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UNITED KINGDOM • CHINA • MALAYSIA

FILAMENTATION OF *CAMPYLOBACTER*

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ABSTRACT

The bacterial pathogen *Campylobacter jejuni is* a leading cause of foodborne gastroenteritis worldwide. Consumption of contaminated poultry meat is considered a major source of infection. Changes in cell morphology were demonstrated to occur spontaneously on entry in to stationary phase, with development of filamentous cells amongst short spiral morphotypes. The aim of this study was to investigate differences between the morphotypes of *C. jejuni* and *C. coli* and gain insights into their development. Using a minimal culture medium filament formation was observed to be wide spread amongst *C. jejuni* strains tested but was not universal in *C. coli strains*. Filamentation did not appear to arise due to the release of diffusible molecules or the accumulation of either toxic metabolites or oxidative stressors in the medium. Separated filaments exhibited greater intracellular ATP contents compared to spiral cells, and were able to survive longer in water at 4 and 37 C. *C. jejuni* 12661 was identified as producing long filaments but genome sequence analysis provided no clear explanation for the enhanced filament formation.

Using RNA-Seq, transcriptome differences were examined between cells growing in exponential phase and separated cell morphotypes recovered from stationary phase cultures of the *C. jejuni* strains 12661 and PT14. These studies identified profound transcriptional differences between the cell morphotypes present at stationary phase, and highlighted problems of interpreting such data without separation of these sub-populations. Stationary phase cells of either morphotype were impaired in motility,

which likely resulted from down-regulation of *rpoN* encoding sigma factor 54, and several key motility associated genes. The *spoT* gene of *C. jejuni* mediates the synthesis of ppp(G)pp as part of the stringent response to stress. The *spoT* gene was differentially regulated in the stationary phase morphotypes recovered in this study, as were the genes encoding the phosphohydrolases PPX1/PPX2 and polyphosphate kinases PPK1/PPK2 that control cellular (p) pp(G)pp pools. Prominent heat-shock and oxidative stress responses were evident in stationary phase cells compared to cells in exponential growth phase but transcription of the ribosomal proteins was not down-shifted. The transcript levels of several cell division associated genes were down-regulated in stationary phase spiral and filamentous cells. The formation of long filamentous cell morphotypes by *C. jejuni* 12661 corresponds with reduced expression of *maf* (inhibitor of septum formation), *mreB* (actin-like rod-shape determining protein), *mreC* (rod-shape determining protein), *parA*, *parB* (chromosome partitioning proteins), *ftsA* (actin-like function in cytokinesis), *ftsH* (ATP-dependent zinc metallopeptidase), *ftsW* (lipid II-linked peptidoglycan transporter) and *ftsZ* (tubulin-like z-ring formation) that collectively function in septum formation between daughter cells.

Conferences, workshopes and courses

1. Nacheervan M. Ghaffar, Phillippa L. Connerton, and Ian F. Connerton, 2015. Filamentation of *Campylobacter* in broth cultures. 2nd Midlands Molecular Microbiology Meeting. Nottingham University, UK. September 14th and 15th 2015 (Poster presentation).
2. The 2nd International Scientific Conference for Kurdistan students, Nottingham University, UK, 15th September 2014 (Attendance).
3. Post-genomics and bioinformatics, Nottingham University, UK on the 16, 17 and 18/12/2014 (Attendance).
4. Personal Skills for the Working Environment: A Conference for Postgraduate Students, Nottingham University, UK, 21st January 2014. (Attendance).
5. Society for general microbiology (SGM) and society for applied microbiology (SfAM) policy workshop, 2014. Nottingham, UK.
6. Nacheervan M. Ghaffar and Ian F. Connerton, 2013. Viability of *C. jejuni* Under Oxidative Stress Conditions at Different Temperature. The 1st International Scientific Conference for Kurdistan students, Nottingham University, UK 16th September 2013 (Poster presentation).
7. The basic laboratory technique Module and pass the test. Nottingham university,UK
8. Attend some usefull short courses, Nottingham university,UK
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1 . Introduction

1.1 *Campylobacter*

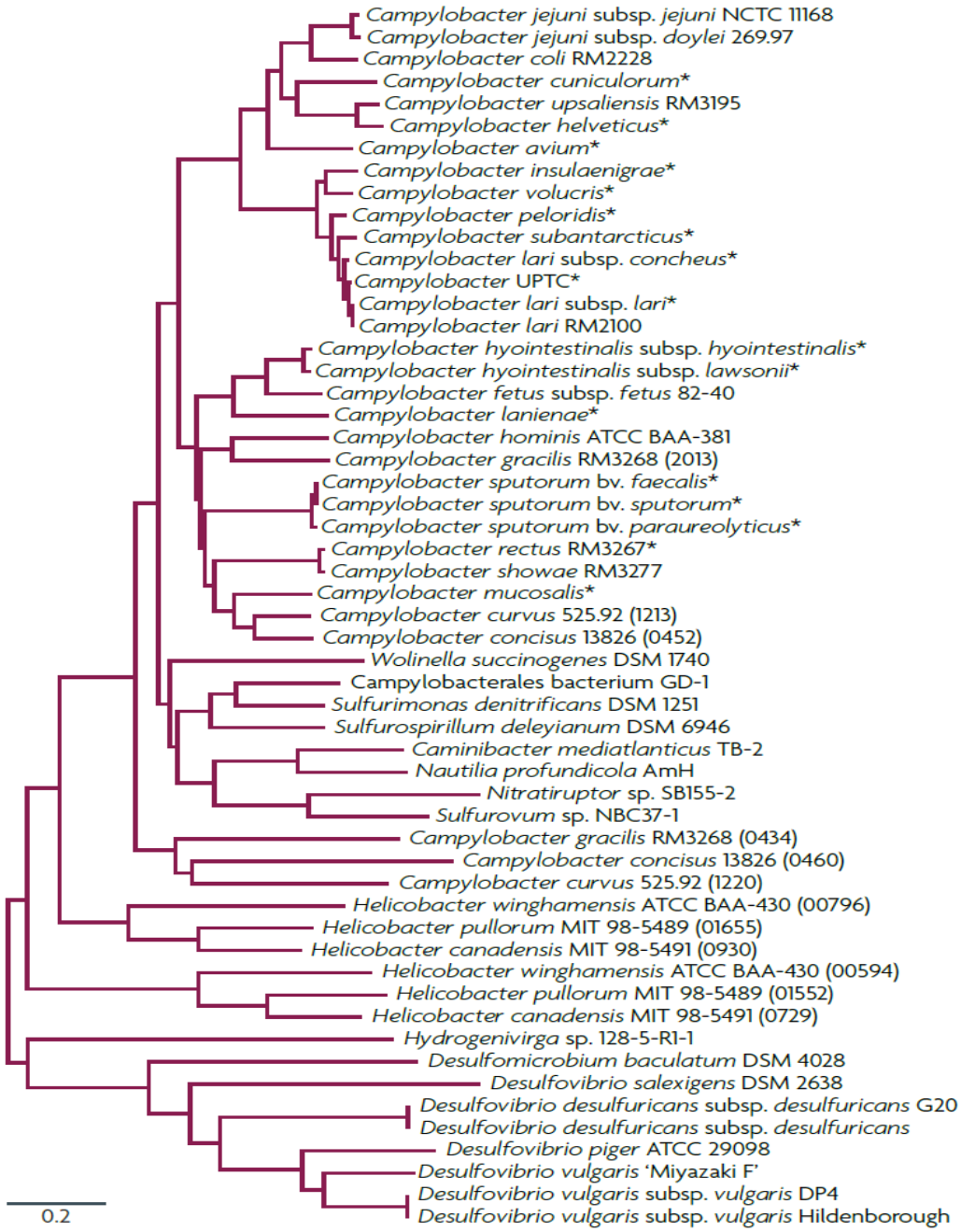
1.1.1 Early history overview

The first description of *Campylobacter* was probably when bacteria described as *Vibrio*-like spiral shaped organisms, were recovered from the stools of children who had died from diarrhoeal disease (Escherich, 1886). Spiral shaped bacteria were later observed from aborted bovine foetuses and were named "*Vibrio fetus",* because of their morphology and source (McFadyean and Stockman, 1913, Smith et al., 1919). "*V. jejuni "* isolated from the jejunum of calves (Jones et al., 1931) and later "*V. coli* " isolated from pigs (Doyle, 1944) were described following this nomenclature. The first well documented "*vibrio*-related" case of human infection was identified during a diarrhoeal outbreak caused by contaminated milk (Levy, 1946), where organisms similar to "*V. jejuni* " were recovered from blood cultures. These organisms were probably *Campylobacter* spp (Kist, 1985). Isolates of "*V. fetus"* that grew *at* 42°C rather than 37°C, were first identified in 1957 (King, 1957) and were referred to as "related vibrio". The genus *Vibrio* underwent a major taxonomic revision in 1963, with the description of a new genus, *Campylobacter* Greek for "curved rod" (Sebald and Véron, 1963). "*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. It was not until 1972 that *Campylobacter* was recognised as a human pathogen after successful isolation from human faeces using a filtration technique (Dekeyser et al., 1972). This was followed by the development of a suitable selective

medium which enabled similar organisms to be isolated from human faeces (Skirrow, 1977). Partial 16S sequences of *Campylobacter* spp. were compared a decade later (Lau et al., 1987) to generate the first phylogenetic data. A revision of *Campylobacter* taxonomy was suggested by Goodwin et al. (1989) in which genotypic and phenotypic arguments were summarised to eliminate the gastric species *C. pylori* and *C. mustelae* from the genera *Campylobacter* and *Wolinella*, and a new genus called *Helicobacter* was suggested to accommodate these.

1.1.2 *Campylobacter* taxonomy and physiology

The *Campylobacter*aceae family includes three closely related genera, *Campylobacter*, *Arcobacter* and *Sulfospirillum*. The genus *Campylobacter* was first proposed by Sebald and Véron (1963) and involved only two species, previously categorised as *Vibrio* spp. Figure 1 shows the members of this family along with their phylogenetic relationships based protein glycosylation B (Pglb) sequences of all sequenced deltaproteobacteria and epsilonproteobacteria (Nothaft and Szymanski, 2010). 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 there are presently 26 species of *Campylobacter* and 9 sub species (On, 2013, Koziel et al., 2014, Fitzgerald et al., 2014, Gilbert et al., 2015) , with the type species being *C. fetus.* Many members of the *Campylobacter* genus have been implicated in human disease. However, the two species *C. jejuni and C. coli are* recognised as the foremost causative agents of human *Campylobacter* infections.



** New species are being described all the time

Figure 1-1: Phylogenetic tree comparing protein glycosylation b (Pglb) Sequences of all sequenced deltaproteobacteria and epsilonproteo bacteria.

(Nothaft and Szymanski, 2010)

Members of the family *Campylobacter*icae are, Gram-negative, curved, S-shaped, or spiral rods, 0.2 to 0.8 μm wide and 0.5 to 5 μm long (Vandamme, 2000). . Most *Campylobacter* species are typically motile, with a characteristic corkscrew-like motion achieved by a single polar unsheathed flagellum at one or both ends of the cell (Vandamme, 2000). *Campylobacter* cannot produce energy from carbohydrates but utilises amino acids or tricarboxylic acid cycle intermediates, whilst carbohydrates are neither fermented nor oxidised (Debruyne et al., 2008). In general, *Campylobacter* and *Arcobacter* cells only grow under microaerobic conditions, with low levels of oxygen (5%) and it has shown that the metabolism of these bacteria is respiratory and chemoorganotrophic (Fernandez et al., 1985, Debruyne et al., 2008).

The majority of *Campylobacter* species cannot grow at temperatures below 30°C or above 45°C. *C. jejuni, C. coli* and *C. lari* are described as thermophilic *Campylobacter*s because they have the ability to grow at temperatures above 37°C and grow well at 42°C. Members of the *Campylobacter* genus are indole-negative and are able to reduce nitrate, with the exception of *C. jejuni subsp*. *doylei*. They are also generally oxidase-positive, with the exception of *C. gracilis*, and unable to hydrolyse hippurate, with the exception of *C. jejuni* which possesses the gene encoding hippurate hydrolase, *hipO* (Mohammed et al., 2004). The permissive pH for *Campylobacter* growth extends from 4.9 to 9.0 with the ideal between pH 6.5 and 7.5 (ICMSF, 1996).

1.2 Morphological adaptation of bacteria in response to different stress conditions

Bacteria have developed complex systems to maintain consistent cell morphologies. However, in some cases, bacteria change this highly regulated process to transform into filamentous organisms. Accumulating evidence attributes important biological roles to filamentation in stressful environments, including, the sites of interaction between pathogenic bacteria and their hosts. Filamentation could represent an intended response to specific environmental cues that promote survival within the threats of consumption and killing (Justice et al., 2008). Filamentation has been implicated in bacterial survival during exposure to environmental stresses, such as host effectors, protist predators and antimicrobial therapies (Justice et al., 2008).

The typical size and shape of bacterial cells is dependent on numerous variables that are unique to the particular environments that are associated with the lifestyle of each species (Young, 2006). Bacteria have retained genes that encode proteins that are dedicated to the purposeful alteration of the overall bacterial cell length under certain conditions (Harry et al., 2006). There are many conditions that lead to bacterial filamentation, including metabolic changes and DNA damage (Rothfield et al., 1999).

Pathogenic bacterial filaments occur due to subversion of innate immunity in vivo, such as in *Proteus mirabilis* which is an opportunistic urinary pathogen that forms elongated, filamentous cells in response of

flagellar contact with solid surfaces, such as urinary catheter materials (Belas and Suvanasuthi, 2005). It is unknown whether the observed filamentous morphology reflects bacterial responses to intracellular stress, nutrient limitation or inhibited metabolic pathways. The survival of *S*. *typhimurium* within intracellular compartments of macrophages *in vitro* is associated with a filamentous phenotype. Cell-division inhibition occurs in response to macrophage production of nitric oxide radicals, which suggests the SOS response (Rosenberger and Finlay, 2002).

In addition to resistance to biological stresses that are native to the host, filamentation may also contribute to a pathogen's resistance to antimicrobial agents, which has obvious implications for therapeutics. Filamentous organisms are commonly observed in clinical specimens taken from patients who are undergoing antibiotic treatment. Miller et al. (2004b) reported that exposure to β-lactam antibiotics, induces the SOS response in *E. coli* . Exposure of *B. pseudomallei* to levels of ceftazidime resulted in the appearance of filamentous bacteria that remained viable within the vacuoles of immortalized human macrophages. By Contrast, the bacillary form was susceptible to killing under the same experimental conditions (Chen et al., 2005). Thus, the developmental changes that accompany filamentation may provide additional survival advantages.

Or may be due to other environmental factor, such as Prolonged growth of *C. crescentus* results in a decrease in the FtsZ concentration, which leads to the formation of long, helical, filamentous organisms that have acquired resistance to heat, oxidative stress and changes in alkalinity

(Wortinger et al., 1998). However, it is found that the filamentation in *C. jejuni* appears when enters to stationary phase (Griffith, 1993) or under the hyperosmotic stress condition (Cameron, 2012). *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).

1.3 Epidemiology and occurrence in human population

Many studies suggest that *Campylobacters* may be considered as the most common bacteria causing human enteritis in the industrialised world (Tauxe, 1992). In 2007 and 2008 the incidence of these bacteria in Europe was 45.2/100 000 and 40.7/100, 000 of inhabitants, respectively (EFSA, 2009, EFSA, 2010a). The reported incidences of confirmed cases are significantly different between different countries. However, it is difficult to make a direct comparison of *Campylobacter* incidence due to the wide variety of monitoring and reporting methodologies in use. In Europe, generally the majority of *Campylobacter* cases were of local origin. Similarly, in Spain, Malta, Slovakia, Czech Republic and Hungary 99-100% of the identified cases were of national origin. In other countries such as Finland, Sweden and Norway the largest proportion of cases (51-70%) were described as imported cases acquired whilst travelling abroad (EFSA, 2009, EFSA, 2010a). Countries in Northern Europe that have free health care and good laboratory support, report the highest incidence of *Campylobacter*iosis, ranging from 50 to 90 cases per 100,000 habitants per year (Friedman et al., 2000). A study undertaken in England in 1999 to detect the causative agents in

infectious diarrhoea found that *Campylobacter* species was the most common organism isolated, which accounted for 12.2% of 2893 cases (Tompkins et al., 1999). A similar epidemiological picture was reported in Scotland, where *Campylobacter* species were also the most frequently reported pathogens (Noone et al., 2000). Public Health England (2013) has reported more than 600,000 of *Campylobacter* laboratory confirmed cases in England and Wales in patients with enteric and non-enteric infections during the period between 2000-2012 with a peak in 2012 **(Figure 1-2)**.

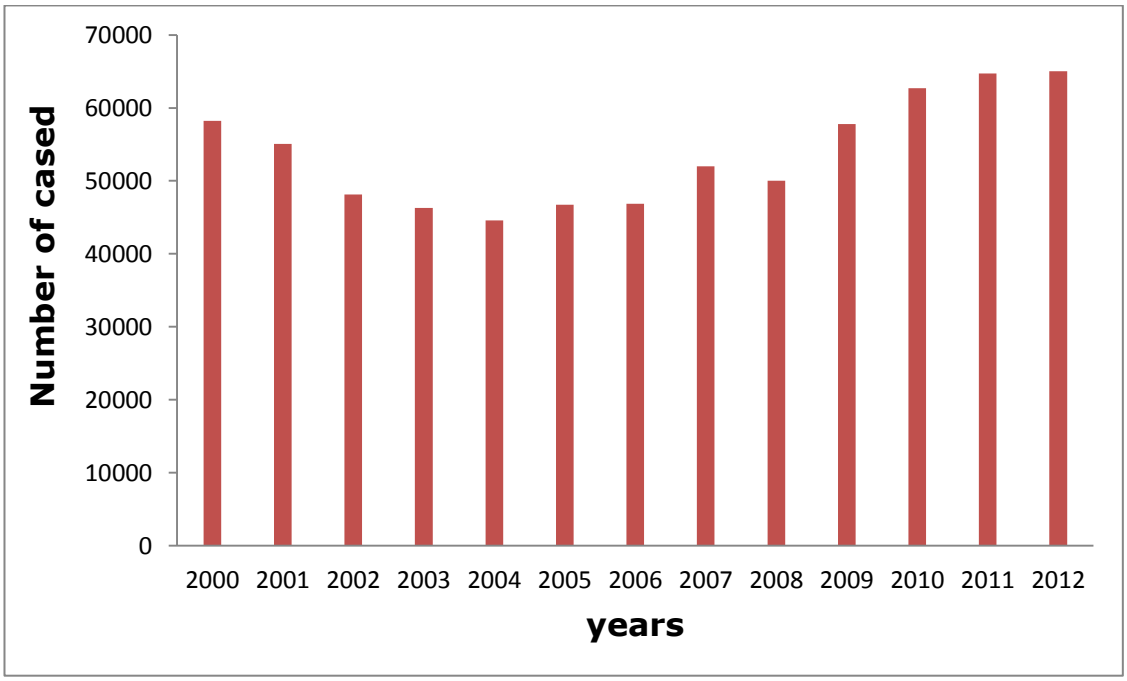


Figure 1-2: Laboratory reports of *Campylobacter* spp infections by year in England and Wales, 2000-2012.

Campylobacter spp cases reported to the Public Health England for England and Wales. It includes patients with enteric and non-enteric infections and includes isolates from all body sites (Public Health England, 2013).

It is generally believed that the number of reported cases is an underestimate of the total disease burden. For example, within England

and Wales over 45,000 of laboratory-confirmed *Campylobacter*iosis cases per annum, was estimated to represent a disease burden of 500,000 cases (Tam et al., 2006). In the United States, it has been recognized that 2 million people were infected with *Campylobacter* species each year during the period 1996-1999 (Samuel MC et al., 2004). Generally, 80-90% of *Campylobacter* infections are thought to be due to *C. jejuni* in industrialised countries and 5-10% to *C. coli* when the diagnosis is achieved only on selective media (Fitzgerald et al., 2008). In Europe *C. jejuni has* been reported as the most frequent *Campylobacter* species accounting for 40-44% of the isolates, whereas just 2-3% was *C. coli* and other *Campylobacter* species accounted for 7-9% of the total. The remaining 46-49% was not characterised to species level (EFSA, 2009, EFSA, 2010a). In the United Kingdom as a whole, there is growing evidence indicate that *C. jejuni accounts* for about 93% of the cases of *Campylobacteriosis*, with *C. coli constituting* 7%, *C. lari* <1%, and *C. fetus* <1% (Gillespie et al., 2002, Workman et al., 2006). The number of reported *Campylobacter* cases in most countries exhibits a steady increase **(Table1.1)**.

Table 1-1: Reported cases and notification rates per 100,000 of human *Campylobacter*iosis in the EU/EEA, 2009–2013.

(EFSA, 2015)

	2013					2012		2011		2010		2009	
	National Coverage[a]	Data Format[a]	Total Cases	Confirmed Cases & Rates		Confirmed Cases & Rates		Confirmed Cases & Rates		Confirmed Cases & Rates		Confirmed Cases & Rates	
				Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate
Austria	Y	C	5726	5726	67.7	4710	56.0	5129	61.0	4404	52.6	4502	53.9
Belgium[b]	N	C	8148	8148	-	6607	-	7716	-	6047	-	5697	-
Bulgaria	Y	A	124	124	1.7	97	1.3	73	1.0	6	0.1	26	0.3
Croatia[c]	Y	A	1379	-	-	-	-	-	-	-	-	-	-
Cyprus	Y	C	56	56	6.5	68	7.9	62	7.4	55	6.7	37	4.6
Czech Republic	Y	C	18389	18267	173.7	18287	174.1	18743	178.7	21075	201.5	20259	194.3
Denmark	Y	C	3772	3772	67.3	3720	66.7	4060	73.0	4037	72.9	3353	60.8
Estonia	Y	C	385	382	28.9	268	20.2	214	16.1	197	14.8	170	12.7
Finland	Y	C	4066	4066	74.9	4251	78.7	4267	79.4	3944	73.7	4050	76.0
France[d]	N	C	5198	5198	39.6	5079	38.9	5538	42.6	4324	33.5	3956	30.7
Germany	Y	C	63636	63271	77.3	62504	76.5	70812	86.8	65110	79.8	62787	76.7
Greece[e]	-	-	-	-	-	-	-	-	-	-	-	-	-
Hungary	Y	C	7250	7247	73.5	6367	64.4	6121	62.4	7180	72.9	6579	66.6
Ireland	Y	C	2288	2288	49.8	2391	52.2	2433	53.2	1660	36.5	1810	40.0
Italy[b]	N	C	1178	1178	-	774	-	468	-	457	-	531	-
Latvia	Y	C	9	9	0.4	8	0.4	7	0.3	1	0.0	0	0.0
Lithuania	Y	C	1142	1139	38.3	917	30.5	1124	36.8	1095	34.9	812	25.5
Luxembourg	Y	C	675	675	125.7	581	110.7	704	137.5	600	119.5	523	106.0
Malta	Y	C	246	246	58.4	220	52.7	220	53.0	204	49.3	132	32.1
Netherlands[f]	N	C	4182	3702	42.4	4248	48.8	4408	50.9	4322	50.1	3782	44.1
Poland	Y	C	552	552	1.4	431	1.1	354	0.9	367	1.0	359	0.9
Portugal[e]	-	-	-	-	-	-	-	-	-	-	-	-	-
Romania	Y	C	218	218	1.1	92	0.5	149	0.7	175	0.9	254	1.3
Slovakia	Y	C	5953	5845	108.0	5704	105.5	4565	84.7	4476	83.0	3813	70.8
Slovenia	Y	C	1027	1027	49.9	983	47.8	998	48.7	1022	49.9	952	46.8
Spain[g]	N	C	7064	7064	50.4	5548	47.4	5469	46.9	6340	54.6	5106	44.2
Sweden	Y	C	8114	8114	84.9	7901	83.3	8214	87.2	8001	85.7	7178	77.5
United Kingdom	Y	C	66465	66465	104.0	72560	114.3	72150	115.3	70298	113.2	65043	105.5
EU Total	-	-	**217242**	**214779**	**64.8**	**214316**	**65.9**	**223998**	**69.0**	**215397**	**67.0**	**201711**	**62.8**
Iceland	Y	C	101	101	31.4	60	18.8	123	38.6	55	17.3	74	23.2
Liechtenstein	-	-	-	-	-	-	-	-	-	-	-	-	-
Norway	Y	C	3291	3291	65.2	2933	58.8	3005	61.1	2682	55.2	2848	59.3
Switzerland[h]	Y	C	7481	7481	93.1	8432	106.0	7963	101.2	6611	84.9	7803	101.3

(a): Y, yes; N, no; A, aggregated data; C, case-based data;-, no report.
(b): Sentinel surveillance; no information on estimated coverage. Thus, notification rate cannot be estimated.
(c): All cases of unknown case classification.
(d): Sentinel surveillance; notification rates calculated based on an estimated coverage of 20 %.
(e): No surveillance system.
(f): Sentinel surveillance; notification rates calculated based on an estimated coverage of 52 %.
(g): Sentinel surveillance; notification rates calculated based on an estimated coverage of 30 % in 2013 and 25 % in 2009-2012.
(h): Switzerland provided data directly to EFSA.

Variation of incidence and the number of confirmed cases from different countries or areas within the same country may be significantly different (Kaakoush et al., 2015) **(Figure 1.3).**

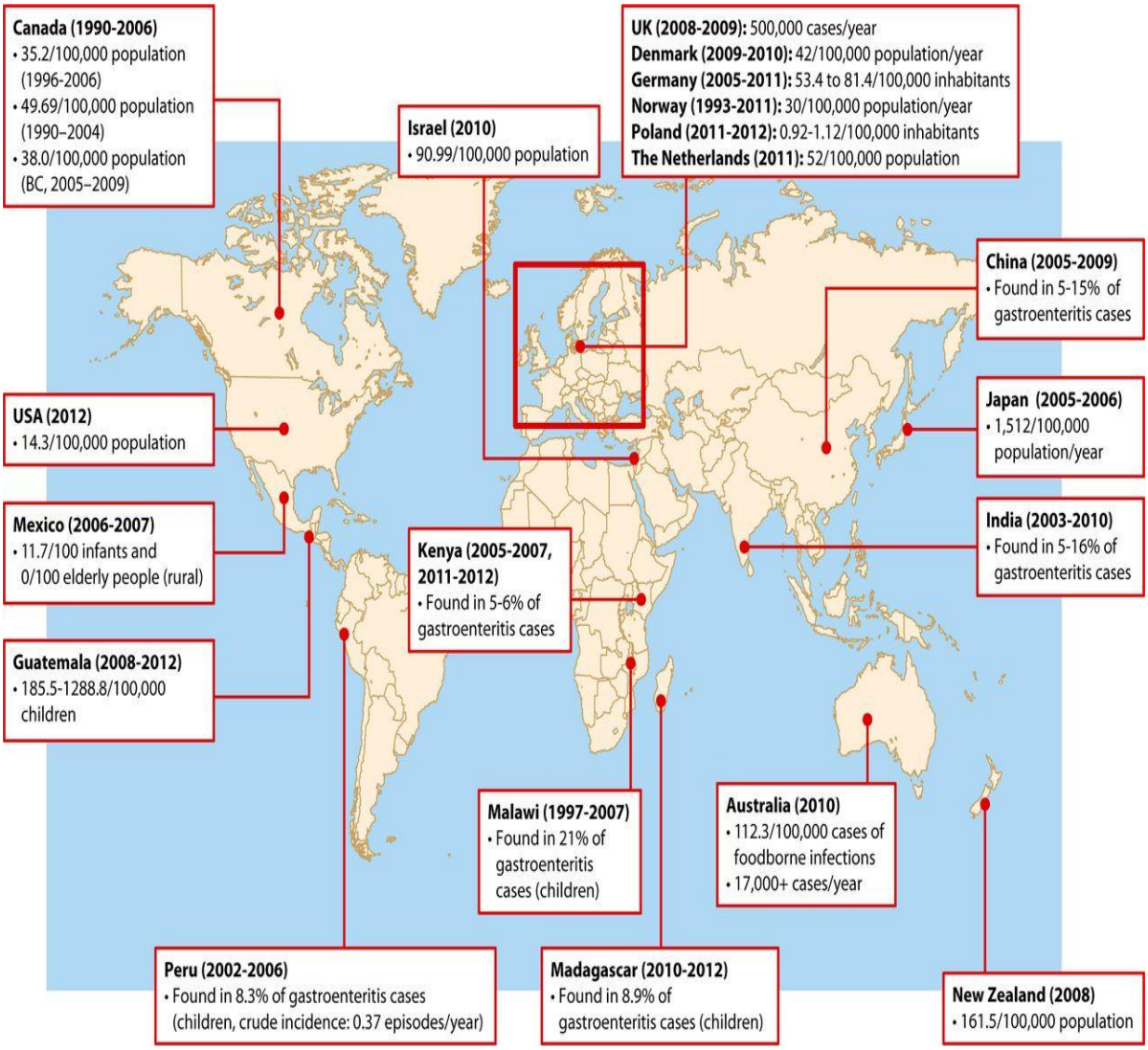


Figure 1-3: Incidence and prevalence of *Campylobacter*iosis (*C. jejuni /C. coli*), the latest information on the global epidemiology of *Campylobacter*iosis.

(Map adapted from an image from Wikimedia Commons [http: //commons. wikimedia. Org/ wiki / File: A_ large _ blank_ world_ map_ with_ oceans_ marked_ in_ blue.PNG]. (The Figure was taken from Kaakoush et al., 2015)

Various factors could explain the increase in infection rates observed from the 1980s to the 1990s, such as increased physician consciousness, an increase in the number of diagnostic laboratories and improved detection methods, whereas the increase detected since the 1990s most likely suggests a true increase in the number of infections (Friedman et al., 2000).

In industrialized countries the following patterns have been observed: 1. The incidence of *Campylobacter* is higher in males than females, which may be partially due to a sex-specific behaviour (Friedman et al., 2000); 2. Seasonal effects on *Campylobacter* distribution with the highest numbers of cases reported in summer months (Friedman et al., 2000, Nylen et al., 2002); 3. *Campylobacter* infects all age groups but the highest incidences have a bimodal age distribution with children younger than 5 years of age followed by young adults of 20-40 years of age (ECDC, 2009, Nylen et al., 2002, Samuel MC et al., 2004). Whilst, most of *Campylobacter*iosis cases are sporadic that affect one or few people. Outbreaks of *Campylobacter* species are not uncommon.

Since 2007, the numbers of individuals reported in published outbreaks of *Campylobacter*iosis have ranged from 10 to more than 100 and have associated with incidents and environmental sources of infection **(Figure 1.4)** (Kaakoush et al., 2015).

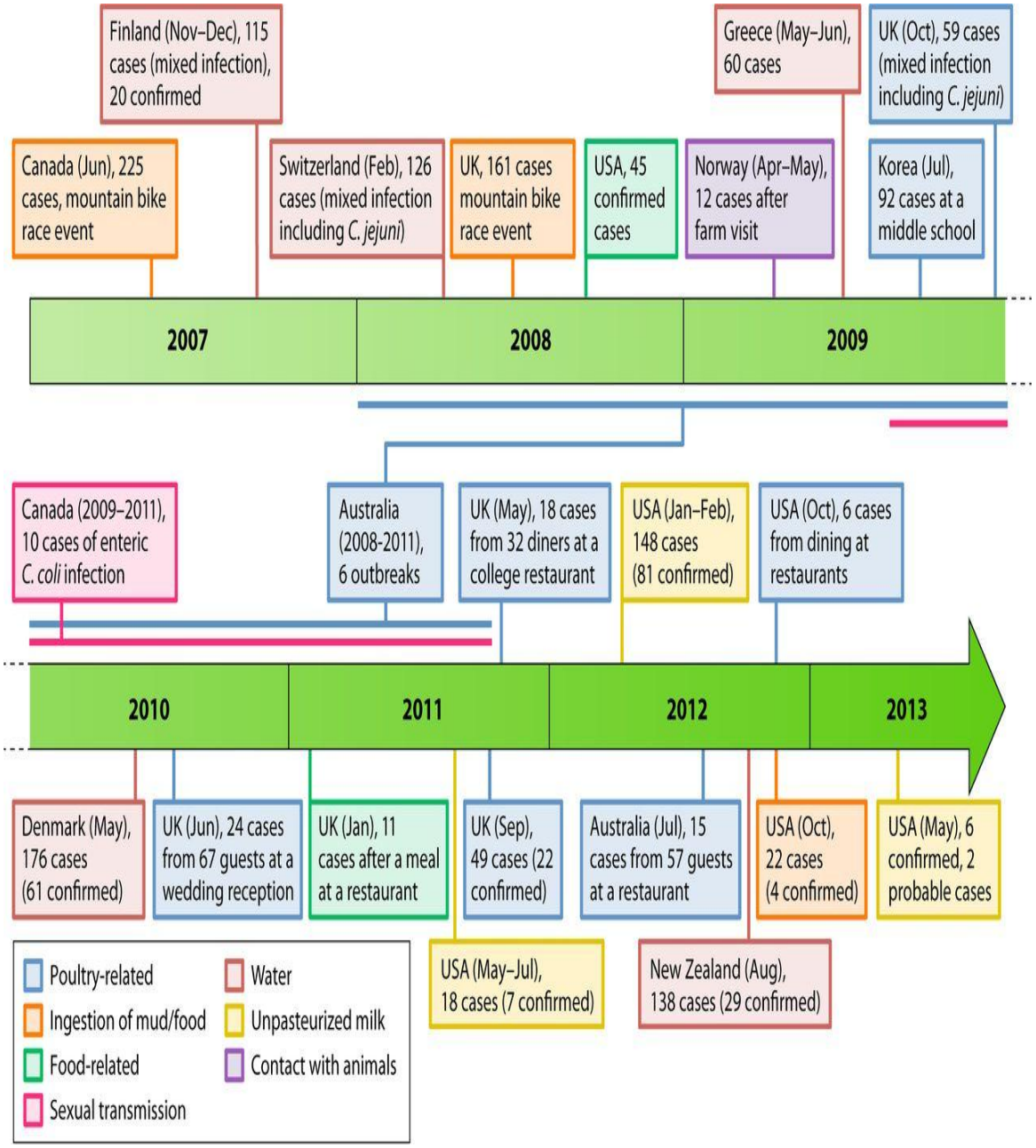


Figure 1-4: Timeline of published *Campylobacter*iosis outbreaks since 2007.

(Bartholomew et al., 2014, Castrodale et al., 2013, Longenberger et al., 2013, Yu et al., 2010). The figure was reproduced from Kaakoush et al., 2015.

1.3.1 Clinical characterization and treatment of *Campylobacter* infection

Once *Campylobacter* has been ingested the incubation period ranges between 18 hours to 8 days. The clinical symptoms of infection have been suggested to be dependent upon the virulence of the infecting strain, challenge dose and the susceptibility of the patient (Skirrow and Blaser, 2000). Generally, the infective dose in human is relatively low between 500 and 800 cells (Black et al., 1988, Robinson, 1981). The clinical signs caused by *C. coli* , *C. hyointestinalis, C. upsaliensis,* and *C. lari* are similar to *C. jejuni* (Edmonds et al., 1987, Lastovica, 2006, Patton et al., 1989, Rennie et al., 1994).

Campylobacteriosis is an acute diarrhoeal disease with clinical manifestations that include diarrhoea, fever and abdominal cramps. However, these symptoms are not distinctive from symptoms exhibited by other organisms, so the detection of *Campylobacters* in the faeces is considered the definitive diagnosis. The symptoms often start with abdominal pain followed by diarrhoea. Other signs, such as fever, headache, myalgia, dizziness, vomiting and rigors can rarely appear (Peterson, 1994)**(Figure 1.6)**.

Table 1-2: Species within the genus *Campylobacter* and their clinical relevance to humans (as of December 2014) [c]

***Campylobacter* species**[a]	**Clinical manifestations**
C. coli	Established pathogen in enteritis; also found in blood, meningitis, and acute cholecystitis
C. concisus	Emerging pathogen associated with enteritis and IBD (Crohn's disease and ulcerative colitis); also found in Barrett's esophagitis, blood, and brain abscess
C. curvus	Found in enteritis, ulcerative colitis, Barrett's esophagitis, blood, liver, and bronchial abscesses
C. fetus[b]	Associated with bacteremia; also found in enteritis, brain abscesses, epidural abscess aspirate, cerebrospinal fluid, cellulitis, endocarditis, mycotic aneurysm of the abdominal aorta, and peritonitis
C. gracilis	Potential periodontal pathogen; also found in IBD, head and neck infection, and brain abscess
C. hominis	Found in blood and IBD (possibly a commensal in the intestine)
C. helveticus	Found in enteritis
C. hyointestinalis	Found in enteritis and blood
C. insulaenigrae	Found in enteritis and blood
C. jejuni	Established pathogen in enteritis and possible predisposing agent in IBD, post infectious IBS, and celiac disease; infection may result in sequelae in the forms of Guillain-Barré syndrome, Miller Fisher syndrome, Bell's palsy (unilateral facial paralysis), and reactive arthritis; found in IBD, blood, myocarditis, meningitis, acute cholecystitis, urinary tract infection, and acute febrile illnesses associated with leukopenia or thrombocytopenia
C. lari	Associated with enteritis; also found in blood
C. mucosalis	Found in enteritis
C. lari	Associated with enteritis; also found in blood
C. mucosalis	Found in enteritis
C. rectus	Putative periodontal pathogen; also found in enteritis, IBD, vertebral abscess, blood, necrotizing soft tissue infection, and pus
C. showae	Found in IBD, intraorbital abscess, and blood
C. sputorum	Found in enteritis, axillary abscess, and blood
C. upsaliensis	Emerging pathogen in gastroenteritis; also found in breast abscess, blood, and placenta
C. ureolyticus	Associated with gastroenteritis and IBD; also found in oral, perianal, and soft tissue abscesses, soft tissue or bone infections, and ulcers or gangrenous lesions of the lower limb

[a] No disease association in humans has been reported for *C. avium*, *C. canadensis*, *C. corcagiensis*, *C. cuniculorum*, *C. lanienae*, *C. lari* subsp. *concheus*, *C. peloridis*, *C. subantarcticus*, *C. troglodytis*, *C. volucris*, "*Campylobacter* sp. Dolphin DP," and "*Campylobacter* sp. Prairie Dog" (as of December 2014).

[b]Includes *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp.*testudinum*.[c]The table was updated from (Man, 2011) and reproduced by (Kaakoush et al., 2015).

A compendium of clinical signs connected with *Campylobacter* infection is presented in Table 1.2. *Campylobacter*iosis is usually self-limiting so antibiotic therapy is seldom recommended, unless the patient is immunocompromised or has other underlying conditions. When appropriate, erythromycin is considered a drug of choice due to its effectiveness, low toxicity and low cost, and is particularly effective against the disease in children if administered early in the course of the illness (Anders et al., 1982, Pai et al., 1983, Salazar-Lindo et al., 1986). Other macrolide antibiotics as well as quinolones such as ciprofloxacin are usually recommended for adults (Dryden et al., 1996). The mortality rate is relatively low and frequently limited to elderly patients or patients suffering from another underlying disease. *Campylobacter* bacteraemia may occur but is generally rare, with only 0.2-0.5 per 100,000 inhabitants/year. However bacteraemia is more common in infections caused by *C. fetus*, particularly in immunocompromised and elderly patients (Tremblay et al., 2003, Monno et al., 2004).

