj0031 (Figure 5-1). After successful transformation to produce the PT14Δ00150::kan mutant, bacteriophage propagation was examined with this strain using a selection of group II and III *Campylobacter* phages. Interestingly, the EOP results for propagation on the wild-type was not what would be expected for a restricting host and contrary to findings reported for phage-host combinations in other species. Functional tests were carried out to assess motility, adherence to and invasion of human cells and colonisation ability *in vivo*. Phenotypic differences between the wild-type and mutant were observed for all tests performed. In addition, examination of gene regulation in PT14 revealed that A911_00150 plays a regulatory role within this strain and influences expression of key genes. These data support the phenotypic findings.

5.3.1 A group of bacteriophages appears to require A911_00150 for replication

The plaque forming ability of a large number of the bacteriophages tested was reduced in the absence of A911_00150 (Table 5-1). It has previously been found that the efficiency of plating calculated for phages grown on a non-restricting host was close to 1 whilst growth on a host containing a functional restriction system that recognises and restricts unmodified bacteriophage DNA was lower (Arber & Dussoix 1962). However, the findings of the current study provide a mechanism that is different to what has previously been established

as the EOP values for bacteriophages propagated on the wild-type, which should act as a restricting host if its R-M system is functional, were calculated to be 1 while values for those grown on the mutant, a non-restricting host, were lower than 1 (Table 5-2). This suggests that a class of phages replicate more efficiently in the presence of the type II R-M system represented by A911_00150 in PT14.

5.3.2 Unmodified bacteriophages were not restricted by PT14

Under normal circumstances a particular bacterial host strain will be infected with phages that carry the strain-specific DNA modification produced by that host. As the majority of the phages tested were originally propagated on *C. jejuni* PT14, they would have already been modified for the associated recognition sequence and were therefore equipped to escape restriction. It was however interesting to see that bacteriophages CP8 and CP30 that were previously propagated on alternative host strains, namely NCTC 11168, NCTC 11168H and HPC5, were able to escape restriction and successfully infect and lyse PT14 cells, producing either confluent or semi-confluent growth on both the wild-type and mutant strains (Table 5-1). This may suggest that the PT14 R-M system could be inactive against these two phages as the wild-type strain was unable to restrict them. This may also imply that these phages do not possess restriction recognition sites for A911_00150. This finding also illustrates that the Type I R-M system present in PT14 (3.2.8) may be also inactive against these unmodified phages.

Some bacteriophages can escape bacterial restriction by reducing or eliminating the number of recognition sites present in their genome or by changing the orientation of the recognition sequence (Labrie et al. 2010; Samson et al. 2013). However, Hill et al. (1991) were the first to report of a system where phage could escape restriction via genetic exchange between phage and bacterial host. They showed that phage Φ50 was able to methylate its own genome and escape restriction by lactococcal strains because it acquired the amino domain of the type II methyltransferase, *Lla*I by recombination with the plasmid pTR2030. Similarly, a putative DNA methylase and a hypothetical protein noted to be similar to a putative methyltransferase in phage NCTC12673 have been identified in phage CP8, therefore making such an occurrence possible with this phage. However, methylase genes have not identified in phage CP30A.

5.3.3 Inactivation of A911_00150 in *C. jejuni* PT14 leads to loss of motility, adhesion, invasion and colonisation ability

Previous colonisation studies have shown that flagella play a major role in the ability of *C. jejuni* to colonise the gut of chickens (Nachamkin et al. 1993; Wassenaar et al. 1993), mice (Morooka et al. 1985; Newell & Mcbride 1985), rabbits (Caldwell et al. 1985; Pavlovskis et al. 1991) and hamsters (Aguero-Rosenfeld et al. 1990). A study with human volunteers also found flagellar motility to be important in human disease (Black et al. 1988). Similarly, *in vitro* studies have suggested that flagella play a role in adherence (Newell & Mcbride 1985) and invasion (Wassenaar et al. 1991; Grant et al. 1993; Yao et al. 1994) of human epithelial cells.

The A911_00150 deletion mutation in *C. jejuni* PT14 caused a significant decrease in the ability of the mutant to swarm on motility agar compared to the wild-type strain (Figure 5-4). Inactivation of this gene in PT14 also caused hypoadherence to HCA-7 cells by almost 3-fold (Figure 5-5). However, complementation restored the wild-type adhesion phenotype confirming that the absence of this gene was responsible for the decrease in adherence to the HCA-7 cell monolayer. Similarly, invasion of HCA-7 cells by PT14Δ00150::kan decreased by almost 8-fold compared to the wild-type and complementation restored wild-type invasion efficiency (Figure 5-6). This therefore suggests that the A911_00150 gene in PT14 is important for invasion of human colonic cells. The ability of the mutant to colonise chickens was also tested using three different concentrations of PT14Δ00150 inoculum. The results showed that the mutant was unable to colonise the chicken gut while the wild-type was successful in doing so. Again, this illustrates that inactivation of this genes affects the colonisation ability of *C. jejuni* PT14.

5.3.4 Stress-responsive and ribosomal protein synthesis genes were up-regulated in response to A911_00150 mutation

A very specific set of genes were activated in response to the A911_00150 deletion mutation in PT14. The most notable gene clusters to be up-regulated were the *hrcA-grpE-dnaK* operon, along with co-chaperones *clpB* and *cbpA* as well as a large number of ribosomal proteins (*rplCDFNORWX*, *rpsE*). Molecular chaperones within bacterial cells are known to promote efficient folding of newly synthesised polypeptides and allow their translocation, while ensuring the stability of *de novo* folding under conditions of stress and the re-

folding of denatured proteins following exposure to stress (Hartl & Hayer-Hartl 2002). Chaperone activity of ClpB in *E. coli* was investigated by Zolkiewski et al. (1999) who found that this heat shock protein worked with the DnaK-DnaJ-GrpE system to suppress and reverse aggregation of a luciferase protein substrate. It was later shown that the DnaK system possessed a size-dependent aggregation mechanism whereby small protein aggregates could be readily dissolved and refolded into active proteins while large aggregates required the catalytic action of ClpB and ATP (Diamant et al. 2000).

Many chaperones are known to be up-regulated in response to heat shock or any other condition that causes mis-folding of cellular proteins. It therefore appears that inactivation of A911_00150 in PT14 triggered protein mis-folding which then caused an increased need for the chaperones to function and for new proteins to be synthesised. As a result, up-regulation of the stress response genes and the ribosomal proteins occurred.

5.3.5 Flagellar biogenesis genes down-regulated in response to A911_00150 mutation

Several structural genes required for flagellar biogenesis in *C. jejuni* were found to be negatively regulated in response to the A911_00150 mutation in PT14, including those involved in formation of the filament (*flaB* and *flaG*), hook (*flgE*, *flgE2*, *flaD* and *flgK*) and rod (*flgB*, *flgG*, *flgG2*, *flgH* and *flgI*,). Regulation of these genes supports the findings of decreased motility of the mutant as well as hypoadherence to and reduced penetration of HCA-7 human colonic epithelial cells.

In *C. jejuni*, regulation of expression of a number of flagellar genes is known to be controlled by the two alternative sigma factors *rpoN* (σ^{54}) and *fliA* (σ^{28}) (Guerry et al. 1991; Hendrixson et al. 2001; Jagannathan et al. 2001; Hendrixson & Dirita 2003; Carrillo et al. 2004). However, the primary sigma factor σ^{70} (Wösten et al. 1998), encoded by *rpoD*, plays a role in housekeeping functions. Flagellar structural genes are temporally transcribed in the order in which the proteins are required for assembly of the flagellum, ending with production of the structural protein, flagellin (Macnab 1992; Macnab 1996; Soutourina & Bertin 2003). Early genes which are needed for assembly of the basal body and flagellar export apparatus are controlled by σ^{70} promoters (Petersen et al. 2003). However late genes, such as those required to complete assembly of the hook and basal body complex are activated by σ^{54} promoters while extension of the flagellum is controlled by σ^{28} promoters (Hendrixson et al. 2001). The σ^{54}-associated transcriptional activators, FlgR and FlgS, may also be essential for regulation of flagellar gene expression (Jagannathan et al. 2001; Hendrixson & Dirita 2003; Boll & Hendrixson 2013). In addition, the transcriptional regulator FlgM is an anti-σ factor protein which inhibits σ^{28}-dependent transcription and has an important role to play as well (Wösten et al. 2010).

All of the flagellar genes that were repressed in response to the A911_00150 deletion mutation in PT14 were under σ^{54} control, with the exception of *flaG* which requires σ^{28} to initiate transcription. All three sigma factors were expressed at similar levels in the wild-type and mutant. The major flagellin encoding gene *flaA*, which also requires a σ^{28} promoter, was significantly

down-regulated but did not meet the fold change cut-off of -1.5 fold. Jagannathan et al. (2001) previously found that expression of FlaA was repressed when genes activated by σ^{54} or FlgR were not expressed and this is similar to what was observed in the PT14Δ00150::kan mutant. On the other hand, Hendrixson & Dirita (2003) found that transcription of *flaA* remained unaffected in flgDE2 mutants while *flaB* gene expression was significantly reduced. They also found weak repression of σ^{28}-dependent transcription by *flgM*.

5.3.6 Possible effect of HspR transcriptional regulator

It has previously been reported that inactivation of the negative transcriptional regulator for heat shock genes, *hspR*, resulted in increased expression of *dnaK*, *grpE*, *clpB* and *hrcA* along with down-regulation of many flagellar structural genes (*flgD*, *flgE*, *flgE2*, *flaA* and *flaB*) (Andersen et al. 2005). They also found that the *hspR* mutant displayed reduced motility, showed changes in morphology compared to the wild-type and there was a decrease in its ability to adhere to and invade human epithelial INT-407cells in vitro. Holmes et al. (2010) also obtained similar findings when they deleted both *hrcA* and *hspR* in *C. jejuni* NCTC 11168. These results overlap considerably with what has been seen for the PT14Δ00150 strain. This may therefore suggest that the A911_00150 gene could be interacting in some way with HspR in PT14. However the expression level of this gene remained unchanged.

5.3.7 The A911_00150 gene is important for pathogenesis

Kim et al. (2008) looked at the role of the putative methyltransferase gene *cj1461* in gene regulation and virulence in *C. jejuni*. They found that mutation

of this gene caused a significant reduction in motility and resulted in a 7-fold increase in adherence to but a 50-fold decrease in invasion of INT-407 human epithelial cells. They also found that the *cj1461* mutation caused a defect in flagellar appearance and suggested the potential of *cj1461*-regulated epigenetic control of flagellar biosynthesis. Several studies have also demonstrated that inactivation of genes encoding flagellar structural components, such as *flaA* and *flaB*, resulted in reduced motility which led to changes in adhesion and invasion efficiencies (Wassenaar et al. 1991; Grant et al. 1993; Yao et al. 1994). Wassenaar et al. (1991) found that expression of *flaA* but not *flaB* was necessary for motility and that non-motile *C. jejuni* had lost their ability to adhere to and invade human intestinal cells *in vitro*. Other studies have suggested that the presence of adhesins such as CadF, JlpA and PEB1 are important for *C. jejuni* adherence to and invasion of human epithelial cells and for intestinal colonisation (Konkel et al. 1997; Pei et al. 1998; Jin et al. 2001; Monteville et al. 2003).

Inactivation of the A911_00150 gene in *C. jejuni* PT14 caused changes in several properties related to pathogenesis and virulence such as motility, colonisation and adhesion to and penetration of human epithelial cells. The reduced expression of the flagellar biogenesis genes is therefore likely to be responsible for these changes in phenotypes observed. This suggests that although *C. jejuni* PT14 does not possess a Dam methyltransferase homologue, there appears to be A911_00150-regulated epigenetic control of flagellar biogenesis.

Chapter 6 : Synopsis

6.1 GENERAL DISCUSSION

C. jejuni is a primary cause of human bacterial gastroenteritis worldwide. Consumption of contaminated poultry meat is considered to be one of the main sources of infection. With the increasing emergence of antibiotic-resistant *Campylobacter* isolates, the use of virulent bacteriophages to reduce this pathogen in poultry is a potential biocontrol strategy that could limit its entry into the human food chain. To help achieve this, a better understanding of the mechanisms involved in phage-host interactions, particularly at the molecular level, is important. The main objective of this study was to therefore examine such interactions between *Campylobacter* and its virulent phages. This was achieved by investigating the transcriptional response of *C. jejuni* to phage infection as well as examining the role of a Type II R-M system during phage infection of *Campylobacter*. The highly phage-sensitive *Campylobacter* strain, *C. jejuni* PT14, was selected for this study along with a number of group II and III bacteriophages (*Eucampyvirinae*).