*Campylobacter*s not only cause diarrhoea but infection can also lead to serious post-infection sequelae, including Guillain-Barré syndrome (GBS) and reactive arthritis (RA). The incidence of reactive arthritis due to *Campylobacter* infection has been estimated to be 1-5% (Pope et al., 2007), whereas, it is reported that the *C. jejuni* infection leads to 20-50% of Guillain-Barré syndrome cases in Europe, North and South America, Japan and Australia (Jacobs-Reitsma et al., 2008). Guillain-Barré syndrome is characterised by limb and respiratory muscle weakness which may appear one to three weeks after infection. *C. jejuni*

is considered the most common known causative agent of this disease. It is believed that GBS is an autoimmune disorder where the adaptive immune system is provoked to attack myelin or axons of the peripheral nervous system. This may be due to the surface of *C. jejuni being* composed of polysaccharides that are similar to gangliosides of human nerve tissues. The disease is self-limiting with limited or full recovery over weeks to months (Adams and Victor, 1993). Reactive arthritis (RA) is defined as an immune-mediated inflammation of the joints. The common symptoms are pain, stiffness, redness and swelling in the joints of the lower limbs and hardly ever in the joints of the upper limbs (Havelaar et al., 2000). The estimated interval between the appearance of pain and swelling of the joints and the onset of *Campylobacter*iosis is 14 days (Peterson, 1994). The disease may persist from several weeks to several months, sometimes a year, but complete recovery is usual (Skirrow and Blaser, 2000) **(Figure1.5)**.

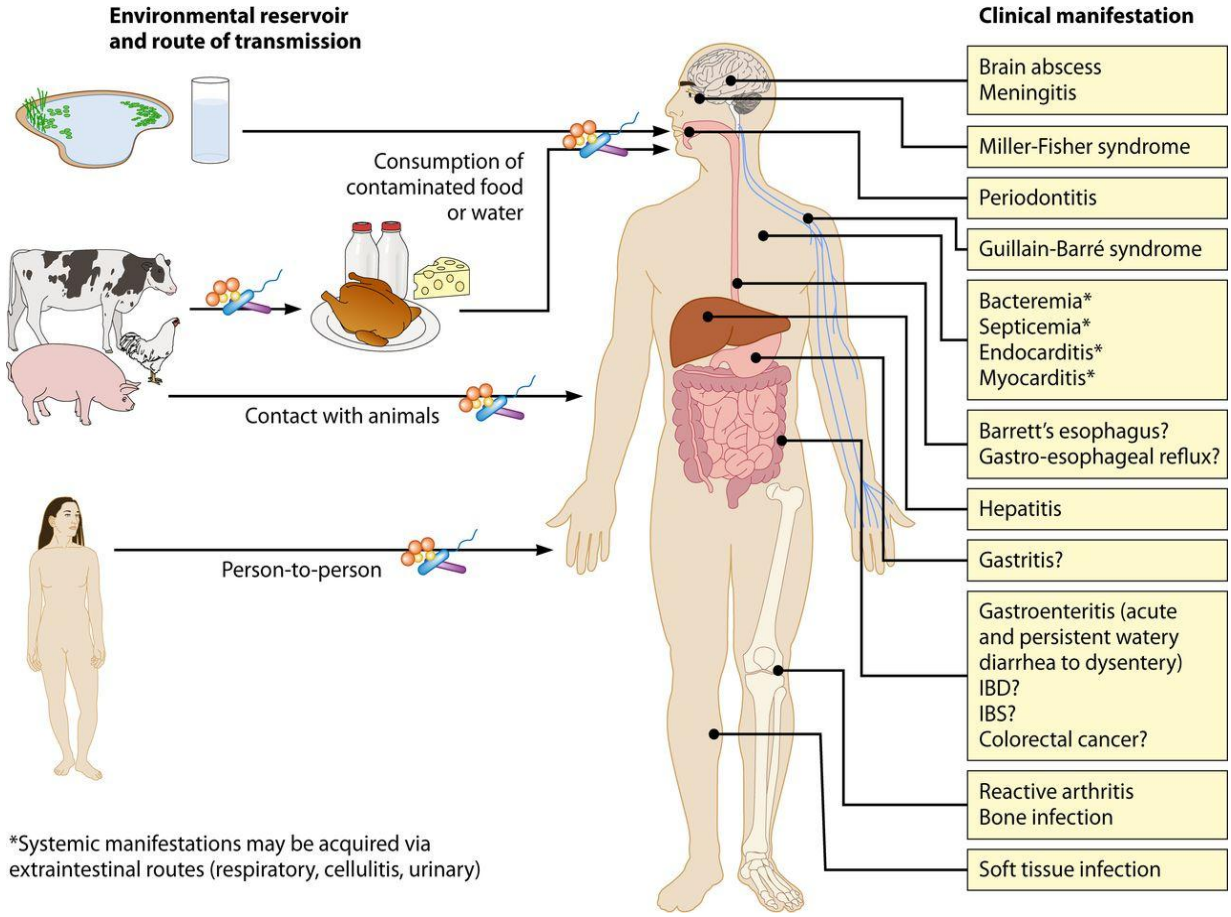


Figure 1-5: Environmental reservoirs, routes of transmission, and clinical manifestations associated with *Campylobacter* species.

Campylobacter species can be transmitted to humans through consumption of undercooked or contaminated food or via contact with animals. Tap, bore, and pond waters are also sources of *Campylobacter* species. Person-to-person transmission (fecal-oral or via fomites) can occur. Ingestion of a sufficient dose of organisms via the oral-gastric route may lead to one or more gastrointestinal and/or extra gastrointestinal manifestations; the outcome is dependent on the species or strains of *Campylobacter* involved in the infection. Abbreviations: IBD, inflammatory bowel diseases; IBS, irritable bowel syndrome. Question marks indicate conditions for which a role for *Campylobacter* is implicated but not certain. Reproduced from (Kaakoush et al., 2015).

1.4 Epidemiology and occurrence of *Campylobacter* in poultry

1.4.1 Epidemiology of poultry flock colonisation

C. jejuni is extremely prevalent in poultry flocks throughout the world. Whilst it cannot usually be detected in hatchlings but once birds are 2-3 weeks of age, broiler chicken flocks are frequently fully colonised (Newell and Fearnley, 2003). Nevertheless flocks can test positive after just 7 days when routine surveillance methods have been used (Van Gerwe et al., 2005, Katsma et al., 2007). As soon as the first bird becomes colonized in a flock, it will be the source of transmission to the whole flock through coprophagy after faecal shedding. Within the first days the small number of birds colonized may not be detectable but quickly go on to infect the entire flock. This may explain the observed lag period between hatch and first detection. Other factors have also been suggested to contribute to the lag period, such as maternal immunoglobulin and feed consumption.

The prevalence rate of positive flocks between different countries may be dissimilar and difficult to compare with each other because sampling, transmission and analytical methods may be different. It has been reported that the prevalence rate of positive *Campylobacter* flocks within different EU countries ranged between 3-91% in 2004 (Europe Food Safety Authority, 2005). Currently within EU countries the monitoring of *Campylobacter* has become compulsory with regulated strategies and procedures for sampling, transport, culture and reporting to provide a comparative baseline survey of *Campylobacter* in broiler chickens (Zoonoses Directive 2003/99/EC). Within Great Britain differences have

been noted according to season and geography on the prevalence of *Campylobacter* subtypes present in housed broiler flocks between 2004 and 2006 (Jorgensen et al., 2011). The prevalence of positive groups was significantly higher in the northern part of Great Britain compared to southern and central areas, and was significantly higher in July, August and September compared to other times of the year. Some countries in the European Union revealed similar seasonal patterns of *Campylobacter* prevalence, with the highest levels of infection also being experienced during the months of July to September (EFSA, 2010). A number of different parameters may affect the colonisation frequency of poultry, for example: the number of birds in the flock, the presence and number of other animals on the farm, the distance between farms and the prevailing environmental conditions. In addition there are differences in management practices between northern EU countries and other parts of the world.

It has been demonstrated in many studies that the colonisation species in broiler chickens is largely *C. jejuni* with a small proportion of *C. coli* stains and rarely other strains detected. This proportion of different species differs from one country to another. For example, in the UK and Netherlands the proportion of *C. jejuni* to other species is reported to be 95 and 65 % of *Campylobacter* positive flocks respectively with *C. coli* making up the remainder (Evans, 1997, Nielsen and Nielsen, 1999). The reason for the different abundance of the two species is not fully understood.

In order to discover how *Campylobacter*s enter a flock attention has focussed on the role of vertical transmission from parent flock to progeny (Cox et al., 2012). This was previously thought to be a very rare event (Sahin et al., 2003, Fonseca et al., 2006) and remains a highly debatable issue amongst researchers (Newell and Fearnley, 2003). Studies have found 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). The role of the horizontal transmission (bird to bird) has been well established with the rapid spread of *Campylobacter* within and between flocks. As soon as a flock becomes colonised, bird to bird transmission rapidly occurs and up to 100% of the flock being colonised within few days (Shreeve et al., 2000, van Gerwe et al., 2009). Several transmission routes have been suggested within the broiler house environment. These include aerosols caused by poor air-ventilation systems and transmission from wild life that occupy the surroundings of the broiler house such as rodents, insects, livestock and domestic pets (Moreno et al., 1993, Newell and Fearnley, 2003, Bull et al., 2006). Chickens are coprophagic and therefore the faecal-oral route is probably the main route of transmission within an already infected flock **(Figure 1.6)**.

1.4.2 Colonisation of broilers

Different kinds of poultry may become colonised with *Campylobacter* including: broilers, layers, turkeys, (Jacobs-Reitsma et al., 1995, Rivoal et al., 1999) and ducks (Borck and Pedersen, 2005, Yogasundram et al., 1989). To date research has mainly concentrated on colonisation of intensively reared broilers, because this sector is the largest market and considered to cause the majority of poultry associated cases of *Campylobacter*osis. As discussed in section 1.3.1 broiler flocks become *Campylobacter* positive at 2 to 3 weeks of age which is known as the lag phase. Infected chickens start to shed the pathogen 2-3 days after infection. The ability of these bacteria to colonise the chicken intestine varies from strain to strain (Cawthraw et al., 1996, Chen et al., 2006, Ringoir and Korolik, 2003). The highest levels of colonisation appear 5 days after challenge and decrease slightly after 4 weeks, at which time negative birds may occur. Mixed infections of flocks have been described (Jacobs-Reitsma et al., 1995, van de Giessen et al., 1998). Moreover potential dominant strains have the ability to replace other strains during colonisation (El-Shibiny et al., 2007). Where a mixed infection occurs, DNA may exchange between the resident strains, leading to chimeras, and a greater diversity of the *Campylobacter* genotypes present (de Boer et al., 2002).

C. jejuni has been considered by most researchers to be a commensal organism of broiler chickens, due to its high prevalence and the absence of clinical symptoms and pathological lesions (Dhillon et al., 2006). However, this view has been challenged recently with evidence that

*Campylobacter*s can invoke variable inflammatory responses in broiler chicken breeds (Humphrey et al., 2014). Regardless *Campylobacter* colonisation often persists due to tolergenic immune responses in chickens (Hermans et al., 2012). The caecum is the predominant site of colonisation and is largely limited to the mucous membrane of intestine within the intestinal crypts of the villi (Beery et al., 1988). It has been suggested that this organism has ability to translocate from the intestine to other organs via the blood supply and is recoverable from the liver and spleen (Knudsen et al., 2006).

It is recognised that following colonisation, mucosal and systemic antibody reactions are stimulated and that these amplify with time (Cawthraw et al., 1996, Rice et al., 1997). Temporary and acquired protective immunity may play a role in the dynamics of flock infection. Chicks at day of hatch may have high levels of maternal immunoglobulin (IgG) because the majority of parent flocks of broilers are colonised with *Campylobacter*. These antibodies will decline after (2-3) weeks of hatching. Studies show a delay of 2-4 days in the colonisation of 3-day old chicks from hens of colonised flocks compared with chicks from non-colonised flocks (Sahin et al., 2001). This signifies the protective role of maternal immunoglobulin (IgG), which may contribute to the 2-3 weeks lag phase observed before broiler flock infections become apparent.

1.4.3 Prevalence of Campylobacter on poultry meat

C. jejuni and *C. coli* are frequently isolated from broiler carcasses and poultry meat and as such are a major food safety concern (Newell, 2001, Cui et al., 2005, Moran et al., 2009, Ridley et al., 2011, Adak et al., 2005, Fhogartaigh and Edgeworth, 2009). A large survey was conducted in Europe in 2008 from January to December to determine the prevalence of *Campylobacter* in broiler flocks and relate this data to the prevalence on the carcasses immediately after slaughter (EFSA, 2010). A total of 10,132 broiler batches were tested from 561 slaughter houses 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 samples being collected from neck and breast skin immediately after chilling (EFSA, 2010). This study demonstrated that a high prevalence of *Campylobacter* within the flock can be related to a high level of contamination of the final meat product.

1.5 Isolation, culture and storage of *Campylobacter*

Isolation of *Campylobacter* is generally carried out from faeces samples that contain many other bacteria that grow more vigorously on laboratory culture media. When first discovered there was no selective media available to inhibit the normal intestinal flora. In order to isolate *Campylobacter* a filtration method that exploits there small size and active motility was developed (Butzler et al., 1973, Dekeyser et al., 1972). Although largely superseded when antibiotic containing selective

media were developed, it is still used to isolate *Campylobacters* that are unable to grow under selective conditions.

There are many different selective media available but not all these media support the growth of all *Campylobacter* species (Aspinall et al., 1993, Butzler et al., 1983, Griffiths and Park, 1990, Griffiths, 1993). Probably the most commonly used selective medium to isolate *C. jejuni* and *C. coli* is modified charcoal, cefoperazone, desoxycholate agar (CCDA) which contains cefoperazone and amphotericin B. Samples are usually streaked directly onto an agar plate which is then incubated in an atmosphere of approximately 5% oxygen v/v, 10% carbon dioxide v/v, and 85% nitrogen v/v at 43°C (Skirrow, 1977) for 48 hours. Direct plating is usually sufficient for the isolation of *Campylobacter* from human faeces or poultry intestinal material. Enrichment may be used to isolate low numbers of C. *jejuni* and *C. coli ,* usually from food where the bacteria may be stressed due to sub-optimum temperature and gaseous conditions (Gharst et al., 2013). Many enrichment broths have been suggested but Bolton Broth is probably the most well-known. Once inoculated, the broth is incubated in a microaerobic atmosphere at 37 °C for four to six hours to allow recovery, then transferred to 42 °C for 48 hours followed by inoculation of selective agar for example CCDA or Campy Food agar (CFA) and finally incubated microaerobically for 48 hours. For growth in pure cultures Mueller Hinton broth with or without agar is considered a good growth medium for *Campylobacter*. To store isolates at -80°C high cell concentrations are suspended in Mueller

Hinton broth with 10% glycerol or dimethyl sulfoxide (DMSO) added (Davis and DiRita, 2008).

1.6 Identification of *Campylobacter* species

As mentioned previously most human disease is caused by the two species C. *jejuni* and *C. coli* and usually a diagnosis of "*Campylobacter"* is sufficient information for health professionals. Selective media and high incubation temperatures tend to favour isolation of only these two species so isolation of other species is prejudiced.

In order to confirm the presence of C. *jejuni* or *C. coli* a Gram stain showing typical morphology is usually sufficient. Several different immunological tests have been developed to aid confirmation. This includes latex agglutination tests used for the rapid and simple confirmation of *Campylobacter* colonies. Polyclonal antibodies are bound to latex particles which agglutinate in the presence of *Campylobacter* antigens such as outer membrane proteins or flagella. The sensitivities and specificities of the tests differ (Miller et al., 2008). Similar ELISA based immunoassays have also been developed to confirm presumptive colonies or in some cases detect *Campylobacter*s directly in faecal samples reviewed by (Oyarzabal and Battie, 2012).

Correct identification of *Campylobacter* species allows a significant amount of information about the prevalence of different species to be collected, which may be exploited in epidemiological and risk assessment studies. The main phenotypic test differentiating *C. jejuni and C. coli is* hippurate hydrolysis. The majority of *C. jejuni strains* have ability to

hydrolyse hippurate to glycine and benzoic acid while a few lack this characteristic (Totten et al., 1987). Both false-negative and false-positive hippurate test results of C*. jejuni* strains have been reported in several studies (Cacho et al., 1989, Denis et al., 1999, Kos et al., 2006, Steinhauserova et al., 2001, Totten et al., 1987, Waino et al., 2003). The API Campy (bioMérieux, Marcy l'Etoile, France) is a two-part system (Elharrif and Mégraud, 1986) using enzymatic, assimilation, and susceptibility tests. The ability of this test to identify different *Campylobacter* strains has been the subject of debate (Martiny et al., 2011).

Genotypic methods for species discrimination have largely superseded other techniques in recent years. A number of different polymerase chain reaction (PCR) approaches have been reported. These include both conventional and quantitative PCR, which both rely on differences between species-specific genes that can be used for species identification. Examples include: *cad*F (Cunningham et al., 2010), *hip*O (Persson and Olsen, 2005, Wang et al., 2002), *map*A (Best et al., 2004), *ceu*E (Houng et al., 2001), 23S rDNA (Eyers et al., 1993), *ipx*A (Klena et al., 2004) and *cdt*A (Asakura et al., 2008) for *C. jejuni* ; and alternative forms of *cad*F (Cunningham et al., 2010), *asp*A (Linton et al., 1997), *ceu*E (Houng et al., 2001, Gonzalez et al., 1997) *gly*A (Wang et al., 2002), 23S rDNA (Eyers et al., 1993) and *cdt*B (Asakura et al., 2008) for *C. coli* . It should be noted that many of the PCR methods have been used directly for the detection and often enumeration of *Campylobacter* species from human, animal, food or environmental specimens. However,

the sensitivity and specificity of the PCR assays are limited, due to the high levels of heterogeneity within species (On and Jordan, 2003). Although partial 16S rDNA sequencing is extensively used for species identification for a range of enteric bacteria, for *Campylobacter* is less practical due to the absence of discrimination between *C. jejuni , C. coli* and some *C. lari* strains but remains useful for the identification of alternative *Campylobacter* species (Gorkiewicz et al., 2003). DNA sequencing of the housekeeping gene *groEL*/cpn60 has been reported to be effective for identifying *Campylobacter* species (Hill et al., 2006, Karenlampi et al., 2003).

1.6.1 Typing methods

Strain typing is necessary in order to carry out epidemiological studies and trace outbreaks. In order to discriminate between the different isolates of the two species, *C. jejuni* and *C. coli ,* both phenotypic and genotypic typing methods have been used and these have evolved as new techniques have become available (Eberle and Kiess, 2012). From the 1980s to the 1990s serotyping and biotyping were the only discriminative methods available. From the 1990s a number of different techniques have been proposed including: phage typing (Frost et al., 1999), multilocus sequence typing (MLST) (Dingle et al., 2001b), pulse-field gel electrophoresis (Ribot et al., 2001), ribotyping (Gibson et al., 1995), flagellin typing (Nachamkin et al., 1993) and amplified fragment length polymorphisms (Cardarelli-Leite et al., 1996). In general genotyping methods have overtaken phenotypic methods in popularity and are discussed in more detail in the next section.

1.6.2 Genotyping methods

Typing methods based on the genetic composition of bacteria have the advantage of being rapid, reproducible and can be standardised between laboratories.

1.6.2.1 Pulsed Field Gel Electrophoresis (PFGE)

In this technique large fragments of chromosomal DNAs are separated after digestion with restriction enzymes, such as *Sma*I, *Sal*I, *Apa*I, *Kpn*I, *Xho*I and *Bss*HII. These enzymes cut *Campylobacter* DNA infrequently, such that a small number of large fragments are produced which, represent molecular fingerprint that can be used to compare different isolates (Wassenaar and Newell, 2000).

1.6.2.2 Restriction Fragment Length Polymorphism (RFLP)

Another technique that has been used for typing isolates of *C. jejuni* and *C. coli* is restriction fragment length polymorphism (Takahashi et al., 2006). The technique involves the digestion of PCR amplicons, such as the *fla*A gene, using restriction enzymes to produce a reproducible, fingerprint type pattern, allowing isolate comparison (Nachamkin et al., 1993).

1.6.2.3 Amplified Fragment Length Polymorphism (AFLP)

The amplified fragment length polymorphism (AFLP) technique was developed in the 1990s (Duim et al., 1999) and had the advantage of sampling the whole genome rather than a single gene. The bacterial

genome was digested to generate a large number of fragments using a frequently cutting enzyme. Mutations that generate or eliminate restriction endonuclease recognition sites generate diversities in the restriction fragment lengths which were amplified using fluorescently labelled primers that helped automate the analysis and facilitate higher throughput. AFLP has been used in epidemiological investigations of multiple *Campylobacter* species (Duim et al., 2000, Islam et al., 2009).

1.6.2.4 Multi-locus sequence typing (MLST)

Multi-locus sequence typing (MLST) is based on direct nucleotide sequencing of PCR amplicons of specific regions of seven housekeeping genes of *C. jejuni* (Kinana et al., 2007, Levesque et al., 2008, Dingle et al., 2001b) and has been extended for the typing of *C. coli* , *C. lari*, *C. upsaliensis* and *C. helveticus* (Miller et al., 2005a). The MLST technique has provided a highly reproducible typing scheme for *Campylobacter*s Internet-based MLST databases assist a standardised nomenclature and exchange of MLST results between laboratories. It has been used for the genetic investigation and improved understanding of genetic structure of *Campylobacter* populations for outbreak analysis (Clark et al., 2005, Lastovica and Allos, 2008, Sails et al., 2003).

1.6.2.5 Fla typing

The motility of *C. jejuni is* due to the presence of a flagellum at one or both ends of organism. The flagellar filaments are constructed from flagellin protein subunit encoded by two genes (*fla*A and *fla*B). These

genes are arranged together in tandem and are separated by approximately 170 nucleotides containing the short variable regions (SVR). Fluorescently labelled PCR oligonucleotide probes have been designed to amplify the SVR in a real-time PCR methodology to discriminate between different isolates of *C. jejuni* and *C. coli* (Jensen et al., 2005, Ridley et al., 2008). Earlier typing techniques focused on the amplification of regions of the actual flagellin genes, with differences in the primer design, annealing temperatures and restriction enzymes used. These techniques were useful at this time because they were cheaper, more widely accessible, time saving and less labour intensive than other procedures (Harrington et al., 2003). Nevertheless, one of the major drawbacks of *fla* typing was the genetic instability of isolates. Harrington et al. (1997a) supplied evidence for inter genomic recombination between *fla*A genes of various strains. To overcome this it was recommended to combine *fla* typing with another genotypic technique (Wassenaar and Newell, 2000).

1.6.2.6 Whole genome sequencing (WGS)

As the cost of sequence determination has reduced, it has become possible to examine the whole genome of a bacterial isolate and compare it to other isolates in minute detail (Taboada et al., 2013) However, bioinformatics is a field that will require further developments to cope with routine *C. jejuni* subtyping (Pendleton et al., 2013).

1.7 Quantification of viable Campylobacter

The influence of pathogenic microorganisms to public health can be estimated by their quantification in food, environmental and clinical samples. To estimate the health risk posed by pathogenic microorganisms it is essential that dependable methods to enumerate viable cells are available. Doubt has been cast on classical growth-dependent approaches for viable cell calculation because of the idea that various kinds of bacteria may enter a state called viable but non culturable (VBNC) that renders them incapable of growth on the culture media but still able to infect (Colwell and Grimes, 2000).

However, recovery in animal models remains controversial as the results could not always be reproduced (van de Giessen et al. 1996). To clarify the difference between culturable *in vitro* and culturable *in vivo*, molecular techniques have been suggested. The terms 'viability' and 'culturability' are often equated but there are differences which should be better understood (Kelly, 2001). One measure of viability is the detection of quantifiable mRNA (Coutard et al., 2005, Liu et al., 2010, Rijpens et al., 2002, Sheridan et al., 1998). The presence of 16S rRNA is the most widely used biomarker for bacterial viability (Buswell et al., 1998, Churruca et al., 2007, Li et al., 2008). Another measure suggested to be of use in the molecular quantification of viability, is to examine the integrity of the bacterial cell membrane. Methods which combine the use of photo reactive dyes such as propidium monoazide (PMA) and ethidium monoazide (EMA) with DNA amplification have been developed.

These compounds can only penetrate cells with damaged membranes (Nogva et al., 2003). Once inside the cell the compounds intercalate with bacterial genomic DNA, inhibiting PCR amplification. Thus when PCR amplification is carried out from the sample, only DNA from cells with intact membranes can be amplified allowing quantification of viable cells (Josefsen et al., 2010, Rudi et al., 2005). However, several studies have indicated that the PMA-PCR may not be a fully effective approach to avoid the signal from dead cells. Recently studies suggest that using longer PCR amplicons sizes could be a feasible technique to develop the utility of the intercalating dye (EMA-PCR and PMA-PCR). More work is required before these techniques can be generally accepted.

1.8 General features of *Campylobacter* pathogenesis

There are three major possible mechanistic steps involved in *Campylobacter* pathogenesis: 1. colonisation of the intestinal tract; 2. adhesion to intestinal epithelial cells, 3. invasion (Hu and Kopecko, 2008). It is generally thought that the capability of *Campylobacter* to arrive and live within non phagocytic cells plays a significant role in the development of pathogenesis. Flagella probably have a crucial role in chemotaxis and motility in both the commensal and pathogenic lifestyles of *C. jejuni* and may also have a role in adhesion (Hendrixson and DiRita, 2003, Hu and Kopecko, 2000, Hugdahl et al., 1988). It has been shown that a number of proteins have been implicated in the pathogenesis mechanism, such as outer membrane protein CadF (Krause-Gruszczynska et al., 2007b), GTPases (Krause-Gruszczynska et al., 2007a), regulatory proteins (Kamal et al., 2007), surface-exposed

lipoprotein JlpA (Jin et al., 2001), secreted protein CiaB (Konkel et al., 1999) and cytolethal distending toxin (Hu and Kopecko, 2000). *C. jejuni* cells have ability to produce a polysaccharide capsule, which is essential for both adhesion and invasion of epithelial cells, and for serum resistance (Bachtiar et al., 2007, Karlyshev et al., 2001, Bacon et al., 2001a, Parkhill et al., 2000). *C. jejuni* is one of the Gram-negative enteric organisms that produce low molecular weight lipooligosaccharide (LOS) not lipopolysaccharide (LPS) (Fry et al., 2000). In this section the general structures and the main virulence factors that regulate the development of the *Campylobacter* disease process will be discussed.

1.8.1 Adhesion and Colonisation

Like other enteric pathogens such as *Salmonella*, *Yersinia* species, *Shigella* and *Escherichia coli ,* the role of flagella, adhesins, cellular and extracellular matrix proteins are all important in the pathogenesis processes of *C. jejuni* (Ketley, 1997). In addition motility, chemotaxis, iron acquisition, quorum sensing as well as host immune evasion have also been identified as playing different roles in *C. jejuni* pathogenesis (Elvers and Park, 2002, Ketley, 1997).

1.8.2 Invasion

C. jejuni has been demonstrated to invade intestinal epithelial cells after colonisation of the intestinal mucus layer where it may modify signal transduction pathways supporting entry and passage through the epithelial monolayer (Konkel et al., 2005). This invasion process may be started through signalling actions on the surface of host cells, associated

with a unique caveolae-dependent entry pathway and downstream signals that activate microfilament and microtubule depolymerisation. This leads to membrane disturbance and internalization of the bacterium (Hu et al., 2006, Watson and Galan, 2008, Wooldridge and Ketley, 1997). Depolymerisation or stabilisation of microtubules (Figure 1.6), as well as inhibition of host cell tyrosine kinases, receptor-mediated endocytosis, endosome acidification, and phosphatidylinositol-3-kinases, has been shown to result in reduction in or inhibition of, invasion by *C. jejuni* (Biswas et al., 2000, Biswas et al., 2003, Oelschlaeger et al., 1993, Wooldridge et al., 1996). Pseudopods of epithelial cell membrane, that contain microtubules, have also been shown to expand and cover *C. jejuni* (Biswas et al., 2000, Hu and Kopecko, 1999, Kopecko et al., 2001).

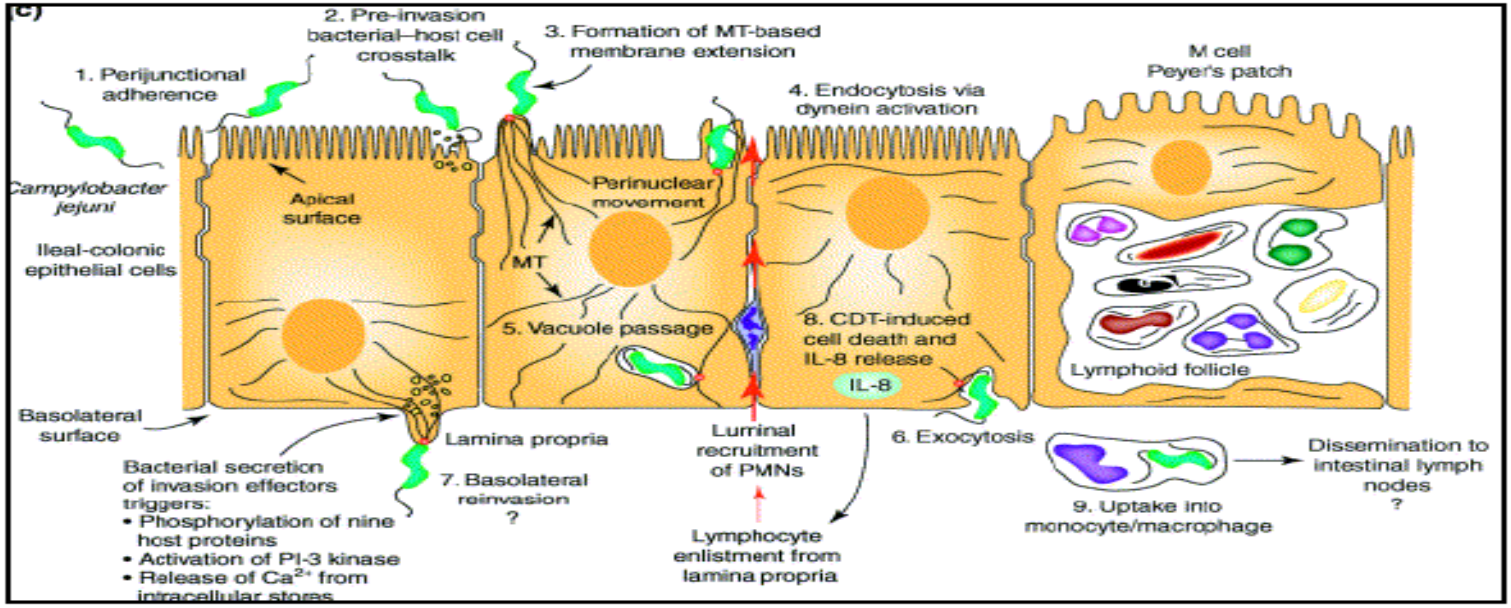


Figure 1-6: Illustration of the current working model of *C. jejuni* pathogenesis.

(1) Initial adhesion of *C. jejuni is* believed to start at the apical cell surface at the peri-junctional region. (2) Where it secretes putative "invasion" effectors into the host cell, which in turn trigger phosphorylation of host proteins and the release of Ca2+ from intracellular stores. (3) Host signalling cascades trigger a localized disruption of cortical actin filaments and an extension of microtubules (MTs) to form a membrane protrusion to meet the adjacent bacterium. (4) Host-pathogen interaction at caveolae leads to an endocytosis of the bacterium via membrane invagination. (5) The engulfed *C. jejuni within* a membrane-bound vacuole moves via dynein along MTs to the basolateral surface for exocytosis (6), and then re-enter the epithelium basolaterally (7). (8) Infected epithelial cells secrete interleukin (IL)-8 basolaterally, which leads to proliferation of lymphocytes from the lamina propria. (9) *C. jejuni* apparently survive in macrophages/monocytes for several days, which aids their local dissemination (Kopecko et al., 2001).

Furthermore, it has been reported that caveolae which originate from the plasma membrane of many types of vertebrate cell are also involved in the internalisation of *C. jejuni* (Schnitzer et al., 1994, Wooldridge et al., 1996). Transport of *C. jejuni* across caveolae may inhibite by filipin III, a sterol-binding agent (Wooldridge et al., 1996, Schnitzer et al., 1994). Although, this model has become well accepted, there have been other studies which proposed alternative mechanisms of *C. jejuni invasion* (Kopecko et al., 2001, van Alphen et al., 2008). Early studies of *C. jejuni* invasion revealed that at least eight proteins were secreted into the culture medium during co-cultivation with human internal epithelial (INT) 407 cells or in an INT 407 cell-conditioned medium (Konkel et al., 1999). All these proteins together are known as *Campylobacter* invasion antigens (Cia), and include the 73-kDa protein CiaB. It was proposed that the CiaB gene belonged to a type III secretion system (Konkel et al., 1999). However, in the *C. jejuni* genome no type III system could be recognisedRecently, a functional type VI secretion system has been identified in *C. jejuni* but the genes encoding this system are only reported as present in 10% of the isolates tested, all of which contained the CJIE3 integrative element (Bleumink-Pluym et al., 2013).

1.8.3 Intracellular Survival

After entry or invasion of this pathogen through caveolae-dependent pathway, the vacuoles that contain *C. jejuni* appear to roll along microtubules towards the peri-nuclear area of the epithelial cell through interactions with dynein. Consequently, they escape from the canonical

endocytic pathway, and therefore preventing being released into lysosomes (Hu and Kopecko, 1999, Watson and Galan, 2008). Although some bacteria have been noted as free in the cytoplasm, the majority of intracellular *C. jejuni* remain restricted to membrane bound vacuoles (Konkel et al., 1992, Russell et al., 1993). The cytotoxic effect of *C. jejuni* surviving within epithelial cells leads to cytoplasmic swelling, loss of microvilli and premature apoptosis. The dead cells are then exfoliated into intestinal lumen, disrupting the intestinal epithelium (Konkel et al., 1992, Russell et al., 1993).

1.8.4 Toxin production

The exact role of toxins in the pathogenesis of *Campylobacter* enteritis remains unclear. Although various cytotoxins and enterotoxins have been described in *Campylobacter*s in the litrature (Wassenaar, 1997) the only one that to be characterised is the cytolethal distending toxin (CDT) (Pickett et al., 1996, Whitehouse et al., 1998). Many *Campylobacter* species have been shown to produce CDT, including: *C. jejuni, C. coli, C fetus, C. lari*, and *C. upsaliensis.* The signs of toxicity on sensitive cells are progressive cell elongation, swelling and eventual death. Three structural genes, *cdtA*, *cdtB* and *cdtC*, are essential for CDT cytotoxicity (Pickett et al., 1996, Pickett and Whitehouse, 1999). The enzymatically active subunit of the toxin is CdtB (Pickett and Whitehouse, 1999).

The functions of both CdtA and CdtC have been demonstrated to be essential as when the individual purified components CdtA, B or C were applied to cells, no toxic activity was detected (Lara-Tejero and Galán,

2001). However they interacted to form a fully active tripartite complex when applied together (Lara-Tejero and Galán, 2001).

1.8.5 Iron acquisition

A key step of *Campylobacter*s ability to cause infection is the ability to acquire iron within their animal host. The availability of free iron is usually very limited because it is only present as a complex with iron-binding and transport proteins such as haemoglobin, transferrin in serum or lactoferrin in mucosal secretions (Andrews et al., 2003). Iron limitation acts as a non-specific host defence mechanism against pathogenic bacteria (Andrews et al., 2003). In order to overcome this limitation, different mechanisms have been developed by bacterial pathogens, including the use of small iron-chelating compounds known as siderophores, which enable them to scavenge iron from their extracellular environment. *C. jejuni* have also been shown to be able to utilise haem-containing compounds (Pickett et al., 1992). Iron may exist in two different forms ferrous (Fe^{2+}) or ferric (Fe^{3+}) states and depending on the redox and pH conditions of the surrounding environment, soluble ferrous iron can be rapidly oxidised to produce insoluble ferric iron. Iron in the ferrous state is considered as a simple iron form and can diffuse through outer membrane porins (size limit ~600 Da) but ferric-siderophore complexes are too big to pass through porins. Therefore in Gram-negative bacteria the (Fe^{3+}) iron acquisition system involves an outer-membrane (OM) receptor protein, which specifically binds to an associated ferric-siderophore or free iron and transports it through the OM to a periplasmic binding protein and

then to a cytoplasmic membrane (CM) (Braun et al., 1998). *C. jejuni* typically acquire iron as ferri-enterochelin through the CfrA OM receptor, which displays high homology to a number of different ferric siderophore receptors (Palyada et al., 2004). The *cfrA* gene is considered to be an iron regulator (Guerry et al., 1997).

1.9 Host associated *Campylobacter*

C. jejuni is commonly found in the intestinal tract of poultry and thus is a frequent contaminant on the surface of poultry meat. It is however found in other animals and in the environment so if a human infection occurs it is difficult to track the origin of infection (Falush, 2009). Therefore, a great deal of effort has been expended to isolate bacteria from a number of different reservoirs and to obtain their genotypes by multilocus sequence typing (MLST section 1.5.2.4) in order to correlate these types with those recovered from human infection. It has been shown that when comparing types isolated from chickens and cattle, numerous genotypes are recognised at high frequency such as the MLST type ST-21, whereas other lineages are less frequent (McCarthy et al., 2007). The strains that were isolated from different farm animals were more closely related to each other compared with the strains isolated, for instance, from wild birds (Colles et al., 2009). The diversity of the digestive system and body temperature of chickens from the other animal species such as cattle raises the question of how the different *Campylobacter* lineages have the ability to compete successfully in diverse hosts. There are three possible mechanisms suggested that can help rapid phenotypic adaptation: First: "in built regulatory mechanisms" that enables bacterium to change gene

expression in response to new environmental conditions (Gressmann et al., 2005); Second the presence of ‘‘contingency loci’’ “that can mutate rapidly, creating phenotypic variation amongst bacteria that are otherwise genetically identical” (Moxon et al., 2006); Thirdly the importation of DNA from strains that have previously adapted to the environment (Falush, 2009).

The linking of strains and host origin based on their genome sequence is considered a first step towards understanding the evolution of host specificity. A group of strains that are phylogenetically related to each other in one host species may reflect a common ancestor with the traits necessary to survive in that species. Because *C. jejuni strains* show a high level of genetic recombination, the origins of imported DNA may be traced. The expect value as to the occurrence of a variant allele could be found in chicken or cattle is substantially better than random assignment (McCarthy et al., 2007). Additionally, the high recombination level within specific hosts represents a mechanism by which complex adaptations to specific host species can be gained quickly subsequent to a host switch (Falush, 2009). It is estimated that host effects are stronger than geographic and temporal effects, which should be considered when analysing epidemiologic data. Notably the differences between cow and sheep isolates is much weaker than the differences between cattle and chicken (McCarthy et al., 2007).

1.10 Survival of *Campylobacter* under different environmental stresses

1.10.1 Thermal stress

*Campylobacter*s are exposed to a variety of temperatures within host species and in extra-intestinal environments, for example 42°C within avian reservoirs, 37°C within human and other animal hosts and chilled temperature ranges from 4°C to -20°C within food products (water, milk or on poultry meat). The ideal temperature range for growth is between 37-42°C, where temperatures above this initiate a thermal stress response in *C. jejuni.*

*Campylobacter*s can be easily inactivated within foods by heat treatments. For example, they were completely inactivated within ground beef cooked in the oven at 190 and 218 °C where the internal meat temperature reached 70 °C (Stern and Kotula, 1982). Thermal death times (D-values) determined from different growth conditions and media show marked variation (Blankenship and Craven, 1982). In these studies the D-values in chicken meat were greater than those recorded from peptone water. *Campylobacter* spp also differ markedly in their heat resistance (Christopher et al., 1982, Waterman, 1982). However, although differences in the D-values between strains were noted, they were considered to be of minimal significance when the strains were inactivated in meat at cooking temperatures (Blankenship and Craven, 1982).