The genome of *C. jejuni* PT14 was firstly characterised at the nucleotide level and examined for possible reasons to explain the susceptibility of this strain to phage infection based on its genome content and arrangement. However, despite the nature of this strain to be more vulnerable to predation by a wide range of *Campylobacter* bacteriophages compared to other *C. jejuni* strains, the genome analysis identified no obvious mechanisms that could explain this phenomenon. In fact, several features related to bacterial defence were found to be present including multiple R-M systems, an intact minimal type II-c CRISPR-*cas* system containing Cas1, Cas2 and Cas9 proteins as well as the

presence of homopolymeric tract repeats that constitute phase variable regions within genes that have the potential to impact on bacteriophage replication. BLAST searches of the spacer regions of the PT14 CRISPR array found no obvious nucleotide matches to phage or plasmid DNA, but instead identified matches to the PT14 chromosome and sequences of other *C. jejuni* strains. This suggests that no phage-derived protospacer sequences have yet been integrated into the CRISPR system of PT14. It was therefore not surprising that exposure of *C. jejuni* PT14 to 60 virulent bacteriophages as part of this study, resulted in only 15% of the phages being unable to form plaques, which included the group II phage CP220 (Timms et al. 2010) and a number of the UK typing phages (Frost et al. 1999). However, there was no obvious rational to explain the resistance of PT14 to these phages compared to the others.

Examination of the transcriptional response of *C. jejuni* PT14 to infection by the group III phages CP30A and CPX enabled host-phage interactions to be explored immediately prior to host cell lysis. The mechanisms required by the phages as they redirected host metabolism for successful production of progeny were examined, along with the physiological state of the host. The results revealed coordinate changes in host gene expression in response to phage infection. However, despite similarities between the two phage genomes, CPX had more of a measurable effect on host gene transcription than CP30A as almost five times more genes were differentially regulated. This may have been solely due to the length of time the phage-infected cells were able to grow before cell lysis occurred because the CPX-infected PT14 population crashed one hour later than the CP30-infected population.

The phage-infected host transcriptome was consistent with that of an iron-limited aerobic environment. In response to phage infection there was up-regulation of several genes that were previously found to be up-regulated during iron limitation (Palyada et al. 2004; Holmes et al. 2005; Butcher & Stintzi 2013). These included genes encoding products related to siderophore-based iron acquisition systems such as the ferri-enterochelin (*cfrA*, *ceuBDE*) (Palyada et al. 2004), haem (*chuABC*) (Ridley et al. 2006), transferrin (*cfbpBC*) (Miller et al. 2008) and rhodotorulic acid (A911_07990/*cj1658*, A911_07995/*p19*, A911_08000, A911_08005, A911_08010 and A911_08015/*cj1660-cj1663*) uptake systems. Up-regulation of these iron acquisition genes in response to bacteriophage infection of *C. jejuni* PT14 is consistent with the model of Fur as a transcriptional repressor of iron acquisition genes during iron-replete conditions. The results therefore suggest that the Fur protein, in its iron-dissociated apo-Fur form, derepressed transcription of the iron acquisition genes in what was perceived to be iron-limited conditions. The up-regulation of a number of oxidative stress defence genes (*katA*, *perR*, *sodB*, A911_02725/*cj0559*, A911_06735/*cj1386*, A911_08020/*cj1664* and A911_08025/*cj1665*) and down-regulation of several flagellar biogenesis genes (*flaB*, *flaD*, *flaG*, *flgB*, *flgE*, *flgE2*, *flgG*, *flgG2*, *flgH* *flgI* and *flgK*) in response to phage infection were also indicative of apo-Fur gene regulation. (Palyada et al. 2004; Holmes et al. 2005; Butcher & Stintzi 2013) .

The general pattern of down-regulated gene expression was consistent with what would be expected for an aerobic environment. There was strong

repression of genes associated with a number of anaerobic electron transport pathways that utilise alternative electron acceptors to oxygen. An increase in oxygen availability could also explain the increased transcription of the oxidative stress defence genes as well as up-regulation of the NO-responsive haemoglobins, Cgb and Ctb, which have previously been found to be up-regulated in response to the presence of oxygen (Monk et al. 2008). The increased demand for iron and oxygen during phage infection of PT14 was thought to have occurred as a consequence of the reduction of ribonucleotides to form 2'-deoxyribonucleotides during phage replication. This process is solely catalysed by the enzyme RNR, which requires iron and oxygen during formation of a reactive di-iron tyrosyl radical cofactor (Y_{122}•) before initiation of the nucleotide reduction process.

Based on the RNA-seq data, it does not appear that host defence mechanisms were completely disabled by either phage. Instead, up-regulation the CRISPR-associated *cas* 2 gene (A911_07325), the gene encoding a member of the Type I R-M system of PT14, *hsdS* (A911_07470) as well as genes encoding an adenine specific (A911_01010) and site-specific DNA methyltransferase in response to CPX infection suggest a possible attempt by the host to defend itself against the incoming threat. However, in response to infection by either phage, there was up-regulation of the gene A911_06918/*cj1421c*, which was previously found to encode a product that was identified as a specific target for bacteriophage F336 (Sørensen *et* al., 2011). Therefore, if CP30A and CPX utilise this target, the phages may have counteracted the effort of the host to

up-regulate their defence genes by increasing the possibility of them adsorbing to this potential host receptor.

To conclude this study, a putative type IIs R-M system in *C. jejuni* PT14, encoded by A911_00150, was examined during bacteriophage infection. The nearest homologue to this gene in *C. jejuni* NCTC 11168, *cj0031*, is phase variable and can therefore reversibly switch "on" and "off" the expression of the reading frame. However, although A911_00150 is not phase variable, a BLAST search showed that there is 98% sequence identity to the N-terminal end of *cj0031* and 90% identity to its C-terminal end. An A911_00150 deletion mutant was constructed by naturally transforming PT14 using genomic DNA from the knockout mutant NCTC 11168Δcj0031::kan. The plaque forming ability of a number of selected phages was then determined in the presence and absence of A911_00150. The results revealed a mechanism that is different to what has previously been established because the phages generally appeared to replicate more efficiently in the presence of this type II R-M system. The EOPs for bacteriophages propagated on the wild-type, which was assumed to have a functional R-M system that could recognise and restrict phage DNA, were calculated to be 1. In addition, phages that were previously propagated on alternative hosts and therefore should not have been modified to enable their escape from the R-M system, were not restricted by PT14 and instead formed plaques. EOPs calculated for phages propagated on the mutant were less than 1, despite this host expected to be a non-restricting host due to deletion of the A911_00150 gene, which should have allowed the phages to replicate and therefore form plaques. EOPs previously reported for phages propagated on a

host containing a functional restriction system were lower than 1 whilst in the presence of a non-restricting host, EOPs were calculated to be close to 1 (Arber & Dussoix 1962). The results of the current study therefore suggest that A911_00150 in *C. jejuni* PT14 may not be functional, but the data do not provide a rational for the low plaque forming ability of the phages in the absence of this type II R-M system. Perhaps another R-M system or underlying mechanism was responsible for the observed outcome.

Functional tests were conducted to assess the effect of the A911_00150 gene deletion in *C. jejuni* PT14. Significant changes ($p<0.05$) were observed between the wild-type and PT14Δ00150::kan mutant in several properties related to pathogenesis and virulence such as motility, colonisation as well as adhesion and invasion of human epithelial cells. Complementation of the mutant was able to restore the wild-type phenotype for motility as well as adhesion and invasion of human cells. The transcriptional response of *C. jejuni* PT14 to inactivation of the A911_00150 gene was also investigated. It was found that A911_00150 plays a regulatory role within PT14 and influences expression of key genes involved in stress response, ribosomal protein synthesis and flagellar biogenesis. These data support the phenotypic findings and in particular, down-regulation of the flagellar biogenesis genes is consistent with some of the differences observed in phenotypes between the wild-type and mutant.

In conclusion, the findings presented in this thesis provide a better understanding of the interactions that occur during bacteriophage infection of

Campylobacter. Phage infection of bacterial cells is generally known to cause changes in the functioning of host cells, along with modifications during transcription and translation. This study identifies the regulatory mechanisms involved during phage infection of *Campylobacter*, and provides a base to determine how such mechanisms may impact survival or escape from phage predation. These findings will also contribute to the growing research involved in bacteriophage therapy in an effort to control this important food-borne pathogen.

6.2 FUTURE WORK

Due to time constraints, it was not possible to complete all aspects of this work. Although strand-specific sequencing was applied to both of the transcriptome studies conducted within this thesis, the presence of novel transcripts and antisense transcription within *C. jejuni* PT14 were not extensively examined and were therefore not reported. In addition, subsequent to the work in this thesis being carried out, the transcriptomes of uninfected and phage-infected PT14 were reanalysed using a dRNA-seq approach to enable identification of host and phage-specific TSSs. Once processed, these data will likely provide an invaluable contribution to the current findings.

Experimental validation of the expression patterns identified by the transcriptome data from Chapters 4 and 5 is still required. This will likely be performed using quantitative reverse transcription PCR (RT-qPCR) on remaining aliquots of the total RNA that was used for the RNA-seq analysis. Primers will be designed to amplify genes that displayed an increase, decrease or no change in gene expression levels between the uninfected and phage-infected cultures as well as the PT14 wild-type and PT14Δ00150::kan mutant strains.

Finally, bacteriophage infection of a *C. jejuni* PT14 Fur-deficient mutant should be investigated since the transcriptome data suggests Fur-regulation of key genes that were differentially expressed between the uninfected and phage-infected cultures. These results would supplement the findings of the current

study and also highlight the importance of the Fur protein during phage infection of *Campylobacter*.

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APPENDIX 1

Genes up-regulated in *C. jejuni* PT14 during bacteriophage CP30 infection

	Gene designation	Product	Fold Change	p_{adj}[a]
1	A911_00180 (Cj0037c)	cytochrome C	1.664	4.72E-02
2	A911_00230 (Cj0046)	C4-dicarboxylate transporter	2.3	7.14E-03
3	A911_00695 (Cj0144)	methyl-accepting chemotaxis signal transduction protein	1.549	3.30E-10
4	A911_00865 (exbB1)	TonB system transport protein ExbB	2.078	4.47E-02
5	A911_01060	pathogenicity -like protein	1.829	3.46E-03
6	A911_01395 (peb3)	major antigenic peptide PEB3	1.8	3.49E-27
7	A911_01670 (trpE)	anthranilate synthase component I	1.599	2.01E-07
8	A911_01675 (trpD)	anthranilate synthase component II	1.756	4.69E-16
9	A911_01680 (trpF)	N-(5'phosphoribosyl)anthranilate isomerase	1.956	7.33E-07
10	A911_01685 (trpB)	tryptophan synthase subunit beta	1.667	4.92E-02
11	A911_01690 (trpA)	tryptophan synthase subunit alpha	1.95	9.59E-14
12	A911_02220 (Cj0455c)	hypothetical protein	1.548	3.16E-04
13	A911_02270 (ctb)	group III truncated haemoglobin	7.915	7.23E-23
14	A911_02340 (rpoC)	DNA-directed RNA polymerase subunit beta	1.549	7.66E-14
15	A911_02730 (Cj0560)	MATE efflux family protein	1.625	9.56E-06
16	A911_02735 (Cj0561c)	hypothetical protein	1.56	3.40E-08
17	A911_02995 (pstS)	phosphate ABC transporter substrate-binding protein	1.63	2.66E-05
18	A911_03000 (pstC)	phosphate ABC transporter permease	1.596	1.67E-02

Appendix 1 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
19	A911_03005 (pstA)	phosphate ABC transporter permease	1.821	3.33E-05
20	A911_03010 (pstB)	phosphate ABC transporter ATP-binding protein	1.925	1.50E-07
21	A911_03555 (Cj0728)	periplasmic protein	1.701	2.59E-02
22	A911_03560 (Cj0729)	type I phosphodiesterase/nucleotide pyrophosphatase	1.756	1.82E-02
23	A911_03565 (Cj0730)	putative ABC transport system permease	1.796	1.01E-02
24	A911_03685 (Cj0761)	hypothetical protein	7.424	3.27E-150
25	A911_04025 (Cj0830)	hypothetical protein	2.842	3.45E-02
26	A911_04030 (trmA)	tRNA (uracil-5-)-methyltransferase	1.949	7.06E-05
27	A911_04425 (Cj0916c)	hypothetical protein	1.914	1.47E-14
28	A911_04430 (cstA)	carbon starvation protein A	1.608	7.98E-07
29	A911_04885 (Cj1012c)	hypothetical protein	1.501	2.91E-16
30	A911_05610 (Cj1158c)	hypothetical protein	1.628	4.63E-02
31	A911_06295 (Cj1297)	aminoglycosidase N3'-acetyltransferase	1.593	2.89E-04
32	A911_06305 (Cj1298)	putative N-acetyltransferase	1.912	1.64E-11
33	A911_06450 (neuB2)	legionaminic acid synthase	1.546	4.39E-10
34	A911_06455 (neuC2)	UDP-N-acetylglucosamine-2-epimerase	1.552	7.23E-06
35	A911_06895 (Cj1417c)	putative class I glutamine amidotransferase	1.502	2.86E-05
36	A911_06918 (Cj1421c)	hypothetical protein	1.593	3.74E-06
37	A911_07230 (Cj1501)	putative inner membrane protein	1.611	4.80E-25
38	A911_07630 (cgb)	single domain haemoglobin	63.974	6.19E-25