The heat resistance of different *C. jejuni* and *C. coli strains* were examined between 50 and 60°C by the use of differential scanning

calorimetry (DSC). This study demonstrated that isothermal methods yielded mean D55 values and z-*values* (temperature change causing a 1-log change in thermal death time) as a reliable measurement of relative heat resistance (Nguyen et al., 2006). The results indicated that cell death coincides with heat denaturation of the most thermally sensitive parts of the ribosome. The authors speculate that within the same temperature range a variety of other proteins important for the survival of a cell also unfold (Nguyen et al., 2006).

The primary response of bacteria to thermal stress is the stimulation of the HSPs (heat shock proteins), which are the most conserved protein coding genes. HSPs are components of the cellular complement of molecular chaperones, for which the mechanisms of action have been well characterised (Winter and Jakob, 2004). The expression of these proteins in many bacteria is increased under various stress conditions and are not reserved for heat stress, for example in *C. jejuni* show marked increases in expression of the chaperones under alkaline conditions (Wu et al., 1994) and combined heat and oxidative stress (Takata et al., 1995). The response of *C. jejuni* to heat stress has been investigated, showing that *C. jejuni* directly synthesises a number of HSPs after thermal stress including 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 a role in vivo *since* a *dnaJ* mutant was demonstrated to be unable to colonise chickens (Konkel et al., 1998).

1.10.2 Survival of cold stress

C. jejuni has the ability to survive better at 4°C than at higher temperatures such as 25°C or 30°C (Blaser et al., 1980, Kelana and Griffiths, 2003). The reason for this is not fully understood but is probably due to reduced oxygen solubility leading to a reduction in oxidative stress at lower temperatures. There is considerable strain variation between *Campylobacter* isolates with human isolates suggested to be more resilient to refrigeration temperature than poultry strains (Chan et al., 2001). Tolerance of *Campylobacter* to freezing at -20°C is also reported to be strain specific (Chan et al., 2001). The survival of all *Campylobacter* strains was enhanced when chilled rapidly (cooling at−20 °C/min) to 4 °C compared to standard refrigeration (El-Shibiny et al., 2009). Freezing to−20°C reduced viable counts by 2.2–2.6 log10 CFU/cm2 in 24 h. Rapid cooling to−20 °C (at −30 °C/min) enhanced the survival of *C. coli* 99/367 compared to freezing in a domestic freezer (El-Shibiny et al., 2009). Substantial interactions between particular strains were detected in mixed inoculums chilled to−20 °C by freezing in a domestic freezer or by rapid chilling to −20 °C (El-Shibiny et al., 2009).

Oxidative damage has been suggested as the mechanism involved in freeze thaw injury (Park et al., 1998) as superoxide dismutase (SOD)-deficient mutants are particularly sensitive to freeze thaw cycles (Stead and Park, 2000). Moreover, the oxygen concentrations available in microenvironments may reduce by competitor microorganisms such as those found on the surface of meat, and consequently protect related pathogens from cold stress (Aldsworth et al., 1998). This could be

essential for *Campylobacter*s, which are sensitive to oxidative stress. Otherwise the presence of competing microflora may affect the survival of *Campylobacter* and this may also be strain specific. Investigation of metabolic activities such as oxygen consumption and protein synthesis at 4°C have revealed that *C. jejuni* can elicit a cold shock response which regulates gene expression at low temperatures (Lazaro et al., 1999). The cold shock protein CspA acts as an RNA chaperone to allow more efficient protein translation at cold temperatures (Qoronfleh et al., 1992). Investigation of transcription profiles at 10 and 4°C showed that genes involved in flagellar motility, flagellin biosynthesis, and chemotaxis were down regulated (Stintzi and Whitworth, 2003). A number of genes involved in energy metabolism were upregulated at 5°C compared to 25°C (Moen et al., 2005). This suggests that *C. jejuni* requires more energy for metabolism at lower temperatures and the ability to regulate these genes enhances their ability to survive low temperatures.

1.10.3 Survival of UV stress

One of the stress factors encountered by *Campylobacter* spp outside the host is UV radiation in the form of sunlight. It has been shown that *E.coli* is more resistant than *C. jejuni* , suggesting that *Campylobacter* spp would be particularly sensitive to UV radiation (Butler et al., 1987). Survival of *Campylobacter* in sea and river water has also been studied and shown to be species dependant with *C. lari* being better able to survive than *C. jejuni* (Obiri-Danso et al., 2001). It was also shown that expression of RecA (a protein involved in DNA repair) is increased

indicating that resistance to UV radiation and DNA damage may be increased (Murphy et al., 2006).

1.10.4 Survival of acid stress

The ideal pH for *Campylobacter* is the range between 6.5–7.5, nevertheless, all strains have the ability to grow within the pH range 5.5–8.0 (Doyle and Roman, 1981). Survival at pH 5.0 is less than 24 hours, while at pH 9.0 it is less than 3 days (Christopher et al., 1982). An adaptive tolerance response (Goodson and Rowbury, 1989), stimulated by acid and/or oxidative environment is found in *C. jejuni* (Murphy et al., 2003). This response protects them from subsequent exposure to lethal stress and leads to increased survival against lethal pH. Upregulation of general stress proteins such as the heat shock chaperones DnaK and GroEL was also found in response pH stress. These chaperones play a role in the repair of proteins from aerobic and acid damage. In addition, they play a role in avoiding subsequent DNA damage and in the restoration of previously damaged DNA after mild stress.

1.10.5 Survival of desiccation

C. jejuni is extremely sensitive to desiccation. The ability of the organism to survive depends very much on the surface on which they are exposed to drying conditions. For example on ceramic tiles, Oosterom et al. (1983) noted that *C. jejuni* only remained viable when the surface of the tiles was visibly wet. The situation is different when residing on the exposed surfaces meat or poultry skin. Drying due to ventilation in cooling rooms

plays an important role in reducing viable *Campylobacter* spp. from the pig carcasses (Oosterom et al., 1983). However, this does not apply to poultry carcasses possibly because the cooling time may be shorter than for pork, but probably more importantly, the skin texture and cavities present on poultry skin may provide a suitable refuge where *C. jejuni* can avoid desiccation.

1.10.6 Survival of aerobic stress

As previously highlighted *Campylobacter* spp are generally microaerophilic and thermo-tolerant organisms. The ideal growth environments for *C*. *jejuni* are low oxygen tensions but the exact requirements may depend on other factors. For example it was shown that at high cell densities, the bacterium was able to grow in oxygen concentrations between 5-19% v/v, and this varied between different strains (Kaakoush et al., 2007). As microaerophiles, these bacteria are well suited to the intestinal tract of birds but once outside this environment they have to manage oxidative stress and the toxic products created by oxygen metabolism. These products are mainly represented by reactive oxygen species (ROS), including superoxide anions (O_2), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH•) which have the ability to damage DNA, protein and lipids (Farr and Kogoma, 1991). Even within the host oxidative stress may be a problem since macrophages use oxidative stress as a defence mechanism (Urban et al., 2006). *Campylobacter*s have several defence mechanisms to protect them from reactive oxygen species. There are three suggested proteins involved in the inactivation of ROS. The first one is iron-

containing superoxide dismutase B (SodB) which eliminates superoxide anions ($O_{2^{\cdot}}$) through their dismutation into hydrogen peroxide and oxygen (Purdy and Park, 1994, Purdy et al., 1999). The second stress defence protein is catalase (KatA) which catalyses the decomposition of hydrogen peroxide to water and oxygen. The third protein is the iron-regulated alkyl hydroxide reductase (AhpC), which plays a significant role in the resistance of *C. jejuni to* alkyl hydroperoxides because it can eliminates toxic hydroperoxide products. AphC proteins can play a role in detoxification because they can repair injured molecules that have been peroxidised (Baillon et al., 1999). Temperature has been linked to survival of oxidative stress in *Campylobacter* where several oxidative stress proteins, including superoxide dismutase are over expressed during cold shock (Stintzi and Whitworth, 2003). Therefore, there may be a link between temperature, level of oxidative stress and regulation of the oxidative-stress response.

1.10.7 Nitrosative stress response

The levels of nitric oxide (NO) and other agents that cause nitrosative stress increase during human infection by *Campylobacter* (Forte et al., 1999, Enocksson et al., 2004). The main sources of NO in human hosts are NO synthases, 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). *C. jejuni* expresses a single-domain haemoglobin (Cgb) in response to the NO stress, which plays a role in scavenging and detoxification of NO (Elvers et al., 2004, Lu

et al., 2007). Sensitivity to NO and nitrosative stress was found to be elevated in a Cgb-deficient mutant (Elvers et al., 2004). It was found that the NO-responsive expression of *cgb* was regulated by the NO-sensing regulator NssR (nitrosative stress sensing regulator, *cj0466*), which is a member of the Crp-Fnr superfamily of transcription factors (Elvers et al., 2005). *C. jejuni* similarly expresses a second globin (Ctb), which has also been established to be under the control of NssR (Elvers et al., 2005). The role of Ctb role has not been fully elucidated but it has a very high affinity for oxygen and has also been associated with a role in oxygen metabolism (Wainwright et al., 2005, Wainwright et al., 2006) and may play a role in cytochrome c peroxidase or cytochrome P450-like oxygen chemistry (Lu et al., 2007).

1.11 Diversity of *Campylobacter*

Widespread genetic differences have been observed among *Campylobacter* strains. Phenotypic variations for traits like invasion, sialylation of lipooligosaccharide, colonisation of chickens, toxin production, serum resistance and natural transformation have been identified (Wiesner and DiRita, 2008). This diversity may occur through insertions and deletions of multiple genes that might arise by homologous recombination (Taboada et al., 2004). Intra genomic recombination of *C. jejuni* during the colonisation of chickens has been noted (Scott et al., 2007, Hänninen et al., 1999).

Horizontal acquisition of DNA by mechanisms such as natural transformation, conjugation and transduction also result in genetic diversity (Wiesner and DiRita, 2008).

1.11.1 Horizontal DNA transfer

Natural DNA transfer in *Campylobacter* has been observed in the laboratory (Taylor, 1992, Wassenaar et al., 1995) and under environmental circumstances (Avrain et al., 2004). It has been suggested that the phenotypic diversity of *Campylobacter* may be largely due to horizontal DNA transfer*,* including changes in antibiotic resistance (Wittwer et al., 2005), capsular polysaccharide synthesis (Karlyshev et al., 2005), lipooligosaccharide synthesis (Gilbert et al., 2004) and flagella synthesis (Harrington et al., 1997b). Horizontal gene transfer events are widely reported where more than one *Campylobacter* type co-colonise chickens (Boer et al., 2002, Dingle et al., 2001a, Schouls et al., 2003).

1.11.2 Intra genomic recombination

C. jejuni has two flagellin genes *flaA and flaB,* which are sited adjacent to each other and share sequence similarity but have different promoters (Guerry et al., 1990, Nuijten et al., 1990). It is observed that *flaB* is not essential for motility and has been suggested as a sink for antigenic diversity or for motility changes under several environmental circumstances. Recombination has been observed between these genes *in vitro* (Alm et al., 1993, Wassenaar et al., 1994, Wassenaar et al., 1995) and *in vivo* (Nuijten et al., 2000). There is an apparent minimal role of *flaB* product in flagellin structure and cell motility. In *C. fetus* homologous recombination has been observed at the *sap* locus, which encodes genes for the S-layer proteins (SLP).

Five to nine *sapA* homologues encode the SLP, which are in close association on the chromosome (Dworkin et al., 1995, Tu et al., 2001, Tu et al., 2003). A unique *sapA* promoter is responsible for the potential expression of each *sapA* homologue (Dworkin and Blaser, 1997). To place this promoter in front of different *sapA* homologues, the *sap* locus undergoes high frequency recombination, consequently phenotypic switching of SLP occurs (Dworkin and Blaser, 1997, Garcia et al., 1995, Ray et al., 2000). Chromosomal reorganisations have also been suggested as a reason for *in vivo* changes to macro-restriction profiles noted by pulsed field gel electrophoresis (de Boer et al., 2002, Hänninen et al., 1999, Stern et al., 1994, Wassenaar et al., 1998). Likewise, *C*. *jejuni* and *C*. *coli* isolates have been reported to show macro-restriction profile variability in vitro (Dickins et al., 2002).

1.12 Metabolic requirements for growth and colonisation of *Campylobacter*

*Campylobacter*s is a member of the Epsilonproteobacteria which need a microaerobic environment for growth. In the intestines of host species this is provided in the mucosa that lies adjacent to a single cell layer of epithelial cells that can supply of oxygen from capillary blood. Catabolic activity is extremely limited compared to other enteropathogenic bacteria because a number of carbohydrate utilization pathways are either absent or incomplete (Hofreuter, 2014). Inspite of these restrictions, *C. jejuni* can colonize several animal hosts efficiently as a commensal intestinal inhabitant (Hofreuter, 2014). On the basis of comparative genome sequences, transcriptome and metabolome imply to know how the

metabolic ability of *C. jejuni* drives its persistence in the intestinal habitats of different hosts. Moreover, new understandings into the metabolic necessities that support intracellular survival strategies for *C. jejuni* have been found. Because *C. jejuni* uses different properties to establish infections compared to other pathogenic Enterobacteriaceae, it represents an opportunity for clarifying new characteristics of the dynamic and metabolic interaction between a bacterial pathogen, the microbiota and the host (Hofreuter, 2014).

Many differences in the disease outcome could be related to the virulence potential of individual *C. jejuni* isolates and are probably connected to variations of motility and surface structures (Wilson et al., 2010). Interestingly, not only surface structures are variable but also metabolic traits are extremely variable between *C. jejuni* isolates. For example, in robust-colonizing strains compared to poor-colonizing strains, the genes that support oxygen-independent respiration and the catabolism of amino acids and peptides are over-represented (Ahmed et al., 2002, Hofreuter et al., 2006, Hepworth et al., 2007, Seal et al., 2007, Hiett et al., 2008).

1.13 Nutritional niche determination of *C. jejuni*

1.13.1 The non-glycolytic nature of *C. jejuni*

C. jejuni is a chemoheterotrophic bacterium; with metabolic features that obviously differentiate it from other enteropathogenic bacteria. This is most noticeable in the limitation of this bacterium to catabolise carbohydrates. Early substrate utilization studies of *Campylobacter* have identified its inability to utilize glucose and other carbohydrates for growth. *Campylobacter* is largely considered to be a non-saccharolytic

microorganism. Genomic sequence analysis supported these findings (Parkhill et al., 2000, Velayudhan and Kelly, 2002, Gundogdu et al., 2007). Growth-independent BIOLOG phenotype microarrays established that pentoses and hexoses like glucose, fructose, galactose, and rhamnose and the disaccharides lactose, maltose, trehalose and sucrose do not improve the respiratory activity of *C. jejuni* (Line et al., 2010, Gripp et al., 2011, Muraoka and Zhang, 2011). Nonetheless, these studies found that some *C. jejuni* strains like NCTC 11168 are able to utilize fucose compared to the other isolates. This ability is conferred by the occurrence of a 9 kb genomic island present in *C. jejuni* NCTC 11168, which is absent in *C. jejuni* 81–176 (Muraoka and Zhang, 2011, Stahl and Stintzi, 2011, Hofreuter et al., 2006). Interestingly, it has been identified that the fucose-catabolizing *C. jejuni* strains do not secrete fucosidase enzymes to cleave fucose residues from glycosylated host substrates. These isolates may depend on fucose released from intestinal mucins and host glycans by commensal bacteria like *Bacteroides thetaiotaomicron* as established for enterohaemorrhagic *Escherichia coli* (Pacheco et al., 2012). *C. jejuni* and *H. pylori* have an incomplete glycolysis pathway due to the absence of glycolytic enzyme phosphofructokinase, which catalyses the fructose-6-phosphate to fructose-1, 6-bisphosphate.

C. jejuni possesses all enzymes of the reductive branch of the pentose phosphate pathway but do not hold the glucose-6-phosphate dehydrogenase and the 6-phosphogluconolactonase of the oxidative pentose phosphate pathway branch (Parkhill et al., 2000, Fouts et al.,

2005). Subsequently, gluconeogenesis appears to be essential for the biosynthesis of glucose and its products that are precursor substrates for the lipopolysaccharide and capsule biosynthesis, in addition to the N- and O-glycosylation of several proteins (Karlyshev et al., 2005).

Parkhill et al. (2000) found that the genome of *C. jejuni* holds all the essential enzymes required for gluconeogenic synthesis of glucose from phosphoenolpyruvate (PEP), however experimentally gluconeogenesis has not been proven as yet. It was identified that an important metabolic junction between catabolism and anabolism is constituted in *C. jejuni* by PEP carboxykinase PckA, the pyruvate kinase Pyk and the pyruvate carboxylase (Velayudhan and Kelly, 2002). These enzymes are necessary for the oxaloacetate—PEP—pyruvate conversion (Sauer and Eikmanns, 2005). Interestingly, *C. jejuni* is missing a PEP synthase to enable the first ATP-consuming gluconeogenic reaction that generates PEP from pyruvate and do not have the PEP carboxylase, which catalyses the synthesis of oxaloacetate from PEP. Therefore, the PEP carboxy kinase PckA, catalysing the generation of PEP by the decarboxylation of oxaloacetate, plays a crucial role in the intermediary metabolism of *C. jejuni* (Velayudhan and Kelly, 2002).

1.13.2 Organic acids and amino acids fuel the growth *C. jejuni*

Gluconeogenesis appears to have an important role in the physiology of *C. jejuni* , which raises questions regarding the substrates used by these bacteria to fuel its intermediary metabolism and cope with the need for carbohydrate, lipid and protein generation. A number of studies have

demonstrated that *C. jejuni* has the ability to catabolize organic acids like lactate, pyruvate, acetate and intermediates of the TCA cycle as well as a limited number of amino acids (Elharrif and Mégraud, 1986, Westfall et al., 1986, Velayudhan and Kelly, 2002, Mohammed et al., 2004, Hinton, 2006, Guccione et al., 2008, Wright et al., 2009). Organic acids are important for *C. jejuni multiplication*, and studies have identified pyruvate, 2-oxoglutarate, fumarate, succinate, malate and lactate as chemoattractant for *C. jejuni* (Hugdahl et al., 1988, Vegge et al., 2009). Consequently, imported L-lactate is oxidized to form pyruvate, which is considered as a growth substrate of *C. jejuni* (Mendz et al., 1997, Velayudhan et al., 2004). Pyruvate-flavodoxin oxidoreductase (POR) catalyses the synthesis of acetyl-CoA that fuels the TCA cycle of *C. jejuni* and is used for fatty acid biosynthesis (Leach et al., 1997, Gundogdu et al., 2007, Kirkpatrick et al., 2009). Moreover, acetyl-CoA can be converted to acetyl-P and then to acetate by successive actions. The acetate is released by an unidentified transporter in the logarithmic growth phase (Wright et al., 2009). At the beginning of stationary phase the "acetate switch" which is well described for *E. coli* (Wolfe, 2005), may be found for *C. jejuni* , enabling the organism to utilize secreted acetate as a growth substrate after conversion to acetyl-CoA by the acetyl-CoA synthetase (ACS) (Wright et al., 2009).

Utilization of amino acids plays a significant role in fuelling the central metabolism of *C. jejuni* . However, the pathogen is limited to glucogenic amino acids to support its propagation, and amino acids such as arginine and lysine are reported to be chemorepellents (Rahman et al., 2014). The

growth-promoting amino acids for most *C. jejuni* isolates are aspartate, glutamate, proline and serine (Leach et al., 1997, Guccione et al., 2008, Hofreuter et al., 2008). Metabolic profiling of supernatants from *C. jejuni* liquid cultures indicate considerable reductions of these four amino acids from Mueller-Hinton (MH) medium or nutrient-rich brain heart infusion (BHI) (Guccione et al., 2008, Wright et al., 2009).

Hofreuter et al. (2008) demonstrated that not all *C. jejuni* isolates examined were able to grow with serine alone as a carbon source; therefore utilization of this amino acid seems to be a different catabolic trait of *C. jejuni.* The molecular basis for this metabolic diversity is not known as yet, because *C. jejuni* strains unable to use serine are not mutated in the *sdaA* or *sdaC* genes although the serine dehydratase activity was decreased (Hofreuter et al., 2012). Compared to serine catabolism, proline usage looks to be a more conserved metabolic trait of *C. jejuni ,* as also shown for the utilization of aspartate and glutamate (Hofreuter et al., 2008). The growth of *C. jejuni* 81–176 with proline is mediated by the *PutP* transporter and the enzyme *PutA,* which has been established by directed mutagenesis approaches (Hofreuter et al., 2012). The *PutA* enzyme of *C. jejuni* is expected to have both delta-1-pyrroline-5-carboxylate dehydrogenase and proline dehydrogenase activities to use NADH and FAD as cofactors, respectively, which catalyses the oxidation of the proline to glutamate. Furthermore, the PutA protein of *C. jejuni* does not feature the N-terminal DNA-binding domain, which functions in the repression of the *putAP* gene group in *Salmonella* Typhimurium in the absence of proline (De Spicer and Maloy, 1993). *C. jejuni* utilize

glutamate via an ABC transporter system encoded by the *peb* locus containing genes for two permeases, one periplasmic substrate-binding protein Peb1A and one ATP-binding protein. Furthermore, the Peb ABC transporter, mutations of the permease PaqP and the ATPase PaqQ of the Paq (pathogenesis-associated glutamine) ABC transporter system adversely influenced the uptake of glutamate in *C. jejuni* 81–176 (Lin et al., 2009).

Due to the absence of glutamate dehydrogenase in *C. jejuni,* the conversion of glutamate seems not to proceed directly to 2-oxoglutarate through deamination, which exists in other representatives of the *Campylobacteriaceae*. As an alternative, glutamate could either be transferred to glutamine by the type I glutamine synthetase GlnA or constitute a substrate for the aspartate: glutamate transaminase AspB, catalyzing the synthesis of aspartate and 2-oxoglutarate from oxaloacetate and glutamate (Guccione et al., 2008). AspB plays a significant role in the intermediary metabolism of *C. jejuni* and its proliferation, because growth in the nutrient rich media MH or BHI, or in defined medium with 20 mM glutamate or serine as single energy sources is disrupted by inactivation of *aspB* (Guccione et al., 2008, Novik et al., 2010). In addition to serine, proline and glutamate catabolism, the uptake of aspartate is essential for the metabolic activities of *C. jejuni.* This is indicated by the recognition of two chemoreceptors, which interact with aspartate, Tlp1 (Hartley-Tassell et al., 2010) and CcmL (Tlp3) (Rahman et al., 2014).

Genome sequence analysis of *C. jejuni* indicates that the pathogen encodes an asparaginase (AnsB) which enables the transfer of asparagine to aspartate and also for glutamate synthase (GltBD) catalysing the synthesis of glutamate from glutamine and 2-oxoglutarate (Gundogdu et al., 2007). These studies indicate that *C. jejuni* has the ability to catabolize asparagine and glutamine and both amino acids are chemoattractants for *C. jejuni* (Vegge et al., 2009). Even though the conserved Paq transporter system makes *C. jejuni* able to utilize glutamine (Lin et al., 2009), growth trials illustrate that not all *C. jejuni* isolates can utilize glutamine as a lone carbon and energy source (Hofreuter et al., 2008).

The majority of the examined *C. jejuni* strains do not have the ability to utilize asparagine as a single source of carbon and energy although all carry an L-asparaginase gene that encodes a cytoplasmic AnsB enzyme (Hofreuter et al., 2008). This suggests that a concerted pathway for asparagine import does not exist in *C. jejuni* . Interestingly, a number of gamma-glutamyl transpeptidase (*ggt)*-positive *C. jejuni* strains able to metabolize glutamine and glutathione as a source of amino acids, encode a secreted asparaginase but are missing the *fucP*-gene group, whilst *C. jejuni* isolates that lack GGT and AnsB-mediated extended amino acid catabolism carry the genes essential for fucose uptake (Gripp et al., 2011, Zautner et al., 2011, de Haan et al., 2012).

Further to the growth-promoting amino acids serine, proline, aspartate and glutamate transporter systems, *C. jejuni* have homologs of the LIV

(leucine, isoleucine and valine) system (Ribardo and Hendrixson, 2011). However, no one of these can directly fuel growth of *C. jejuni* (Guccione et al., 2008, Hofreuter et al., 2008). In *C. jejuni* the LIV-locus consists of six genes encoding two permeases LivH and LivM, periplasmic binding proteins LivJ and LivK, and the cytoplasmic ATPases LivG and LivF. Mutation studies established that the individual LIV ABC transporter constituents are responsible for the utilization of these acids, mainly of leucine (Ribardo and Hendrixson, 2011). The presence of leucine does not inhibit the expression of the *liv* genes in *C. jejuni* (Ribardo and Hendrixson, 2011) as noted for *E. coli* (Quay and Oxender, 1976). Within the sequenced *C. jejuni* genomes some additional putative amino acid transporters exist, for instance, the putative sodium/serine-threonine symporter. *C. jejuni* also have three further periplasmic proteins CjaA, CjaC and GlnH, which belong to the family 3 of solute-binding proteins, and may also assist the amino acids utilization. CjaA is an N-glycosylated 30 kDa lipoprotein connected to the inner membrane (Pawelec et al., 1997, Wyszyńska et al., 2008) that binds cysteine. Based on growth experiments it is proposed that CjaA may not be involved in the uptake of cysteine alone. CjaC is an N-glycosylated 28 kDa protein that shows homology to several periplasmic binding proteins of the ABC-type amino acid transporters (Pawelec et al., 1997, Wyszynska et al., 2007). Interestingly, *hisJ* mutant of *S. Typhimurium* can be complemented by heterologous expression of the *C. jejuni cjaC* gene (Garvis et al., 1996). The GlnH family, 3 solute-binding proteins, remain uncharacterized.

The significance of amino acid utilization for the growth of *C. jejuni* is well recognized, a few studies have identified the *de novo* biosynthesis of amino acids, such as the involvement of *cysM* and *ilvE* in the generation of cysteine and leucine, respectively (Ribardo and Hendrixson, 2011, Vorwerk et al., 2014). Other studies have tested the ability *C. jejuni for* amino acid biosynthesis by heterologous expression of the genes for the arginine (Hani and Chan, 1994, Hani et al., 1999), cysteine (Garvis et al., 1997), leucine (Labigne et al., 1992) and aromatic amino acid (Wösten et al., 1996) biosynthesis pathways in respective auxotrophic mutants of *Escherichia coli* . Compared with the biosynthetic capability of *H. pylori*, *C. jejuni* shows deficiencies which leads to the requirement for the following amino acids for growth: arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine (Doig et al., 1999).

1.13.3 Peptidases and peptide catabolism of *C. jejuni*

The fundamental role of amino acid catabolism for *C. jejuni* propagation indicates that peptides may also be essential growth-promoting substances, particularly, as different peptides besides free amino acid can be acquired from proteins in the gastrointestinal tract of host organisms (Adibi and Mercer, 1973). The *C. jejuni* genome encodes many putative peptidases and proteases (Hofreuter et al., 2006, Gundogdu et al., 2007), and some of these have been included with the virulence of *C. jejuni* , including ClpP, HtrA, CJJ81176_1086, CJJ81176_1228, Cj0511 or Pgp1 (Brøndsted et al., 2005, Cohn et al., 2007, Novik et al., 2010, Boehm et al., 2012, Frirdich et al., 2012, Karlyshev et al., 2014). Based on

analyses using the BIOLOG phenotype microarray of *C. jejuni* it is proposed that dipeptides like glycyl-glutamine and glycyl-proline improve the respiratory activity and could be utilized as carbon sources, however, strain specific variations exist (Gripp et al., 2011, Muraoka and Zhang, 2011). The diversity in peptide catabolism could be due to the presence of different numbers of peptidases within the *C. jejuni* isolates (Zautner et al., 2011, Champion et al., 2005, Hofreuter et al., 2006, Hepworth et al., 2007, Gonzalez et al., 2009). Hofreuter et al. (2008) found that only *ggt*-positive *C. jejuni* strains can properly utilize the tripeptide glutathione as a carbon, energy and cysteine source (Vorwerk et al., 2014). Furthermore, the *Campylobacter* peptide transporter A (CptA) has been demonstrated to support the growth of *C. jejuni* 81–176 with the dipeptides Cys-Gly, Arg-Trp and Arg-Ile (Vorwerk et al., 2014).

Altogether, *C. jejuni* displays fascinating metabolic variation. Growth properties vary in *C. jejuni* strains with the presence or absence of metabolic genes notably in the utilization of specific substances such as fucose, asparagine or glutamine and peptides. *C. jejuni* strains are armed with several sets of groups A chemoreceptor *tlp* genes that respond to diverse group of potential nutrients (Day et al., 2012, Rahman et al., 2014).

1.14 Aim of study

The aim of this study was to identify the cues that give rise to filamentation of *C. jejuni growing* in broth cultures. This study also aimed to investigate the viability of component cells of spiral and filamentous population using vital staining, to determine any strain dependency and any possible differences in the ability of the two different morphotypes to survive unfavourable conditions. Functional changes in gene expression leading to these morphological and phenotypic differences were investigated by transcriptome analyses (RNA-Seq) of the morphotypes separated from stationary phase cultures of *C. jejuni* PT14 and 12661.

2 . Material and Methods

2.1 Preparations of growth, storage media and chemical solutions

All growth and storage media, unless otherwise specified, were prepared with reverse osmosis (RO) water and were treated by autoclaving at 121°C and 15 psi for 15 minutes on a liquid cycle. Before adding of any supplements or antibiotics, agar media, molten agar was initially tempered at 45-50°C. A laminar flow cabinet was used for the preparation of microbiological plates. Once they dry, the plates were stored at 4°C for up to four weeks. Unless otherwise stated all other chemicals were kept at room temperature after autoclaving and cooling.

2.1.1 Blood agar (BA)

Blood agar base No. 2 (Oxoid, Basingstoke, UK) (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 according to manufacturer's instructions. Following sterilisation, defibrinated horse blood (TCS, Buckingham, UK) was added to a final concentration of 5% (v/v) before dispensing into Petri dishes. A 1 Litre of BA contained 40 g of blood agar No. 2 and 50 ml of defibrinated horse blood.

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

Campylobacter blood-free selective agar (Oxoid) (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 according to manufacturer's instructions and occasionally supplemented with 2% (w/v) bacteriological agar No. 1

(Oxoid). One Litre of CCDA contained 55 g of *Campylobacter* blood-free selective agar.

2.1.3 Nutrient broth No. 2 (NB2)

Nutrient broth No. 2 (Oxoid) (Lab-Lemco' powder 10.0 g/l, peptone 10.0 g/l, and sodium chloride 5.0 g/l) was prepared according to manufacturer's instructions by suspending 25 g of Nutrient broth No. 2 powder into 1 litre of RO water. After sterilisation, it was kept at room temperature for up to eight weeks.

2.1.4 Mueller-Hinton (MH) Broth

Mueller-Hinton broth (Oxoid) (dehydrated beef infusion 300 g/l, casein hydrolysate 17.5 g/l, starch 1.5 g/l, Ca2+ 3.854 mg/l, Mg2 5.072 mg/l) was prepared according to manufacturer's instructions. Following sterilisation, MH broth was kept in dark at room temperature for a maximum of 8 weeks.

2.1.5 Mueller-Hinton (MH) agar

Mueller-Hinton agar was prepared by adding bacteriological agar No. 1 (Oxoid) to MH broth (2.1.4) to a final agar concentration of 1.5% (w/v).

2.1.6 *Campylobacter* MH motility agar

MH motility agar was prepared as already described for MH agar (2.1.5) but instead the bacteriological agar was added to a final concentration of 0.4% (w/v).

2.1.7 Bacterial storage medium

Nutrient broth (NB) No. 2 (2.1.3) (Oxoid) was dispensed according to manufacturer's instructions and dissolved in RO water supplemented with 20% (v/v) glycerol (Fisher Scientific, Loughborough, UK). This was dispensed in 30 ml aliquots to sterile glass universals and, following sterilisation, stored at room temperature for a maximum of 8 weeks. One Litre of bacterial storage medium contained 23 g of nutrient broth and 200 ml of glycerol. This was stored at room temperature until ready for use.

2.1.8 Maximal Recovery Diluents (MRD)

Maximum recovery diluent (Oxoid) (peptone 1.0 g/l, sodium chloride 8.5 g/l) was prepared according to manufacturer's instructions. Following sterilisation, MRD was stored at room temperature for a maximum of 8 weeks. One litre of RO water contained 9 g of MRD.

2.1.9 Phosphate Buffer Saline (PBS)

Phosphate buffered saline (Oxoid) (0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.115% (w/v) Na2HPO4 and 0.02% (w/v) KH2PO4) was prepared according to manufacturer's instructions to a pH of 7.3±0.2 at 25°C. Following sterilisation, PBS was kept at room temperature for a maximum of 8 weeks.

2.1.10 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, and aliquoted

into 10 ml volumes prior to autoclaving and was stored at room temperature until ready for use.

2.1.11 50X Tris-acetate, EDTA buffer (TAE buffer)

A stock solution of 50X TAE buffer was prepared by dissolving 242 g/l of Trisma® base (Sigma-Aldrich), 57.1 ml/l of glacial acetic acid, 18.61 g/l of EDTA and adjusting the pH to 8.0 by adding NaOH (Fisher Scientific). Following sterilization, TAE buffer was stored at room temperature for a maximum of 8 weeks.

2.1.12 Tris-acetate buffer 10X (TA buffer)

A stock solution of 10X TAE buffer was prepared by dissolving 0.121g (Mr=121.42 g/l) of Trisma® base (Sigma-Aldrich), in 90 ml of pure water and adjusting the pH to 7.74 by adding glacial acetic acid (Fisher Scientific), and then volume adjusted to 100ml and the pH to 7.75 a 25 ºC. Following sterilization, TA buffer was stored at room temperature for a maximum of 8 weeks.

2.1.13 Magnesium sulphate

Magnesium sulphate (1 M) was prepared by dissolving of magnesium sulphate heptahydrate (Sigma-Aldrich) to final concentration 246.7 g/l.

2.1.14 MEM, no phenol, no glutamate

MEM (Minimum Essential Medium) (Life Technologies Ltd, Paisley, UK) is a commonly used medium for cell culture. MEM can be used with a variety of suspension and adherent mammalian cells; however, in this study it was

used as a minimum medium to understand the effect of limiting nutrients on the viability and the morphology of different *Campylobacter* strains compared with other rich media such as NB2. This medium used alone or supplemented with (10mM) of sodium pyruvate as an energy source (Sigma Aldrich, UK).

2.1.15 Sodium Pyruvate

Sodium pyruvate (1M) was prepared by dissolving the solid crystals (Sigma-Aldrich) to final concentration 110 g/l, and filter sterilising. To obtain 10mM $CH_3COCOONa$ was produced by dilution in sterile MEM (2.1.14).

2.1.16 Trichloroacetic acid (TCA)

To measure the ATP concentration in *Campylobacter* efficiently without degradation, TCA is recommended as the best extractant because it efficiently releases ATP from microorganisms and inactivates any enzymes that may degrade the ATP before measurement, usually 0.5% to 2.5% is recommended as a final concentration. From this 1% solution of TCA was found to be the suitable concentration for ATP extraction for this study. To get 100% (w/v) solution of TCA dissolves 125g of TCA in 56.75 ml of sterile water.

2.2 *Campylobacter*

2.2.1 Bacterial strains

The *Campylobacter* strains used in this thesis are detailed in Table 2-1

Table 2-1: *Campylobacter* strains used in this thesis

Campylobacter species	Strain Name	Source	References
C. jejuni	**HPC5**	poultry isolate	(Scott et al., 2007)
C. jejuni	**HF5-4A-4-190712S**	Poultry isolate	Nottingham University
C. jejuni	**NCTC11168**	Human isolate	(Parkhill et al., 2000)
C. jejuni	**NCTC12661**	Human isolate	(Kropinski et al., 2011)
C. jejuni	**81-176**	Human isolate	Nottingham University
C. jejuni	**PT14**	Human isolate	(Brathwaite et al., 2013)
C. jejuni	**NCTC81116**	Human isolate	(Pearson et al., 2007)
C. coli	**OR4451C**	poultry isolate	Nottingham University
C. coli	**OR5482C**	poultry isolate	Nottingham University
C. coli	**FB1**	Human isolate	Nottingham University

2.3 Growth conditions

Campylobacter strains were stored at -80°C either in Microbank vials (Pro-Lab Diagnostics, United Kingdom) or in bacteriological storage medium (2.1.7). For routine growth they were subcultured on Blood Agar base no. 2 (Oxoid) supplemented with 5% defibrinated horse blood (TCS) as described in (2.1.1), the plates were incubated in 3.5 L jars (Oxoid, Basingstoke, UK). Before incubation the pressure was reduced to -22 psi and atmospheric pressure was restored with a gas mixture containing of 85% v/v nitrogen, 10% v/v carbon dioxide and 5% v/v hydrogen (BOC Limited, Surrey, UK).

This resulted in a microaerobic growth condition containing roughly 5.6% v/v oxygen, 3.6% v/v hydrogen, 7.3% v/v carbon dioxide and 83% v/v nitrogen. Alternatively, *Campylobacter*s were grown in a Modular Atmospheric Controlled System (MACS) cabinet (Don Whitely Scientific, Shipley, UK) containing a gas mixture of 85% v/v Nitrogen, 10% v/v carbon dioxide and 5% v/v oxygen. Incubation in either the gas jars or MACS cabinet was for 18-24 hours at 42°C unless otherwise stated.

2.3.1 Storage

2.3.1.1 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 (2.1.7). These were stored at -80°C until ready for use.

2.3.1.2 Short-term storage

Long-term stocks were revived regularly 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 (2.3). Plates with overnight growth were then stored microaerobically (2.3) 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 (2.3) at 42°C for 18-24 hours.

2.3.2 Enumeration of *Campylobacter*

Campylobacter enumeration was carried out using the technique described by Miles and Misra (1938). *Campylobacter* suspensions were enumerated by performing ten-fold serial dilutions using 90µl of MRD (2.1.8) and 10 µl of suspension. Five 10 microliter spots of each dilution were dispensed onto *Campylobacter* Blood-Free Selective Agar (CCDA; Oxoid) containing addition of agar to 2% (w/v) (2.1.2) to prevent swarming. The use of the multiple spot methodologies was adopted to ensure dispersal of the *Campylobacter* colonies and reduce swarming that is obvious when using spread plates to obtain reliable colony counts. The plates 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 dilution factor and by 100.

2.3.3 Confirmatory test

2.3.3.1 Gram stain

Gram stain reagents were firstly prepared according to the manufacturer's instructions immediately prior to use (Pro-lab Diagnostics, Bromborough, Wirral, UK). Smears of colonies to be tested were then prepared aseptically on a glass microscope slide (BDH Lab Supplies, Leicestershire, UK) in PBS (2.1.9) 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) 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 Fuchsin 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. Small, Gram negative, pink coloured, curved cells indicating that the isolate was likely to be *Campylobacter.*

2.3.3.2 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 (2.3). 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. The dye is normally colourless in the reduced state, but is rapidly oxidised to a purple colour by 'oxidase-positive' species (which contain a c-type cytochrome).

2.3.3.3 Hippurate test

Sodium hippurate (Sigma-Aldrich) was prepared 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, whilst a light purple colour or no colour change was considered a negative result.

2.4 Motility assay

Using a sterile 1 µl loop, an inoculum of exponential growth cells and stationary phase cells of *Campylobacter* was stabbed into the centre of a MH motility agar plate (2.1.6), 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 (2.3). The diameter of the motility zone was subsequently measured.

2.5 *In vitro* growth curves

Campylobacter were cultured on blood agar plates at 42°C under microaerobic conditions for 24 h and the growth from whole plate was swabbed into 2 ml PBS. A 1 ml sample was removed to a 1.5 ml plastic cuvette (Sarstedt) and, with PBS as a zero, an OD600 reading was taken.

Approximate *Campylobacter* counts were calculated using a formula, derived from a standard *Campylobacter* dilution series:

Campylobacter titre in cfu/ml= (OD600 * 2 x 10^9) - 6 x 10^6

The remaining sample was diluted with PBS to give a count of roughly Log_{10} 7 cfu/ml, unless other specified. A 1 ml aliquot of this was added to 100 ml of liquid media prepared in a 250 ml conical flask and the broth incubated at 42°C with 100 rpm orbital shaking under microaerobic conditions for 24 h, unless otherwise stated. Starting at time point zero, 1 ml samples were removed every 2 h for 24 h. The *Campylobacter* titre was determined as described in section (2.3.2).

2.6 Assessing *Campylobacter* morphotypes

2.6.1 Growth in liquid cultures

Nutrient Broth Number 2 (2.1.3) and Mueller Hinton Broth (2.1.4) were prepared according to manufacturer's instructions. MEM (minimum essential medium) without glutamine and phenol red (2.1.14), with and without addition of 10 mM sodium pyruvate (2.1.15), as an energy source were also tested. The *Campylobacter* inoculum was prepared by making a suspension containing approximately 10^8 cfu/ml from an overnight culture grown on blood agar (2.1.1) containing 5% (v/v) of defibrinated horse blood (TCS, Buckingham, UK) at 37°C incubated under microaerobic conditions (2.3). To prepare growth curves, 0.1 ml of the *Campylobacter* inoculum was added to three individual 250 ml conical flasks containing 50 ml of medium and the flasks placed in anaerobic jars (Oxoid) under microaeobic conditions generated as previously described above, unless otherwise stated. The jars were placed in an orbital shaker and shaken at 100 rpm at 37°C with

sampling at appropriate intervals. For each time point, an aliquot was serially diluted in maximal recovery diluent (2.1.8) and the microaerobic atmosphere re-generated. Enumeration of *Campylobacter*s was carried out by the Miles and Misra method on *Campylobacter* blood-free selective agar plates without supplement (2.3.2) and incubated microaerobically at 42°C for 48 h. The morphology of the cells was examined microscopically over 10 independent fields using bright field, epifluorescence and Gram-stain at each time point. Pre-used NB2 was prepared by carrying out the above procedure with incubation for 48 h and confirmation of the formation of filamentous cells. The cell growth was removed by centrifugation at 13,000 g for 15 min, and the supernatant filtered through a 0.2 µm filter (cellulose acetate, Sartorius Stedim Biotech, Epsom, UK). The pre-used filter sterilized medium was inoculated as described above to prepare growth curves. To reduce the potential accumulation of free radicals in MEM with pyruvate, the medium was supplemented with 0.15% w/v starch (Sigma Aldrich) prior to sterilization. Statistical differences were assessed by ANOVA from the Excel Data Analysis package (Microsoft Corporation, Redmond, WA, USA).

2.6.2 Fluorescent cell staining (Syto9/Propidium Iodide)

Bacterial suspensions (1ml) were mixed with 1.5 µl of Syto 9 (absorption /emission 485/498 nm) and 1.5 µl propidium iodide (absorption/emission 535/617 nm) from the LIVE/DEAD® BacLightTM Bacterial Viability Kit (Life Technologies) and incubated in the dark for 20 min at room temperature. A 5 µl aliquot was applied to the centre of a clean glass microscope slide. An 18 mm^2 coverslip was placed over the suspension. The slides were

examined over 10 min at a magnification of 1,250 (100×, plan Apo) with an epifluorescence microscope (Labophot; Nikon, Tokyo, Japan) and images captured from 10 independent fields using a vertical mounted digital camera (Cannon EOS 600D, Taiwan).

2.6.3 Separation of short spiral and filamentous morphotypes

In order to separate the short spiral and filamentous forms, three independent biological replicates of *C. jejuni* 12661 or PT14 were taken from -80°C as described in (2.3.1.1) and inoculated into 500 ml of sterile MEM (2.1.14) with sodium pyruvate (2.1.15), to a final density of approximately 10^5 CFU/ml. The flasks were incubated in a shaking incubator at 100 rpm, at 37°C under microaerobic conditions (2.3) for 30 h. The short spiral cells were separated from filamentous cells by collecting the filtrate from the culture that was passed through a 0.8 µm sterile nitrocellulose membrane (Sigma–Aldrich). The filtration was repeated with a fresh filter. The filamentous forms were obtained by flushing the first filter with fresh MEM (with pyruvate) and collecting the eluate. The suspensions were examined microscopically and diluted to contain approximately 5×10^6 CFU/ml in either sterile water (reverse osmosis) or NB2 to examine their survival characteristics.

2.6.4 Comparison of the survival of the short spiral and filamentous morphotypes

Suspensions of the separated morphotypes in either water or NB2 (prepared as described above) were either incubated at 37 or 4°C for 96 h under microaerobic conditions. Viable counts were performed as described above at 24 h intervals (2.3.2).

2.7 Determination of intracellular ATP

The ATP concentrations were measured by luciferase luminescence using a commercial kit according to the manufacturer's instructions (Promega, Southampton, UK). Either suspension of selected morphotypes prepared as above (2.6.3) or culture suspensions collected at various time points were centrifuged at 13,000 g for 15 min and the cell pellets washed in 1ml of TA buffer (20 mM Tris-acetate buffer pH 7.75)(2.1.12) before re-pelleting. The cell suspensions were lysed with 1% (w/v) trichloroacetic acid in TA buffer. The ATP assays were performed by adding 10 µl of the cell extract to a polypropylene tube containing 100 µl of recombinant luciferase/luciferin reagent, followed by gentle mixing and immediate reading in a pre-blanked luminometer (Turner Designs TD 20e, Promega). The signal was integrated over 10 s with a 2 s delay, and reported in relative light units (RLU) that could be converted to ATP concentrations using a pre-prepared standard curve. ATP contents were normalized according to either viable counts or microscopic cell counts or protein content (Bradford reagent; Pierce).

2.8 TEM imaging of *Campylobacter* morphotypes

Transmission electron microscopy (TEM) was performed for different *Campylobacter* morphotypes suspensions that were prepared as previously described (2.6.3). The samples were fixed by adding 2.5ml of 0.2M Cacodylate buffer and 1.9ml distilled water to the tube already containing 600ul of 25% EM Glutaraldehyde and kept at 4 ºC until ready to use. After centrifugation of the sample suspension, approximately $1mm^3$ of the pellet was resuspend in 1ml of fixative and incubated at room temperature in fume hood for 1h, after centrifugation the supernatant was discarded, and the pellet was washed twice with MRD before final suspension in 1ml of MRD (2.1.8) for TEM. The samples were adsorbed onto copper-Formvar-carbon grids (Ted Pella, Inc.) for 10 min, negatively stained with 4% phosphotungstic acid (buffered to pH 6.8) for 2 min, air dried, and examined under a transmission electron microscope (Philips Morgagni 268; FEI Company). Images were captured with the charge-coupled device camera and controller (Gatan, Inc.), and processed using Digital Micrograph (Gatan, Inc.). All steps were performed at room temperature.

2.9 Genomic DNA isolation

Campylobacter genomic DNA was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions. Briefly, 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 suspended 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 2ml 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.

2.10 Polymerase chain reaction (PCR)

2.10.1 Primer design

The primers used within this thesis are listed in Table 2.2

Table 2-2: The primers used within this thesis

Gene ID	Description	Primer Sequences (5'-3')	~Size	References	Annealing temperature
katA (A911_06730)	catalase	ACCGTTCATGCTAAGGGAAG	200	Xie et al 2011	57.5 °C
		CCTACCAAGTCCCAGTTTCC			57.5 °C
groES (A911_05920)	groES co-chaperonin	AAACAACAGCCTCAGGCATAA	150	Xie et al 2011	57.5 °C
		TTCTGTTCCACCGTATTTAGCA			57.5 °C
mreB (UC78_0263)	Rod shape protein	GAGCCTTCTGTTGTGGCAGTT	200	Xie et al 2011	57.5 °C
		AGCGGATCATTTTTTCAGTCAT			57.5 °C
Hup (A911_07770)	hemin uptake system outer membrane receptor	TGGTACTAGAGGTGGCGTGA	350	This thesis	57.5 °C
		GGATCGCTTTGAGCTTGTGC			57.5 °C
omrf (A911_03360)	Outer membrane receptor for ferrienterochelin and *coli* cins	TTGATTGATGGGCGTCGTCA	414	This thesis	57.5 °C
		TGCTTTTGTGGGACTTTGAGCC			57.5 °C
aroE	shikimate 5-dehydrogenase	GCTGATGTGAAGGATGATTTTGCT	200	This thesis	57.5 °C
		CTGTACCTCCAGCACCCAAA			57.5 °C
arp (UC78_1332)	ankyrin repeat protein	GGACAAACCCCATTAGCAGGA	200	This thesis	57.5 °C
		TGCCACATCTTTGCGTCCA			57.5 °C
iup (UC78_0175)	iron-uptake protein	GCGATCACAAAAAGAGCAAGA	105	This thesis	57.5 °C
		AGGAGCTGTAGCACTTCTCATA			57.5 °C
Hpo (UC78_0207)	hypothetical protein	AGTAGCCATAGGATCTGGAACTT	113	This thesis	57.5 °C
		AGCTTACATAACCCACATTAGTCTT			57.5 °C
cu chup (UC78_0865)	Cu chaperone protein	CAGACTTAAAAGAGGAAACCACCC	100	This thesis	57.5 °C
		AGAGTTGCTGGACAAACATCAG			57.5 °C

Table 2.2 continued

Gene ID	Description	Primer Sequences (5'-3')	~Size	References	Annealing temperature
clpB (A911_02485)	*clpB* protease	AGCGAATCATTGCTTTTTCATC	150	Amy J. 2011	57.5 ˚C
		CTATAGGAAGCTTTTTATTTTTAG			57.5 ˚C
petA (A911_05750)	putative ubiquinol-cytochrome C reductase cytochrome B subunit	AGCTCTCCATCTTGCATTCC	100	Amy J. 2011	57.5 ˚C
		CAAGTGTCAAAGCTGCAGG			57.5 ˚C
flag (A911_02665)	flagellar protein *FlaG*	ATTCAAACCGATAGAAGTCAAG	150	Amy J. 2011	57.5 ˚C
		ATATCAGCCAACTTTTCACTCA			57.5 ˚C
Cj1491 (A911_07185)	*cheY*-like receptor	AATCTCTCTTGCCATATCCAG	150	Amy J. 2011	57.5 ˚C
		AATCGGAGCTCAAAATGGAG			57.5 ˚C
hisA (A911_07705)	phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	ACCATGCAAGGAGTAAATGTAA	150	Amy J. 2011	57.5 ˚C
		CCTACGATAACCCCACTACAA			57.5 ˚C
icd (A911_02585)	isocitrate dehydrogenase (internal control) (PT14)	TCTCCAGCTCTTGCGACTTA	284	This thesis	57.5 ˚C
		GCCGCCTTAAGCTGAGGAA			57.5 ˚C
rplA (12661) (UC78_0461)	Ribosomal protein L1 (internal control) (12661)	ACCTGCTGGAACGGGTAAAA	83	This thesis	57.5 ˚C
		CTGCACCAGCATTTTTCGCT			57.5 ˚C
Hypo (PT14) (A911_07055)	hypothetical protein	CCTATACAACAAAGTTATGTGGC	200	This thesis	57.5 ˚C
		GTCAGCAATCGCAGCAGCTG			

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 primers were 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. 1):

Tm = 4 (G+C) + 2 (A+T)

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

2.10.2 PCR condition

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 0.2 ml thin-walled PCR tubes (Fisher Scientific) in 20 µl volumes with 50-100 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 6 minutes. This was followed by 30 cycles each of denaturation at 95°C for 30 seconds, annealing of the primers at 55-58°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 5-7 minutes.

2.10.3 Agarose Gel Electrophoresis

DNA was routinely analysed 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 ethylene diamine tetra acetic 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 Bio labs, Ipswich, UK). Samples were run for 1hours 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* 12661

Genomic DNA from *C. jejuni* 12661 was isolated as described previously (2.9). DNA sequencing was performed using the Illumina MiSeq platform (Northumbria University, Newcastle, UK). The data consisted of a total of 514,564 paired-end reads of 250 bp for *C. jejuni* 12661. Initial

processing of the raw data and assembly was performed using CLC Genomic Workbench v6.0 (Qiagen, Aahus, Denmark).

2.11.2 *C. jejuni* 12661 accession numbers

The complete genome was annotated and deposited into GenBank by Prof. I. Connerton under the accession number 35925B2. The 12661 genome was *de novo* assembled by Prof. I. Connerton using CLC Genomics Workbench software (Qiagen, Aahus, Denmark).

2.11.3 Annotation of *C. jejuni 12661*

The complete genome of *C. jejuni 12661* was annotated automatically using the program Prokka version 1.07 (Seeman, 2014) running on a virtual unix machine (unbuntu). Frame shifts 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 frame shifts. Once all potential frameshifts were confirmed the complete genome of *C. jejuni* 12661 was deposited in GenBank under the accession number 35925B2.

2.11.4 Genome Analysis

The complete annotated genome of *C. jejuni 12661* was analysed using CLC Genomics Workbench software to identify the major genetic

features. Genome comparisons were also made between *C. jejuni* 12661 and other *Campylobacter* strains using the mVISTA (Frazer et al., 2004).

2.12 Transcriptome analysis

2.12.1 RNA isolation and purification

C. jejuni (PT14 and 12661) cultures were grown on blood agar as previously described (2.1.1). Three independent biological replicates of each tested strain were prepared in 500 ml of sterile MEM sublimanted with 10mM sodium pyruvate to a final density of roughly 10^5 cfu/ml as described in (2.6.3). Culture flasks were incubated in a shaking incubator at 100 rpm (Fisher Scientific) at 37 °C under microaerobic conditions (2.3) for about 30 h to produce the long filaments. Filaments were separated from spiral cells by filtration using a filter membrane 0.8µm (nitrocellose, Sigma Aldrich, UK) (2.6.3), from which the RNA was extracted (exponential spiral cells, separated stationary phase spiral cells and separated stationary phase filaments). After centrifugation at 12,000 x *g* for 15 min, at 4 °C, in a Beckman JS-21 centrifuge using a JA14 rotor, the pellets were pooled. Total RNAs were isolated by using TRIzol® Max™ Bacterial RNA isolation kit (Invitrogen, Paisley, UK) following manufacturer's instructions. Pre-heated Max Bacterial Enhancement Reagent at 95 °C was added by pipetting up and down to inhibit RNase activity and incubated at 95 °C for 4 min. TRizol® reagent was added to the lysate and incubated at room temperature for 5 min. Phase separation was proceeded by adding cold chloroform (Sigma-Aldrich) and mixed by shaking the tube vigorously by vortex for 15 sec. The mixture

was incubated at room temperature and centrifuged at 12,000 g for 15 min at 4 °C. The mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colourless aqueous phase containing RNA. The aqueous phase (400 µl) was transferred into a fresh tube before precipitating the RNA by adding 0.5 ml cold isopropanol (Fluka) and mixing by inversion at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was removed carefully without disturbing the RNA pellet. The pellet was suspended in 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 min at 4 °C. The RNA pellet was resuspend in 50 µl RNase free water by pipetting up and down after air-dry. The RNA was treated by DNase I (QIAGEN) to digest away contaminating DNA and purified by using the RNeasy mini kit (QIAGEN) following manufacturer's instructions. Briefly, the RNA was cleaned up by buffer RLT (containing quinidine thiocynate) and subsequently with 96-100% of ethanol by using RNeasy mini spin column and centrifuge at 8000 x g for 15 sec. at each step. The purified RNA was quantified using a NanoDrop ® ND-lOOOUV-Vis Spectrophotometer and the quality was assessed using Agilent gels (Agilent BioAnalyzer 2100).

2.12.2 Depletion of rRNA and library construction

Depletion of 23S, 16S and 5S rRNA from samples was achieved using Ribo-Zero™ Magnetic Kit (Bacteria) (from Illumina, sold by Cambio, Cambridge, UK). According to the manufacturer's instructions, depletion of the rRNA was confirmed using a BioAnalyzer 2100 (Agilent).

NEBNext ® Ultra™ Directional RNA Library Prep Kit (sold by NEB, UK) used for Library preparation. Depletion of rRNA, Library preparation and sequencing were carried out at (Welcome Trust Centre for Human Genetics, Oxford, UK).

To prepare the cDNA libraries, the NEBNext Ultra directional Prep with a Universal Bacteria Ribozero kit were used according to the manufacturer's instructions. Briefly, 1 µg of rRNA-depleted RNA was chemically fragmented by using the RNA fragmentation component of each kit (composition confidential) and incubating for 15 minutes at either 94°C.

The fragmentation reaction was stopped by placing the mixture on ice. All samples were pooled based on the concentration peak size, which was about 290-300bp and a final 10nM pool was created. Fragments were reverse transcribed to a single-stranded cDNA library using ProtoScript II Reverse Transcriptase and random or oligo (dT) primers. End repair, dA-Tailing and Adaptor Ligation: End repair of the cDNA library followed by optional dA-tailing followed by ligation to 3´ & 5´ adaptors. The library was then amplified and sequenced using an Illumina HiSeq 2500 sequencer. The RNA libraries were subjected to single end sequencing with a target read length of 100 nucleotides that resulted in ~20 million reads per library. Sequence reads were mapped against a reference genome using CLC Genomics Workbench.

2.12.3 RNA-Seq analysis

Single-end, directional sequencing was carried out using the Illumina HiSeq 2500 sequencer. The indexed libraries were pooled and loaded onto a single lane of a HiSeq 2500 flow cell at a concentration of 10 nM. Cluster formation, primer hybridisation as well as 100 bp, 36 cycles sequencing at Welcome Trust Centre for Human Genetics, 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.

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, the morphotype sample reads were mapped to the genome sequences of *C. jejuni* PT14 or 12661 (GenBank accession numbers CP003871 and 35925B2 respectively).

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 Quantitative Real Time PCR (qPCR)

2.13.1 RNA extraction and cDNA synthesis for real time qPCR

Cultures were grown under the same growth condition as described above for transcriptomic analysis (2.6.1) and the RNAs were extracted (2.12.1) from the same morphotype cells as previously highlighted (2.6.3).

2.13.2 Strand specific cDNA construction for qPCR

Total RNA extraction was achieved as previously mentioned (2.12.1). First-strand cDNAs were synthesized using the modified protocol of Untergasser (Untergasser, 2008). Briefly, approximately 1µg of RNA, 25ng/µl of reverse primer of the specific genes were mixed and the reaction made to 20 µl with RNAase free water. The primer RNA mixes were denatured and annealed in a thermocycler (Alpha Laboratories, Hampshire, UK) at 70ºC 10 minutes then 25ºC 10 minutes before storage at 4ºC. Then 20ul of the enzyme mix preparation (5x first strand buffer (Invitrogen), 0.1M dithiothreitol (DTT), 10Mm dNTPs (Promega, UK), RNAase inhibitor, superscript II (200 units/1ul) (Invitrogen) and RNAase free water) added to each reaction as a complete master mix then incubated in thermocycler at (25ºC 10min, 37ºC 45min,42ºC 45min, 70ºC 15min and finally stored at 4ºC), the reaction mix was then diluted according to the cDNA concentrations synthesised, and 2 µl of each dilution was used as template for qPCR reactions.

2.13.3 Relative quantification of gene expression using SYBR Green Real Time PCR

RNAs were treated with DNase I (Qiagen) to eliminate residual DNA. DNase I was then deactivated by adding EDTA (15 mM), followed by heating at 75 °C for 5 min and total elimination of chromosomal DNA was checked by PCR. Specific Primers for real time PCR were designed (Table 2.2) with an average length of 18-24 bp and melting temperature ranging between 58-60 °C. Primer3 primer design software (http://primer3.sourceforge.net/) was used to select primer sequences. Secondary structures and dimer formation were checked using the Beacon Designer Free Edition software (http://www. premierbiosoft. com/qOligo/). Aliquots of cDNA were used as the template for qRT PCR. The internal control gene showed no change in the expression and the negative control was DEPC treated water, which matched the purity of the master mix. An optical 96 well micro titre plate (Applied Bio systems) was used with a 20 ul reaction volume consisting of 2x SYBR Green master mixes (Applied Bio systems), 50 nm of the gene specific primers and the template cDNA. An ABI Prism 7000 sequence detector (Applied Bio systems) was programmed for an initial set up of 6min at 95°C, followed by 40 cycles of 30s at 95°C and 30s at 57 - 58 °C. In each run a negative control was included. SYBR Green detects double stranded DNA. A melt curve was obtained from a first step starting from 50- 95°C to control the specificities of the qPCR reactions for each primer pair. Cycle threshold (Ct) values were determined using Light Cycler 480 software (BioRad). The comparative threshold cycle (Ct) method was used to

calculate change (n fold) where samples were normalized to the control gene, since it was selected to be constitutively expressed in all conditions in the RNA-Seq experiments. Reactions were performed in triplicate. The fold changes were calculated using the method:

ΔCt = Ct (Target, spirals/filaments) – Ct (Ref, spirals/filaments)

ΔCt = Ct (Target expo) – Ct (Ref expo)

ΔΔ Ct = Δ Ct (spirals/filaments) – Δ Ct (expo)

Normalized target gene expression level (fold change) = $2^{(-\Delta\Delta Ct)}$

(Schmittgen and Livak, 2008).

The data were presented as ratios of the amount of normalized transcript in the treatment to that from the control. The gene ID and their related primer sequences used for real-time qPCR analysis were listed in table (2.2).

3 . Filamentation of *Campylobacter* in broth cultures

3.1 Introduction

Campylobacter is frequently responsible for food borne bacterial gastroenteritis worldwide (WHO, 2013) *Campylobacter* cells are usually slender, spiral shaped rods measuring 0.2–0.8 μm wide and 0.5–5 μm in length (Vandamme, 2000) but like many other microorganisms, filamentous forms have been observed under certain circumstances (Griffiths, 1993, Thomas et al., 1999, Apel et al., 2012, Cameron et al., 2012). Filamentation has been identified in many different bacteria and is thought to occur through inhibition of cell division, metabolic changes, or DNA damage which includes the SOS response resulting in the inhibition of septum formation whilst the chromosome is repaired (Justice et al., 2008).

It has frequently been associated with stress and starvation conditions during which it may confer survival advantages (Justice et al., 2008). Moreover, it has been suggested that filamentation could represent a programmed response to unfavourable environments that aids the bacterium's survival (Justice et al., 2008) and may enhance virulence (Mulvey et al., 2001, Stackhouse et al., 2012). Alternatively, filamentation may simply occur through an inadvertent loss of control of the normal cell division process. Whichever scenario is true may depend on the species of bacteria and the type of environmental stress encountered. Examples of environmental signals that have been identified to induce filamentation include: starvation, exposure to oxidative stress, reduced water activity, the presence of quorum sensing molecules,

antibiotics, or host immune effectors (Allison et al., 1992, Jones et al., 1996, Janion, 2001, Miller et al., 2004a). Filamentation as a response to sub-lethal stress has been observed in a number of food borne bacteria, which have led to concerns that these bacteria may rapidly divide once the growth conditions become permissive to cause spoilage or disease (Jones et al., 2013). Filamentation in *Campylobacter* has also been observed in response to mutation of the response regulator RacR and its sensor RacS, which are involved in the heat shock response (Apel et al., 2012), in response to treatment with certain antibiotics such as sitafloxacin (Yabe et al., 2010) and as a general response to hyperosmotic stress (Cameron et al., 2012). Importantly, for *C. jejuni and Helicobacter pylori* broth cultures, grown in a microaerobic atmosphere, filamentation occurred spontaneously on entry in to stationary phase (Griffiths, 1993, Fawcett et al., 1999, Thomas et al., 1999, Wright et al., 2009). Here nutrients may become depleted, potentially leading to starvation stress or there may be a build-up of inhibitory metabolites present in the spent medium but the lack of a specific stress trigger distinguishes filamentation of these two related bacterial genera from other bacteria where stationary phase cells are generally reduced in size (Nyström, 2004). Moreover, elongated cells can be readily identified in scanning electron micrographs of *Campylobacter* biofilms suggesting filamentation may occur naturally in situations where biofilms form (Kalmokoff et al., 2006, Brown et al., 2014).

The aim of this study was to investigate the cues that give rise to filamentation of *C. jejuni growing* in broth cultures. We also aimed to investigate the viability of the individual component cells of the filament using vital staining, determine any strain dependency and any possible differences in the ability of the two different morphotypes to survive unfavourable conditions.

3.2 Results

3.2.1 Confirmation of filamentation on entry to stationary phase and the imaging of filament component cells by vital staining

Growth curves and microscopic images of *C. jejuni* strains HPC5 and HF5 growing in NB2 over 48 h are shown in **(Figures 3.1 A–C)**. These data confirm previous observations that on entry to stationary phase, after 24 h, progressively longer filaments are formed (Griffiths, 1993). The morphological changes coincided with different phases of growth. Exponentially growing cells showed typical short spiral forms (1.5–2.5 µm), while mid-stationary phase cells had become elongated. Cell populations in decline phase featured long filaments and the appearance of coccal forms. Vital staining was carried out using a combination of Syto9, which stains intact cells green and propidium iodide, which only penetrates membrane damaged cells, staining them red. This did not produce the expected result, with many motile and therefore essentially live red-stained cells, appearing in the exponentially growing population. It appears that propidium iodide was able to enter live cells and was not a good indicator of viability for *Campylobacter*. Vital staining of the stationary phase cells showing filamentation was variable, with the cell filaments either predominantly red (staining with PI) or predominantly green (stained with Syto-9) with relatively few cells containing both fluors (integrated images coloured yellow). Interestingly comparison of the bright field and fluorescent Microscope images presented in **(Figures 3.1 B, C)** clearly showed that the filaments contained cells that were

unstained between the stained cells, and remarkably these often appeared in a regular interspersed pattern as demonstrated at the 72 h time points for *C. jejuni* strains HPC5 and HF5 in Figure 3.1.

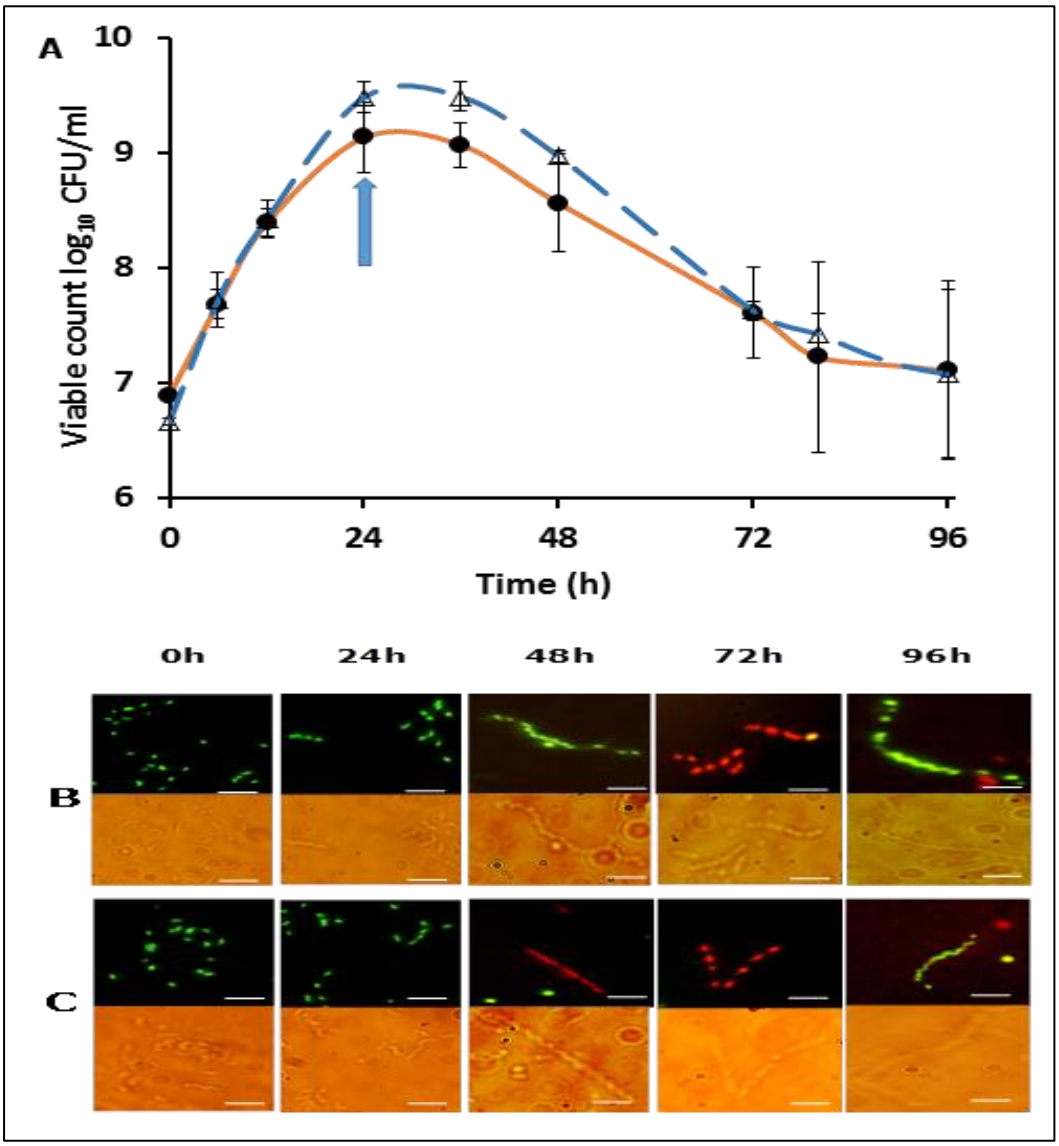


Figure 3-1: Growth curves (A) of *C. jejuni strains* HPC5 (Δ) and HF5 (●) and morphological changes during growth of *C. jejuni HPC5* (B), and HF5 (C) microaerobically in NB2 at 37 °C.

Arrow in (A) indicates the time point at which the filamentous cells were first observed (on entry to stationary phase). Error bars are ±SD for *n* = 3 biological replicates. In (B) and (C) the upper row shows cells stained with fluorescent stains (Green=syto9 and Red=propedium Iodide), whilst the lower row shows bright field microscopy image of the same cells. Scale bar, 5 μm.

3.2.2 Growth of *Campylobacter* in pre-used medium to investigate the potential role of quorum sensing, depletion of nutrients

The exponential growth rates of *C. jejuni* HF5 and HPC5 in the pre-used NB2 medium (k = 0.14 /h for both strains), were marginally reduced compared to fresh medium (k = 0.18 /h for HF5 and k = 0.25 / h for HPC5) (**Figure 2)**. This difference was significant for HPC5 (p = 0.03; ANOVA) but not significant for HF5 (p = 0.4; ANOVA). Although not optimum, the pre-used medium was able to support exponential growth, suggesting that essential nutrients had not become significantly depleted in the course of achieving stationary phase in the previous culture. Moreover the growth period required for filamentation to occur were similar for fresh and pre-used NB2, suggesting that the *Campylobacter*s were not responding to the release of soluble signal molecules as observed for auto inducer molecules involved in quorum sensing. To investigate further the role of nutritional limitation as a possible cause of filamentation, growth curves were prepared using MEM for *C. jejuni* HF5 and HPC5. Under these conditions filamentation was observed as early as 2h (**Figure 3.2; indicated by arrows**), but with no increase in viable count and no filaments greater than 10 µm in length **(Figures 3.3A–C)**. Some individual cells were stained both red and green **(Figure 3.3A)**. Addition of sodium pyruvate, as an energy source, produced an increase in the viable count and filamentation was delayed such that filaments appeared after 4–6h of incubation. Longer incubation periods produced filaments greater than 10 µm in length, which stained with both Syto9

and PI. This dual staining was particularly evident in a third strain investigated, *C. jejuni 12661* (**Figures 3.3D, E**).

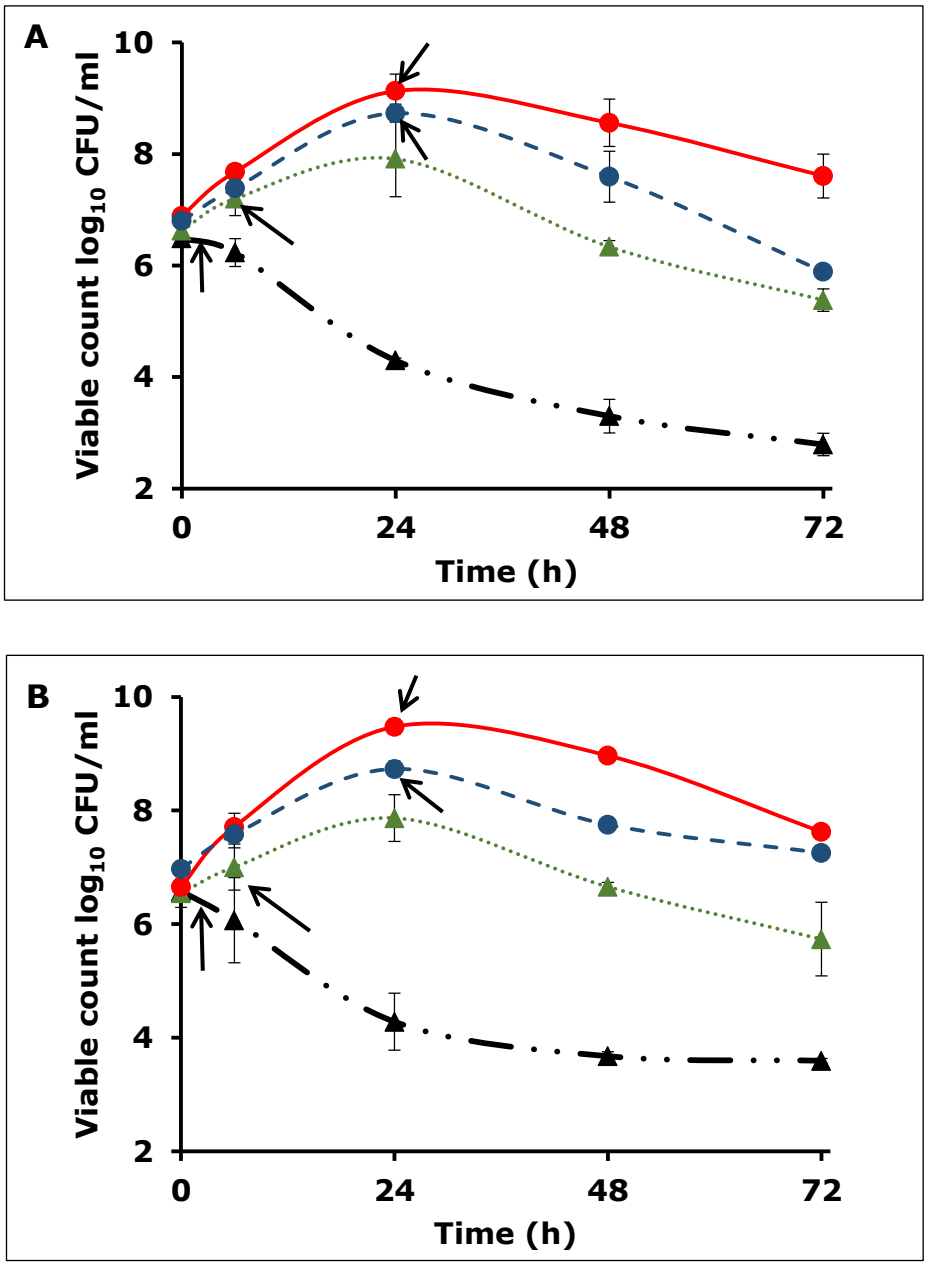


Figure 3-2: Comparison of the growth of *C. jejuni strains* (A) HPC5 and (B) HF5 in: NB2 (red), pre-used NB2 (blue), minimum essential medium (MEM; black), and MEM supplemented with 10mM sodium pyruvate (green) incubated microaerobically at 37°C.

Arrows indicate the time points at which filamentous cells were first observed. Error bars are ±SD for *n* = 3 biological replicates.

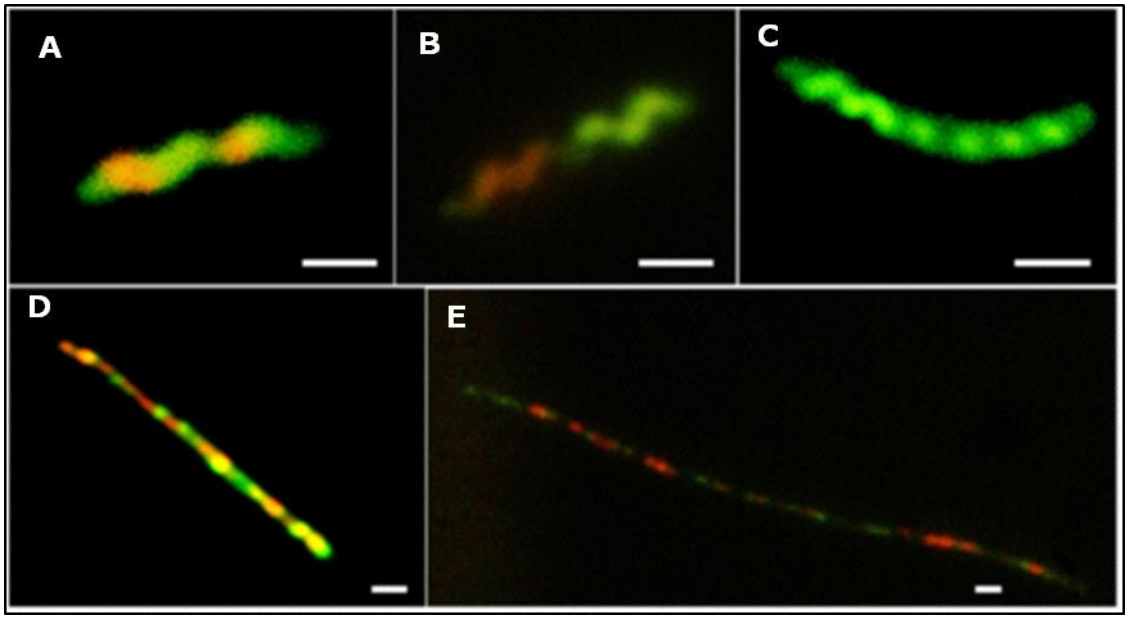


Figure 3-3: Examples of variation in vital staining observed during growth at 37°C microaerobically in MEM and in MEM supplemented with10mM sodium pyruvate.

(A) *C. jejuni* HPC5 after 2h incubation in MEM, (B) *C. jejuni* HPC5 after 4h incubation in MEM, (C) *C. jejuni* HPC5 after 6h incubation in MEM;(D) *C. jejuni* 12661 after 48 h incubation in MEM supplemented with10mM sodium pyruvate; and (E) *C. jejuni* 12661 after 72h incubation microarobically at 37 °C in MEM supplemented with 10mM sodium pyruvate. The cells stained with fluorescent stains (Green=syto9 and Red=propedium Iodide), Scale bar1 µm.