Appendix 1 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
39	A911_02735 (Cj0561c)	hypothetical protein	1.560	3.40E-08
40	A911_08020 (Cj1664)	putative periplasmic thioredoxin	1.555	1.70E-03
41	A911_06455 (neuC2)	UDP-N-acetylglucosamine-2-epimerase	1.552	7.23E-06
42	A911_02340 (rpoC)	DNA-directed RNA polymerase subunit beta	1.549	7.66E-14
43	A911_00695 (Cj0144)	methyl-accepting chemotaxis signal transduction protein	1.549	3.30E-10
44	A911_02220 (Cj0455c)	hypothetical protein	1.548	3.16E-04
45	A911_06450 (neuB2)	legionaminic acid synthase	1.546	4.39E-10
46	A911_08015 (Cj1663)	ABC transporter ATP-binding protein	1.541	1.56E-03
47	rplR	50S ribosomal protein L18	1.506	9.35E-03
48	A911_06895 (Cj1417c)	putative class I glutamine amidotransferase	1.502	2.86E-05
49	A911_04885 (Cj1012c)	hypothetical protein	1.501	2.91E-16

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – $p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

APPENDIX 2

Genes down-regulated in *C. jejuni* PT14 during bacteriophage CP30 infection

	Gene designation	Product	Fold Change	p_{adj}[a]
1	A911_00020 (Cj0004c)	hypothetical protein	-2.444	0.00E+00
2	A911_00025 (Cj0005c)	Molybdopterin oxidoreductase family protein	-4.325	0.00E+00
3	A911_00035 (gltB)	glutamate synthase, large subunit	-2.293	6.67E-08
4	A911_00040 (gltD)	glutamate synthase subunit beta	-2.513	5.71E-10
5	A911_00055 (rrc)	rubrerythrin	-1.507	1.56E-07
6	A911_00410 (aspA)	aspartate ammonia-lyase	-1.502	1.05E-06
7	A911_01265 (Cj0262c)	methyl-accepting chemotaxis protein	-2.344	1.78E-12
8	A911_01275 (Cj0264c)	hypothetical protein	-3.185	0.00E+00
9	A911_01280 (Cj0265c)	trimethylamine N-oxide reductase, cytochrome c-type subunit	-3.038	0.00E+00
10	A911_01735 (Cj0358)	cytochrome c551 peroxidase	-2.824	0.00E+00
11	A911_01985 (frdC)	fumarate reductase cytochrome b-556 subunit	-1.638	7.86E-05
12	A911_01990 (frdA)	fumarate reductase flavoprotein subunit	-1.865	8.83E-04
13	A911_01995 (frdB)	fumarate reductase iron-sulfur subunit	-1.502	0.00E+00
14	A911_02130 (sdhA)	succinate dehydrogenase flavoprotein subunit	-2.296	1.87E-02
15	A911_02135 (sdhB)	succinate dehydrogenase, iron-sulfur protein subunit	-2.669	3.85E-06
16	A911_02140 (sdhC)	succinate dehydrogenase subunit C	-2.284	0.00E+00
17	A911_02185 (Cj0448c)	methyl-accepting chemotaxis protein	-2.064	0.00E+00
18	A911_02190 (Cj0449c)	hypothetical protein	-1.907	0.00E+00

Appendix 2 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
19	A911_02450 (Cj0501)	ammonium transporter	-3.82	1.07E-03
20	A911_02790 (ribA)	bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein	-1.753	1.50E-07
21	A911_02795 (Cj0573)	putative GatB/Yqey family protein	-1.534	1.30E-02
22	A911_02800 (ilvI)	acetolactate synthase 3 catalytic subunit	-1.522	2.77E-04
23	A911_02950 (Cj0604)	polyphosphate kinase	-1.655	7.98E-07
24	A911_02990 (cft)	nonheme iron-containing ferritin	-1.553	1.50E-06
25	A911_03270 (dcuB)	anaerobic C4-dicarboxylate transporter	-2.834	0.00E+00
26	A911_03515 (flaC)	flagellin	-1.591	6.13E-07
27	A911_03665 (hrcA)	heat-inducible transcription repressor	-1.663	4.83E-06
28	A911_03780 (napA)	nitrate reductase catalytic subunit	-1.634	9.41E-08
29	A911_03785 (napG)	quinol dehydrogenase periplasmic component	-1.707	7.00E-05
30	A911_03790 (napH)	quinol dehydrogenase membrane component	-1.676	6.66E-05
31	A911_03795 (napB)	periplasmic nitrate reductase small subunit	-1.802	1.64E-11
32	A911_04050 (acnB)	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	-1.664	3.13E-04
33	A911_04190 (Cj0864)	hypothetical protein	-1.762	1.34E-06
34	A911_04195 (dsbB)	disulfide oxidoreductase	-2.343	0.00E+00
35	A911_04390 (Cj0909)	hypothetical protein	-2.032	2.04E-10
36	A911_04395 (Cj0910)	hypothetical protein	-1.569	2.79E-05
37	A911_04520 (Cj0935c)	putative sodium:amino-acid symporter family protein	-1.71	8.13E-05
38	A911_04820 (Cj0999c)	hypothetical protein	-2.492	0.00E+00

Appendix 2 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
39	A911_05740 (petC)	ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit	-1.508	7.41E-04
40	A911_05920 (groES)	co-chaperonin GroES	-1.586	3.56E-09
41	A911_06155 (hydA)	Ni/Fe-hydrogenase small chain	-1.516	2.37E-05
42	A911_06590 (Cj1356c)	hypothetical protein	-1.751	0.00E+00
43	A911_06595 (nrfA)	Cytochrome c552	-2.194	9.94E-15
44	A911_06600 (nrfH)	putative periplasmic cytochrome C	-2.365	0.00E+00
45	A911_07155 (Cj1485c)	hypothetical protein	-1.788	3.37E-07
46	A911_07160 (Cj1486c)	periplasmic protein	-1.774	1.47E-12
47	A911_07165 (ccoP)	cytochrome c oxidase, cbb3-type, subunit III	-1.853	7.62E-10
48	A911_07170 (ccoQ)	cytochrome c oxidase, cbb3-type, subunit IV	-2.187	0.00E+00
49	A911_07175 (ccoO)	cbb3-type cytochrome c oxidase subunit II	-2.025	0.00E+00
50	A911_07180 (ccoN)	cbb3-type cytochrome c oxidase subunit I	-1.902	4.35E-11
51	A911_07520 (Cj1564)	methyl-accepting chemotaxis signal transduction protein	-1.59	5.30E-04

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – $p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

APPENDIX 3

C. jejuni PT14 genes up-regulated during bacteriophage CPX infection

	Gene designation	Product	Fold Change	p_{adj} [a]
1	A911_00005 (dnaA)	chromosomal replication initiation protein	1.934	4.61E-24
2	A911_00030 (Cj0006)	histidine permease YuiF	2.73	1.88E-15
3	A911_00060 (ilvD)	dihydroxy-acid dehydratase	1.521	5.42E-09
4	A911_00110 (purB)	adenylosuccinate lyase	1.772	1.51E-14
5	A911_00120 (Cj0025c)	sodium/dicarboxylate symporter	2.077	1.74E-09
6	A911_00155 (Cj0033)	Putative integral membrane protein	1.644	5.30E-07
7	A911_00165 (Cj0034c)	periplasmic protein	1.892	9.38E-08
8	A911_00170 (Cj0035c)	Bcr/CflA subfamily drug resistance transporter	3.486	2.72E-24
9	A911_00180 (Cj0037c)	cytochrome C	5.042	6.65E-34
10	A911_00230 (Cj0046)	hypothetical protein	2.156	6.39E-03
11	A911_00235	hypothetical protein	4.722	8.42E-03
12	A911_00340 (Cj0072c)	hemerythrin family non-heme iron protein	2.171	2.90E-05
13	A911_00365 (cdtC)	cytolethal distending toxin C	1.983	4.76E-12
14	A911_00370 (cdtB)	cytolethal distending toxin subunit CdtB	1.699	2.13E-06
15	A911_00380 (Cj0080)	C4-dicarboxylate transporter	2.156	3.93E-03
16	A911_00405 (ung)	uracil-DNA glycosylase	2.406	2.42E-13
17	A911_00465 (fmt)	methionyl-tRNA formyltransferase	2.476	1.73E-33
18	A911_00480 (Cj0101)	parB family protein	1.688	3.85E-26

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
19	A911_00525 (exbD3)	exbD/tolR family transport protein	1.655	7.07E-09
20	A911_00530 (Cj0111)	periplasmic protein	1.556	4.01E-10
21	A911_00590 (Cj0123c)	transcriptional regulator	1.725	1.50E-15
22	A911_00595 (Cj0124c)	hypothetical protein	1.935	8.93E-14
23	A911_00600 (Cj0125c)	dksA-like protein	2.406	2.50E-09
24	A911_00605 (Cj0126c)	hypothetical protein	2.073	7.46E-34
25	A911_00610 (accD)	acetyl-CoA carboxylase subunit beta	1.629	9.84E-07
26	A911_00650 (Cj0135)	hypothetical protein	1.617	1.78E-16
27	A911_00695 (Cj0144)	methyl-accepting chemotaxis signal transduction protein	4.993	2.77E-12
28	A911_00730 (Cj0151c)	Putative periplasmic protein	1.5	9.97E-03
29	A911_00745 (Cj0154c)	tetrapyrrole methylase family protein	1.593	2.10E-09
30	A911_00750 (rpmE)	50S ribosomal protein L31	1.894	2.28E-44
31	A911_00755 (Cj0156c)	16S ribosomal RNA methyltransferase RsmE	1.68	6.75E-06
32	A911_00775 (Cj0160c)	radical SAM domain protein	1.72	7.14E-05
33	A911_00780 (moaA)	molybdenum cofactor biosynthesis protein A	1.789	3.64E-18
34	A911_00785 (Cj0162c)	hypothetical protein	1.75	1.87E-16
35	A911_00790 (Cj0163c)	hypothetical protein	1.515	1.75E-06
36	A911_00815 (sodB)	superoxide dismutase	1.615	1.56E-13
37	A911_00835 (cfbpC)	iron ABC transporter ATP binding subunit	1.524	5.02E-07
38	A911_00840 (cfbpB)	iron ABC transporter permease	1.536	2.16E-02

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
39	A911_00865 (exbB1)	TonB system transport protein ExbB	2.348	3.93E-03
40	A911_00955 (dapB)	dihydrodipicolinate reductase	1.678	1.15E-15
41	A911_00960 (Cj0198c)	recombination factor protein RarA	3.351	5.12E-19
42	A911_00965 (Cj0199c)	periplasmic protein	5.022	1.07E-09
43	A911_00985 (Cj0142c)	citrate transporter	2.651	3.13E-09
44	A911_00990 (Cj0204)	OPT family oligopeptide transporter	2.174	1.83E-47
45	A911_01000 (thrS)	threonyl-tRNA ligase	1.58	4.31E-17
46	A911_01010 (Cj0208)	D12 class N6 adenine-specific DNA methyltransferase	1.526	2.34E-07
47	A911_01080 (argC)	N-acetyl-gamma-glutamyl-phosphate reductase	2.856	3.50E-47
48	A911_01085 (Cj0225)	acetyltransferase	2.651	4.75E-31
49	A911_01090 (argB)	acetylglutamate kinase	1.988	1.45E-10
50	A911_01095 (argD)	acetylornithine/succinyldiaminopimelate aminotransferase	1.798	8.85E-18
51	A911_01100 (pcm)	protein-L-isoaspartate O-methyltransferase	1.649	1.92E-06
52	A911_01155 (Cj0239c)	iron-sulfur clusteraseembly scaffold protein NifU	1.944	3.48E-49
53	A911_01160 (iscS)	cysteine desulfurase	2.249	1.35E-38
54	A911_01165 (Cj0241c)	putative iron-binding protein	3.45	3.40E-15
55	A911_01190 (Cj0247c)	hypothetical protein	2.104	4.23E-02
56	A911_01230 (exoA)	exodeoxyribonuclease III	1.626	1.17E-30

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
57	A911_01260 (Cj0261c)	hypothetical protein	1.546	1.40E-07
58	A911_01265 (Cj0262c)	methyl-accepting chemotaxis protein	2.184	1.99E-06
59	A911_01285 (Cj0266c)	membrane protein	1.706	3.00E-07
60	A911_01305 (Cj0270)	4-oxalocrotonate tautomerase	1.554	7.36E-04
61	A911_01315 (Cj0272)	hypothetical protein	1.607	1.46E-09
62	A911_01355 (tal)	transaldolase	1.647	7.59E-18
63	A911_01380 (Cj0286c)	hypothetical protein	1.7	1.73E-08
64	A911_01385 (greA)	transcription elongation factor GreA	1.692	5.14E-17
65	A911_01395 (peb3)	major antigenic peptide PEB3	1.728	2.04E-09
66	A911_01415 (Cj0294)	MoeB/ThiF family protein	1.52	2.15E-04
67	A911_01465 (bioC)	biotin biosynthesis protein BioC	1.698	1.74E-04
68	A911_01470 (Cj0305c)	hypothetical protein	2.428	4.62E-05
69	A911_01475 (bioF)	8-amino-7-oxononanoate synthase	2.628	2.86E-35
70	A911_01525 (pheA)	chorismate mutase/prephenate dehydratase	1.689	2.91E-23
71	A911_01555(perR)	FUR family transcriptional regulator	2.143	7.53E-38
72	A911_01635 (polA)	DNA polymerase I	1.695	5.12E-14
73	A911_01650 (Cj0341c)	hypothetical protein	1.628	1.70E-08
74	A911_01660 (Cj0343c)	hypothetical protein	2.394	2.79E-46
75	A911_01680 (trpF)	N-(5'phosphoribosyl)anthranilate isomerase	1.616	4.70E-04