3.2.3 Growth of *Campylobacter* in starch-containing media to reduce oxidative stress

To assess if the provision of starch as an antioxidant, in the culture media could prevent or delay the onset of filamentation, *Campylobacter*s were cultured in either Mueller Hinton broth or MEM (with pyruvate) containing starch. Neither of these alternative growth media altered the time of entry to stationary phase or the subsequent observation of filamentous morphotypes **(Figures 3.4 A,B and 3.5 A,B)**.The accumulation of growth related oxidative stressors in the culture medium was probably not a causal effect of filamentation.

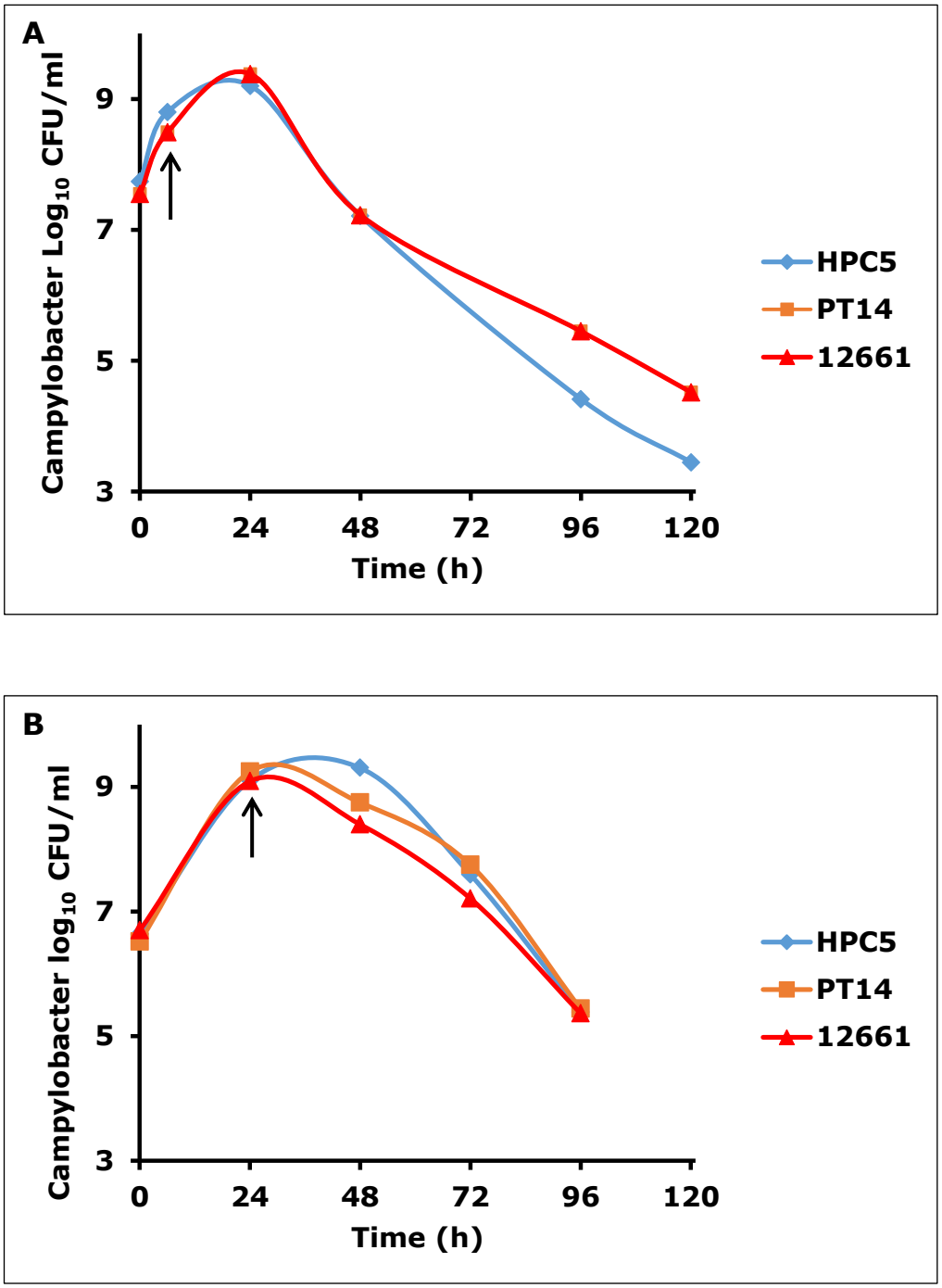


Figure 3-4: Comparison of the growth of *C. jejuni* strains HPC5 (blue), 12661(red) and PT14 (brown) lines in; (A) MEM with 10mM sodium pyruvate and 0.15% (W/V) starch, (B), MHB with 0.15% (W/V) starch incubated microaerobically at 37°C.

Arrows indicate the time points at which filamentous cells were first observed. Error bars are ±SD for *n* = 3 biological replicates.

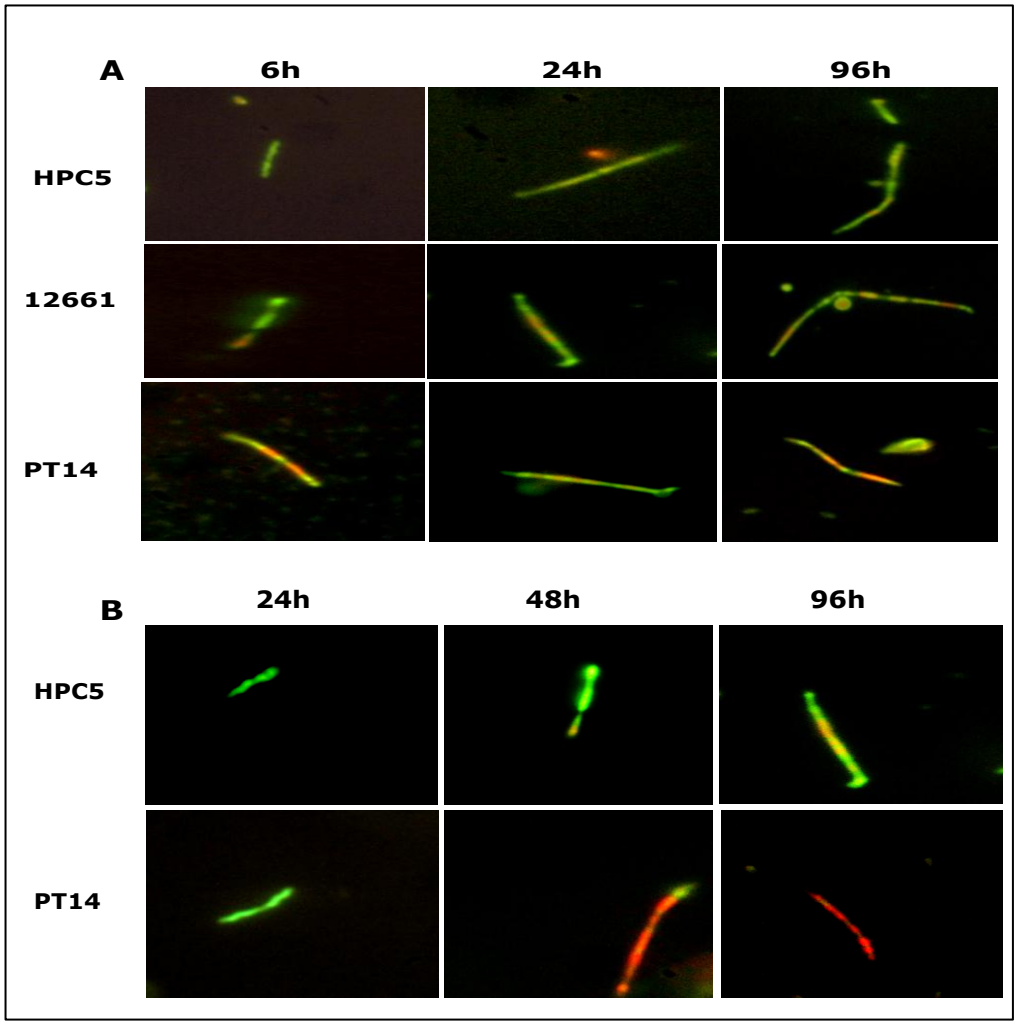


Figure 3-5: Morphological changes during growth of *C. jejuni* HPC5, 12661 and PT14 microaerobically at 37°C in (A) MEM with 10mM sodium pyruvate and 0.15% (W/V) starch, (B) HPC5 and PT14 in MHB with 0.15% (W/V) starch.

The cells stained with fluorescent stains (Green= syto9 and Red=propedium Iodide). Scale bar5 µm.

3.2.4 Filamentation in different strains of *C. jejuni* and *C. coli*

To determine if filamentation was widespread amongst *C. jejuni and C. coli a* larger group of strains was examined. These were grown in MEM with sodium pyruvate and the development of morphological changes compared in terms of the time taken for filamentation to be first observed (**Table 3.1**). Differences in timing were observed, but filamentation was a feature of all the strains tested (cells > 5 µm) with the exception of *C. coli* strain FB1, which did not form long filaments, did not increase the viable count, and produced coccal forms as early as 6 h under these conditions **(Figure 3.6)**. One strain, *C. jejuni 12661*, produced particularly long filaments and was therefore chosen for further experiments involving separation of filaments from other morphotypes.

Table 3-1: Viability and filamentation of *Campylobacter* strains growing in different media

Media	Strain	Time to achieve stationary phase (h)*	Time to first filaments (h)†	Viable count at first filamentation (CFU/ml)
NB2	HPC5	30	24	3.0×10^9
	HF5	30	24	1.3×10^9
Pre-used filtered NB2	HPC5	24	24	2.5×10^9
	HF5	24	24	8.3×10^8
MEM (with sodium pyruvate)	HPC5	24	6	1.0×10^7
	HF5	24	6	1.6×10^7
	FB1$	24	6	4.0×10^6
	OR4451C$	24	6	3.32×10^7
	OR5482C$	24	6	6.0×10^7
	81116	24	6	6.2×10^7
	11168	24	6	1.7×10^7
	12661	24	6	2.1×10^7
	81-176	24	6	1.3×10^7
	PT14	24	6	5.2×10^7
MEM (without sodium pyruvate)	HPC5	--	2	2.6×10^6
	HF5	--	2	1.6×10^6

* Determined from growth curve inoculated with ~10^6 CFU

† Determined microscopically ǂ Double length cells $*C. coli*

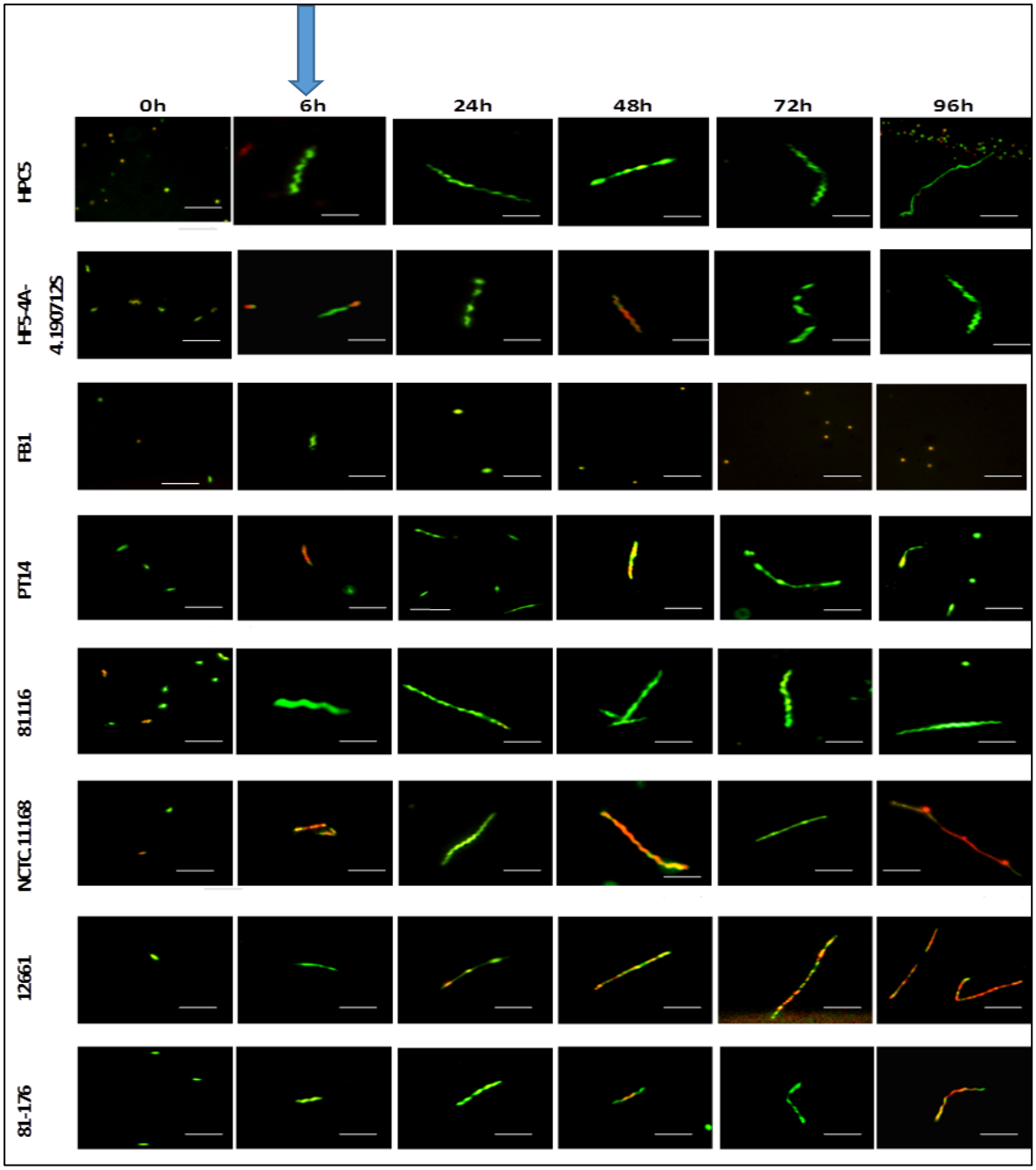


Figure 3-6: Morphological changes during growth of (7) *C. jejuni* strains and one *C. coli* (FB1) in MEM supplemented with 10mM sodium pyruvate incubated microaerobically at 37°C.

Arrows indicate the time point at which the filamentous cells were first observed. The cells stained with fluorescent stains (Green= syto9 and Red=propedium Iodide). Scale bar of is 5 µm but 81116 is 1 µm.

3.2.5 Filamentous forms show increased survival in water

In order to compare the characteristics of the filamentous and spiral cell forms coexisting in stationary phase, cultures of *C. jejuni 12661* (exaggerated filamentation phenotype) were separated using membrane filtrations. A 0.8 μm filter prevented the passage of long filamentous forms but did not hinder the passage of short spiral forms present after 30 h incubation in MEM with sodium pyruvate at 37◦C under microaerobic conditions. The survival characteristics of the separated morphotypes were examined in both NB2 and in water. There was no significant difference (p > 0.5; ANOVA) in the ability of the two morphotypes to survive in NB2 at either 37 or 4◦C (**Figures 3.7 A, B**). However, marked differences were observed in the survival of the two morphotypes incubated in water at either temperature (**Figures 3.8A, B**). The viable count of the short spiral forms fell below the limit of detection after 72h at 37◦C and 96h at 4◦ C, whilst the filamentous forms remained detectable at 37 ◦C and experienced only a modest fall in viability of 1.5 $\log_{10}$ over 96 h at 4◦C.

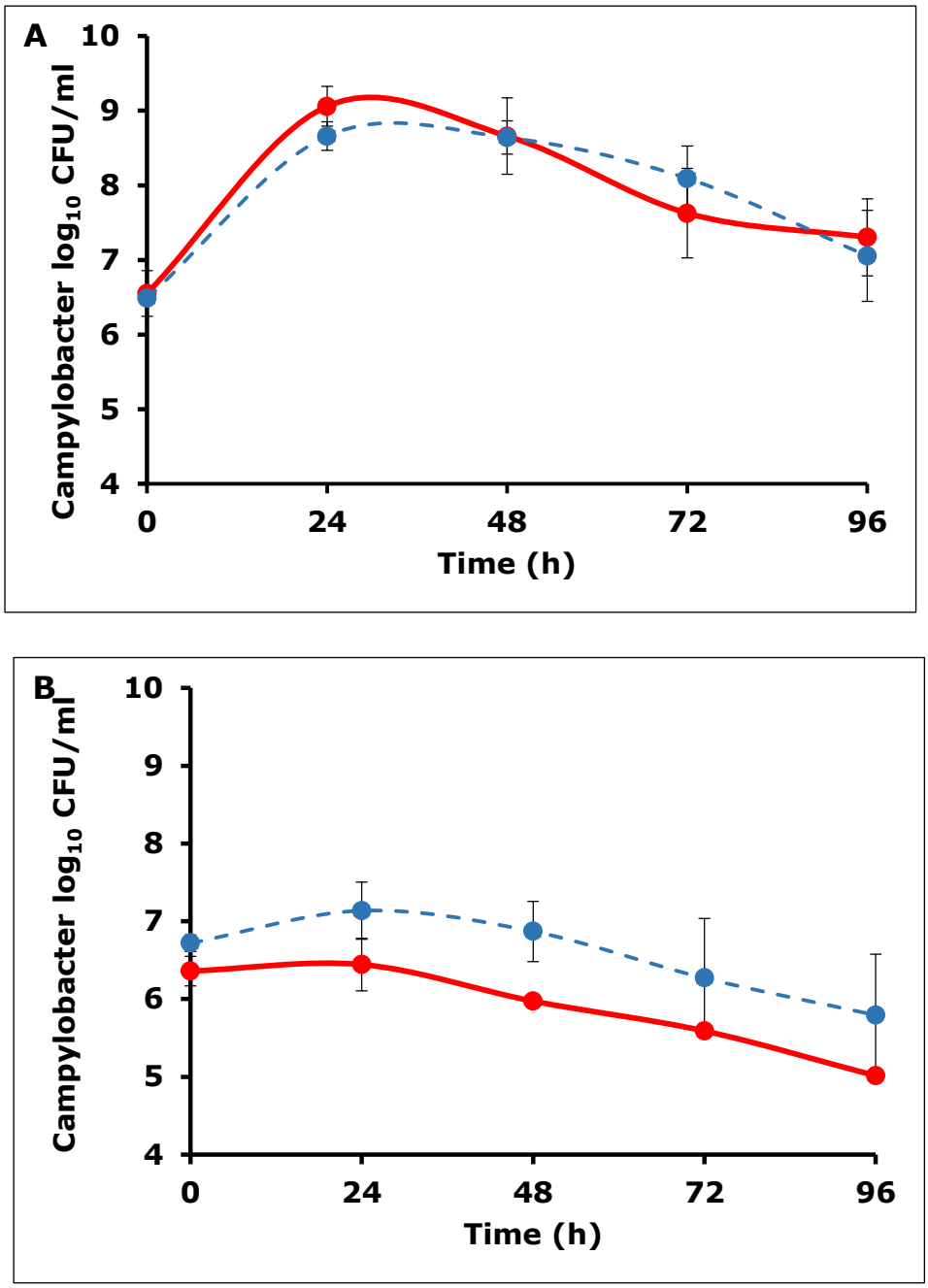


Figure 3-7: Comparison of the survival of *C. jejuni* 12661 morphotypes incubated microaerobically at: (A) 37◦C in NB2; (B) 4◦C in NB2.

Short spiral forms (red line) were separated from filamentous forms (blue line) using 0.8µ filter membrane, the samples were collected at 30h of incubation microarobically at 37◦C in MEM+sodium pyrovate, these cells then reinoculated to NB2 and incubated at different temperature as stated above. Error bars are ±SD for *n* = 3 biological replicates.

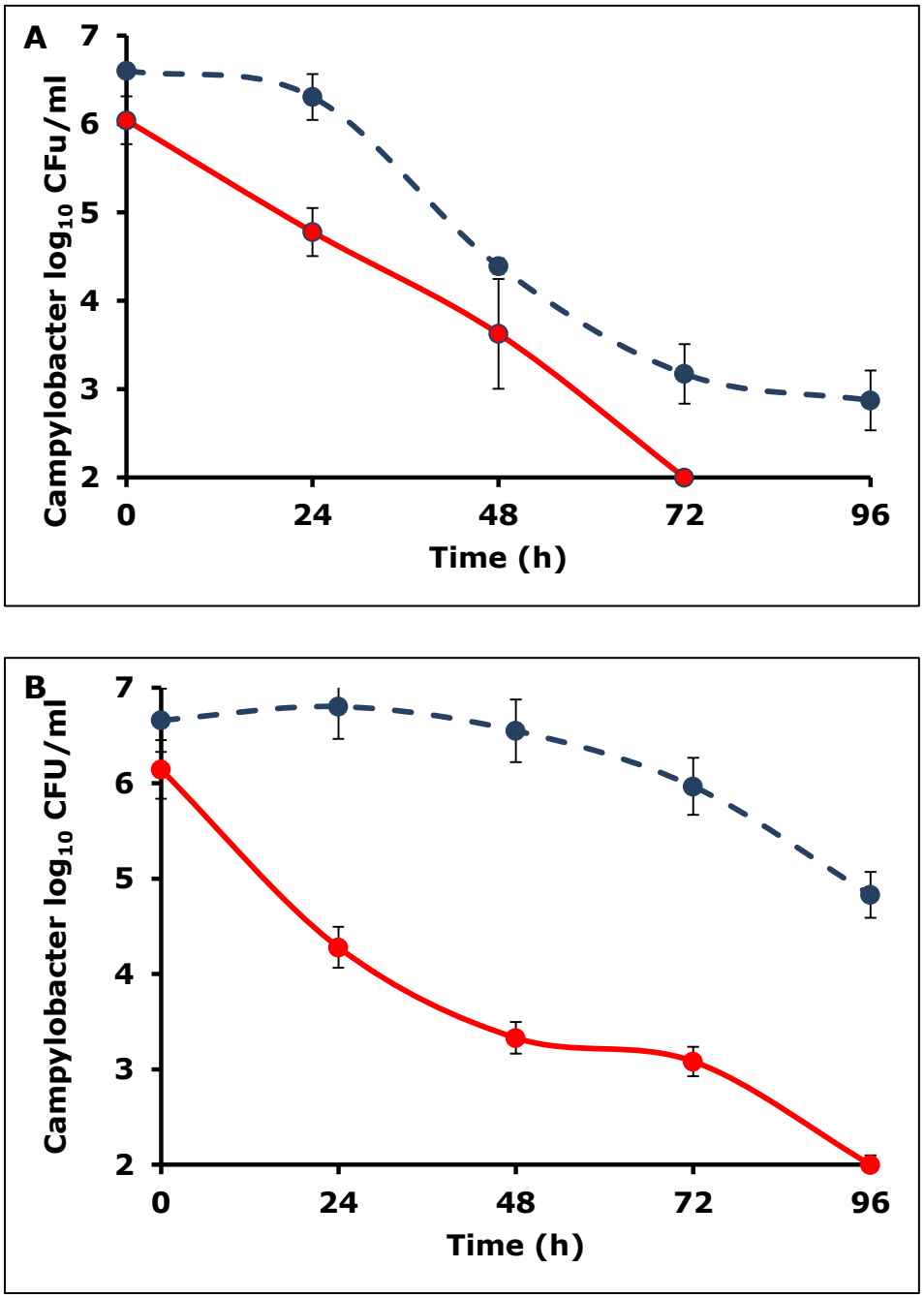


Figure 3-8: Comparison of the survival of *C. jejuni* 12661 morphotypes incubated microaerobically at: (A) 37◦C in water; (B) 4◦C in water.

Short spiral forms (red line) were separated from filamentous forms (blue line) using 0.8u filter membrane, the samples were collected after 30h of incubation microarobically at 37◦C in MEM+sodium pyrovate, these cells then reinoculated to water and incubated at different temperature as stated above. Error bars are ±SD for n = 3 biological replicates.

3.2.6 ATP Contents of spiral, filamentous and coccal cell types

In order to assess the metabolic viability of the observed morphotypes the ATP contents of separated populations of spiral, filamentous and coccal cell types of *C. jejuni* 12661 (exaggerated filamentation phenotype) and *C. jejuni* PT14 (a strain that showed a typical filamentation phenotype) were determined. Viable and microscopic counts of microaerobic cultures at 37◦C of *C. jejuni* 12661 and PT14 in MEM with sodium pyruvate were collected over 216 h to encompass exponential, stationary and decline phases of growth **(Figure 3.10).** Spiral cells harvested in mid-exponential phase at 14 h were serially diluted and the ATP contents of these cells estimated from luciferase/luciferin luminescence after lysis (Table 2). ATP concentrations were calculated using a pre-prepared standard curve (section 2.6) **(Figure 3.9).** The ATP contents of the exponential phase spiral cells between 0.99 and 1.7fg ATP per CFU were similar to earlier estimates reported for *C. jejuni* (Ng et al., 1985). Recovery and separation of the filamentous and spiral forms in decline phase at 96 h enabled determination of the ATP contents of these cell populations before the appearance of coccal forms. Beyond 168h the majority of the cells appeared coccal in these cultures, and estimates of viability required the plating of 0.2 ml culture volumes on multiple blood agar plates and/or recovery at end point dilution in broth cultures. Microscopic evaluation of 216 h cultures revealed that 61% of the *C. jejuni* 12661 cells had become coccal compared with 58% for *C. jejuni* PT14. After correction for the ATP contents of decline phase spiral and filamentous cells present in these cultures **(Figure 3.10),** estimates

of the ATP contents of the dominant coccal cells were calculated **(Table 3.2).** The low or undetectable ATP contents of the coccal cells would support the conclusion that they are no longer viable, and that the viable *Campylobacter*s recovered from these cultures represent the remaining spiral and filamentous forms.

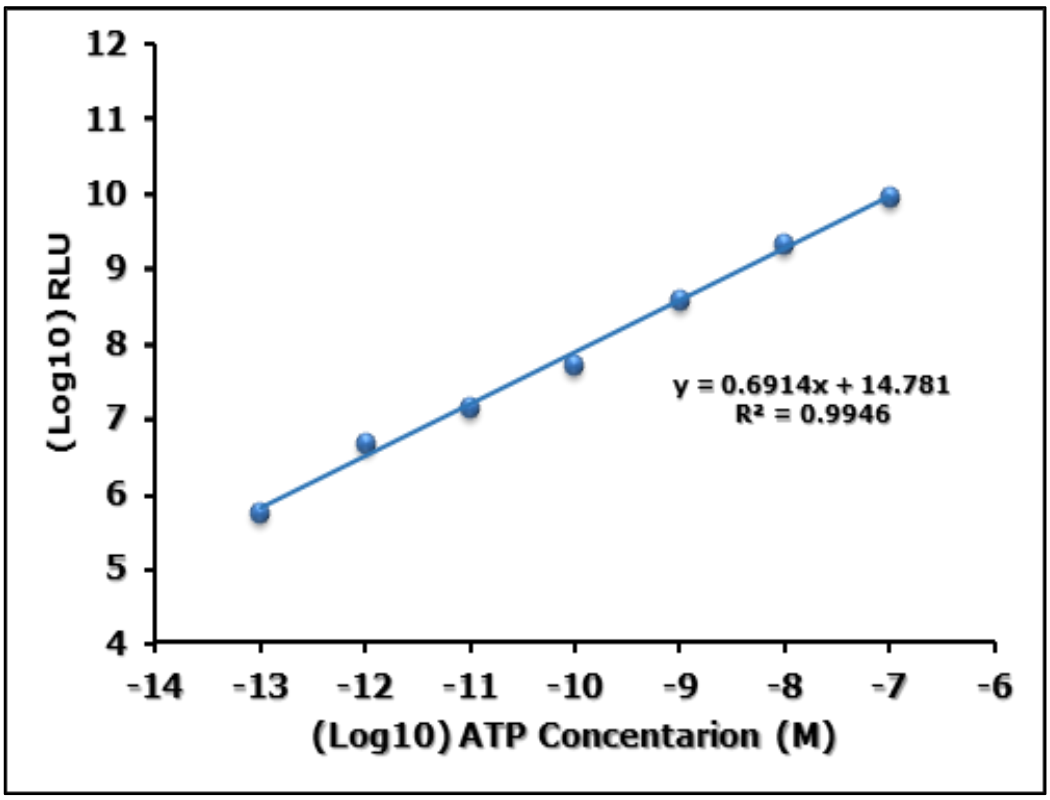


Figure 3-9: A representative ATP standard curve obtained using the ENLITEN® ATP Assay System.

Ten microliters of ATP diluted to the respective concentrations were added to a cuvette and assayed using rL/L Reagent according to the Turner luminometer protocol. A 2-second delay time after rL/L Reagent injection and a 10-second RLU signal integration time were used.

The cell volume values of filaments 0.13 and 0.09 of both 12661 and PT14 respectively, was significantly higher than the value of separated spirals 0.03 at the same time points. However, it is no necessary that all the cells within the filaments are metabolically active and having ATP.

Table 3-2: Intracellular ATP content of *C. jejuni* morphotypes at different phases of growth

Cell Forms*	***C. jejuni* 12661** **ATP (fg/cell)†**	***C. jejuni* PT14** **ATP (fg/cell)†**
Exponential phase spirals (14h)	1. 7 ± 0.35	0.99 ± 0.12
Decline phase spirals (96h)	1.19 ± 0.20	0.84 ± 0.14
Decline phase filaments (96h)	17.4 ± 2.2	2.66 ± 0.54
Decline phase coccoid (196h)	0.01 ± 0.005	ND
Decline phase coccoid (216h)	0.01 ± 0.004	ND

*Cell morphotypes observed at different time points (h) in microaerobic culture of MEM with pyruvate. †±standard deviation n=3. ND–not detectable

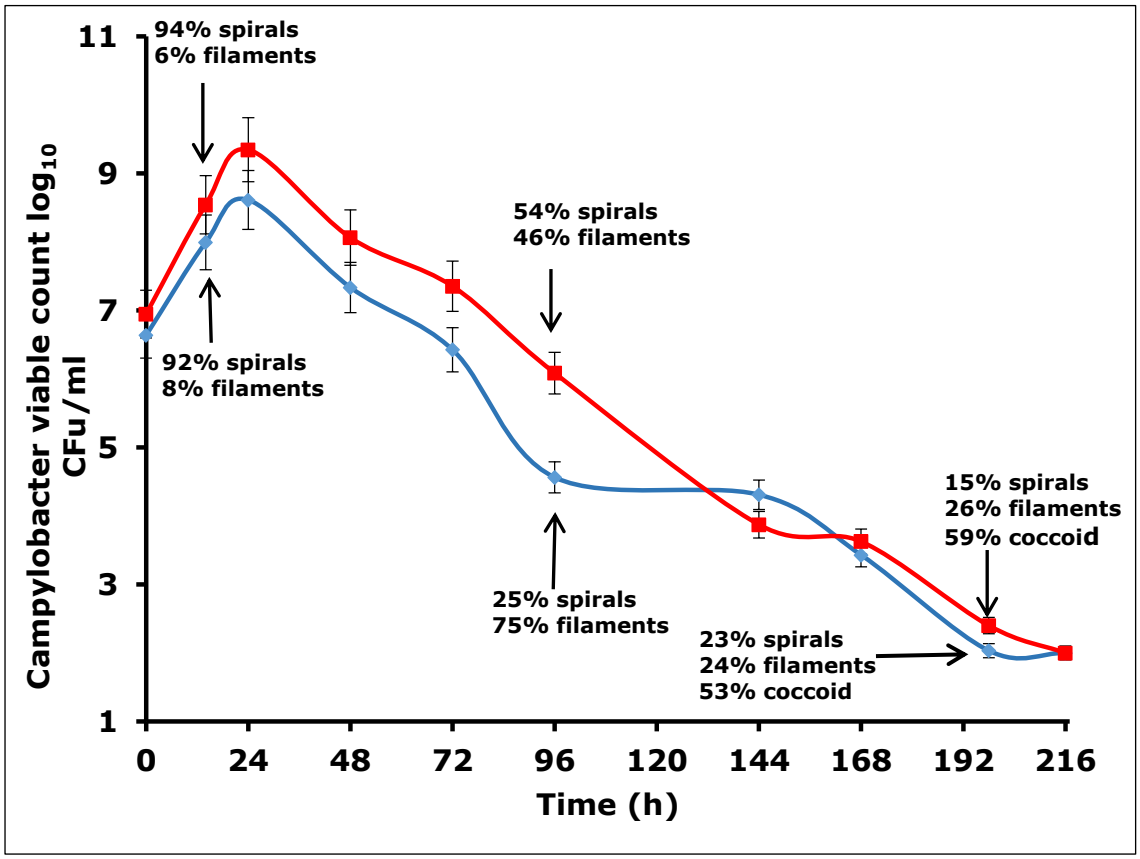


Figure 3-10: Viable counts and microscopic enumeration of the cell morphotypes observed during microaerobic growth of *C. jejuni* PT14 and 12661.

C. jejuni PT14 (blue line) and 12661 (red line) were cultured in MEM supplemented with 10mM sodium pyruvate under microaerobic conditions at 37◦C, from which samples were taken for microscopic examination and estimates of cell bound ATP. Error bars are ±SD for *n* = 3 biological replicates.

3.3 Discussion

Growth curves and microscopic examination of broth grown cultures confirmed that filamentous morphotypes were formed upon entry to stationary phase. Vital staining revealed interesting structural features of the filamentous cells and that this method may not be suitable for distinguishing live *Campylobacter* cells from dead ones. The staining patterns observed suggest growth and septa formation occur within the filaments, and that this leads to differential dye permeability preventing dye migration between cells. Moreover, the interspersed pattern would suggest cell division may be taking place at the predefined cell poles within the filament suggesting at least some of the component cells were viable. Cameron et al. (2012) observed irregular patterns of cells remaining unstained by PI within *Campylobacter* filaments formed in response hyperosmotic stress. This prompted the authors to investigate septa formation using Vanco-FL stain that binds D-Ala-D-Ala moieties of peptidoglycan indicative of septa and/or sites of new peptidoglycan synthesis. The Vanco-FL stain produced a punctuated staining pattern in hyperosmotic-induced filaments that did not co-localize with PI. This observation is also indicative of internal septa formation and as the authors conclude phenotypic differences between cells that compose the filament.

Quorum sensing bacteria produce and release auto inducers that increase in concentration as a function of cell density leading to an alteration in gene expression (Miller and Bassler, 2001) and in some cases

filamentation (Allison et al., 1992). As no change in the time taken to form filaments occurred when pre-used medium was inoculated with a fresh culture, it seems unlikely that quorum sensing was involved in the process. The relatively proficient growth of *Campylobacter*s in the pre-used medium was unexpected, as nutrient depletion on entry to stationary phase was suggested as a possible trigger for filamentation. This raises questions as to what actually triggers entry to stationary and decline phases in *Campylobacter* broth cultures. This is not well understood in other bacteria and probably depends on a combination of factors including the species of bacteria and growth medium. For *Salmonella typhimurium* and *Escherichia coli* it has been shown that increased levels of acetate (Wilson et al., 2003) or carbon starvation (Sezonov et al., 2007) are associated with the cessation of growth. The transition to stationary phase for *Campylobacter* is highly dynamic with a switch from acetate production to utilization together with a peak in motility and numerous gene expression changes (Wright et al., 2009). In addition, the bacteria do not appear to exhibit enhanced stress resistance in stationary phase, unlike many other bacteria, which is consistent with the absence of RpoS homologues (Kelly et al., 2001). Despite the absence of RpoS, *Campylobacter*s retain a stringent response that assists survival in the stationary phase of many bacterial species. Mutation of the *spoT* gene results in aberrant cell morphologies and early coccoid cell formation in stationary phase cultures (Gaynor et al., 2005). As a microaerophile, *Campylobacter* is particularly sensitive to the presence of free radicals and may suffer oxidative stress when grown in broth media despite being

supplied with a reduced oxygen atmosphere (John et al., 2011). Strictly anaerobic conditions in the presence of alternative electron acceptors nitrate or fumarate have also been shown to induce filamentation in *Campylobacter* indicating an oxygen requirement for DNA synthesis (Sellars et al., 2002). The presence of starch in *Campylobacter* culture media acts as an antioxidant and affords a degree of protection against oxidative stress caused by free radicles that can accumulate during exponential growth (Mehlman and Romero, 1982). The provision of starch in the culture media did not influence the timing or degree of filamentation, which suggests that the accumulation of reactive oxygen species (ROS) in the growth medium is not a predisposing factor to the appearance of the filamentation morphotype. However, this does not rule out a role for oxidative stress at a cellular level. Endogenous ROS produced as a consequence of cellular metabolism have been suggested to play a role as signaling molecules and effectors in the development of microbial multi-cellularity, including programed cell death (Čáp et al., 2012).

*Campylobacter*s were unable increase in viable count in MEM, without a carbon source but some increase in cell size was observed. The addition of pyruvate to the MEM allowed growth but with the early onset of filamentation as compared with growth in nutrient rich medium. Nutritional differences clearly impact on the observed morphological changes but since pyruvate remained in excess during the growth period as a carbon and energy source, it is unlikely that filamentation is a

response to carbon starvation in these experiments. Bacteria also require sufficient iron, phosphorous, sulfur, nitrogen, and other trace elements for growth, and it is possible that one, or a combination of these, become quickly exhausted in minimal medium resulting in filamentation, compared to rich media. However, the response to nutrient limitation even within a well-mixed *Campylobacter* broth culture is not uniform, in most cultures filamentous types arise among spirals that continue to divide. This implies that once the growth rate has become limited due to nutritional availability, then the formation of the filaments is either a stochastic process or developmentally controlled to generate a subpopulation that are more able to survive nutritional depletion and/or environmental stresses. In the wider environment other limiting physiological factors may also trigger the filamentation response. *C. jejuni* cultured in MEM with pyruvate exhibit an ability to retain a viable subpopulation through decline phase at 37◦C under microaerobic conditions despite a fall in viable count from >8 $\log_{10}$ cfu/ml at the end of exponential phase (24 h) to 3 $\log_{10}$ cfu/ml at 168 h. The ATP contents of the cell morphotypes recovered from the decline phases of these cultures demonstrate significant increases in the cellular ATP contents of the filamentous types as compared with spiral forms, sampled in either exponential phase, or separated from filaments in decline phase. These increases may be accounted for because the filaments appear to consist of multiple cells, joined in an ordered conglomerate. Consistent with this view, is the observation that the *C. jejuni* strain 12661 produces notably longer filaments and has a higher ATP content (17.4 fg ATP/CFU) than

C. jejuni PT14 (2.66 fg ATP/CFU). However, we have noted interspersed staining patterns and cell division within filaments, which could represent cells with greater metabolic activity within a single filament. In the later stages of these cultures (>168h) the majority of the cells become coccoid. ATP content estimates of coccal cells were either extremely low or non-detectable, suggesting they are not viable as concluded in previous reports (Moran and Upton, 1986, Boucher et al., 1994). Differences in the ability of *Campylobacter* isolates to survive in microcosm waters have been documented but without reference to the formation of filaments (Buswell et al., 1998) . The increased ability of the filamentous morphotype to survive in water compared to the short spiral form suggests that further research is necessary to assess the impact this may have on the transmission of the *Campylobacter*s from the environment to farm animals and on the safety of post-process foods. Based on these observations caution is advised when applying mathematical models that predict the survival *Campylobacter*, but do not take into account that changes in cell morphology and physiology that can increase their probability of survival. The presence of multicellular filaments may also lead to underestimates of viable cell numbers in cultures, since a single filament can form a single colony despite a multicellular origin, and filaments can exhibit differing refraction properties to non-dispersed cell suspensions that can make the interpretation of optical density measurements problematic (Wright et al., 2009).