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
76	A911_01690 (trpA)	tryptophan synthase subunit alpha	1.523	2.50E-02
77	A911_01705 (Cj0352)	transmembrane protein	1.719	4.39E-10
78	A911_01760 (Cj0363c)	coproporphyrinogen III oxidase	1.997	5.44E-15
79	A911_01775 (cmeB)	RND efflux system, inner membrane transporter CmeB	1.531	5.43E-07
80	A911_01845 (Cj0380c)	hypothetical protein	1.95	4.57E-03
81	A911_01850 (pyrF)	orotidine 5'-phosphate decarboxylase	1.717	2.97E-22
82	A911_01870 (Cj0385c)	integral membrane protein	1.842	1.07E-16
83	A911_01915 (Cj0394c)	pantothenate kinase	1.696	7.71E-11
84	A911_02015 (Cj0414)	hypothetical protein	2.499	7.06E-23
85	A911_02020 (Cj0415)	oxidoreductase	2.393	3.84E-30
86	A911_02040 (Cj0419)	histidine triad (HIT) family protein	1.557	6.07E-03
87	A911_02100 (Cj0431)	hypothetical protein	1.873	5.66E-06
88	A911_02105 (murD)	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	1.555	8.63E-06
89	A911_02110 (mraY)	phospho-N-acetylmuramoyl-pentapeptide transferase	2.775	3.22E-15
90	A911_02215 (Cj0454c)	hypothetical protein	1.806	1.51E-06
91	A911_02220 (Cj0455c)	hypothetical protein	1.726	9.44E-08
92	A911_02250 (Cj0461c)	putative MFS (Major Facilitator Superfamily) transport protein	1.787	6.50E-10
93	A911_02345 (Cj0480c)	IclR family transcriptional regulator	1.689	6.05E-05
94	A911_02355 (uxaA)	putative altronate hydrolase N-terminus	1.652	1.22E-01
95	A911_02375 (Cj0485)	short chain dehydrogenase	2.299	1.73E-12

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
96	A911_02380 (Cj0486)	L-fucose permease	1.611	3.08E-04
97	A911_02390 (Cj0488)	hypothetical protein	1.797	3.66E-05
98	A911_02395 (ald')	aldehyde dehydrogenase A	1.574	6.03E-06
99	A911_02455 (hemH)	ferrochelatase	1.884	7.31E-12
100	A911_02460 (Cj0504c)	Gfo/Idh/MocA family oxidoreductase	2.386	5.64E-23
101	A911_02465 (Cj0505c)	putative aminotransferase	1.949	5.63E-13
102	A911_02485 (clpB)	ATP-dependent Clp protease ATP-binding subunit	1.666	1.04E-15
103	A911_02490 (Cj0510c)	hypothetical protein	1.676	2.38E-09
104	A911_02515 (Cj0515)	periplasmic protein	1.716	8.72E-29
105	A911_02520 (plsC)	1-acyl-sn-glycerol-3-phosphate acyltransferase	1.572	1.10E-19
106	A911_02525 (crcB)	CrcB protein	1.573	5.82E-03
107	A911_02575 (Cj0529c)	putative aminodeoxychorismate lyase family protein	1.62	5.36E-05
108	A911_02625 (Cj0539)	hypothetical protein	1.664	3.19E-13
109	A911_02635 (Cj0541)	octaprenyl-diphosphate synthase	1.523	1.91E-12
110	A911_02705 (Cj0555)	hypothetical protein	1.765	8.77E-18
111	A911_02710 (Cj0556)	putative amidohydrolase family protein	1.819	3.80E-11
112	A911_02725 (Cj0559)	Pyridine nucleotide-disulfide oxidoreductase family protein	1.548	3.68E-16
113	A911_02735 (Cj0561c)	hypothetical protein	2.519	1.51E-28
114	A911_02770 (Cj0568)	hypothetical protein	2.192	4.97E-01
115	A911_02820 (tatC)	Sec-independent protein translocase TatC	1.776	2.63E-14

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
116	A911_02825 (Cj0579c)	Sec-independent translocase	1.755	4.85E-45
117	A911_02830 (Cj0580c)	coproporphyrinogen III oxidase	2.296	3.45E-04
118	A911_02895 (Cj0593c)	hypothetical protein	3.177	8.22E-07
119	A911_02900 (Cj0594c)	DNA/RNA non-specific endonuclease	3.889	9.05E-31
120	A911_02905 (nth)	endonuclease III	3.672	1.37E-19
121	A911_02975 (Cj0609c)	hypothetical protein	2.923	5.55E-24
122	A911_02980 (Cj0610c)	periplasmic protein	2.503	1.32E-17
123	A911_02985 (Cj0611c)	poly(beta-D-mannuronate) O-acetylase	2.005	1.32E-16
124	A911_02995 (pstS)	phosphate ABC transporter substrate-binding protein	4.141	6.61E-09
125	A911_03000 (pstC)	phosphate ABC transporter permease	5.204	4.23E-21
126	A911_03005 (pstA)	phosphate ABC transporter permease	4.135	2.54E-40
127	A911_03010 (pstB)	phosphate ABC transporter ATP-binding protein	3.552	1.08E-16
128	A911_03030 (Cj0621)	hypothetical protein	1.509	4.69E-12
129	A911_03035 (hypF)	(NiFe) hydrogenase maturation protein HypF	1.719	7.89E-11
130	A911_03050 (hypD)	hydrogenase isoenzymes formation protein HypD	1.563	2.01E-14
131	A911_03080 (Cj0631c)	ribonuclease R	2.52	6.61E-11
132	A911_03095 (dprA)	DNA processing protein A	1.532	2.80E-05
133	A911_03335 (cipA)	invasion protein CipA	1.714	1.26E-13
134	A911_03375 (Cj0692c)	hypothetical protein	2.704	9.32E-21
135	A911_03380 (mraW)	16S rRNA m(4)C1402 methyltransferase	1.7	3.57E-15

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
137	A911_03415 (Cj0700)	hypothetical protein	1.549	5.28E-25
138	A911_03420 (Cj0701)	peptidase, U32 family protein	1.714	9.24E-34
139	A911_03425 (purE)	phosphoribosylaminoimidazole carboxylase catalytic subunit	1.515	2.93E-11
140	A911_03430 (Cj0703)	hypothetical protein	1.703	1.28E-24
141	A911_03465 (rpsP)	30S ribosomal protein S16	1.57	2.93E-10
142	A911_03555 (Cj0728)	periplasmic protein	1.73	7.96E-03
143	A911_03570 (Cj0731)	ABC transporter permease	2.033	2.07E-02
144	A911_03595 (Cj0736)	hypothetical protein	1.748	2.46E-01
145	A911_03650 (Cj0752)	transposase	1.873	2.68E-02
146	A911_03655 (tonB3)	TonB transport protein	3.079	3.20E-09
147	A911_03660 (cfrA)	ferric enterobactin uptake receptor	2.011	1.57E-04
148	A911_03680 (Cj0760)	hypothetical protein	1.557	4.35E-17
149	A911_03685 (Cj0761)	hypothetical protein	1.597	3.76E-17
150	A911_03695 (cysE)	serine acetyltransferase	1.746	3.70E-07
151	A911_03700 (speA)	arginine decarboxylase	1.643	2.75E-06
152	A911_03705 (hisS)	histidyl-tRNA ligase	1.897	2.01E-17
153	A911_03710 (tmk)	thymidylate kinase	2.478	6.28E-15
154	A911_03715 (coaD)	phosphopantetheine adenylyltransferase	2.048	8.60E-23
155	A911_03720 (Cj0768c)	3-octaprenyl-4-hydroxybenzoate carboxylase	3.155	5.10E-30
156	A911_03725 (flgA)	flagellar basal body P-ring biosynthesis protein FlgA	2.376	6.71E-26

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
157	A911_03760 (Cj0776c)	periplasmic protein	1.536	2.48E-23
158	A911_03765 (Cj0777)	ATP-dependent DNA helicase UvrD/PcrA/Rep, epsilon proteobacterial type1	1.933	4.00E-16
159	A911_03815 (Cj0787)	hypothetical protein	1.715	8.18E-14
160	A911_03860 (Cj0796c)	putative hydrolase	2.214	1.78E-07
161	A911_03865 (Cj0797c)	hypothetical protein	2.116	5.13E-20
162	A911_03875 (ruvA)	Holliday junction DNA helicase RuvA	1.649	2.99E-10
163	A911_03880 (Cj0800c)	putative ATPase	2.289	1.07E-17
164	A911_03960 (glnH)	putative glutamine binding periplasmic protein	1.847	1.07E-06
165	A911_03965 (Cj0818)	putative lipoprotein	2.324	9.26E-15
166	A911_03970 (Cj0819)	small hydrophobic protein	3.057	2.14E-29
167	A911_03975 (fliP)	flagellar biosynthesis protein FliP	3.17	1.36E-16
168	A911_04030 (trmA)	tRNA (uracil-5-)-methyltransferase	1.626	2.71E-14
169	A911_04035 (Cj0832c)	Na+/H+ antiporter family protein	1.548	6.53E-10
170	A911_04080 (mobB)	molybdopterin-guanine dinucleotide biosynthesis protein B	1.96	9.17E-16
171	A911_04100 (gltX)	glutamyl-tRNA ligase	1.775	5.14E-12
172	A911_04120 (Cj0849c)	hypothetical protein	1.679	8.56E-06
173	A911_04175 (pabA)	para-aminobenzoate synthase glutamine aminotransferase, component II	3.043	3.57E-03
174	A911_04180 (pabB)	para-aminobenzoate synthase glutamine aminotransferase, component I	2.647	1.96E-09
175	A911_04185 (xerD)	integrase-recombinase protein	2.262	6.79E-19

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
176	A911_04270 (Cj0883c)	RrF2 family protein, putative	1.606	7.65E-15
177	A911_04280 (ftsK)	putative cell division protein	1.838	5.74E-21
178	A911_04320 (ispH)	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	1.609	1.02E-19
179	A911_04325 (aroA)	3-phosphoshikimate 1-carboxyvinyltransferase	1.591	8.49E-12
180	A911_04355 (Cj0901)	amino-acid ABC transporter permease	2.35	2.01E-18
181	A911_04380 (Cj0906c)	hypothetical protein	1.841	9.28E-26
182	A911_04420 (Cj0915)	putative acyl-CoA thioester hydrolase	1.836	3.64E-22
183	A911_04425 (Cj0916c)	hypothetical protein	2.92	5.23E-41
184	A911_04430 (cstA)	carbon starvation protein A	2.11	5.82E-13
185	A911_04565 (Cj0944c)	hypothetical protein	1.533	1.31E-07
186	A911_04625 (Cj0957c)	hypothetical protein	1.577	8.58E-08
187	A911_04630 (Cj0958c)	membrane protein insertase	1.593	2.43E-13
188	A911_04635 (Cj0959c)	hypothetical protein	1.708	3.97E-39
189	A911_04640 (rnpA)	ribonuclease P protein component	1.636	1.09E-16
190	A911_04650 (Cj0962)	GNAT family acetyltransferase	1.948	2.50E-02
191	A911_04655 (Cj0963)	hypothetical protein	2.101	8.63E-12
192	A911_04710 (Cj0976)	tRNA mo(5)U34 methyltransferase	1.768	5.32E-06
193	A911_04735 (cjaB)	transport protein CjaB	1.747	3.76E-20
194	A911_04745 (Cj0983)	surface-exposed lipoprotein JlpA	1.901	2.17E-09

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
195	A911_04825 (Cj1000)	putative transcriptional regulator (lysR family) protein	1.625	5.20E-07
196	A911_04875 (tgt)	queuine tRNA-ribosyltransferase	1.675	9.90E-12
197	A911_04990 (cmeF)	AcrB/AcrD/AcrF family protein	1.723	3.21E-23
198	A911_04995 (Cj1034c)	adenylosuccinate lyase	1.51	2.63E-03
199	A911_05015 (Cj1038)	cell division protein FtsW	1.674	4.14E-11
200	A911_05020 (murG)	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	1.836	1.03E-14
201	A911_05040 (Cj1042c)	Putative transcriptional regulatory protein	1.519	2.65E-03
202	A911_05070 (dapE)	succinyl-diaminopimelate desuccinylase	1.701	1.34E-05
203	A911_05105 (Cj1055c)	putative integral membrane protein	1.71	1.74E-09
204	A911_05210 (ctsT)	transformation system protein	2.137	2.57E-06
205	A911_05300 (lnt)	apolipoprotein N-acyltransferase	1.622	1.09E-02
206	A911_05310 (Cj1097)	serine/threonine transporter SstT	2.042	8.62E-16
207	A911_05315 (pyrB)	aspartate carbamoyltransferase catalytic subunit	1.92	6.61E-12
208	A911_05320 (Cj1099)	oligoendopeptidase F	1.7	1.39E-14
209	A911_05325 (Cj1100)	hypothetical protein	1.781	6.25E-20
210	A911_05330 (Cj1101)	ATP-dependent DNA helicase UvrD/Rep family protein	1.793	3.22E-15
211	A911_05335 (truB)	tRNA pseudouridine synthase B	1.835	1.21E-09
212	A911_05490 (waaC)	lipopolysaccharide heptosyltransferase I	2.295	4.41E-22
213	A911_05495 (htrB)	lipid A biosynthesis lauroyl acyltransferase	1.923	1.26E-15
214	A911_05500 (Cj1135)	putative two-domain glucosyltransferase	1.628	2.17E-11

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
215	A911_05515 (Cj1138)	putative glycosyltransferase	1.792	5.79E-04
216	A911_05600 (rho)	transcription termination factor Rho	2.474	2.31E-18
217	A911_05605 (dnaX)	DNA polymerase III subunits gamma and tau	2.658	5.27E-26
218	A911_05610 (Cj1158c)	hypothetical protein	2.795	5.84E-10
219	A911_05645 (Cj1165c)	hypothetical protein	1.905	4.59E-16
220	A911_05650 (Cj1166c)	hypothetical protein	2.121	4.86E-11
221	A911_05685 (Cj1173)	SMR family multidrug efflux pump	2.518	4.24E-19
222	A911_05690 (Cj1174)	putative efflux protein	1.786	4.04E-08
223	A911_05755 (arsB)	arsenical pump membrane protein	1.526	3.05E-05
224	A911_05765 (cetB)	Signal transduction protein CetB	1.533	2.56E-13
225	A911_05770 (cetA)	bipartate energy taxis response protein CetA	1.557	1.59E-15
226	A911_05875 (Cj1211)	ComEC/Rec2 family competence protein	1.638	1.35E-02
227	A911_05940 (Cj1224)	hemerythrin-like iron-binding protein	1.78	6.70E-07
228	A911_05975 (kefB)	Sodium/hydrogen exchanger family protein	1.635	3.86E-13
229	A911_05980 (Cj1232)	hypothetical protein	2.751	3.67E-16
230	A911_05985 (Cj1233)	putative HAD-superfamily hydrolase	2.177	9.88E-20
231	A911_05995 (Cj1235)	peptidase, M23/M37 family protein	1.549	5.92E-18
232	A911_06025 (Cj1241)	transporter	1.855	1.96E-03
233	A911_06050 (uvrC)	excinuclease ABC subunit C	2.056	2.79E-20
234	A911_06060 (guaA)	GMP synthase	1.792	2.84E-33

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
235	A911_06070 (Cj1249)	hypothetical protein	1.786	1.57E-17
236	A911_06085 (Cj1252)	hypothetical protein	2.028	2.03E-17
237	A911_06090 (pnp)	polynucleotide phosphorylase/polyadenylase	1.638	4.79E-14
238	A911_06095 (Cj1254)	G:T/U mismatch-specific uracil/thymine DNA-glycosylase	1.678	8.57E-17
239	A911_06110 (Cj1258)	phosphotyrosine protein phosphatase	2.064	2.59E-09
240	A911_06120 (dnaJ)	chaperone protein DnaJ	1.567	3.95E-08
241	A911_06130 (racS)	two-component sensor histidine kinase	1.655	6.45E-20
242	A911_06225 (mrdB)	RodA protein	2.164	3.95E-15
243	A911_06240 (Cj1285c)	hypothetical protein	1.841	6.20E-15
244	A911_06245 (upp)	uracil phosphoribosyltransferase	1.504	2.10E-12
245	A911_06265 (accC)	biotin carboxylase	1.755	1.57E-38
246	A911_06270 (accB)	acetyl-CoA carboxylase, biotin carboxyl carrier protein	2.264	1.69E-62
247	A911_06435 (Cj1324)	hypothetical protein	1.509	2.74E-04
248	A911_06440 (Cj1325)	Putative methyl transferase	1.676	3.56E-03
249	A911_06450 (neuB2)	legionaminic acid synthase	2.138	7.63E-28
250	A911_06455 (neuC2)	UDP-N-acetylglucosamine 2-epimerase	2.078	5.37E-22
251	A911_06460 (Cj1329)	putative sugar-phosphate nucleotide transferase	2.302	2.43E-19
252	A911_06465 (Cj1330)	hypothetical protein	2.053	6.03E-22
253	A911_06470 (ptmB)	legionaminic acid cytidylytransferase	2.032	3.52E-12

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
254	A911_06475 (ptmA)	flagellin modification protein A	1.696	4.68E-13
255	A911_06480 (pseD)	PseD protein	1.701	1.27E-06
256	A911_06560 (mobA)	putative molybdopterin-guanine dinucleotide biosynthesis protein	2.574	5.35E-12
257	A911_06565 (pldA)	hemin uptake system outer membrane receptor	2.395	1.50E-13
258	A911_06570 (ceuB)	enterochelin ABC transporter permease protein	5.562	3.16E-14
259	A911_06580 (ceuD)	enterochelin ABC transporter, ATP-binding protein	4.475	8.74E-16
260	A911_06585 (ceuE)	enterochelin ABC transporter, periplasmic enterochelin-binding protein	3.119	3.43E-34
261	A911_06615 (ruvB)	Holliday junction DNA helicase RuvB	2.497	2.40E-12
262	A911_06645 (Cj1368)	hypothetical protein	2.09	9.38E-28
263	A911_06650 (hydA2)	xanthine/uracil permease family protein	1.887	5.25E-14
264	A911_06655 (Cj1370)	phosphoribosyltransferase	1.69	7.65E-14
265	A911_06665 (Cj1372)	putative periplasmic toluene tolerance protein	1.754	1.12E-07
266	A911_06670 (Cj1373)	putative integral membrane protein	2.14	7.80E-15
267	A911_06675 (Cj1374c)	dlTP/XTP pyrophosphatase	1.939	6.00E-20
268	A911_06680 (Cj1375)	multidrug efflux transporter	1.759	5.53E-19
269	A911_06685 (Cj1376)	hypothetical protein	1.748	1.98E-18
270	A911_06695 (selA)	selenocysteine synthase	2.501	1.19E-26
271	A911_06700 (selB)	putative selenocysteine-specific elongation factor	2.698	8.35E-13
272	A911_06720	hypothetical protein	1.741	1.23E-01

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
273	A911_06730 (katA)	catalase	1.507	6.83E-03
274	A911_06735 (Cj1386)	ankyrin repeat protein	2.082	1.85E-05
275	A911_06830 (nadD)	nicotinate (nicotinamide) nucleotide adenylyltransferase	1.922	4.99E-09
276	A911_06835 (Cj1405)	hypothetical protein	1.928	4.88E-16
277	A911_06875 (kpsS)	capsule polysaccharide export protein KpsS	1.564	1.23E-14
278	A911_06908	capsular polysaccharide biosynthesis protein	1.565	2.87E-15
279	A911_06909	capsular polysaccharide biosynthesis protein	1.83	1.44E-05
280	A911_06910	capsule biosynthesis phosphatase	1.614	8.13E-17
281	A911_06918 (Cj1421c)	hypothetical protein	1.646	9.88E-08
282	A911_06965 (Cj1449c)	hypothetical protein	1.916	1.89E-39
283	A911_06990 (tilS)	tRNA(Ile)-lysidine synthetase	1.695	1.89E-07
284	A911_07000	sugar transferase	2.069	2.99E-06
285	A911_07020 (truD)	tRNA pseudouridine synthase D	1.553	1.21E-06
286	A911_07025 (thiL)	thiamine monophosphate kinase	2.316	6.58E-21
287	A911_07035 (Cj1460)	hypothetical protein	1.724	2.35E-11
288	A911_07040 (Cj1461)	site-specific DNA methyltransferase	2.022	8.20E-18
289	A911_07075 (Cj1468)	hypothetical protein	1.534	6.96E-06
290	A911_07090 (Cj1472c)	hypothetical protein	1.585	3.79E-04

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
291	A911_07095 (ctsP)	putative ATP/GTP-binding protein	1.696	6.34E-04
292	A911_07100 (ctsD)	general secretory pathway protein D	1.555	3.53E-08
293	A911_07105 (ctsR)	transformation system protein	1.783	1.17E-02
294	A911_07225 (Cj1500)	hypothetical protein	1.572	1.85E-03
295	A911_07230 (Cj1501)	putative inner membrane protein	1.608	1.26E-10
296	A911_07240 (putA)	bifunctional putA protein	1.825	6.81E-19
297	A911_07290 (Cj1513c)	Tat pathway signal sequence domain-containing protein	1.558	2.21E-50
298	A911_07295 (Cj1514c)	hypothetical protein	1.51	2.05E-05
299	A911_07320 (moeA2)	molybdopterin biosynthesis protein	1.752	5.89E-08
300	A911_07325 (Cj1521c)	CRISPR-associated Cas2 family protein	1.589	6.48E-17
301	A911_07365 (coaE)	dephospho-CoA kinase	2.778	2.36E-05
302	A911_07400 (acs)	acetyl-CoA synthetase	1.807	7.32E-04
303	A911_07405 (Cj1538c)	anion-uptake ABC-transport system ATP-binding protein	1.61	1.81E-02
304	A911_07445 (Cj1546)	putative transcriptional regulator	1.672	8.83E-16
305	A911_07450 (Cj1547)	Blc protein-like protein	2.806	2.73E-25
306	A911_07470 (hsdS)	putative type I restriction enzymeS protein	1.783	3.55E-08
307	A911_07475 (mloB)	hypothetical protein	1.614	1.53E-05

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
308	A911_07495 (Cj1558)	membrane protein	1.803	7.06E-03
309	A911_07590 (nuoB)	NADH dehydrogenase subunit B	1.566	3.79E-03
310	A911_07595 (nuoA)	NADH dehydrogenase subunit A	1.818	8.04E-08
311	A911_07600 (Cj1580c)	peptide ABC transporter ATP-binding protein	2.278	1.95E-09
312	A911_07630 (cgb)	single domain haemoglobin	1.982	1.97E-07
313	A911_07635 (Cj1587c)	multidrug transporter membrane component/ATP-binding component	1.672	6.93E-06
314	A911_07730 (mrp)	putative ATP/GTP-binding protein	1.736	5.38E-09
315	A911_07735 (ispDF)	bifunctional 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase/2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase protein	4.044	2.94E-107
316	A911_07740 (Cj1608)	putative two-component regulator	3.02	1.99E-05
317	A911_07745 (Cj1609)	sulfate adenylyltransferase	2.449	2.16E-20
318	A911_07750 (pgpA)	putative phosphatidylglycerophosphatase	2.351	5.72E-15
319	A911_07755 (rpsT)	30S ribosomal protein S20	1.805	4.21E-32
320	A911_07765 (Cj1613c)	heme iron utilization protein	2.08	1.46E-09
321	A911_07770 (chuA)	hemin uptake system outer membrane receptor	2.33	2.23E-02
322	A911_07775 (chuB)	putative hemin uptake system permease protein	1.991	3.04E-03
323	A911_07780 (chuC)	putative hemin uptake system ATP-binding protein	3.251	2.50E-02
324	A911_07790 (Cj1618c)	putative radical SAM domain protein	1.558	1.11E-07
325	A911_07800 (mutY)	A/G-specific adenine glycosylase	1.626	8.42E-08

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj} [a]
326	A911_07815 (Cj1623)	hypothetical protein	1.681	1.94E-02
327	A911_07820 (sdaA)	L-serine dehydratase	1.89	5.42E-09
328	A911_07840 (exbB2)	exbB/tolQ family transport protein	4.336	9.59E-31
329	A911_07845 (exbD2)	biopolymer transport exbD protein	3.458	2.95E-12
330	A911_07850 (tonB2)	periplasmic TonB transport protein	2.614	1.70E-06
331	A911_07865 (Cj1633)	putative ATP-binding protein	2.032	3.31E-31
332	A911_07900 (Cj1640)	hypothetical protein	1.74	1.97E-04
333	A911_07905 (murE)	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	1.743	1.14E-22
334	A911_07910 (Cj1642)	hypothetical protein	1.582	1.62E-09
335	A911_07915 (Cj1643)	PDZ domain-containing periplasmic protein	1.563	4.84E-08
336	A911_07920 (ispA)	geranyltransterase	1.864	4.93E-17
337	A911_07925 (tkt)	transketolase	1.551	4.79E-11
338	A911_07930 (iamB)	putative ABC transport system permease	1.63	4.70E-13
339	A911_07940 (Cj1648)	ABC transporter, periplasmic substrate-binding protein, putative	1.544	1.47E-06
340	A911_07945 (Cj1649)	putative lipoprotein	1.583	1.46E-04
341	A911_07985	hypothetical protein	4.407	1.55E-07
342	A911_07990 (Cj1658)	putative FTR1 family permease	7.183	4.32E-29
343	A911_07995 (p19)	periplasmic protein p19	5.074	5.91E-33
344	A911_08000 (Cj1660)	hypothetical protein	6.46	9.01E-62