Rapid filamentation was observed in MEM with pyruvate, in all the *C. jejuni tested*, and in all but one of the *C. coli strains* tested regardless of the strains being of either poultry or human origin. The response appears wide spread amongst the two species. The minimal medium employed in these experiments did not support any observable growth of the single *C. coli strain* that did not form filaments, it could therefore not be concluded that this strain lacks the capacity to form filaments.

Laboratory based experiments of protein expression and metabolism of *Campylobacter* demand the use of broth grown cultures to control the phase of growth. Cultures are often harvested in late exponential phase to maximize cell yields, which can contain filamentous forms with potentially different characteristics to exponentially grown short spiral forms. Whilst it has long been recognized that growth *in vitro* cannot necessarily be compared with growth *in vivo*, the observation that *in vitro* grown cells harvested late in the growth cycle show such radically different morphology and survival ability, to cells in exponential phase, requires consideration in the context of experimental design and the methods to be adopted for environmental surveys. The filamentation of *Campylobacter* strains in laboratory broth culture appears to be a common occurrence. Whether filamentation occurs in all environments inhabited by *Campylobacter*s, for example in the intestines of animals and birds, is not known but the presence of filaments in biofilms indicates that this should be further investigated. Similarly there are no reports of filamentous forms observed from environments such as water courses, or

from chilled meat, where *Campylobacter*s survive but have limited growth potential.

4 . Genomic characterisation of *C. jejuni* 12661

4.1 Introduction

C. jejuni strain 12661 is a human isolate which was previously used to propagate bacteriophages in the United Kingdom phage typing scheme (Grajewski et al., 1985, Frost et al., 1999). Comparative morphologic studies reported in chapter three demonstrated that *C. jejuni* 12661 formed unusually long filaments in stationary phase compared with other *C. jejuni.* These filamentous morphotypes also exhibited greater cellular ATP contents as compared with spiral forms. Based on these observations the genome sequence of strain 12661 was determined as a prerequisite to examine transcriptional differences associated with development of the filaments, and to compare these with the morphological development of *C. jejuni* PT14.

Over the last decade, a large number of *Campylobacter* genomes have either been partially or completely sequenced. The first complete *Campylobacter* genome sequence reported was for *C. jejuni* NCTC 11168 (Parkhill et al., 2000, Gundogdu et al., 2007), which subsequently 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). However, based on the phenotypic differences observed between *C. jejuni* 12661 and other *Campylobacter* strains the reference strain selected in this study was *C. jejuni* PT14 (Brathwaite et al., 2013) with comparative genome analysis of the closest database representatives *C. jejuni* strains 81116 and M1.

Filamentation in *Campylobacter* occurs under certain stress conditions including stationary phase. Different genes in prokaryotes contribute to the process and control of cell division. FtsZ encodes the protein that assembles into the Z ring that marks the site of cell division (Yu and Margolin, 1998). Inactivation or depletion of FtsZ results in the rapid disappearance of the Z ring and the inhibition of cell division, under these circumstances long filamentous cells missing Z rings or septa are formed (Addinall et al., 1997). FtsZ is therefore a rate-limiting component of cell division (Bi and Lutkenhaus, 1990, Ward and Lutkenhaus, 1985). FtsA is involved in cytokinesis and functions in cell division by tethering the Z ring to the cytoplasmic membrane via a conserved C-terminal amphipathic helix domain. Loss of the C-terminal helix in *E. coli* results in the formation of long and stable polymer bundles of FtsA that no longer function in cytokinesis (Pichoff et al., 2012). The *ftsA* gene is generally present directly upstream of ftsZ within a cell division cell wall biosynthesis gene cluster in a variety of diverse species. FtsK and FtsW are important cell division proteins that are expected to span the membrane multiple times (Khattar et al., 1994, Boyle et al., 1997). FtsK also deployed to the Z ring and requires both FtsZ and FtsA for recruitment (Khattar et al., 1994, Boyle et al., 1997). Another protein which may influence the cell division is FlhG.

FlhG negatively affects the ability of FtsZ to initiate cell division. Without FlhG proteins, cell division occurs more often at Polar Regions to form

minicells. In *C. jejuni* flhG mutation results in variation in the distribution of the lengths of the cells (Balaban and Hendrixson, 2011).

In *E. coli* and *B. subtilis* MinD or in *Caulobacter crescentus* MipZ, regulate the formation of cell division sites by protecting poles from cell division, but the functional mechanisms by which these proteins achieve this are different. MinD proteins do not directly inhibit cell division. Instead, these proteins localize MinC, the FtsZ inhibitor, to poles or maturing septa to inhibit divisome formation (De Boer et al., 1991, De Boer et al., 1992, Marston and Errington, 1999, Hu and Lutkenhaus, 1999). However, in *C. crescentus* MipZ mediates FtsZ depolymerization directly at polar sites (Thanbichler and Shapiro, 2006). *C. jejuni* FlhG is more homologous to MinD proteins than MipZ, where FlhG and *E. coli* MinD share a 167- amino acid region of homology (Balaban and Hendrixson, 2011).

Balaban and Hendrixson (2011) suggest that amphitrichous bacteria such as *Campylobacter* species positively exploit the placement of flagella at both poles to spatially control an FlhG-dependent mechanism to inhibit polar cell division, thus symmetrical cell division is stimulated to generate large numbers of viable off spring. Moreover, they demonstrated that other polarly-flagellated bacteria produce FlhG proteins that impact cell division, proposing that FlhG and polar flagella may work together in a wide range of bacteria to spatially regulate division (Balaban and Hendrixson, 2011).

C. jejuni strains are known to have hypervariable homopolymeric repeats within genes related to the assembly of surface structures including capsular polysaccharide, lipooligosaccharides (LOS) and flagella structures (Parkhill et al., 2000). During chromosomal replication these repeats make the genes prone to slip-strand mispairing leading to phase variation (Parkhill et al., 2000). However, phase variation of such genes can alter cell surfaces. The *cps* locus of *C. jejuni* is recognized to contain conserved and variable genes (Karlyshev et al., 2005).

The genes involved in heptose biosynthesis and those flanking the kps regions, adjacent to kpsC are highly conserved. Outside these regions the biosynthetic cps genes of strains HS:41 and NCTC11168, were found to be highly variable with the exception of gmhA2, hddA and hddC (Karlyshev et al., 2005).

In this chapter the genome sequence of *C. jejuni 12661* was characterised and an attempt was made to determine what genetic factors that may be contributing to the morphological change and longer filament formation of this strain compared with other *C. jejuni* strains **(Figure 4-1)**.

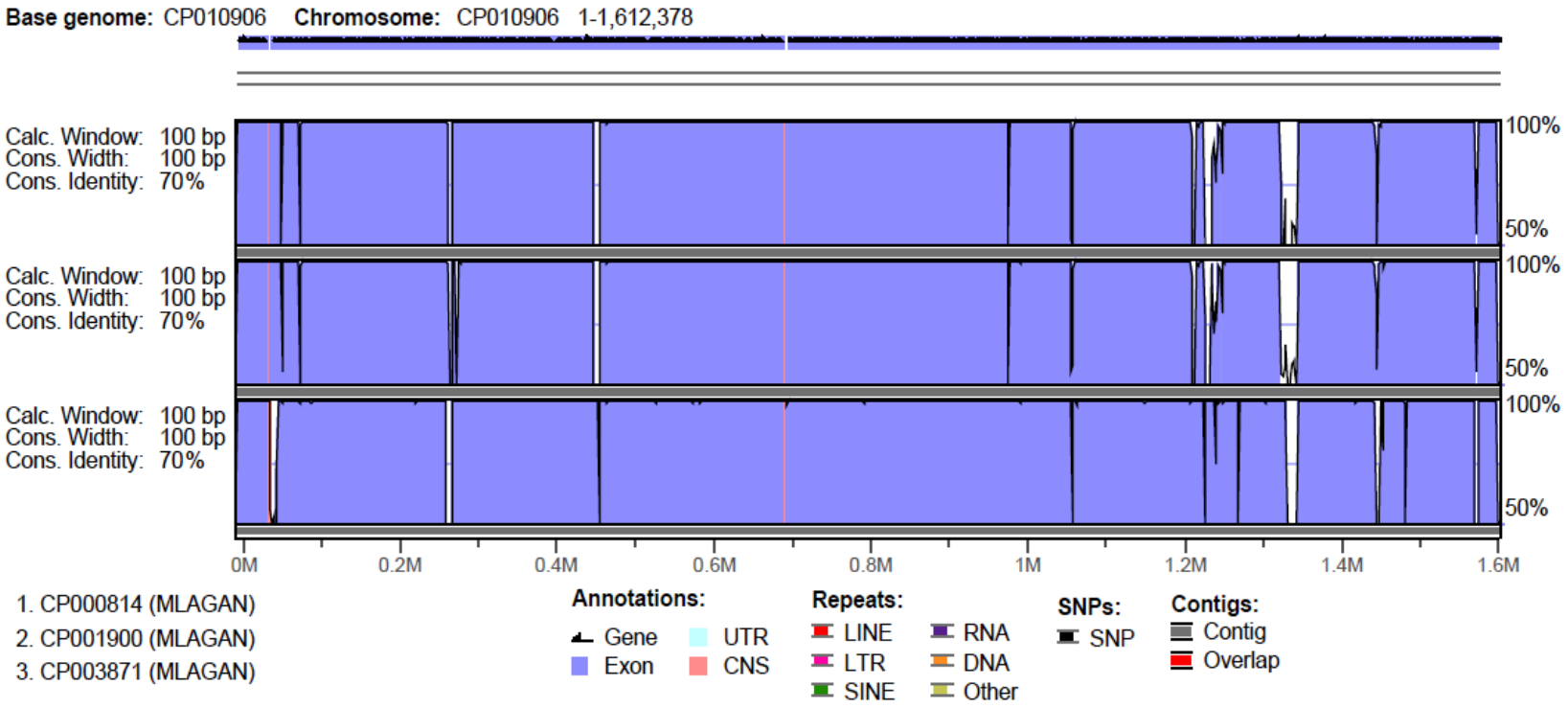


Figure 4-1: Complete genome alignment of *C. jejuni* 12661, NCTC 81116, M1 and PT14.

Plot showing the percentage identity, and the locations of inserted and missing genes across the 12661 genome (CP010906) base aligned with (1) 81116, (2) M1 and (3) PT14. The plot was created using mVISTA (Frazer et al., 2004). Blue represents the conserved exons, pink represent the conserved non-coding sequences (CNC); white represents missing or relocated genes.

4.2 Results and discussion

4.2.1 Genome analysis

The circular genome of *C. jejuni 12661* was found to be 1,612,355 bp in length with 1629 putative open reading frames (ORFs) and an average G+C content of 31.11%. The genome sequence contains 3 copies of the ribosomal RNA operon and 44 tRNA genes covering all amino acids, with arginine, alanine and leucine being represented most frequently followed by isoleucine, glycine and valine. The genome was automatically annotated using Prokka version 1.07 (Seemann, 2014) (2.11.4), from which it was possible to assign functional information to 99.7% of the 1629 ORFs on the basis of what was available in the database. **Figure 4.2** shows a genome map of *C. jejuni 12661* with the annotations related to the CDS and conserved hypothetical proteins. There is no evidence for the presence of any prophage-associated genes or plasmids within this genome.

Possible pseudogenes are annotated with similar gene names but with consecutive numbers to indicate their relationships. All the frameshifts were investigated using the microbial genomes submission tool at NCBI. For example, there is a frame shift between Arylsulfate sulfotransferase (AssT1) and Arylsulfate sulfotransferase (AssT2) compared to the genome sequences but is interrupted in several genomes including PT14 where the reading frames are identifies as a pseudogenes (Brathwaite et al., 2013).

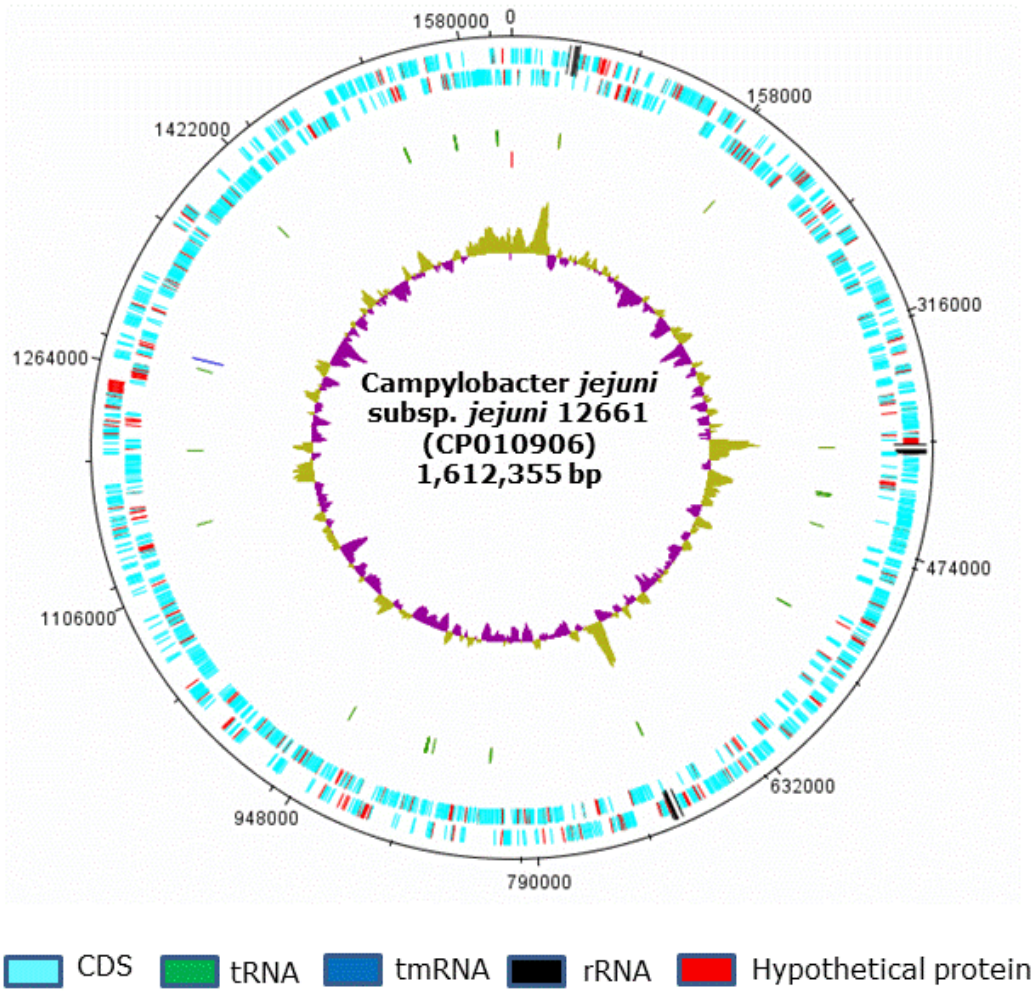


Figure 4-2: Circular representation of the *C. jejuni 12661* genome.

The annotation types are colour coded according to the key. Colours coded are 1625 CDS, 277 conserved hypotheticals, 9 rRNAs and 44 tRNAs. From the outside the concentric circles represent: forward strand; CDS, rRNA and hypothetical protein; reverse strand CDS and hypothetical protein followed by tRNA (green) and tmRNA (blue) and the inner circle is %GC plot.

C. jejuni 12661 was found to contain 19 homopolymeric G:C tracts (defined as containing ≥7 consecutive G:C residues), which bears comparison to 27 reported for PT14 (Brathwaite et al., 2013), 29 for NCTC 11168 (Parkhill et al., 2000) , 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 12661* genome three tracts reside within intergenic regions, eleven in CDS encoding hypothetical proteins and five within genes of known function **(Table 4-1).** Variation in the length of the G:C tracts within genes results in phase variable expression.

Table 4-1: Listing and location of Homopolymeric repeats within the *C. jejuni* 12661 genome

No	Position	Homopolymeric G/C tract	Gene ID/Region affected	Putative function	Locus
1	55619-55627	G (9)(16% 10G)	UC78_0040	Type II S restriction enzyme Eco57I	-
2	74655-74663	C (9)	UC78_0054	Hemerythrin-like metal-binding domain protein	-
3	535309-535318	G (10) (4% 9G)	intergenic	N/A	-
4	642503-642512	G (10) (16% 9G)	UC78_0661	Hypothetical Protein	-
5	696278-696287	C(10)	Intergenic	N/A	
6	901261-901267	G (9) (9%10G, 5%7G)	UC78_0928	Hypothetical Protein	-
7	1069917-1069924	C (8)	UC78_1098	Hypothetical Protein	LOS
8	1217885-1217892	G (9) (5% 10G)	UC78_1247	Hypothetical Protein	Flagellar modiffication
9	1228567-1228576	C(10) (12%9G, 4%11G)	UC78_1259	Hypothetical Protein	Flagellar modiffication
10	1235115-1235123	G (9)	UC78_1267	Pseudaminic acid biosynthesis (PseD)	Flagellar modiffication
11	1239576-1239584	G (10)	Intergenic	N/A	Flagellar modiffication
12	1245479-1245487	G(9)	UC78_1276	Hypothetical Protein	Flagellar modiffication
13	1249312-1249320	G(9)	UC78_1278	Hypothetical Protein	Flagellar modiffication

Table 4-1 continued

No	Position	Homopolymeric G/C tract	Gene ID/Region affected	Putative function	Locus
14	1261000-1261009	C(10)	UC78_1285	Hypothetical Protein	-
15	1281307-1281315	C(9)(11% 10G)	Intergenic	Hypothetical Protein	-
16	1338087-1339095	C(9) (6% 8G)	UC78_1362	Ubiquinone/menaquinone biosynthesis methyltransferase	CPS
17	1340278-1340286	C(9) (5% 10G)	UC78_1363	Hypothetical Protein	CPS
18	1346515-1346523	C(9) (4% 10G)	UC78-1371	F5/8 Type c domain protein	CPS
19	1348740-1348754	C(9)	UC78-1372	Hypothetical Protein	CPS

Note: Homopolymeric G:C tracts are defined as containing ≥7 consecutive G:C residues. The relative chromosomal position within the 12661 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.

LOS: lipooligosaccharide, CPS: Capsular polysacharide

4.2.2 Nucleotide sequence statistics

The nucleotide distribution displayed in **Figure 4-3** is typical of an AT-rich *Campylobacter* genome. The average G+C content of the *C. jejuni* 12661 genome is 31.11% and this percentage is close to that observed for other strains including PT14 30.5% (Brathwaite et al., 2013), NCTC 11168 (30.6%) (Parkhill et al., 2000, Gundogdu et al., 2007), CG8486 (30.4%) (Poly et al., 2007) and 81-176 (30.6%) (Hofreuter et al., 2006).

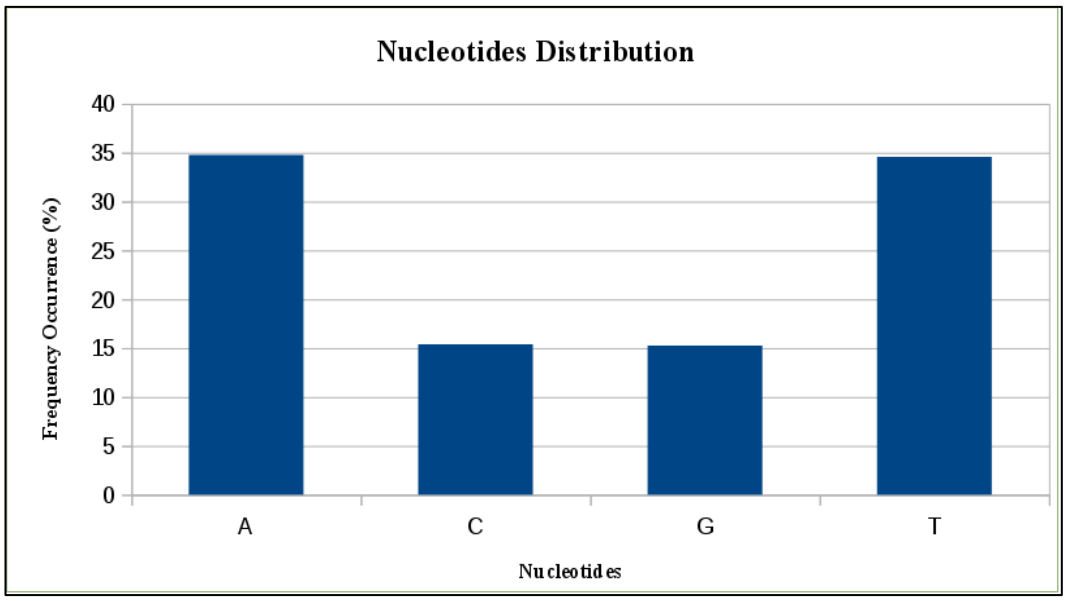


Figure 4-3: Nucleotide distribution histogram for the *C. jejuni* 12661 genome.

The occurrence of each nucleotide within the genome is illustrated. The average G+C content of the genome is shown to be 31.11%.

Table 4-2 shows the nucleotide frequency in codon positions. "A" residues frequency is observed to be the same in three positions 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" 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 4, 1278 times and was the most abundant codon. However, "CGG" encoding arginine was the least frequent, occurring only 52 times.

Table 4-2: Frequency of nucleotide codon positioning for *C. jejuni 12661* genome

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

4.2.3 Phylogenetic relation to other *C. jejuni strains*

Figures 4-4 and 4-5 illustrate the relationship between *C. jejuni 12661* and its nearest neighbours based on genomic BLAST comparisons. **Figure 4-5** shows the closest group of genomes to *C. jejuni 12661,* that includes the genomes of 15 *C. jejuni* that form a group named *C. jejuni* subsp. *jejuni* 1213 (red box). Within this group the genome with the greatest similarity is that of *C. jejuni subsp. jejuni 81116* that was originally isolated from the waterborne outbreak (Manning et al., 2001). *C. jejuni 81116* contains a single gene encoding TonB, compared to two or three genes in other *C. jejuni* strains, and lacks CfrA and Cj0178 outer membrane receptors for iron uptake (Pearson et al., 2007). The second nearest is *C. jejuni* M1 which is isolated from the diarrheic stools of a research team member 9 days after visiting a poultry processing plant in the UK (Friis et al., 2010). However, *C. jejuni* subsp. *jejuni* strain YH001 clusters furthest away from *C. jejuni* 12661, a strain that was originally isolated from a beef liver purchased from a local supermarket (He et al., 2015).

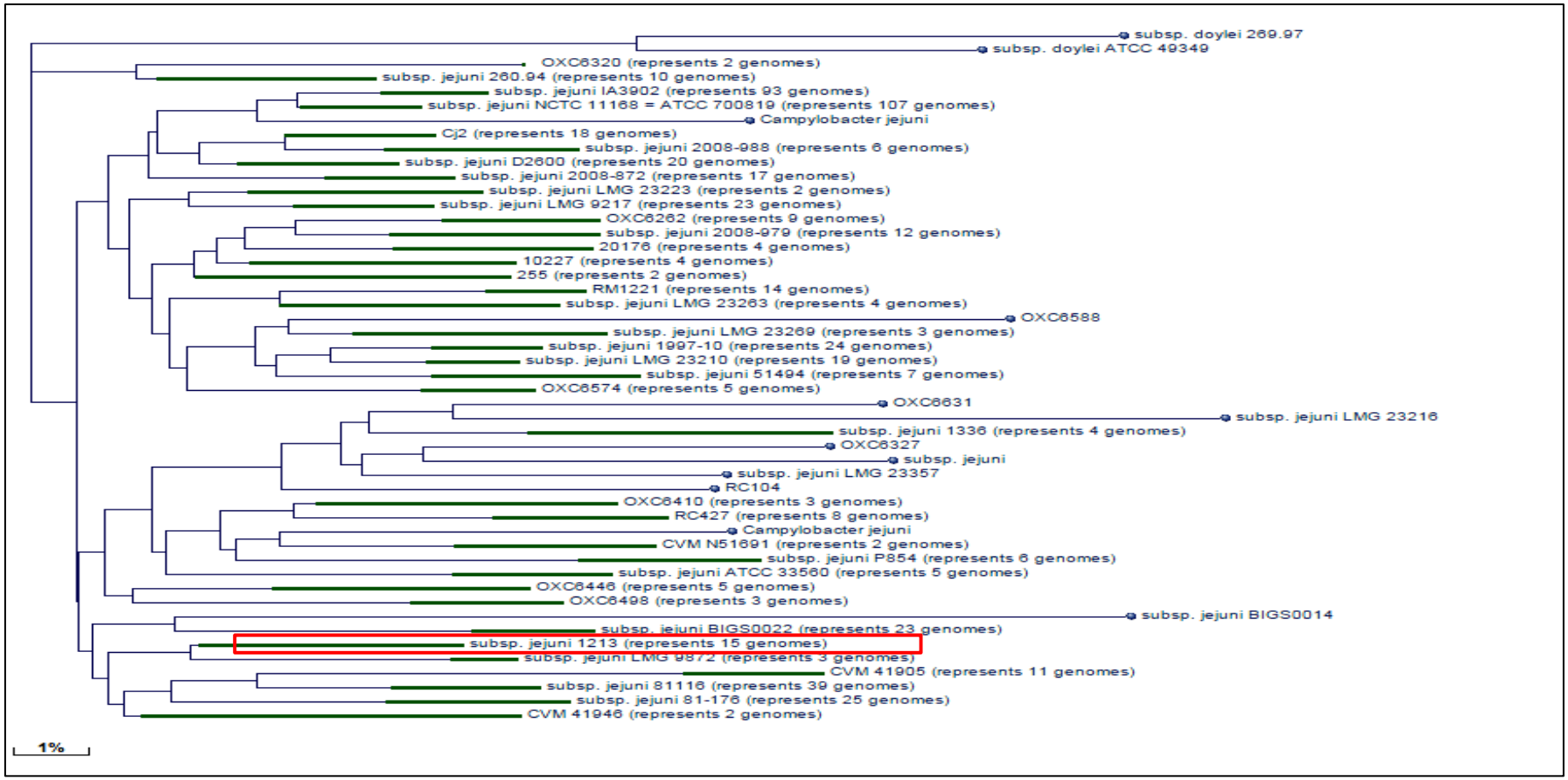


Figure 4-4: Phylogenetic comparison of *C. jejuni* 12661 with the closest relatives.

The *C. jejuni* 12661 (35925B2) is located within *C. jejuni subsp. jejuni* 1213 group (red box). The comparisons are based on genomic BLAST searches. The dendogram was calculated using the Genome feature of the NCBI website.

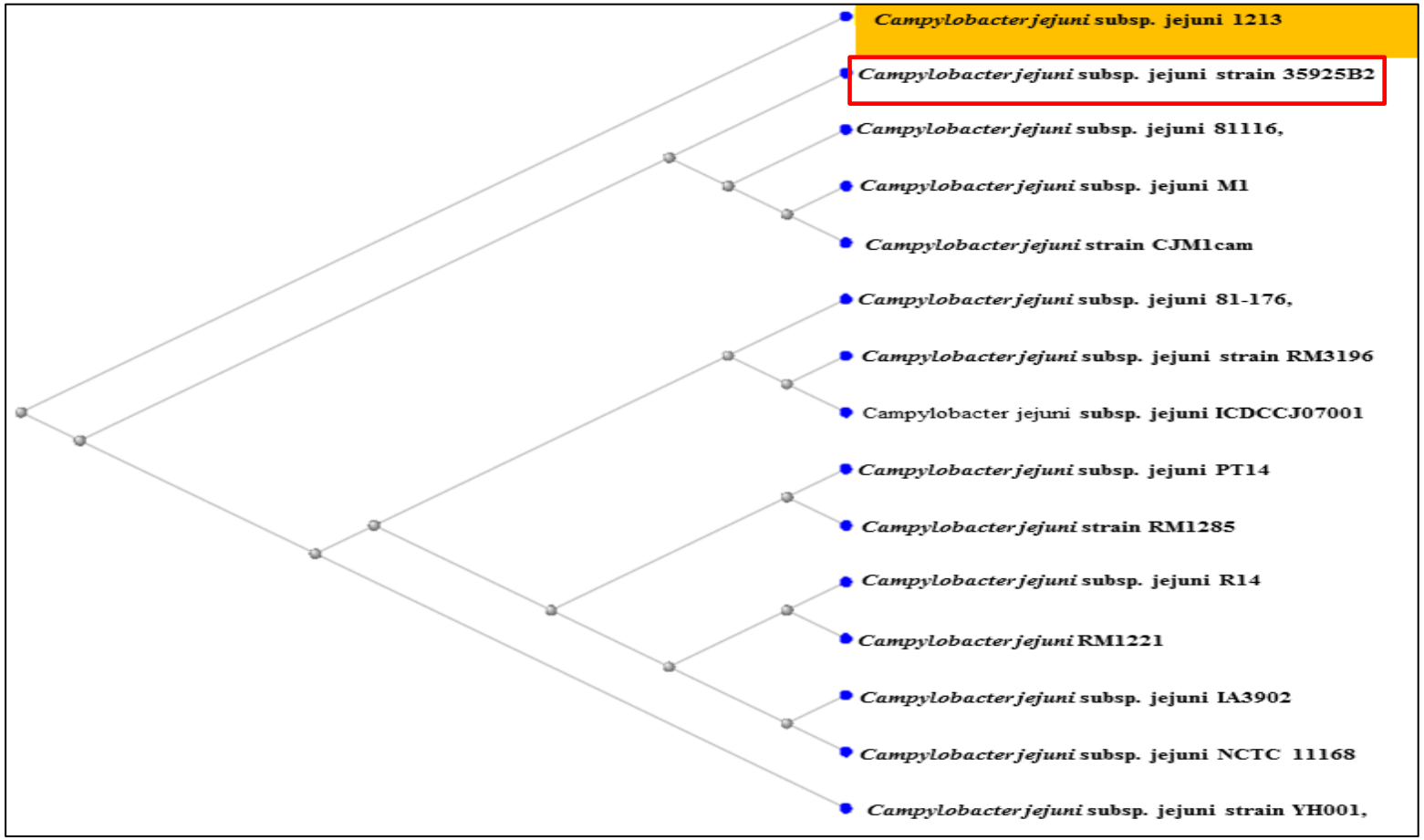


Figure 4-5: Phylogenetic comparison of *C. jejuni 12661* (35925B2) within closest relative's group.

C. jejuni subsp. *jejuni* 1213 (red box) (Figure 4-4).The comparisons are based on genomic BLAST searches. The dendogram was calculated using the Genome feature of the NCBI website.

4.2.4 The CRISPR-*cas* system of *C. jejuni 12661*

The CRISPR repeats of *C. jejuni* have been suggested as the basis for molecular typing of strains (de Cárdenas et al., 2015). The CRISPR-*cas* system within the genome of *C. jejuni 12661* was identified and found to be intact. *C. jejuni 12661* contains a minimal type II-C CRISPR array, with six 36-bp direct repeats (start position of first repeat: 1429028, end position of last repeat: 1429261) flanking six 30-bp spacer sequences (start position of first spacer: 1428997, end position of last spacer: 1429225).The number of spacer sequences are variable in different *C. jejuni* (Table 4-3). A putative transcriptional -10 start site (5'-TAAAAT-3') could be identified 180-bp upstream of the initial direct repeat. The genes encoding three CRISPR-associated Cas proteins, *cas2* (UC78_01449) (position: 1429405-1429836), *cas1* (UC78_1450) (position: 1429829-1430719) and *cas9* (UC78_01451) (position: 1430706-1433669) were also identified, beginning 144-bp downstream of the terminal direct repeat sequence. A tracrRNA sequence was also found that could potentially base-pair with the repeat sequence. The CRISPR array sequences in **Table 4-4.** BLAST searches of the spacer sequences of the *C. jejuni 12661* CRISPR array revealed that the first and third spacer sequences map 100% to the hypothetical gene in *Campylobacter* pro-phage CJIE4 and to the *Campylobacter* CRISPR regions of *C. jejuni* 00-0949, *C. coli* RM466 and *C. jejuni* S3.

The second spacer has identity with the sequences of four other strains, which match to a gene encoding a hypothetical protein of *Ehrlichia ruminantium* str. Welgevonden.

CRISPR spacer 4 overlaps with spacers 1 and 3. Nucleotides 1 to 22 show 100% identity to *Campylobacter* pro-phage CJIE4 and other *Campylobacter* CRISPR regions. Whereas, CRISPR spacer 5 contains a 100% match to a gene encoding a hypothetical protein of two *Campylobacter* strains and the CRISPR region of *C. jejuni* HCC81.CRISPR spacer 6 shares spacer sequences with *Campylobacter* CRISPR regions encoded by *C. jejuni* GB1 and *C. jejuni* 183. This spacer contains nucleotide matches to two non-*Campylobacter* species, *Strongyloides stercoralis* (parasitic roundworm) and *Lactobacillus gallinarum* encoding hypothetical hydrolase family proteins that are surrounded by glycosyl transferase genes.

A BLAST search of the direct repeat sequence displayed 100% nucleotide sequence identity to those of at least 95 other *C. jejuni strains* and 5 *C. coli.* The CRISPR element of *C. jejuni 12661* has a G+C content of 28.1%, which is lower than the average G+C content of the rest of the genome.

The CRISPR locus is normally transcribed as a large single RNA transcript (precursor CRISPR RNA or pre-crRNA), which is processed by CRISPR associated (cas) proteins to produce small antisense RNA molecules (CRISPR RNA or crRNA). The crRNAs have 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). However, it should be noted that in *C. jejuni* internal direct repeat sequences contain cognate promoter motifs that can transcribe each proceeding spacer independently (Dugar et al., 2013). There are three main types of CRISPR-*cas* systems (Type I, II and III), all types have the highly conserved Cas proteins, Cas1 (DNA endonuclease) and Cas2 (RNA endonuclease). Each system is characterised by the particular 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 normally holds genes encoding different configurations of Cas1, Cas2, Cas4 or Csn2/Csn2-like and Cas9 (Makarova et al., 2011, Chylinski et al., 2014). It is primarily linked with pathogenic bacteria such as *C. jejuni* , which has a type II-C system that lacks a Cas4 or Csn2/Csn2-like protein **(Figure 4-6).**

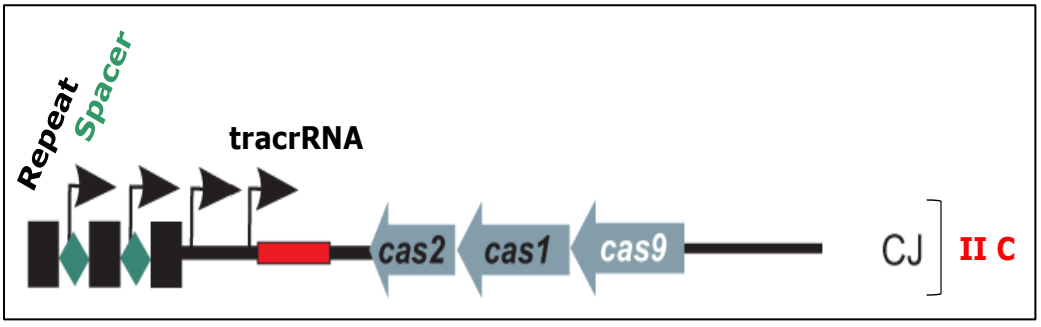


Figure 4-6: Type II-C CRISPR-*cas* systems in *C. jejuni*

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

The crRNA maturation step within type II system is characterised by the 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 revealed that in the presence of Cas9 the mature crRNA can be complementary base-paired to the tracrRNA to procedure an RNA duplex, which then guides the Cas9 protein to cleave the target DNA at positions that are complementary to the crRNA (Jinek et al., 2012). This step is referred to as CRISPR interference.

BLAST searches for each spacer repeat sequence of the *C. jejuni 12661* CRISPR array did not find any plasmid or virulent phage-associated DNA matches or matches to the 12661 chromosome sequence but exhibited nucleotide matches to the CRISPR arrays of other *C. jejuni strains* **(4.2.4)**. The BLAST searches revealed that none of the spacer sequences were unique to 12661; with *Campylobacter* species, *C. jejuni strains* and other prokaryote organisms carrying identical spacers.

Stern et al. (2010) previously observed CRISPR spacers that target chromosomal genes, and identified that amongst 23,550 spacers from 330 CRISPR-encoding prokaryotic organisms, one in every 250 spacers were self-targeting. Consequently, they expected that the CRISPR system may be included in gene regulation and therefore required to recognize conservation amongst self-targeting spacers. Nevertheless, no conserved sequences were found and this hypothesis was rejected. It was found that 37% of the self-targeting spacer sequences were noted to be placed nearby the leader sequence, either in the first or second

position of the array. In general, spacers are joined into the array towards the leader end and this result may recommend that acquisition of self-targeting spacers is a recent event that could probably have harmful effects.

They also identified that the two direct repeats flanking the self-targeting spacer sequence were twice as likely to have mutations compared to those flanking the other spacers (Stern et al., 2010). This may therefore disturb crRNA maturation of the self-targeting spacer, but allowing the rest of the CRISPR array to stay functional. Finally, it was proposed that targeting of chromosomal genes by the CRISPR system may be accidental and this may form the autoimmunity that could finally cause a negative effect to the organism (Stern et al., 2010). Evidence for CRISPR- mediated autoimmunity in *C. jejuni* has recently been reported for the bacteriophage carrier state of PT14, where self-spacers were detected appended to the distal end of the CRISPR array (Hooton and Connerton, 2015).

Table 4-3: Number of spacers in different *C. jejuni* strains, data are taken from this site.

http://crispr.u-psud.fr/crispr/

No.	*C. jejuni* strains	Spacers No.	Direct repeat length
1	81116	7	30
2	11168	3	36
3	PT14	3	36
4	S3	4	37
5	M1	3	36
6	RM1221	2	36
7	00-2544	4	36
8	Doylei 269.97	4	36
9	IA3902	3	36
10	ICDCCJ07001	5	36
11	4031	4	36
12	32488	1	35

Table 4-4: CRISPR array sequences for *C. jejuni* 12661

CRISPR feature	Sequence (5'→3')
Direct repeat	GTTTTAGTCCCTTTTTAAATTTCTTTATGGTAAAAT
Spacer 1	CCTTCAAGTCGTGCAATTTTAATACACTGG
Spacer 2	GTATCAGCTATAGATCTATCAGAATTTACA
Spacer 3	CCTTCAAGTCGTGCAATTTTAATACACTGG
Spacer 4	AATTTTAATACACTGGGGAAACGTTGATAA
Spacer 5	CTTGGTGGTATTATGGCAAATTCTGTTAAT
Spacer 6	ATTTATAAATTTGGATATGAAGCTTTAAGT
tracrRNA	AAGAAATTTAAAAAGGGACTAAAA

4.2.5 The *cps* gene locus of *C. jejuni 12661*

The *C. jejuni 12661* capsular polysaccharide synthesis gene locus (*cps*) extends from UC78_1355 to UC78_1381 and has a G+C content of 28%. On comparing the genomes of 12661 to PT14, 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 encoding the capsule polysaccharide biosynthesis proteins *kpsS* and *kpsC* (UC78_1355 and UC78_1356) while *kpsF*, *kpsD*, *kpsE*, *kpsT* and *kpsM* (UC78_1377, UC78_1378, UC78_1379, UC78_1380 and UC78_1381) make up the second cluster.