Appendix 3 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
345	A911_08005 (Cj1661)	ABC transporter permease	9.499	2.92E-29
346	A911_08010 (Cj1662)	integral membrane protein	8.947	2.76E-23
347	A911_08015 (Cj1663)	ABC transporter ATP-binding protein	5.829	5.65E-21
348	A911_08020 (Cj1664)	putative periplasmic thioredoxin	5.172	1.25E-13
349	A911_08025 (Cj1665)	putative lipoprotein thioredoxin	1.536	9.94E-05
350	A911_08090 (Cj1679)	Hypothetical protein	1.638	7.54E-08
351	A911_08120 (topA)	DNA topoisomerase I	1.73	8.73E-09
352	A911_08240 (Cj1710c)	metallo-beta-lactamase family protein	1.614	3.87E-10
353	A911_08245 (ksgA)	16S ribosomal RNA methyltransferase KsgA/Dim1 family protein	1.599	7.95E-16
354	A911_08260	small hydrophobic protein	1.601	1.62E-05
355	A911_08305 (Cj1725)	hypothetical protein	1.704	5.33E-16
356	A911_08325 (ruvC)	Holliday junction resolvase	2.683	3.75E-40
357	A911_t08352		1.600	1.54E-05
358	A911_t08374		1.643	1.94E-05
359	A911_t08376		1.618	5.38E-03
360	A911_t08410		1.589	2.33E-03

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – $p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

APPENDIX 4

Genes down-regulated in *C. jejuni* PT14 during bacteriophage CPX infection

	Gene designation	Product	Fold Change	p_{adj}[a]
1	A911_00020 (Cj0004c)	hypothetical protein	-3.435	0.00E+00
2	A911_00025 (Cj0005c)	Molybdopterin oxidoreductase family protein	-8.865	0.00E+00
3	A911_00035 (gltB)	glutamate synthase, large subunit	-2.908	1.03E-13
4	A911_00040 (gltD)	glutamate synthase subunit beta	-4.011	0.00E+00
5	A911_00050 (Cj0011c)	competence protein ComEA	-1.888	0.00E+00
6	A911_00055 (rrc)	rubrerythrin	-2.275	0.00E+00
7	A911_00195 (Cj0040)	hypothetical protein	-2.481	1.81E-04
8	A911_00200 (fliK)	hypothetical protein	-1.863	1.09E-05
9	A911_00205 (flgD)	flagellar basal body rod modification protein	-3.335	0.00E+00
10	A911_00210 (flgE2)	flagellar hook protein	-2.426	7.23E-11
11	A911_00220 (Cj0045c)	Hemerythrin-like iron-binding protein	-1.785	1.55E-05
12	A911_00330 (Cj0069)	hypothetical protein	-2.885	2.95E-12
13	A911_00410 (aspA)	aspartate ammonia-lyase	-3.054	0.00E+00
14	A911_00415 (dcuA)	anaerobic C4-dicarboxylate transporter	-2.123	0.00E+00
15	A911_00540 (pal)	peptidoglycan-associated lipoprotein Omp180	-1.672	3.47E-10
16	A911_01275 (Cj0264c)	hypothetical protein	-2.116	4.47E-07
17	A911_01280 (Cj0265c)	trimethylamine N-oxide reductase, cytochrome c-type subunit	-2.377	3.79E-10
18	A911_01610 (fdxA)	ferredoxin, 4Fe-4S	-1.696	0.00E+00
19	A911_01735 (Cj0358)	cytochrome c551 peroxidase	-4.22	0.00E+00

Appendix 4 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
20	A911_01740	hypothetical protein	-2.379	1.77E-15
21	A911_01745 (glmM)	phosphoglucosamine mutase	-1.761	3.81E-07
22	A911_01800 (Cj0371)	hypothetical protein	-2.248	1.41E-11
23	A911_01805 (Cj0372)	glutathionylspermidine synthase	-1.844	0.00E+00
24	A911_01900 (Cj0391c)	hypothetical protein	-1.655	1.13E-04
25	A911_01905 (pyk)	pyruvate kinase	-1.579	0.00E+00
26	A911_02050 (Cj0421c)	hypothetical protein	-2.015	2.77E-05
27	A911_02085 (Cj0428)	hypothetical protein	-1.613	9.21E-09
28	A911_02130 (sdhA)	succinate dehydrogenase flavoprotein subunit	-12.38	1.05E-08
29	A911_02135 (sdhB)	succinate dehydrogenase, iron-sulfur protein subunit	-11.668	0.00E+00
30	A911_02140 (sdhC)	succinate dehydrogenase subunit C	-6.765	0.00E+00
31	A911_02185 (Cj0448c)	methyl-accepting chemotaxis protein	-2.121	0.00E+00
32	A911_02190 (Cj0449c)	hypothetical protein	-2.615	0.00E+00
33	A911_02440 (Cj0499)	HIT family protein	-1.702	2.81E-03
34	A911_02445 (Cj0500)	tRNA 2-selenouridine synthase	-1.535	6.48E-08
35	A911_02450 (Cj0501)	ammonium transporter	-7.558	1.03E-05
36	A911_02570 (flgB)	flagellar basal body rod protein FlgB	-1.683	3.38E-12
37	A911_02665 (flaG)	flagellar protein FlaG	-1.655	3.58E-09
38	A911_02790 (ribA)	bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein	-1.817	7.02E-13

Appendix 4 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
39	A911_02795 (Cj0573)	putative GatB/Yqey family protein	-1.577	4.48E-05
40	A911_02910 (peb4)	major antigenic peptide PEB-cell binding factor	-1.672	0.00E+00
41	A911_02915 (fba)	fructose-bisphosphate aldolase	-1.741	6.74E-13
42	A911_02950 (Cj0604)	polyphosphate kinase	-2.625	0.00E+00
43	A911_02955 (Cj0605)	carboxypeptidase	-1.553	2.45E-04
44	A911_03270 (dcuB)	anaerobic C4-dicarboxylate transporter	-3.766	0.00E+00
45	A911_03345 (flgH)	flagellar basal body L-ring protein	-2.644	0.00E+00
46	A911_03400 (flgG2)	flagellar basal-body rod protein	-3.462	0.00E+00
47	A911_03405 (flgG)	flagellar basal body rod protein FlgG	-2.09	0.00E+00
48	A911_03490 (Cj0715)	transthyretin-like periplasmic protein	-1.535	3.14E-13
49	A911_03495 (Cj0716)	phospho-2-dehydro-3-deoxyheptonate aldolase	-1.606	0.00E+00
50	A911_03510 (Cj0719c)	hypothetical protein	-1.502	3.42E-15
51	A911_03515 (flaC)	flagellin	-2.075	0.00E+00
52	A911_03590 (Cj0735)	periplasmic protein	-1.838	7.98E-03
53	A911_03640 (Cj0747)	hypothetical protein	-5.343	2.09E-10
54	A911_03780 (napA)	nitrate reductase catalytic subunit	-1.74	0.00E+00
55	A911_03785 (napG)	quinol dehydrogenase periplasmic component	-1.693	0.00E+00
56	A911_03790 (napH)	quinol dehydrogenase membrane component	-1.581	7.56E-12
57	A911_03795 (napB)	periplasmic nitrate reductase small subunit	-1.805	0.00E+00
58	A911_03850 (Cj0794)	hypothetical protein	-1.884	2.65E-07
59	A911_03910 (dapA)	dihydrodipicolinate synthase	-1.617	0.00E+00

Appendix 4 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
60	A911_03950 (Cj0814)	hypothetical protein	-1.795	5.71E-05
61	A911_03955 (Cj0815)	hypothetical protein	-1.825	4.39E-06
62	A911_03980 (glmU)	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase	-1.839	0.00E+00
63	A911_04190 (Cj0864)	hypothetical protein	-1.84	1.88E-04
64	A911_04195 (dsbB)	disulfide oxidoreductase	-2.466	0.00E+00
65	A911_04285 (flgL)	Flagellar hook-associated protein FlgL	-1.824	1.20E-06
66	A911_04345 (thiJ)	4-methyl-5(beta-hydroxyethyl)-thiazole monophosphate synthesis protein	-1.502	4.79E-14
67	A911_04350	Small hydrophobic protein	-1.571	0.00E+00
68	A911_04365 (Cj0903c)	Putative amino-acid transport protein	-1.507	0.00E+00
69	A911_04390 (Cj0909)	hypothetical protein	-1.544	7.21E-05
70	A911_04665 (Cj0965c)	putative acyl-CoA thioester hydrolase	-1.999	0.00E+00
71	A911_04755 (hipO)	hippurate hydrolase	-2.191	1.07E-08
72	A911_04820 (Cj0999c)	hypothetical protein	-4.76	0.00E+00
73	A911_05445 (pglC)	galactosyltransferase	-1.511	2.17E-05
74	A911_05480 (gne)	UDP-glucose 4-epimerase	-1.54	0.00E+00
75	A911_05640 (Cj1164c)	hypothetical protein	-1.507	1.37E-08
76	A911_05810 (luxS)	S-ribosylhomocysteinase	-1.607	0.00E+00
77	A911_05815 (Cj1199)	2OG-Fe(II) oxygenase	-2.403	0.00E+00
78	A911_05820 (Cj1200)	putative NLPA family lipoprotein	-1.544	7.72E-05

Appendix 4 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
79	A911_05960 (htrA)	serine protease	-1.69	0.00E+00
80	A911_06030 (Cj1242)	hypothetical protein	-2.25	0.00E+00
81	A911_06400 (pseA)	flagellin modification protein, PseA	-2.003	1.72E-04
82	A911_06500 (flaB)	flagellin	-2.203	1.48E-11
83	A911_06590 (Cj1356c)	hypothetical protein	-3.641	0.00E+00
84	A911_06595 (nrfA)	Cytochrome c552	-5.856	0.00E+00
85	A911_06600 (nrfH)	putative periplasmic cytochrome C	-5.78	0.00E+00
86	A911_06765 (metC')	putative cystathionine beta-lyase, N-terminus	-1.671	1.98E-02
87	A911_06825 (gapA)	glyceraldehyde 3-phosphate dehydrogenase A	-1.53	1.86E-11
88	A911_07045 (flgI)	flagellar basal body P-ring protein	-2.279	1.77E-15
89	A911_07050 (flgJ)	hypothetical protein	-1.843	2.11E-05
90	A911_07055 (flgM)	hypothetical protein	-1.608	2.36E-07
91	A911_07060 (Cj1465)	hypothetical protein	-1.79	4.37E-10
92	A911_07065 (flgK)	flagellar hook-associated protein FlgK	-1.606	2.67E-03
93	A911_07155 (Cj1485c)	hypothetical protein	-1.737	3.20E-09
94	A911_07160 (Cj1486c)	periplasmic protein	-1.752	0.00E+00
95	A911_07165 (ccoP)	cytochrome c oxidase, cbb3-type, subunit III	-1.702	0.00E+00

Appendix 4 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
96	A911_07170 (ccoQ)	cytochrome c oxidase, cbb3-type, subunit IV	-1.756	0.00E+00
97	A911_07175 (ccoO)	cbb3-type cytochrome c oxidase subunit II	-1.599	0.00E+00
98	A911_07810 (ribD)	riboflavin-specific deaminase/reductase	-1.882	6.16E-11
99	A911_07870 (aroC)	chorismate synthase	-1.592	3.25E-04
100	A911_07950 (Cj1650)	hypothetical protein	-1.76	3.70E-06
101	A911_07980 (Cj1656c)	hypothetical protein	-1.578	3.17E-02
102	A911_08035 (Cj1668c)	hypothetical protein	-1.745	0.00E+00
103	A911_08275 (leuC)	isopropylmalate isomerase large subunit	-2.357	7.82E-13
104	A911_08280 (leuB)	3-isopropylmalate dehydrogenase	-2.806	2.10E-12
105	A911_08285 (leuA)	2-isopropylmalate synthase	-3.543	0.00E+00
106	A911_08320 (flgE)	flagellar hook protein FlgE	-2.442	4.78E-10

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – $p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

APPENDIX 5

Bacteriophage CP30A transcription during infection of *C. jejuni* PT14

	Gene designation	Product	RPKM
1	D302_gp001	putative homing endonuclease	4811.44
2	D302_gp002	putative RecA-like protein	13788.2
3	D302_gp003	hypothetical protein	5621.39
4	D302_gp004	hypothetical protein	4012.14
5	D302_gp005	hypothetical protein	4095.69
6	D302_gp006	hypothetical protein	16697.2
7	D302_gp007	DNA polymerase accessory protein sliding clamp loader subunit	15376.4
8	D302_gp008	hypothetical protein	1389.81
9	D302_gp009	hypothetical protein	6420.72
10	D302_gp010	putative dUTP pyrophosphatase	6122.67
11	D302_gp011	hypothetical protein	5449.32
12	D302_gp012	putative NAD/FAD-utilizing dehyrogenase	6675.73
13	D302_gp013	hypothetical protein	6803.97
14	D302_gp014	hypothetical protein	4805.79
15	D302_gp015	hypothetical protein	2757.96
16	D302_gp016	hypothetical protein	14118.6
17	D302_gp017	putative DNA topoisomerase	9038.05
18	D302_gp018	putative Hef-like homing endonuclease	6926.11
19	D302_gp019	topoisomerase II large subunit	8960.18
20	D302_gp020	hypothetical protein	8926.36
21	D302_gp021	hypothetical protein	5946.23
22	D302_gp022	hypothetical protein	235.89
23	D302_gp023	hypothetical protein	7608.72
24	D302_gp024	hypothetical protein	2878.06
25	D302_gp025	hypothetical protein	108.33
26	D302_gp026	hypothetical protein	1905.03
27	D302_gp027	hypothetical protein	1033.96
28	D302_gp028	putative peptidase	1541.19
29	D302_gp029	hypothetical protein	2627.29
30	D302_gp030	hypothetical protein	4071.13
31	D302_gp031	hypothetical protein	1763.17
32	D302_gp032	hypothetical protein	690.95
33	D302_gp033	hypothetical protein	952.92
34	D302_gp034	putative baseplate hub and tail lysozyme	2080.4
35	D302_gp035	hypothetical protein	491.4
36	D302_gp036	hypothetical protein	6477.61

Appendix 5 (continued)

	Gene designation	Product	RPKM
37	D302_gp037	putative Hef-like homing endonuclease	8373.99
38	D302_gp038	hypothetical protein	2652.81
39	D302_gp039	hypothetical protein	4946.73
40	D302_gp040	putative tail tube protein	8853.56
41	D302_gp041	putative tail tube protein	6640.26
42	D302_gp042	hypothetical protein	10177.1
43	D302_gp043	hypothetical protein	1484
44	D302_gp044	putative baseplate wedge subunit	2998.67
45	D302_gp045	putative baseplate wedge subunit	3496.51
46	D302_gp046	putative sliding clamp DNA polymerase accessory protein	20782.1
47	D302_gp047	aminidotransferase family protein	31076.5
48	D302_gp048	DNA primase-helicase subunit	4515.99
49	D302_gp049	hypothetical protein	281.39
50	D302_gp050	putative tail fiber protein	2937.61
51	D302_gp051	RNaseH ribonuclease	8851.63
52	D302_gp052	putative clamp loader subunit	15834.8
53	D302_gp053	putative ssDNA binding protein	52736.8
54	D302_gp054	putative Hef-like homing endonuclease	2193.24
55	D302_gp055	putative DNA end protector protein	2094.03
56	D302_gp056	hypothetical protein	4691.78
57	D302_gp057	hypothetical protein	4484.43
58	D302_gp058	hypothetical protein	8336.65
59	D302_gp059	thymidylate synthetase	4884.46
60	D302_gp060	putative Hef-like homing endonuclease	2678.26
61	D302_gp061	putative Hef-like homing endonuclease	1742.45
62	D302_gp062	tail sheath protein	17963
63	D302_gp063	hypothetical protein	3663.79
64	D302_gp064	hypothetical protein	9217.55
65	D302_gp065	DNA ligase	6095.26
66	D302_gp066	hypothetical protein	1470.91
67	D302_gp067	hypothetical protein	11047.3
68	D302_gp068	hypothetical protein	13901.6
69	D302_gp069	hypothetical protein	19142.3
70	D302_gp070	hypothetical protein	12666.6
71	D302_gp071	putative Hef-like homing endonuclease	823.35
72	D302_gp072	putative Hef-like homing endonuclease	4106.93
73	D302_gp073	ribonucleotide-diphosphate reductase subunit alpha	9594.04
74	D302_gp074	putative Hef-like homing endonuclease	6390.78

Appendix 5 (continued)

	Gene designation	Product	RPKM
75	D302_gp075	major capsid protein	72160.7
76	D302_gp076	hypothetical protein	29652
77	D302_gp077	prohead core scaffold and protease	14162.2
78	D302_gp078	hypothetical protein	6449.2
79	D302_gp079	portal vertex protein	3765.97
80	D302_gp080	hypothetical protein	17412.5
81	D302_gp081	neck protein	9324.66
82	D302_gp082	putative phage DNA packaging protein	3876.32
83	D302_gp083	hypothetical protein	7147.32
84	D302_gp084	putative tail tube protein III	6250.21
85	D302_gp085	putative Hef-like homing endonuclease	8750.53
86	D302_gp086	phosphatidylserine decarboxylase	20784.9
87	D302_gp087	putative co-chaperonin GroES	21107.4
88	D302_gp088	hypothetical protein	17641.7
89	D302_gp089	hypothetical protein	9060.75
90	D302_gp090	putative DEAD/DEAH box helicase	1558.84
91	D302_gp091	ATP-dependent DNA/RNA helicase	8235.29
92	D302_gp092	hypothetical protein	10722.3
93	D302_gp093	putative DNA repair and recombination protein	2814.4
94	D302_gp094	hypothetical protein	6030.29
95	D302_gp095	hypothetical protein	15417.2
96	D302_gp096	hypothetical protein	3486.23
97	D302_gp097	RecB family exonuclease	9989.67
98	D302_gp098	hypothetical protein	23474.7
99	D302_gp099	hypothetical protein	10297.1
100	D302_gp100	DNA polymerase	2225.96
101	D302_gp101	hypothetical protein	5251.56
102	D302_gp102	hypothetical protein	7723.93
103	D302_gp103	thymidine kinase	5212.27
104	D302_gp104	hypothetical protein	3412.75
105	D302_gp105	DNA primase subunit	10198.5
106	D302_gp106	hypothetical protein	37272.6
107	D302_gp107	hypothetical protein	18180.4
108	D302_gp108	tail completion and sheath stabilizer protein	1447.44
109	D302_gp109	hypothetical protein	5077.27
110	D302_gp110	hypothetical protein	10186.3
111	D302_gp111	hypothetical protein	18769.1
112	D302_gp112	hypothetical protein	1691.31
113	D302_gp113	hypothetical protein	1996.02

Appendix 5 (continued)

	Gene designation	Product	RPKM
114	D302_gp114	hypothetical protein	1239.57
115	D302_gp115	hypothetical protein	6755.04
116	D302_gp116	hypothetical protein	14523.9
117	D302_gp117	recombination endonuclease	4234.61
118	D302_gp118	hypothetical protein	44367.2
119	D302_gp119	putative recombination endonuclease subunit	6222.43
120	D302_gp120	sigma factor for late transcription	5227.54
121	D302_gp121	major head protein II	5414.88
122	D302_gp122	putative neck protein	4188.73
123	D302_gp123	putative Hef-like homing endonuclease	2145.02
124	D302_gp124	tail sheath stabilizer and completion protein	2708.37
125	D302_gp125	hypothetical protein	3374.61
126	D302_gp126	hypothetical protein	9031.61
127	D302_gp127	hypothetical protein	3124.71
128	D302_gp128	hypothetical protein	2711.28
129	D302_gp129	hypothetical protein	452.67
130	D302_gp130	hypothetical protein	2770.18
131	D302_gp131	hypothetical protein	6061.99
132	D302_gp132	hypothetical protein	4003.83
133	D302_gp133	hypothetical protein	5949.08
134	D302_gp134	hypothetical protein	5435.62
135	D302_gp135	hypothetical protein	3367.14
136	D302_gp136	putative exonuclease	21024.8
137	D302_gp137	hypothetical protein	5998.39
138	D302_gp138	hypothetical protein	3075.89
139	D302_gp139	hypothetical protein	4986.83
140	D302_gp140	hypothetical protein	1297.1
141	D302_gp141	hypothetical protein	1515.38
142	D302_gp142	hypothetical protein	1078.34
143	D302_gp143	putative sugar-phospahte nucleotidyltransferase	3340.54
144	D302_gp144	hypothetical protein	4337.18
145	D302_gp145	hypothetical protein	1680.82
146	D302_gp146	hypothetical protein	6722.98
147	D302_gp147	hypothetical protein	5499.88
148	D302_gp148	hypothetical protein	6215.47
149	D302_gp149	hypothetical protein	4965.93
150	D302_gp150	head completion protein	5595.21
151	D302_gp151	hypothetical protein	10340.9

Appendix 5 (continued)

	Gene designation	Product	RPKM
152	D302_gp152	hypothetical protein	5864.04
153	D302_gp153	RNA ligase	3250.16
154	D302_gp154	hypothetical protein	5701.31
155	D302_gp155	hypothetical protein	6036.37
156	D302_gp156	hypothetical protein	9963.85
157	D302_gp157	hypothetical protein	1592.82
158	D302_gp158	poly A polymerase	7496.6
159	D302_gp159	ribonucleotide reductase large subunit	8499.36
160	D302_gp160	ribonucleotide reductase small subunit	13252.5
161	D302_gp161	hypothetical protein	5797.58
162	D302_gp162	hypothetical protein	15357.2

APPENDIX 6

Bacteriophage CPX transcription during infection of *C. jejuni* PT14

	Gene designation	Product	RPKM
1	CaPhCPX_gp001	putative homing endonuclease	4811.26
2	CaPhCPX_gp002	hypothetical protein	11591.1
3	CaPhCPX_gp003	hypothetical protein	26863.6
4	CaPhCPX_gp004	hypothetical protein	6593.44
5	CaPhCPX_gp005	hypothetical protein	2208.19
6	CaPhCPX_gp006	thymidylate synthetase	4606.61
7	CaPhCPX_gp007	putative Hef-like homing endonuclease	3244.56
8	CaPhCPX_gp008	putative Hef-like homing endonuclease	1253.69
9	CaPhCPX_gp009	gp18 tail sheath protein	27307
10	CaPhCPX_gp010	hypothetical protein	5855.83
11	CaPhCPX_gp011	hypothetical protein	10586.1
12	CaPhCPX_gp012	gp30 DNA ligase	5355.69
13	CaPhCPX_gp013	hypothetical protein	1917.43
14	CaPhCPX_gp014	hypothetical protein	8301.72
15	CaPhCPX_gp015	hypothetical protein	8720.23
16	CaPhCPX_gp016	hypothetical protein	29930.6
17	CaPhCPX_gp017	hypothetical protein	22605
18	CaPhCPX_gp018	putative Hef-like homing endonuclease	2690.91
19	CaPhCPX_gp019	putative ribonucleotide-diphosphate reductase subunit alpha	9860.65
20	CaPhCPX_gp020	putative Hef-like homing endonuclease	7754.67
21	CaPhCPX_gp021	gp23 major capsid protein	96451.8
22	CaPhCPX_gp022	hypothetical protein	45544.3
23	CaPhCPX_gp023	putative gp21 prohead core scaffold and protease	16957
24	CaPhCPX_gp024	portal vertex protein	6041.46
25	CaPhCPX_gp025	hypothetical protein	35221.6
26	CaPhCPX_gp026	gp13 neck protein	20144.2
27	CaPhCPX_gp027	putative phage DNA packaging protein (terminase)	6609.25
28	CaPhCPX_gp028	hypothetical protein	12952
29	CaPhCPX_gp029	putative tail tube protein	8896.68
30	CaPhCPX_gp030	putative Hef-like homing endonuclease	6154.1
31	CaPhCPX_gp031	phosphatidylserine decarboxylase	16341.2
32	CaPhCPX_gp032	putative co-chaperonin GroES	18224.2
33	CaPhCPX_gp033	hypothetical protein	22403

Appendix 6 (continued)

	Gene designation	Product	RPKM
34	CaPhCPX_gp034	hypothetical protein	11834.9
35	CaPhCPX_gp035	putative helicase	487.69
36	CaPhCPX_gp036	putative ATP-dependent DNA/RNA helicase (uvsW)	2715.61
37	CaPhCPX_gp037	putative repair and recombination protein	730.64
38	CaPhCPX_gp038	hypothetical protein	2963.83
39	CaPhCPX_gp039	hypothetical protein	7414.31
40	CaPhCPX_gp040	hypothetical protein	1469.49
41	CaPhCPX_gp041	putative DNA methylase	1546.55
42	CaPhCPX_gp042	hypothetical protein	10170.8
43	CaPhCPX_gp043	hypothetical protein	8520.81
44	CaPhCPX_gp044	DNA polymerase	2257.11
45	CaPhCPX_gp045	hypothetical protein	1986.78
46	CaPhCPX_gp046	hypothetical protein	3138.24
47	CaPhCPX_gp047	hypothetical protein	7112.93
48	CaPhCPX_gp048	thymidine kinase	2900.37
49	CaPhCPX_gp049	DNA primase subunit	4904.84
50	CaPhCPX_gp050	hypothetical protein	18730.7
51	CaPhCPX_gp051	hypothetical protein	15585.7
52	CaPhCPX_gp052	tail completion and sheath stabilizer protein	4452.92
53	CaPhCPX_gp053	hypothetical protein	9583.85
54	CaPhCPX_gp054	hypothetical protein	28235.8
55	CaPhCPX_gp055	hypothetical protein	1742.83
56	CaPhCPX_gp056	hypothetical protein	4377.56
57	CaPhCPX_gp057	hypothetical protein	9981.04
58	CaPhCPX_gp058	hypothetical protein	2723.74
59	CaPhCPX_gp059	hypothetical protein	16415.6
60	CaPhCPX_gp060	recombination endonuclease	2854.05
61	CaPhCPX_gp061	hypothetical protein	38990.7
62	CaPhCPX_gp062	putative recombination endonuclease subunit	3770.08
63	CaPhCPX_gp063	putative sigma factor for late transcription	4505.27
64	CaPhCPX_gp064	putative gp23 major head protein II	13274.3
65	CaPhCPX_gp065	putative neck protein	8288.32