The region between these two clusters contains many different polysaccharide biosynthetic genes, some similar to those required for the biosynthesis of other bacterial capsules **(Figure 4-7).**

The Kps proteins allow translocation of the capsular polysaccharide to the bacterial surface. The role of the *fcI* gene product present in *C. jejuni* 12661 is currently unknown. However, it is suggested that this enzyme may be involved in the epimerization reaction required for the biosynthesis of the capsular heptopyranose based on alditol acetate analysis (Michael et al., 2002).

This capsular polysaccharide locus in *C. jejuni* 12661 shows differences to other *C. jejuni* strains on the basis that it contains four unique genes that theoretically encode a F5/8 type C domain protein (UC78-1371), glycerophosphotransferase (tagF) (UC78-1373), a dTDP-glucose 4,6-dehydratase (rfbB) (UC78-1374) and a puatative 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase 2 (ispD2) (UC78-1375). **(Figure 4-7)**.

An insertion of thirteen genes between (UC78-1362 to UC78-1376) and substitution of twelve genes has occurred within PT14 compared to 12661 (A911_06908 to A911_06931, excluding A911_06909 and A911_06911) **(Table 4-5, 6) (Figure 4-7).** In addition, within the *cps* locus of 12661 there are two genes containing homopolymeric G: C tract repeats. These are UC78-1371, which encodes a F5/8 type C domain protein (discoidin), and the hypothetical gene represented by UC78-1372.

The reading frame UC78-1371 is unique with no homologues found for this gene in any other strain. UC78-1372 within *C. jejuni* PT14 is annotated as a putative sugar transferase (A911_06906). Figure 4-6 shows an alignment of the capsular polysaccharide loci between *C. jejuni* 12661, NCTC 81116 and M1. These two genomes were chosen for the alignment because of their close phylogenetic relationship with 12661 based on genomic BLAST searches (4.2.3), and also align with PT14 as the reference genome of this study.

There are no identical gene arrangements within this locus compared with the other strains, **(Figure 4-7), (Table 4-6)**.

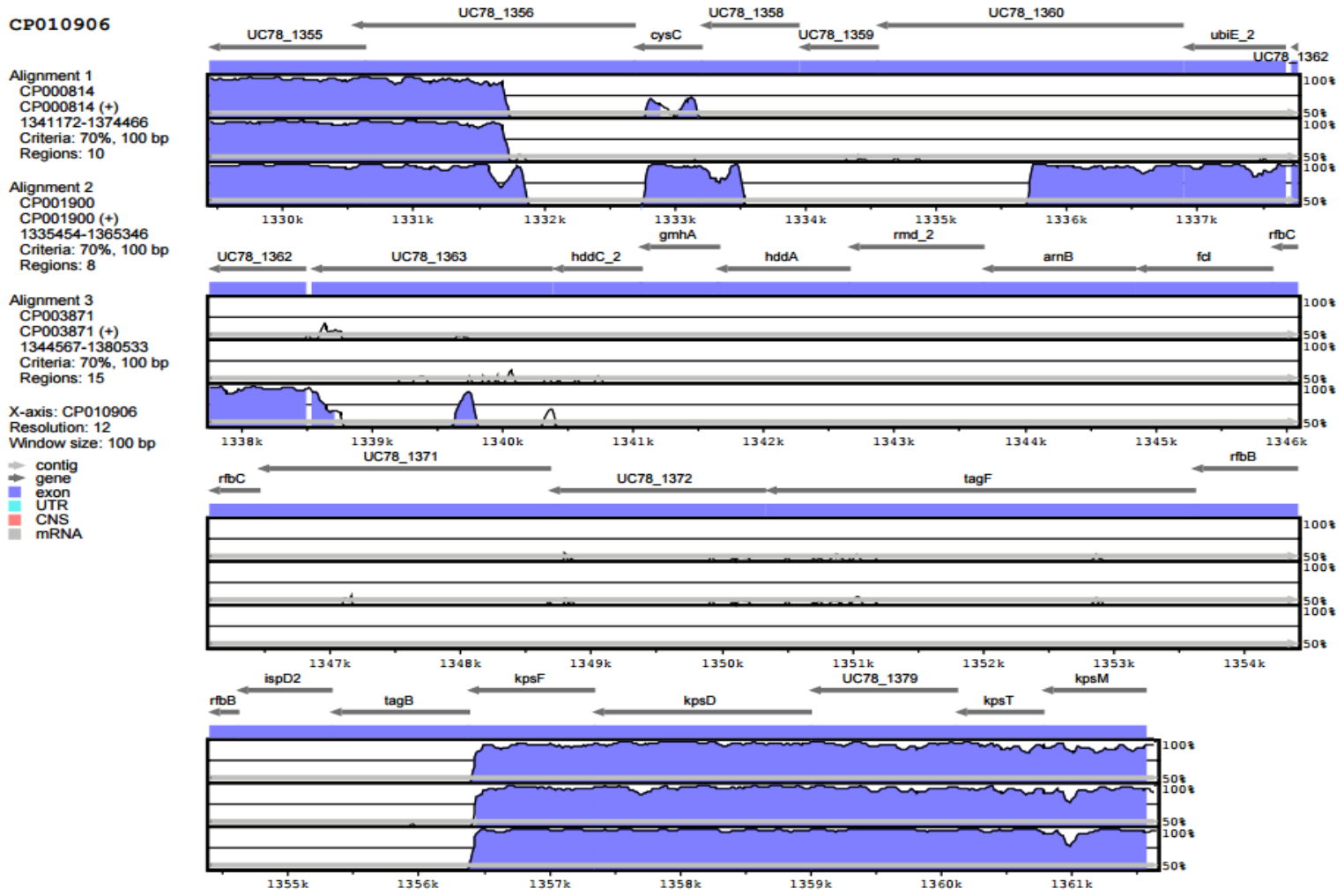


Figure 4-7: Alignment of the capsular polysaccharide loci of *C. jejuni 12661*, NCTC 8116, M1 and PT14.

Gene names are shown over each ORF and the direction of transcription is indicated by an arrow. In 12661, this locus extends from the first gene shown, *kpsS* (UC78_1355) until the *kpsM* (UC78_1381) gene. The plot shows the percentage identity with the positions of inserted and missing genes marked across the capsular polysaccharide locus of the 12661(CP010906), which is a base sequence aligned with (1) 81116, (2) M1 and (3) PT14. The plot was created using mVISTA (Frazer et al., 2004). Blue regions represent conserved cistrons, red regions represent conserved non-coding sequences (CNC) and white sections represent missing or relocated genes.

Table 4-5: Genes missing in *C. jejuni 12661* comparing with reference strains PT14

No.	Gene ID	Description	Location
1	A911_00145	hypothetical protein	43,200
2	A911_00265	hypothetical protein	71,621
3	A911_00855	TonB-dependent *coli* cin lipoprotein, putative	172,712
4	A911_00860	TonB-dependent *coli* cin receptor protein	173,560
5	A911_00865	TonB system transport protein ExbB	175,839
6	A911_00870	biopolymer transport ExbD protein	176,587
7	A911_00875	TonB-dependent *coli* cin receptor protein	176,999
8	A911_01190	hypothetical protein	227,708
9	A911_01845	hypothetical protein	346,907
10	A911_02055	putative H-T-H containing protein	386,542
11	A911_02060	hypothetical protein	386,920
12	A911_02065	putative acidic periplasmic protein	387,156
13	A911_02070	periplasmic protein	387,801
14	A911_02750	hypothetical protein	527,204
15	A911_02755	hypothetical protein	527,728
16	A911_02760	hypothetical protein	528,030

Table 4-5 continue

No.	Gene ID	Description	Location
17	A911_02765	hypothetical protein	529,426
18	A911_02770	hypothetical protein	529,867
19	A911_02775	hypothetical protein	530,112
20	A911_03065	lipoprotein	587,635
21	A911_03640	hypothetical protein	704,379
22	A911_03645	ISCco1, transposase orfB	705,117
23	A911_03650	transposase	705,555
24	A911_03655	TonB transport protein	706,030
25	A911_03660	ferric enterobactin uptake receptor	707,054
26	A911_03965	putative lipoprotein	769,171
27	A911_03970	small hydrophobic protein	769,424
28	A911_05085	Type I restriction modification enzyme	983,514
29	A911_05435	hypothetical protein	1,055,382
30	A911_05505	putative glycosyltransferase	1,070,573
31	A911_05510	putative glycosyltransferase	1,071,729
32	A911_05515	putative glycosyltransferase	1,072,782

Table 4-5 continued

No.	Gene ID	Description	Location
33	A911_05525	alpha-2,3 sialyltransferase	1,074,909
34	A911_05530	sialic acid synthase	1,075,774
35	A911_05535	UDP-N-acetylglucosamine 2-epimerase	1,076,802
36	A911_05540	bifunctional beta-1,4-N-acetylgalactosaminyltransferase/CMP-Neu5Ac synthase	1,077,914
37	A911_05550	Hypothetical protein	1,079,519
38	A911_06440	Putative methyl transferase	1,252,839
39	A911_06630	Putative secreted serine protease	1,297,112
40	A911_06909	Capsular polysaccharide biosynthesis protein	1,356,223
41	A911_06911	Capsular polysaccharide biosynthesis protein	1,358,181
42	A911_06912	Putative sugar nucleotidyltransferase	1,360,094
43	A911_06913	hypothetical protein	1,361,950
44	A911_06914	hypothetical protein	1,362,282
45	A911_06915	putative nucleotidyltransferase	1,362,610
46	A911_06916	HAD superfamily hydrolase	1,363,329

Table 4-5 continued

No.	Gene ID	Description	Location
47	A911_06917	hypothetical protein	1,363,960
48	A911_06920	putative sugar transferase	1,366,766
49	A911_06925	putative CDP glycerol glycerophosphotransferase	1,369,272
50	A911_06930	hypothetical protein	1,372,561
51	A911_06935	putative glycerol-3-phosphate cytidylyltransferase	1,373,766
52	A911_07000	sugar transferase	1,384,219
53	A911_07460	putative type I restriction enzyme R protein	1,472,749
54	A911_07465	putative ATP/GTP-binding protein	1,475,847
55	A911_07470	putative type I restriction enzyme S protein	1,477,624
56	A911_07475	hypothetical protein	1,478,985
57	A911_07480	putative type I restriction enzyme M protein	1,480,448
58	A911_07625	putative oxidoreductase	1,508,975
59	A911_07710	hypothetical protein	1,524,316
60	A911_08080	lipoprotein	1,591,353
61	A911_08090	hypothetical protein	1,594,983
62	A911_08305	hypothetical protein	1,628,646
63	A911_08310	homoserine O-succinyltransferase	1,629,250
64	A911_08315	O-acetylhomoserine sulfhydrylase / O-succinylhomoserine sulfhydrylase	1,630,215

Table 4-6: Genes inserted in *C. jejuni* 12661 comparing with other *C. jejuni* strains including PT14

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
1	UC78-0033	45727	Rodanese-like domain protein	NF	F	F	F	NF
2	UC78-0034	47182	Hypothetical protein	NF	F	F	F	NF
3	UC78-0035	47840	Thiodisulfide interchange protein precursor	NF	F	F	F	NF
4	UC78-0036	48417	ResB-like family protein	NF*	F	F	F	NF*
5	UC78-0038	50297	Cytochrome C biogenesis protein (CcsA) CCA-1	NF	F	F	F	NF
6	UC78-0038	51156	Gammas-glutamyl transpeptidas precursor(ggt)	NF	F	F	F	NF
7	UC78-0041	56778	Hypothetical protein	F	NF	NF*	F	NF*
8	UC78-0042	58194	Hypothetical protein	F	F	NF*	NF	NF*
9	UC78-0058	80641	Hypothetical protein	NF	NF	NF	NF	NF
10	UC78-0059	80889	Hypothetical protein	NF	NF	NF	NF	NF
11	UC78-0060	81367	Hypothetical protein	NF*	NF*	NF*	NF	NF*
12	UC78-0093	111995	Hypothetical protein	NF*	NF	NF*	NF	NF*
13	UC78-0094	112885	Curli production assembly/Transport component	NF*	NF*	NF*	F	NF*
14	UC78-0123	134787	Hypothetical protein	NF*	NF*	NF*	F	NF*
15	UC78-0123	150809	5-methylcytosine-specific restriction enzyme B (mcrB)	F	NF	F	F	F

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
16	UC78-0145	156436	Hypothetical protein	NF*	F	F	F	F
17	UC78-0203	206807	Hypothetical protein	NF	NF	NF	NF	NF
18	UC78-0204	207262	Hypothetical protein	NF	NF	NF	NF	NF
19	UC78-0234	229705	methyle accepting chemotaxis protein (mcpc)	NF	NF	NF	NF	NF
20	UC78-0274	268299	lipid A-disaccharide synthase (lpxB)	NF	F	F	F	NF
21	UC78-0275	269394	Hypothetical protein	NF	NF	NF	NF	NF
22	UC78-0276	269956	Hypothetical protein	NF	NF	NF	NF	NF
23	UC78-0277	271411	putative methyle transferase	NF	NF	NF	NF	NF
24	UC78-0278	273263	type III restriction modification system	NF	NF	NF	NF	NF
25	UC78-0288	283785	Exosortase E/protease, VPEID CTERM system	NF	NF	NF	NF	NF
26	UC78-0474	461018	fucp/L-fucose permease	F	NF	NF	NF	NF*
27	UC78-0475	462258	Putative metal dependent hydrolase of TIM bsrrel fold protein	NF*	NF	NF	NF	NF*
28	UC78-0476	463029	aldA/ L-rhamnose 1-epimerase	NF*	NF	NF	NF	NF*
29	UC78-0466	455590	pectin degradation precurssor protein (kdgp)	F	NF	NF	NF	NF*
30	UC78-0467	456581	4-hydroxy-tetrahydrodipi*coli* nate synthase (dapA1)	F	NF	NF	NF	NF*

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
31	UC78-0468	457486	Altronate hydratase (uxaA 1)	F	NF	NF	NF	NF*
32	UC78-0469	457631	Hypothetical protein	F	NF	NF	NF	NF*
33	UC78-0470	457753	Altronate hydratase (uxaA 2)	F	NF	NF	NF	NF*
34	UC78-0471	458931	Inner membrane transport protein (rhmT)	F	NF	NF	NF	NF*
35	UC78-0472	460218	short chain dehydrogenase	F	NF	NF	NF	NF*
36	xecE	460576	2-(s)-hydroxypropyle-COM dehydrogenase	F	NF	NF	NF	NF*
37	amtB	473277	Ammonia transporter	NF*	NF	NF	NF	NF*
38	UC78-0549	536494	Hypothetical protein	NF	NF	NF	NF	NF
39	UC78-0550	536992	Hypothetical protein	NF*	NF*	NF*	NF*	NF*
40	UC78-0551	538056	HTH domain protain	NF*	F	F*	NF*	F
41	UC78-0610	592064	Hypothetical protein	NF	F	F	F	NF
42	dtpT1	616689	Di-/tripeptide transporter	F	F	F	F	NF*
43	dtpT2	617744	Di-/tripeptide transporter	F	F	F	F	NF
44	UC78-0718	701623	cell wall associtated hydrolas	NF*	F	F	F	NF*
45	UC78-0820	801404	Hypothetical protein	NF*	F	F	F	F

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
46	YicL	801927	putative inner membrane transporter	NF	F	F	F	F
47	UC78-0932	907696	bacteriohemerythrin	NF*	F	NF*	F	NF*
48	UC78-0933	908305	bacteriohemerythrin	NF*	F	NF*	F	NF*
49	UC78-1011	985114	phosphoglycerol transferase I	F	NF	F	NF	F
50	UC78-1012	986277	phosphoglycerol transferase/Alkaline phosphate superfamilly	F	NF	F	NF	F
51	UC78-1026	999243	Xylose isomerase-like TIME barrel	NF	NF	NF	NF	NF
52	yihN-1	1000241	inner membran protein	NF	NF	NF	NF	NF
53	epsH-1	1066195	Putative glycosyltransferase EpsH	NF	NF	NF	NF	NF
54	epsJ-1	1067075	Putative glycosyltransferase EpsJ	NF	NF	NF	NF	NF
55	UC78-1095	1068361	Hypothetical protein	NF	NF	NF	NF	NF
56	epsH-2	10687998	Putative glycosyltransferase EpsH2	F	NF	F	NF	F
57	UC78-1097	1069321	Hypothetical protein	NF	NF	NF	NF	NF
58	UC78-1098	1069887	Hypothetical protein	NF	NF	NF	NF	NF
59	kfoc	1064676	chondritin polymerase	F	NF	F	NF	F
60	UC78-1101	1072065	glycosyltransferase family 25	NF	NF	F	NF	NF

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
61	UC78-1248	1219046	Aminoglycoside 3-N-acetyletransferase	F	NF*	NF	F	F
62	UC78-1249	1219831	Acyl carrier protein	F	NF*	F*	NF	F
63	UC78-1250	1220046	bifunctional 3-demethylubiquinonene-9-3-methyltransferase	F	NF	NF	F	F
64	UC78-1251	1220954	FkbH domain protein	F	NF	F	NF	F
65	fabH-2	1222506	3-oxoacyl-synthase 3	F	NF	F	NF	F
66	UC78-1253	1223567	Hypothetical protein	F	NF	F	NF	F
67	UC78-1254	1223802	Hypothetical protein	F	NF	F	NF	F
68	UC78-1257	12268848	Hypothetical protein	NF*	NF	NF	NF	F
69	UC78-1267	1234947	Hypothetical protein	NF*	NF*	F	F	F
70	rmd1	1236875	GDP-6-deoxy-D-mannose reductas	NF*	NF	NF	NF	F
71	epsN	1237860	Putative pyridoxal phosphate dependent aminotransferase ESPV	F	NF	NF	NF	F
72	UC78-1278	1249144	Hypothetical protein	F	F	NF*	F	F
73	UC78-1282	1256689	Hypothetical protein	NF*	NF*	NF*	F	F
74	UC78-1305	1279127	outer membrane autotransporter barrel domain protein	NF	NF*	NF	F	NF
75	UC78-1306	1279723	Hypothetical protein	NF	NF*	NF	F	NF

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
76	UC78-1307	1280143	Hypothetical protein	NF	F	NF	F	NF
77	UC78-1358	1333204	bifunctional N-acetyleglucosamine-phosphate uridyltransferase	F	NF*	F	F	NF
78	UC78-1359	1333955	GMP synthas subunite A	F	NF*	F	F	NF*
79	UC78-1360	1334548	phosphoenol pyruvate synthase	F	NF*	F	F	NF*
80	ubiE-2	1336902	ubiquinnone/menaquinone biosynthesis methyltrasferase	F	NF*	F	F	F
81	UC78-1362	1337715	ubiquinnone/menaquinone biosynthesis methyltrasferase	F	NF*	F	F	F
82	hddc_2	1338544	Hypothetical protein	NF*	NF	F	NF	F
83	gmhA	1341048	phosphate isomerase	NF	NF	F	NF	F
84	hddA	1341641	bifunctional fukinase	NF	NF	F	NF	F
85	rmd2	1342657	GDP-6-deoxy-D-mannose reductas	NF	NF	F	NF	NF
86	arnB	1343685	UDP-4 amino-4deoxy-L-arabinose--oxoglutarate aminotransferase	NF	NF	NF	NF	NF
87	FCI	1344849	GDP-L-fucose synthase	NF	NF	NF	NF	F
88	rfbc	1345895	dTDP-4-dehydrorhamnose 3,5 epimerase	NF	NF	F*	NF	F
89	UC78-1371	1346458	F5/8 type C domainprotein	NF	NF	NF	NF	NF
90	UC78-1372	1348689	Hypothetical protein	NF	NF	NF	NF	NF

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
91	tagF	1350345	glycerophosphotransferase	NF	NF	NF	NF	NF
92	rfbB	1353610	dTDP-glucose 4,6-dehydratase	NF	NF	NF	NF	NF
93	ispD2	1354621	puatative 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase 2	NF	NF	NF	NF	NF
94	tagB	1355337	glycerophosphotransferase	NF	NF	NF	NF	NF
95	Cas2	1429405	CRISPR-associated endoribonuclease Cas2	F	F	NF	NF	F
96	Cas1	1429829	CRISPR-associated endoribonuclease Cas1	F	F	NF	NF	F
97	Cas9	1430706	CRISPR-associated endoribonuclease Cas9	F	F	NF	NF	F
98	dcuD	1433876	Putative cryptic C4-dicarboxylate transporter	F	F	NF	F	F
99	UC78-1474	1451923	Chloramphenicol acetyletransferase	NF	NF	NF	NF	NF
100	hsdR	1453689	type I restriction enzyme R protein	NF	NF	NF	NF	NF
101	UC78-1477	1455413	Hypothetical protein	NF	NF	NF	NF	NF
102	UC78-1478	1456015	Hypothetical protein	NF	NF	NF	NF	NF
103	UC78-1479	1456949	superfamily II helicase	NF	NF	NF	NF	NF
104	UC78-1480	1458227	Hypothetical protein	NF	NF	NF	NF	NF
105	hsds	1459206	type I restriction enzyme EcokI	NF	NF*	NF	F	NF

Table 4-6 continued

No	Gene ID	location	Description	PT14	81116	18-178	M1	11168
106	UC78-1484	1462010	4-carboxymuconolactone decarboxylase	NF	F	NF	F	NF
107	ydhp_2	1463349	inner membrane transport protein	NF	F	NF	F	NF
108	arSC	1465917	Arsenate reductase	F	F	NF	NF	NF
109	UC78-1490	1466333	Arsenical resistant protein	F	F	NF	NF	NF
110	dmsA	1490715	dimethyle sulfoxide reductase	NF	F	F	F	NF
111	dmsB	1493089	DMSO reductas iron sulfur subunite	NF	F	F	F	NF
112	UC78-1516	1493732	DMSO reductas anchor subunite	NF	F	F	F	NF
113	dmsD	1495222	Twin-argnine leader-binding protein	NF	F	F	F	NF
114	cocE	1582236	Cocaine esterase	NF	NF	F	NF	NF
115	yihN-2	1584232	Inner membrane protein	NF	NF	F	NF	NF

F=Found NF=Not found * Variable or rearrangement gene

4.2.6 The Lipooligosaccharide biosynthesis locus of *C. jejuni* 12661

The *C. jejuni LOS* locus extends from UC78_1088 (*galE*) to UC78_1105 (*gmhB*) (Figure 4-7) and has a G+C content of 28%. Genes involved in the LOS heptose biosynthesis pathway are present, and include *rfaC* (UC78_1090) encoding Lipopolysaccharide heptosyltransferase I, *gmhA* (UC78_1102) encoding phosphoheptose isomerase and *hldD* (UC78_1104) encoding ADP-L-glycero-D-manno-heptose-6-epimerase. Comparing with the reference strain *C. jejuni* PT14, three glycosyltransferases (A911_05505, A911_05510 and A911_05515) were absent in *C. jejuni* 12661 as well as the genes in the *neuBCA* operon (A911_05530, A911_05535 and A911_05540), which are essential for sialic acid biosynthesis. The gene (A911_05525) encoding α-2, 3 sialyltransferase is also absent **(Figure 4-8) (Table 4.5)**. These genes are known to be involved in biosynthesis of sialylated LOS structures with the potential for human ganglioside mimicry (Gilbert et al., 2002).

An insertion of six genes has occurred within *C. jejuni 12661* compared to *C. jejuni* PT14 (UC78-1093 to UC78-1101, excluding UC78-1092, UC78-1099 and UC78-100) **(Figure 4-8) (Table 4.5),** which encode putative glycosyltransferases.

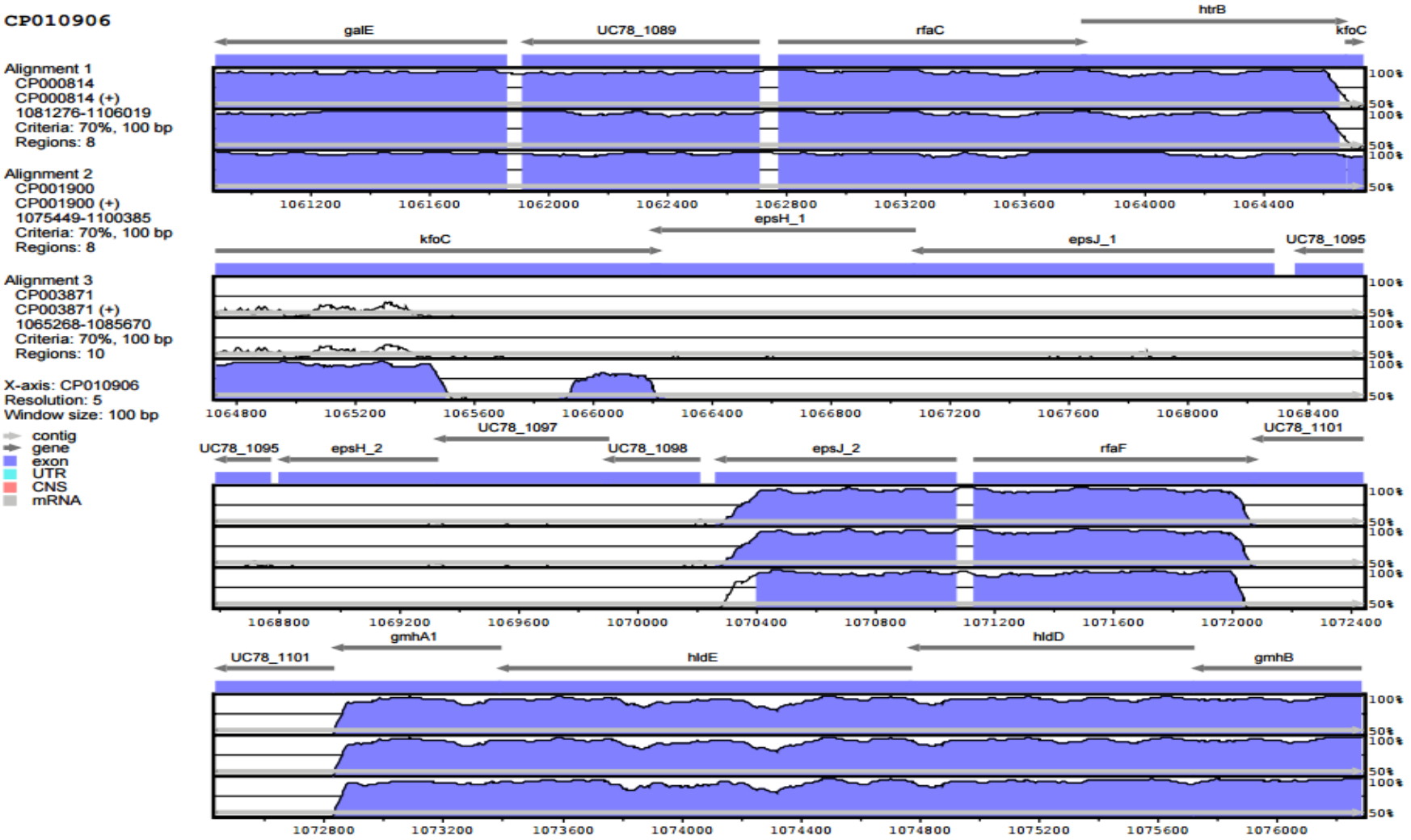


Figure 4-8: Alignment of the LOS biosynthesis loci of *C. jejuni 12661*, NCTC 81116, M1 and PT14.

Gene names are shown over each ORF and the direction of transcription is indicated by an arrow. The LOS locus in 12661 extends from *galE* (UC78_1105) to *gmhB* (UC78_1105). Genes involved in the LOS heptose biosynthesis pathway are present. The glycosyltransferases and genes in *neuBCA* operon are missing. The plot shows the percentage identity, with inserted and missing genes marked across the LOS locus of the *C. jejuni* 12661(CP010906) that is base aligned with (1) 81116, (2) M1 and (3) PT14. The plot was created by mVISTA (Frazer et al., 2004). Blue represents conserved cistrons, red represents conserved non-coding sequences (CNC) and white sections represent the missing or relocated genes.

4.2.7 *C. jejuni 12661* seems to have a class D or E LOS locus

Comparative genome analyses have revealed that the LOS biosynthesis locus of *C. jejuni is* a highly variable region within the genome (Parker et al., 2005, Gilbert et al., 2002, Leonard II et al., 2003, Leonard et al., 2004). These differences are responsible for the modifications found in LOS structures amongst *C. jejuni strains*.

Based on the general organisation of the LOS biosynthetic genes, five classes of the LOS locus have been recognized (A-E) within *C. jejuni* (Gilbert et al., 2002, Godschalk et al., 2004). Godschalk et al. (2004) identified the relationship between expression of ganglioside-like structures and the class of LOS locus assigned as well as the prevalence of the different classes within Guillain-Barre Syndrome-associated *C. jejuni strains*. They observed that classes A, B and C having genes involved in biosynthesis and transfer of sialic acid, while classes D and E lacked such genes. It is also found previously that classes A and B loci also 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). Nevertheless, class C was reported to carry the *cstIII* variant encoding the mono-functional α2,3-sialyltransferase (Gilbert et al., 2002). Based on this distinction, the LOS locus of *C. jejuni* 12661 lacks the sialic acid biosynthesis genes as well as α2, 3-sialyltransferase (*cstIII*), which suggests that this strain has either class (D or E) LOS locus. (Table 4-6).

4.2.8 The flagellar modification locus in *C. jejuni* 12661

In *C. jejuni 12661*, the flagellar modification locus extends from UC78_1245 (*pseB*) to UC78_1284 (hypothetical protein) and has a G+C content of 30 %. Within this locus are five genes and an intergenic region containing homopolymeric G:C tracts. These genes encode a number of hypothetical proteins and one pseudaminic acid biosynthesis (PseD) protein. Comparing with the reference strain PT14 an aminoglycosidase N3'-acetyltransferase found to be phase variable at sequence level and a methyltransferase (A911_06440) is missing in 12661. Compared to the other *C. jejuni* strains 11 genes were found to be inserted in 12661 (UC78_1250 to UC78_1282) as indicated in **Table 4.6 (Figure 4-9).**

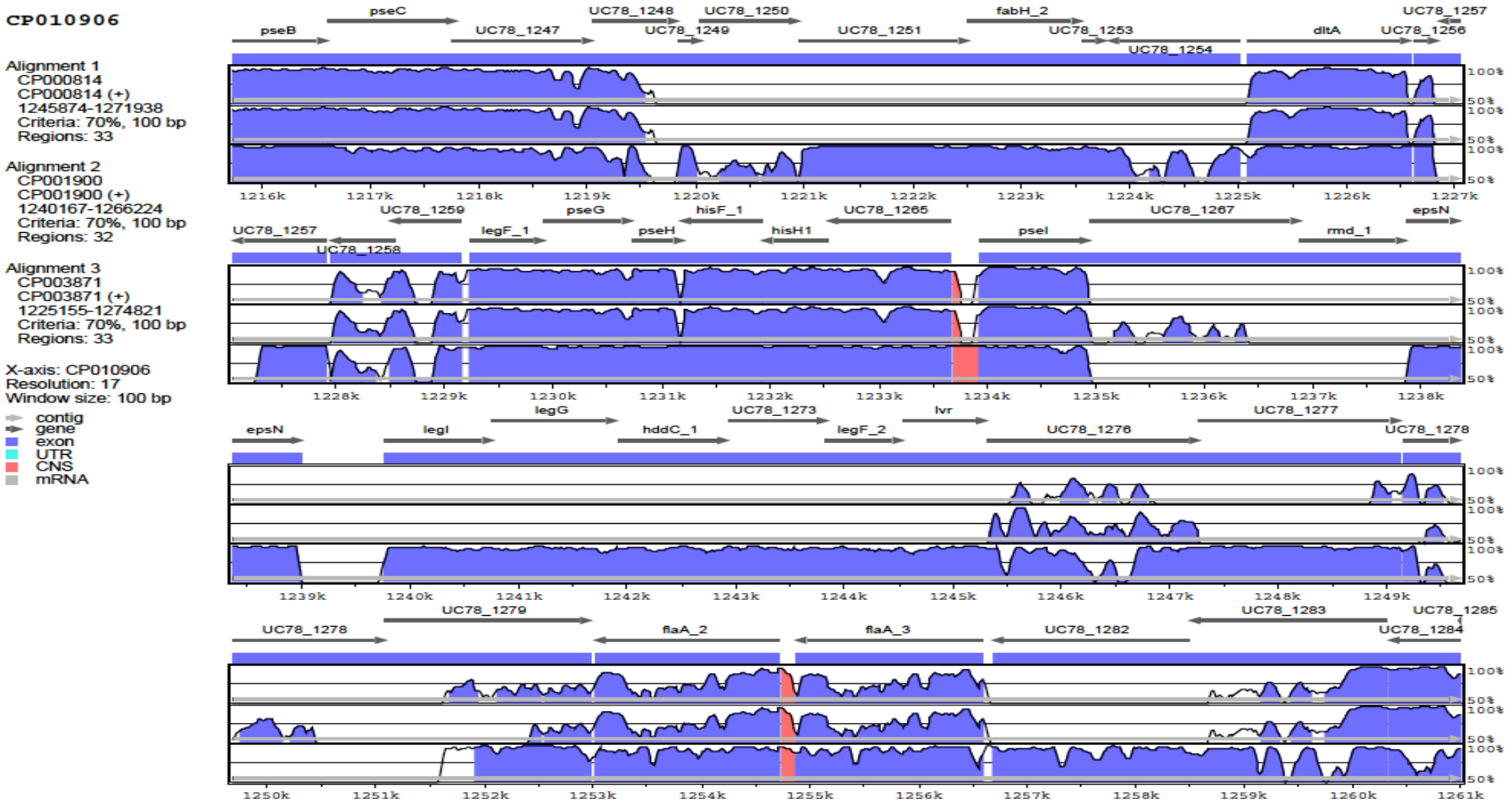


Figure 4-9: Alignment of the flagellar modification locus of *C. jejuni 12661*, NCTC 81116, M1 and PT14

Gene names are marked over each ORF and the direction of transcription is indicated by an arrow. The flagellar modification locus in 12661 extends from UC78_1247 (*pseB*) to UC78_1284 (hypothetical protein). The plot shows the percentage identity, with the inserted and missing genes marked across the flagellar modification locus of the 12661(CP010906) base aligned with (1) 81116, (2) M1 and (3) PT14. The plot was created using mVISTA (Frazer et al., 2004). Blue represents conserved cistrons, red represents conserved non-coding sequences (CNC) and white sections represent missing or relocated genes.

4.2.9 Restriction and modification systems in *C. jejuni* 12661

Restriction-modification (R-M) systems are essential bacterial defence systems that utilises a self/non-self DNA recognition mechanism that destroys incoming unmethylated or unmodified DNA. R-M systems are generally composed of a methyltransferase that catalyses methylation of a particular DNA sequence and a restriction enzyme which cleaves or limits foreign DNA missing that particular methylation. It has been found that about 90% of bacterial genomes contain at least one R-M system and roughly 80% hold multiple R-M systems (Vasu and Nagaraja, 2013).

Four main groups of R-M systems (Types I, II, III and IV) are identified dependent on 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). Each group can be divided into sub-categories, particularly the Type II class.

Type I R-M systems consist of a multi-subunit complex which functions as both a methyltransferase and a restriction endonuclease. It frequently contains two R subunits (restriction), two M subunits (modification) and one S subunit (specificity) (Dryden et al., 2001). The Type I systems genes are *hsdR*, *hsdM* and *hsdS* and the proteins encoded by the latter two are adequate for methyltransferase activity, but all three are essential for restriction activity (Titheradge et al., 2001, Chin et al., 2004).

The most commonly known and simplest systems are the Type II R-M systems. They consist 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 Type III R-M systems consist of two genes, *mod* and *res*, which encode protein subunits M (recognition and modification) and R (restriction). The M subunit can act separately as a methyltransferase but both M and R subunits are needed for restriction activity (Janscak et al., 2001, Mücke et al., 2001). 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 are not associated with methyltransferase activity. *C. jejuni* 12661 possesses multiple R-M systems. A type I R-M system was identified as containing one hsdR (UC78 _1476), one hsdS (UC78_1481) and two hsdM genes (UC78_1482 and UC78_1483).

HsdS proteins consist of four domains: two variable target recognition domains, a central conserved domain, and a conserved C-terminus (Chen et al., 1995, Gubler et al., 1992, Murray et al., 1982). Within the same family HsdM and HsdR proteins are found to be highly conserved, however, sequence identity between *HsdM* proteins from different families is frequently very limited (Titheradge et al., 2001, Sharp et al., 1992). In *C.jejuni* hsd loci are often organised such that the hsdS gene is located between the hsdR and hsdM genes. The gene order *hsdR-hsdS-hsdM* in 15 strains exemplified by 81116 (Miller et al., 2005b), this order is conserved in 12661 and PT14. However, they are ordered as *hsdM-*

hsdS-hsdR in strains RM1047 and NCTC 11168 (Miller et al., 2005a).

C. jejuni 12661 contains one hsd locus; however, it is not identical to the reference strain or even to other *Campylobacter*s such as 11168, (Table, 4-5). In addition, one of the *hsdM* genes present in PT14 is different from that present in 12661 (Table, 4-4).

The *C. jejuni* 12661 genome also contains a type II system (UC78_0040), which contains homopolymeric G:C tract, with the nearest homologue in PT14 being A911_00150. The orthologous gene in NCTC 11168 (Cj0031) also contains a homopolymeric G:C tract and has been demonstrated to be phase variable (Anjum et al., 2016). Adenine-specific (UC78_0205), site-specific (UC78_1394) and methionine-dependent (UC78_0205) DNA methyltransferases were also identified as well as putative methyl transferase (UC78-0277) and a type III restriction modification system (UC78-0278) (Table 4-6).

4.2.10 Putative anaerobic DMSO reductase

C. jejuni is an obligate microaerophile with a remarkably versatile respiratory chain, which makes it able to utilize a large variety of electron donors and acceptors. Under limited oxygen conditions, *C. jejuni* can use different alternative electron acceptors, including fumarate, nitrate, nitrite, trimethylamine-N-oxide, DMSO and sulfite (Sellars et al., 2002, Myers and Kelly, 2005, Pittman and Kelly, 2005). Such versatility is thought to enhance the ability of this pathogen to colonize the limited oxygen environment of the intestinal tract. The *C. jejuni* 12661 genome has additional respiratory functions compared to those encoded by reference strain PT14. For instance, instead of the putative oxidoreductase encoded by A911_07625 in PT14, *C. jejuni* 12661 holds a gene cluster that encodes putative anaerobic DMSO reductase, including dimethyl sulfoxide reductase (DmsA) UC78-1514, DMSO reductas iron sulfur subunit (DmsB) UC78-1515, DMSO reductase anchor subunit (DmsC) UC78-1516 and twin-arginine leader-binding protein (DmsD) UC78-1517 (Table4-6), **(Figure 4-10)**.