Appendix 6 (continued)

	Gene designation	Product	RPKM
66	CaPhCPX_gp066	putative Hef-like homing endonuclease	1281.51
67	CaPhCPX_gp067	tail sheath stabilizer and completion protein	4580.52
68	CaPhCPX_gp068	hypothetical protein	1777.08
69	CaPhCPX_gp069	hypothetical protein	3410.45
70	CaPhCPX_gp070	hypothetical protein	1585.38
71	CaPhCPX_gp071	hypothetical protein	935.32
72	CaPhCPX_gp072	hypothetical protein	3761.45
73	CaPhCPX_gp073	hypothetical protein	643.69
74	CaPhCPX_gp074	hypothetical protein	2870.55
75	CaPhCPX_gp075	hypothetical protein	23347.5
76	CaPhCPX_gp076	hypothetical protein	2946.51
77	CaPhCPX_gp077	hypothetical protein	5485.62
78	CaPhCPX_gp078	hypothetical protein	3334.4
79	CaPhCPX_gp079	hypothetical protein	4779.23
80	CaPhCPX_gp080	hypothetical protein	767.76
81	CaPhCPX_gp081	hypothetical protein	672.78
82	CaPhCPX_gp082	hypothetical protein	421.02
83	CaPhCPX_gp083	hypothetical protein	603.96
84	CaPhCPX_gp084	hypothetical protein	1601.03
85	CaPhCPX_gp085	hypothetical protein	1258.13
86	CaPhCPX_gp086	hypothetical protein	396.15
87	CaPhCPX_gp087	hypothetical protein	3578.38
88	CaPhCPX_gp088	hypothetical protein	3810.34
89	CaPhCPX_gp089	head completion protein	5652.44
90	CaPhCPX_gp090	hypothetical protein	13334.7
91	CaPhCPX_gp091	hypothetical protein	5735.33
92	CaPhCPX_gp092	RNA ligase	3628.88
93	CaPhCPX_gp093	hypothetical protein	2716.3
94	CaPhCPX_gp094	hypothetical protein	3864.59
95	CaPhCPX_gp095	putative poly A polymerase	4675.57
96	CaPhCPX_gp096	ribonucleotide reductase large subunit	6112.23
97	CaPhCPX_gp097	ribonucleotide reductase small subunit	10508.7
98	CaPhCPX_gp098	hypothetical protein	2679.99
99	CaPhCPX_gp099	hypothetical protein	12396.7
100	CaPhCPX_gp100	RecA-like recombination protein	11351.3
101	CaPhCPX_gp101	hypothetical protein	4989.38
102	CaPhCPX_gp102	hypothetical protein	3717.3
103	CaPhCPX_gp103	hypothetical protein	3396.94

Appendix 6 (continued)

	Gene designation	Product	RPKM
104	CaPhCPX_gp104	hypothetical protein	12861.8
105	CaPhCPX_gp105	putative DNA polymerase accessory factor (sliding clamp loader)	6334.87
106	CaPhCPX_gp106	hypothetical protein	542.68
107	CaPhCPX_gp107	putative dUTP pyrophosphatase	6521.39
108	CaPhCPX_gp108	hypothetical protein	3412.18
109	CaPhCPX_gp109	putative NAD(FAD)-utilizing dehydrogenase	3633.69
110	CaPhCPX_gp110	hypothetical protein	3151.93
111	CaPhCPX_gp111	hypothetical protein	1213.27
112	CaPhCPX_gp112	hypothetical protein	8493.78
113	CaPhCPX_gp113	putative DNA topoisomerase II	5374.85
114	CaPhCPX_gp114	putative Hef-like homing endonuclease	4796.95
115	CaPhCPX_gp115	topoisomerase II large subunit	8456.4
116	CaPhCPX_gp116	hypothetical protein	5096.35
117	CaPhCPX_gp117	hypothetical protein	5863.2
118	CaPhCPX_gp118	hypothetical protein	261.18
119	CaPhCPX_gp119	hypothetical protein	11990.2
120	CaPhCPX_gp120	hypothetical protein	5700.71
121	CaPhCPX_gp121	hypothetical protein	1529.77
122	CaPhCPX_gp122	hypothetical protein	1102.38
123	CaPhCPX_gp123	putative peptidase	2220.12
124	CaPhCPX_gp124	hypothetical protein	3366.7
125	CaPhCPX_gp125	hypothetical protein	7772.01
126	CaPhCPX_gp126	hypothetical protein	4721.41
127	CaPhCPX_gp127	hypothetical protein	1405.66
128	CaPhCPX_gp128	hypothetical protein	2003.35
129	CaPhCPX_gp129	putative baseplate hub subunit and tail	3831.9
130	CaPhCPX_gp130	hypothetical protein	421.97
131	CaPhCPX_gp131	hypothetical protein	5086.91
132	CaPhCPX_gp132	putative Hef-like homing endonuclease	7652.2
133	CaPhCPX_gp133	hypothetical protein	1566.19
134	CaPhCPX_gp134	hypothetical protein	7122.74
135	CaPhCPX_gp135	putative tail tube protein	17828.4
136	CaPhCPX_gp136	putative tail tube protein	10268.8
137	CaPhCPX_gp137	hypothetical protein	20861.4
138	CaPhCPX_gp138	hypothetical protein	2031.57
139	CaPhCPX_gp139	putative baseplate wedge protein	4600.27
140	CaPhCPX_gp140	putative sliding clamp DNA polymerase accessory protein	13716.1
141	CaPhCPX_gp141	amidinotransferase family protein	27660.8
142	CaPhCPX_gp142	DNA primase-helicase subunit	3230.93

Appendix 6 (continued)

	Gene designation	Product	RPKM
143	CaPhCPX_gp143	hypothetical protein	159.24
144	CaPhCPX_gp144	hypothetical protein	1062.04
145	CaPhCPX_gp145	hypothetical protein	2964.94
146	CaPhCPX_gp146	hypothetical protein	8543.7
147	CaPhCPX_gp147	RNaseH	5471.79
148	CaPhCPX_gp148	hypothetical protein	8482.53
149	CaPhCPX_gp149	putative ssDNA binding protein lysozyme	41991.4

APPENDIX 7

Genes up-regulated in *C. jejuni* PT14 in response to PT14Δ00150::kan mutation

	Gene designation	Product	Fold Change	p_{adj}[a]
1	A911_00035 (gltB)	Glutamate synthase, large subunit	1.682	8.29E-04
2	A911_00040 (gltD)	Glutamate synthase subunit beta	1.517	3.94E-05
3	A911_01165 (Cj0241c)	Putative iron-binding protein	1.637	9.63E-05
4	A911_02485 (clp)	ATP-dependent Clp protease ATP-binding subunit	1.623	1.11E-04
5	A911_03250 (Cj0667)	S4 domain-containing protein	1.877	1.26E-03
6	A911_03255 (Cj0668)	ATP/GTP-binding protein	1.824	2.12E-09
7	A911_03260 (Cj0669)	ABC transporter ATP-binding protein	1.584	5.18E-06
8	A911_03600 (Cj0737)	Putative hemagglutination activity domain containing protein	1.952	4.37E-02
9	A911_03665 (hrcA)	Heat-inducible transcription repressor	2.039	1.49E-02
10	A911_03670 (grpE)	Heat shock protein GrpE	2.381	2.31E-103
11	A911_03675 (dnaK)	Molecular chaperone DnaK	2.002	1.53E-02
12	A911_03680 (Cj0760)	Hypothetical protein	1.729	6.29E-03
13	A911_04375 (alr)	Alanine racemase	1.65	1.32E-05
14	A911_05550 (Cj1145c)	Hypothetical protein	1.633	1.05E-04
15	A911_05665 (Cj1169c)	Hypothetical protein	1.544	8.31E-20
16	A911_05670 (omp50)	Hypothetical protein	1.661	6.67E-33
17	A911_05965 (cbpA)	Co-chaperone protein DnaJ	1.756	9.75E-04
18	A911_0771 (hisF)	imidazole glycerol phosphate synthase subunit HisF	1.656	4.14E-02
19	A911_07810 (ribD)	Riboflavin-specific deaminase/reductase	1.727	2.61E-02

Appendix 7 (continued)

	Gene designation	Product	Fold Change	p_{adj}[a]
20	A911_08130 (secY)	Preprotein translocase subunit SecY	1.698	3.17E-08
21	A911_08135 (rplO)	50S ribosomal protein L15	1.886	2.78E-22
22	A911_08140 (rpsE)	30S ribosomal protein S5	1.863	6.12E-11
23	A911_08145 (rplR)	50S ribosomal protein L18	1.641	4.18E-09
24	A911_08150 (rplF)	50S ribosomal protein L6	1.535	6.47E-05
25	A911_08170 (rplX)	50S ribosomal protein L24	1.632	3.91E-09
26	A911_08175 (rplN)	50S ribosomal protein L14	1.526	1.64E-16
27	A911_08215 (rplW)	50S ribosomal protein L23	1.639	1.17E-05
28	A911_08220 (rplD)	50S ribosomal protein L4	1.681	1.07E-13
29	A911_08225 (rplC)	50S ribosomal protein L3	1.644	3.06E-03

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – $p_{adj}<0.05$ – corrected p-value representing a false discovery rate of <5%

APPENDIX 8

Genes down-regulated in *C. jejuni* PT14 in response to PT14Δ00150::kan mutation

	Gene designation	Product	Fold Change	p_{adj}[a]
1	A911_00155 (Cj0033)	Putative integral membrane protein	-15.704	0.00E+00
2	A911_00160	Hypothetical protein	-8.301	0.00E+00
3	A911_00170 (Cj0035c)	Bcr/CflA subfamily drug resistance transporter	-1.67	1.25E-02
4	A911_00195 (Cj0040)	Hypothetical protein	-20.63	0.00E+00
5	A911_00200 (flik)	Hypothetical protein	-11.498	0.00E+00
6	A911_00205 (flgD)	Flagellar basal body rod modification protein	-13.954	0.00E+00
7	A911_00210 (flgE2)	Flagellar hook protein	-8.821	4.46E-12
8	A911_00220 (Cj0045c)	Hemerythrin-like iron-binding protein	-1.776	4.66E-03
9	A911_01170 (Cj0243c)	Hypothetical protein	-2.415	3.03E-02
10	A911_01670 (trpD)	Anthranilate synthase component I	-1.569	1.76E-04
11	A911_01675 (trpE)	Anthranilate synthase component II	-1.587	2.22E-10
12	A911_01900 (Cj0391c)	Hypothetical protein	-1.543	2.51E-04
13	A911_02085 (Cj0428)	Hypothetical protein	-1.754	4.68E-09
14	A911_02570 (flgB)	Flagellar basal body rod protein FlgB	-2.26	1.53E-14
15	A911_02665 (flaG)	Flagellar protein FlaG	-1.51	9.37E-04
16	A911_03190 (Cj0653c)	M24 family peptidase	-1.828	0.00E+00
17	A911_03195 (Cj0654c)	Di-/tripeptide transporter	-1.908	0.00E+00
18	A911_03345 (flgH)	Flagellar basal body L-ring protein	-4.099	1.53E-14
19	A911_03400 (flgG2)	Flagellar basal-body rod protein	-2.963	5.83E-12

Appendix 8 continued

	Gene designation	Product	Fold Change	p_{adj}[a]
20	A911_03405 (flgG)	Flagellar basal body rod protein FlgG	-2.123	0.00E+00
21	A911_03850 (Cj0794)	Hypothetical protein	-2.378	4.41E-07
22	A911_03950 (Cj0814)	Hypothetical protein	-2.555	1.01E-03
23	A911_04285 (flgL)	Flagellar hook-associated protein FlgL	-3.836	3.30E-11
24	A911_04425 (Cj0916c)	Hypothetical protein	-1.639	3.71E-06
25	A911_04715 (Cj0977)	Hypothetical protein	-1.615	0.00E+00
26	A911_06030 (Cj1242)	Hypothetical protein	-7.689	3.03E-04
27	A911_06500 (flaB)	Flagellin B	-9.319	0.00E+00
28	A911_06907 (Cj1422c)	Putative sugar transferase	-1.982	7.96E-10
29	A911_07045 (flgI)	Flagellar basal body P-ring protein	-3.535	0.00E+00
30	A911_07050 (flgJ)	Hypothetical protein	-3.425	2.39E-11
31	A911_07060 (Cj1465)	Hypothetical protein	-1.691	1.44E-05
32	A911_07065 (flgK)	Flagellar hook-associated protein FlgK	-3.082	4.16E-11
33	A911_07240 (putA)	Bifunctional putA protein	-1.646	0.00E+00
34	A911_07400 (acs)	Acetyl-CoA synthetase	-1.51	7.35E-04
35	A911_07950 (Cj1650)	Hypothetical protein	-3.084	0.00E+00
36	A911_08320 (flgE)	Flagellar hook protein flgE	-18.171	0.00E+00

Note: The corresponding gene locus for *C. jejuni* NCTC11168 is illustrated within the parentheses.

a – padj<0.05 – corrected p-value representing a false discovery rate of <5