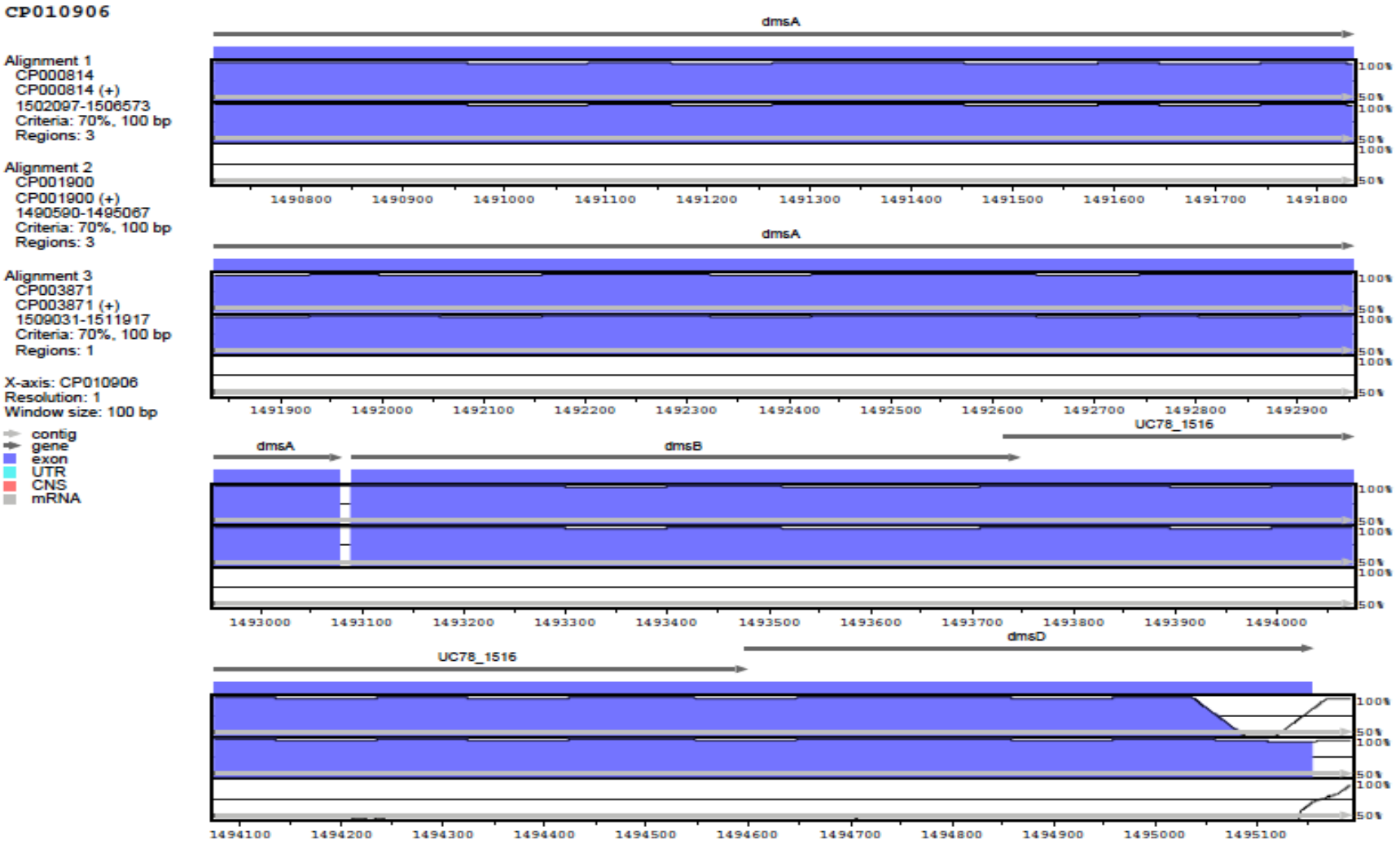


Figure 4-10: Alignment of the DMSO locus of *C. jejuni 12661*, NCTC 81116, M1 and PT14.

Gene names are marked over each ORF and the direction of transcription is indicated by an arrow. The DMSO locus in 12661 extends from UC78-1514 (DmsA) to UC78-1517 (DmsD). In the genome of *C. jejuni 12661*, the putative oxidoreductase gene A911_07625 is replaced by a cluster of four genes whose products show significant sequence similarity to the components of a DMSO reductase complex encoded by an operon of *W. succinogenes*. The plot shows the percentage identity and the positions of the inserted and missing genes across the DMSO locus of the 12661(CP010906) aligned with (1) 81116, (2) M1 and (3) PT14. The plot was created using mVISTA (Frazer et al., 2004). Blue represents conserved cistrons, red represents conserved non-coding sequences (CNC) and white sections represent the missing or relocated genes.

4.2.11 TonB-ExbB-ExbD complex

The TonB-ExbB-ExbD complex interacts with outer membrane receptor proteins that carry out high-affinity binding and energy dependent uptake into the periplasmic space of specific substrates. It might act to transduce energy from the cytoplasmic membrane to particular energy-requiring processes in the outer membrane, resulting in the release into the periplasm of ligands bound by these outer membrane proteins (Postle and Kadner, 2003). In Gram-negative bacteria the outer membrane (OM) serves as a selectively permeable barrier for the exchange of ions and organic molecules. While the transport of small molecules occurs by passive diffusion across the OM, larger solutes require active transport systems which present a unique mechanical challenge (Ferguson and Deisenhofer, 2004). The inner membrane protein complex consists of the proteins ExbB, ExbD, and TonB (Zhai et al., 2003). TonB contains three domains: an N-terminal domain consisting of a single transmembrane helix, a middle proline-rich segment and a C-terminal domain couples the inner membrane to the outer membrane transporter (Wiener, 2005). TonB-dependent outer membrane transporters have a conserved motif, the Ton-box (Lundrigan and Kadner, 1986, Schramm et al., 1987) that interacts with TonB during the active transport cycle.

The *C. jejuni* 12661 genome contains two genes encoding TonB, compared to one in 81116 or three genes in the other comparative *C. jejuni* strains. However, compared with the reference strain PT14, the *C. jejuni* 12661

genome lacks the ferric-enterobactin receptor (CfrA) A911_03660, two TonB-dependent *coli* cin receptor protein (outer membrane receptors for iron uptake) A911_00855 and A911_00875, one TonB-dependent putative *coli* cin lipoprotein A911_00855, one TonB system transport protein ExbB A911_00865, one biopolymer transport ExbD protein A911_00870 and TonB transport protein A911_03655 **(Table 4-5)**.

4.2.12 Genome analysis does not provide obvious rational for the morphological change and longer filaments formation of *C. jejuni* 12661

The genome of *C. jejuni* 12661 shows extended synteny with the genomes of *C. jejuni NCTC* 81116 and M1. However, no apparent features were recognized via the genome analysis that could explain the observations related to cell division inhibition and filaments formation of *C. jejuni* 12661 over other *C. jejuni* strains examined.

4.3 Conclusion

To explain the ability of *C. jejuni* 12661 to be morphologically different from other strains based on its genome content and arrangement. The genome sequence of this strain was characterised and examined for possible reasons.

Genomic characterization of *C. jejuni* 12661 identified many variable loci along the genome, including the characterization of several features related to bacterial capsule, lipooligosaccharide, flagellar modification genes, putative anaerobic DMSO reductase and the TonB-ExbB-ExbD complex. Genes encoding these functions were found to be variable by comparing their presence or absence across the corresponding regions of other *C. jejuni* strain 81116, M1 as closest strains and PT14 as a reference strain. Furthermore, this strain has multiple R-M systems and 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. BLAST searches of the spacer regions of the 12661CRISPR array found no obvious matches to the chromosomal DNA, but instead identified matches to the chromosome and sequences of other C. *jejuni* strains. Despite that this strain forms longer filaments compared to other *C. jejuni* strains, the genome analysis identified no obvious gene content that could explain this phenomenon. Chapter 5 seeks to examine regulatory changes that might provide an explanation for the observations of long filaments developing during the stationary phase of growth for C. *jejuni* 12661 as described in chapter three.

5. Transcriptome analysis of different *Campylobacter* morphotypes

5.1 Introduction

Under various stresses and environmental conditions *C. jejuni* is able to regulate gene expression to enable it to survive (Davis et al., 2009, Woodall et al., 2005, Brás et al., 1999). However, *C. jejuni* encodes only three recognisable sigma factors compared with seven in *E. coli* , and has a total of 34 other recognized transcriptional regulators (Pearson et al., 2007, Parkhill et al., 2000). Alternative mechanisms to modify gene expression have been reported in *C. jejuni ,* such as slip-strand mispairing, leading to phase variation in open reading frames carrying homopolymer G:C tracts. Phase variable genes are recognised in flagella biosynthesis (Hendrixson, 2006, Karlyshev et al., 2002), lipooligosaccharide glycosylation (Guerry et al., 2002), capsule production (Bacon et al., 2001b) and restriction/modification (Anjum et al., 2016).

In Chapter 3 the formation of filamentous *C. jejuni* morphotypes was associated with the stationary phase of growth in laboratory culture. A few studies have evaluated the transcriptome of *C. jejuni during* stationary and exponential phase, some of which examine the effects of these growth phases on the efficiency and outcomes of in *vivo* colonization using animal models. These models include a day-of-hatch chicken model (Woodall et al., 2005)**,** a rabbit ileal loop model (Stintzi et al., 2005) and the colonization of the chicken cecum with two cultures at different phases of growth using strand specific RNA-Seq (Taveirne et al., 2013).

RNA-Seq has revolutionised transcriptome studies by providing greater sensitivity, greater dynamic range, less background noise and the ability to identify transcripts in an annotation-independent manner. Investigations of the transcriptomes of prokaryotic organisms have been able to identify rare or novel transcripts, accurately define operons, discover the expression of putative non-coding 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).

It has been more challenging to study the bacterial transcriptome compared to that of eukaryotes. This has primarily been due to the fact that messenger RNA (mRNA) represents only 5% of the total RNA of bacterial cells. Moreover, the absence of polyadenylation at the 3'-ends of prokaryotic mRNAs prevents specific targeting of bacterial mRNA. To overcome this problem different mRNA enrichment protocols have been used to increase mRNA recovery, including the elimination of ribosomal RNA (rRNA) by hybridisation to complementary oligonucleotide sequences that target specific regions of the 5S, 16S and 23S rRNAs (Chen and Duan, 2011, Giannoukos et al., 2012, Peano et al., 2013).

Two key factors need to be considered for transcriptome studies, the read lengths and the number of reads available in a sequencing reaction. Short read lengths may allow reads to align to multiple locations of the reference genome (Bloom et al., 2009, Passalacqua et al., 2009). Consequently,

subsequent analyses to determine gene expression levels may be affected by such ambiguously mapped reads, and it may be better that they are excluded (Passalacqua et al., 2009).

The bacterial transcriptome usually consists of coding RNA (mRNA) and small ncRNAs (rRNA, tRNA and regulatory RNAs). If the ncRNA transcripts are highly abundant and subsequently more frequently sequenced, this can decrease the number of reads obtainable for low abundance mRNAs (Bloom et al., 2009). A possible limitation of RNA-Seq is the inability of standard cDNA libraries to preserve information on the direction of transcription. Directionality is particularly important in cases where transcripts may be produced from both strands and as a result the determination of gene expression levels would be influenced. In order to allow strand-specific RNA-Seq several protocols have been developed, including; (1) RNA molecule modification prior to reverse transcription by ligation of a 5' RNA linker (Passalacqua et al., 2009), (2) chemical modification during second strand cDNA synthesis by adding particular residues to the 3' end, followed by subsequent degradation of the unmarked strand (Parkhomchuk et al., 2009, Levin et al., 2010). In addition, RNA-Seq has the ability to differentiate 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).

Sharma et al. (2010) have exploited the sequencing approach known as differential RNA-Seq (dRNA-Seq) to enable selective sequencing of primary transcripts without previous rRNA removal, which has been used for automated annotation of transcriptional start sites (TSSs). To prepare the dRNA-seq libraries the RNA sample is divided into two, one is left untreated to capture both 5'PPP and 5'P RNAs, while in the second sample any processed RNAs are depleted by treatment with 5'P-dependent terminator exonuclease (TEX), which degrades the 5'P RNAs.

The transcriptomes of *C. jejuni* strains have been examined using RNA-Seq tools in several studies (Chaudhuri et al., 2011, Dugar et al., 2013, Taveirne et al., 2013, Porcelli et al., 2013, Butcher and Stintzi, 2013, Brathwaite et al., 2015). In some instances, dRNA-seq was used to allow selection of unprocessed RNA species and allowed the primary transcriptome of *C. jejuni to* be determined (Dugar et al., 2013, Porcelli et al., 2013, Hooton and Connerton, 2015), and the identification of a novel type-II CRISPR/Cas system amongst multiple *Campylobacter* strains (Dugar et al., 2013, Butcher and Stintzi, 2013, Hooton and Connerton, 2015). Comparative transcriptome analyses (RNA-Seq) was used to study the host adaption to the bacteriophage carrier state of *C. jejuni* (Brathwaite et al., 2015).

RNA-Seq has been used to characterise the regulon of the sigma factor RpoN (σ54) from *C. jejuni* that regulates the expression of genes involved in flagellar biosynthesis (Chaudhuri et al., 2011). These data were

subsequently compared to previous microarray expression data (Chaudhuri et al., 2011). Differences of strand-specific sequencing were applied in other cases to determine ncRNAs and antisense transcription (Chen and Duan, 2011, Taveirne et al., 2013, Butcher and Stintzi, 2013). RNA-Seq has also been applied to study the transcriptome of *Campylobacter* during colonisation of the chicken caecum (Taveirne et al., 2013), the response of *C. jejuni* to iron limitation (Butcher and Stintzi, 2013). These studies have also prompted re-examination of earlier transcriptome studies using microarray technologies (Palyada et al., 2004, Holmes et al., 2005).

In this chapter, the transcriptome of three different morphotypes of *C. jejuni* from the strains 12661 and PT14 were examined using strand-specific RNA-Seq. Cells were harvested during two different phases of growth (exponential and stationary) that lead to morphological differences in the cell populations, in order to characterize differential gene expression patterns. Thus far, there are no published studies reporting strand-specific transcription for different morphological forms of *Campylobacter* in different growth conditions or for strains grown in minimal medium.

5.2 Results

5.2.1 Preparation of *in vitro* growth curves for RNA isolation

A number of growth experiments were performed with the aim of determining a suitable time course that would encourage cells to reproducibly change their shape to the long filaments observed in Chapter 3. To enable a comparison of these cell types with metabolically active bacteria, cells were also harvested from exponential phase of growth. MEM with sodium pyruvate was selected to achieve these requirements in the *Campylobacter* populations. Further experiments were conducted to establish the conditions and time points when sufficient cells in the same phase of growth could be harvested for RNA isolation as described in section 2.6.3. Briefly, three independent biological replicates of *C. jejuni* 12661 or *C. jejuni* PT14 were inoculated into 500 ml of sterile MEM (2.1.14) with sodium pyruvate (2.1.15); and the flasks incubated under microaerobic conditions in a shaking incubator at 100 rpm (2.3) for 8 and 30 h. The short spiral cells were separated from filamentous cells by collecting the 8 µm filtrates from three independent cultures harvested at 30 h (2.6.3). This approach was adopted taking into account the findings of the preceding experiments where cells were harvested at different time points corresponding with different phases of growth and using various filter sizes for the isolation of different morphotypes. Viable counts of triplicate cultures were performed in parallel to RNA preparation. RNA preparations representing the desired growth phases and morphotypes that were of

sufficient quality (RIN >7), were selected for RNA-Seq analysis. For *C. jejuni 12661* and *C. jejuni* PT14, exponential phase cells were harvested for RNA isolation when the bacterial density reached approximately 6.5 log_{10} CFU ml^{-1} over 8 h periods **(Figure 5-1)**.

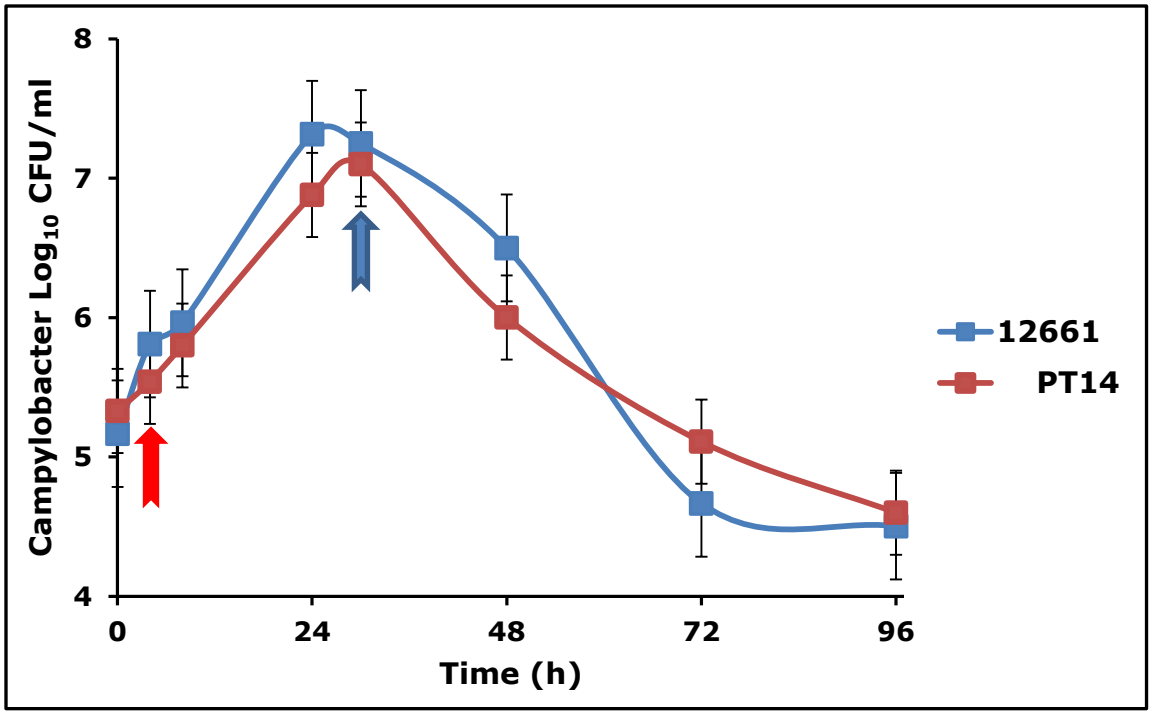


Figure 5-1: Illustrates *in vitro* growth curves of *C. jejuni* PT14 (red line) and 12661 (blue line) in MEM+sodium pyruvate incubated microaerobically at 37 °C.

Cultures were grown in 500 ml of MEM supplemented with sodium pyruvate. The time points at which the different morphotypes were recovered for RNA isolation are marked. The red arrow indicates the time point where bacteria were harvested for exponential growth phase cells. The blue arrow indicates the time at which the samples were collected in order to filter separate short spiral and long filamentous cells at stationary phase. Error bars represent ± SD for n=3 biological replicates. Cells were harvested from three biological replicates for RNA isolation at 8 and 30 h incubation for the *C. jejuni* 12661 and *C. jejuni* PT14.

These experiments yielded bacterial populations of exponential phase spiral cells for comparison with separated stationary phase spiral cells and separated stationary phase filaments. To avoid confusion with the stationary phase spiral morphotype, the spiral cells from exponential growth phase are referred to simply as exponential growth phase cells.

5.2.2 Transcriptome analysis of *C. jejuni* morphotypes

Total RNA was isolated from the various cell morphotypes 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 2.12.2 using the Ribo-Zero™ Magnetic Kit (Bacteria). From this 1 µg of rRNA-depleted RNA was used to prepare cDNA libraries using the NEBNext Ultra directional Prep. The libraries were individually indexed, pooled and then the library was then amplified and sequenced using an Illumina HiSeq 2500 sequencer. Sequence reads were mapped against a reference genome using CLC Genomics Workbench version 8.5. The RNA-sequencing generated an average of approximately 20 million reads per sample (**Tables 5-1** and **2**), each of which was 100 nucleotides in length and from a single-end. CLC Genomics Workbench software was used to

remove the adapter indexes and filter the raw sequence reads (section 2.12.4.1). Principle component analysis of the datasets demonstrated the replicates of sense and antisense clustered according to their origin **(Figures 5-2 to 5-5)**. The filtered reads were mapped against the *C. jejuni* PT14 reference genome (Brathwaite et al., 2013) and the *C. jejuni* 12661 genome annotated in Chapter 4 according to section 2.12.4.2. **Tables 5-1** and **5-2** provide a summary of the number of reads attained after filtering and record the number of reads that aligned uniquely to the to the reference genomes.

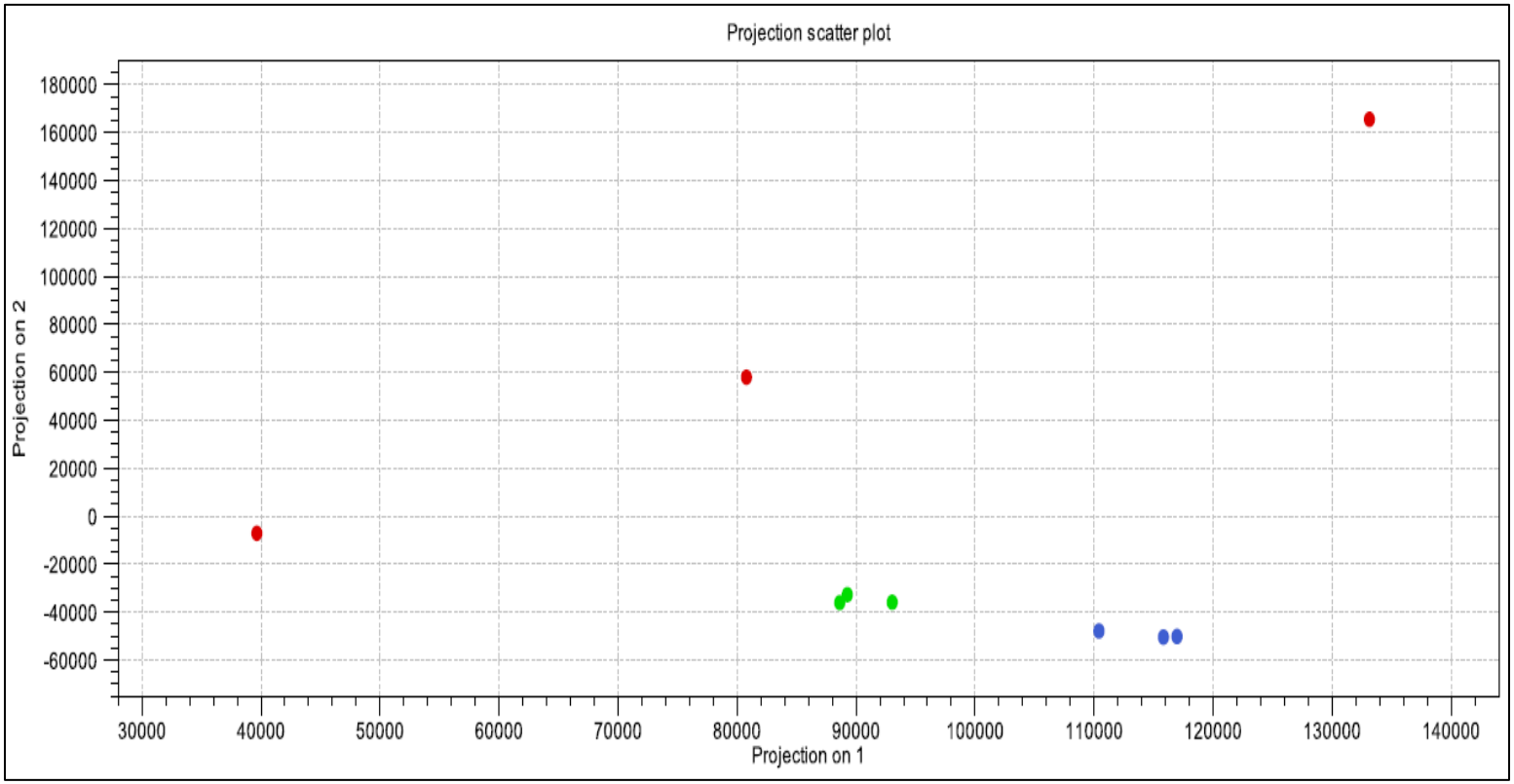


Figure 5-2: Principle component analysis of RNA-Seq reads for *C. jejuni 12661* sense transcription.

The Exponential replicates are illustrated in red while the filament and spiral replicates are shown in blue and green respectively. Each condition is displayed as a distinct group, with patterns of similarity being exhibited in each case.

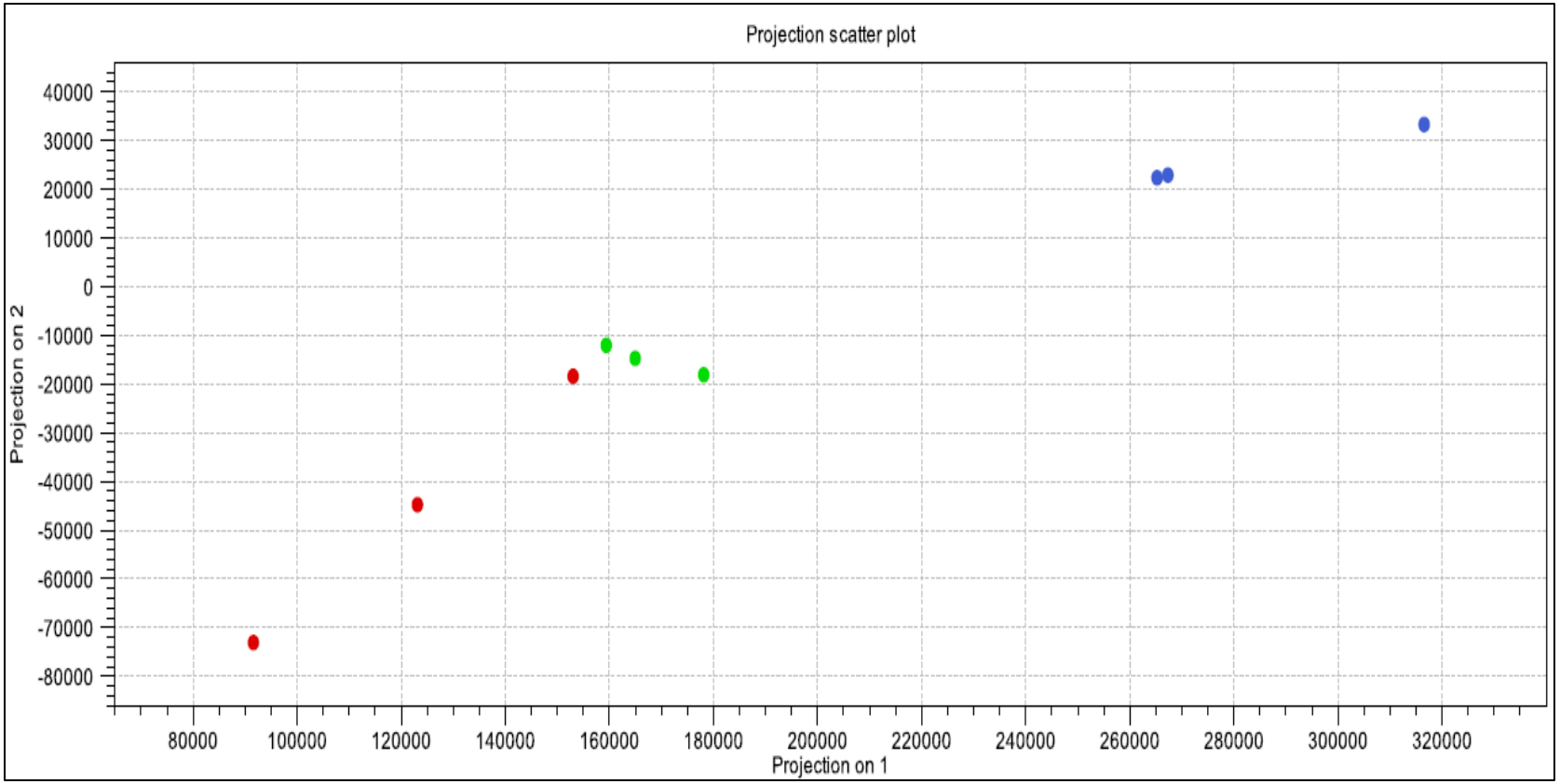


Figure 5-3: Principle component analysis of RNA-Seq reads for *C. jejuni 12661* antisense transcription.

The Exponential replicates are illustrated in red while the filament and spiral replicates are shown in blue and green respectively. Each condition is displayed as a distinct group, with patterns of similarity being exhibited in each case.

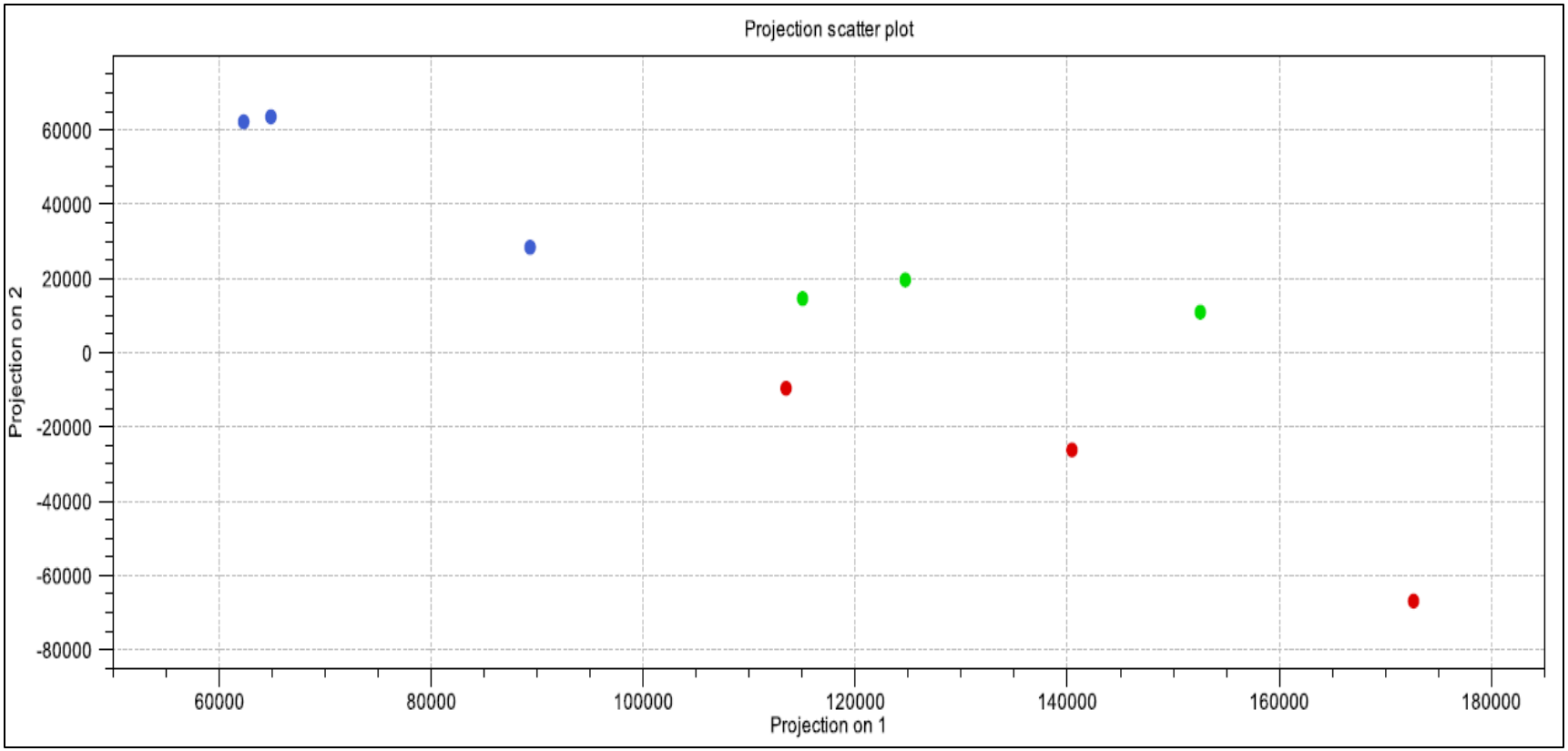


Figure 5-4: Principle component analysis of strand-specific RNA-Seq reads for *C. jejuni PT14* sense transcription.

The Exponential *C. jejuni* replicates are illustrated in red while the filament and spiral replicates are shown in blue and green respectively. Each condition is displayed as a distinct group, with patterns of similarity being exhibited in each case.

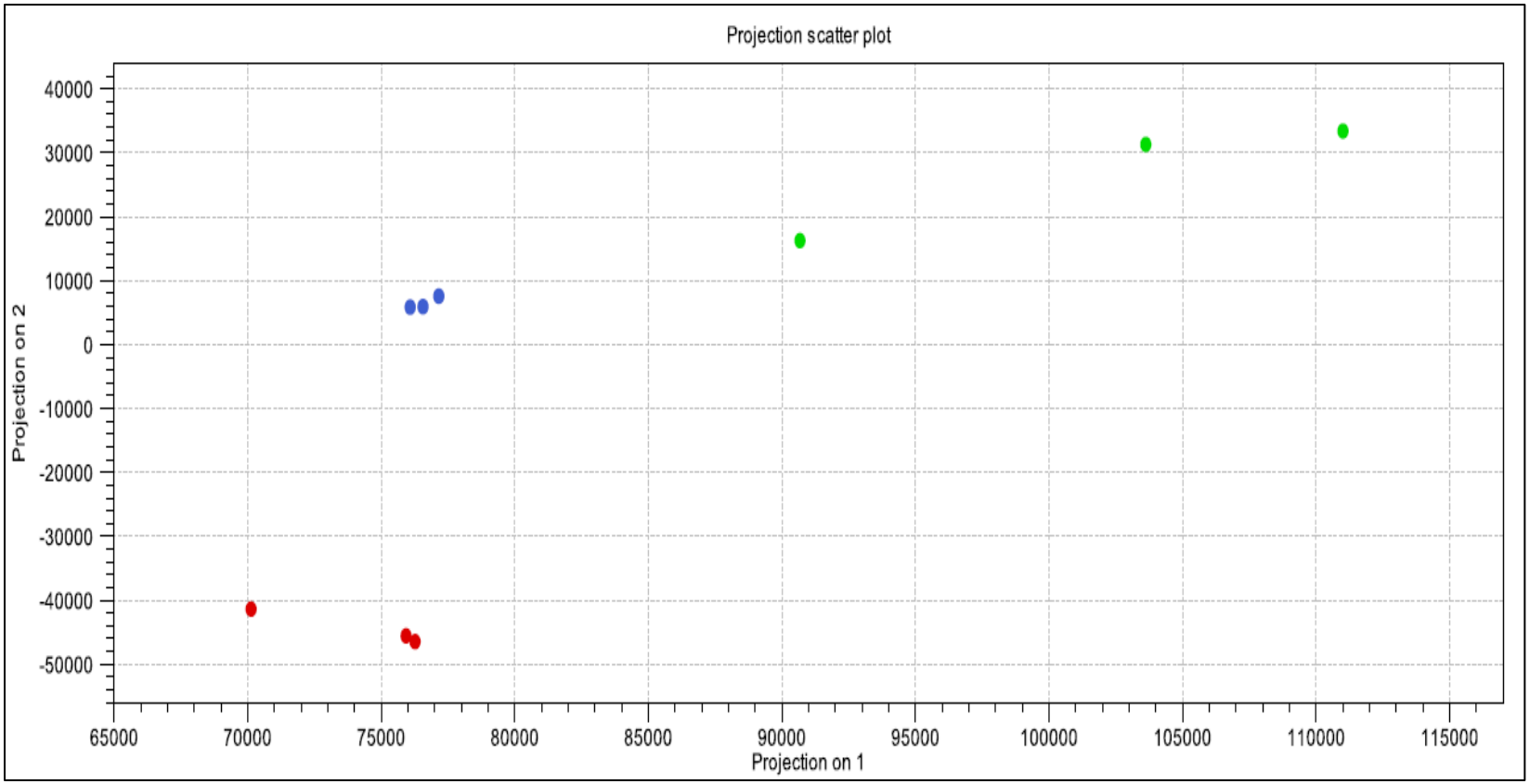


Figure 5-5: Principle component analysis of strand-specific RNA-Seq reads for *C. jejuni PT14* antisense transcription.

The Exponential *C. jejuni* replicates are illustrated in red while the filament and spiral replicates are shown in blue and green respectively. Each condition is displayed as a distinct group, with patterns of similarity being exhibited in each case.

Table 5-1: Analysis for RNA-Seq data for reads mapped against *C. jejuni PT14*

Conditions	Original Read count	Number of reads after trimming	Number of uniquely mapped reads	Read base pairs (bp)
PT14 Exponential 1	35,796,654	32,232,168	30,604,461	single-end 100 bp reads
PT14 Exponential 2	32,209,508	29,184,262	27,849,829	single-end 100 bp reads
PT14 Exponential 3	22,996,829	21,095,789	20,235,624	single-end 100 bp reads
PT14 Spiral 1	31,306,836	25,678,083	23,211,232	single-end 100 bp reads
PT14 Spiral 2	30,884,067	23,344,371	21,368,205	single-end 100 bp reads
PT14 Spiral 3	34,756,319	26,010,340	23,644,555	single-end 100 bp reads
PT14 Filament 1	33,931,360	29,861,813	28,500,727	single-end 100 bp reads
PT14 Filament 2	43,775,133	38,029,287	35,617,930	single-end 100 bp reads
PT14 Filament 3	23,574,998	20,648,985	19,299,620	single-end 100 bp reads

Table 5-2: Analysis for RNA-Seq data for reads mapped against *C. jejuni 12661*

Conditions	Original Read count	Number of reads after trimming	Number of uniquely mapped reads	Read base pairs (bp)
12661 Exponential 1	29,270,615	27,401,734	26,735,798	single-end 100 bp reads
12661 Exponential 2	27,204,634	24,540,784	22,724,881	single-end 100 bp reads
12661 Exponential 3	27,173,235	25,006,685	23,821,125	single-end 100 bp reads
12661 Spiral 1	21,391,862	19,785,647	19,422,930	single-end 100 bp reads
12661 Spiral 2	28,028,179	26,166,400	25,651,247	single-end 100 bp reads
12661 Spiral 3	26,029,625	24,332,558	23,828,146	single-end 100 bp reads
12661 Filament 1	32,928,741	29,811,661	27,669,206	single-end 100 bp reads
12661Filament 2	28,365,150	25,699,352	25,255,447	single-end 100 bp reads
12661 Filament 3	51,076,059	46,237,126	45,262,896	single-end 100 bp reads

5.2.3 Regulated gene expression of *C. jejuni* 12661 and *C. jejuni* PT14 comparing both spiral shaped cells and filamentous cells harvested at 30 h, with cells in exponential growth phase harvested at 8 h

The RNA-Seq data was processed using the CLC Genomics Workbench package (2.12.4.3). Reads that mapped uniquely to the *C. jejuni* 12661 and *C. jejuni* PT14 genomes 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. Baggerley's test on expression proportions (Baggerly et al., 2003) was used to determine genes with significant differential expression between filaments, exponential and spirals cells. To account for multiple testing, p-values were adjusted using the false discovery rate controlling method of Benjamini and Hochberg (1995).

5.2.3.1 Transcriptome analysis of *C. jejuni* 12661 morphotypes compared to each other and to exponential growth phase cells

The total number of genes that exhibited significant changes in their expression levels (≥ 1.5-fold, padj<0.05) for the two different *C. jejuni* 12661 morphotypes compared to each other and to exponential growth phase cells are summarised in **Table 5-3.**

Table 5-3: Number of differentially regulated genes between the different *C. jejuni* 12661 morphotypes

Regulation	Number and percentage of differentially regulated genes within the C. *jejuni* 12661 genome		
	Spiral morphotype compared to exponential cells	**Filamentous morphotype compared to spiral morphotype**	**Filamentous morphotype compared to exponential cells**
Up-regulated	**53 (3.18%)**	**162 (9.71%)**	**132 (7.91%)**
Down-regulated	**107 (6.41%)**	**782 (46.9%)**	**628 (37.65%)**

Figure 5-6 presents a summary of the distribution of the regulated genes in the two different *C. jejuni* 12661 morphotypes compared to each other and to exponentially growth phase cells.

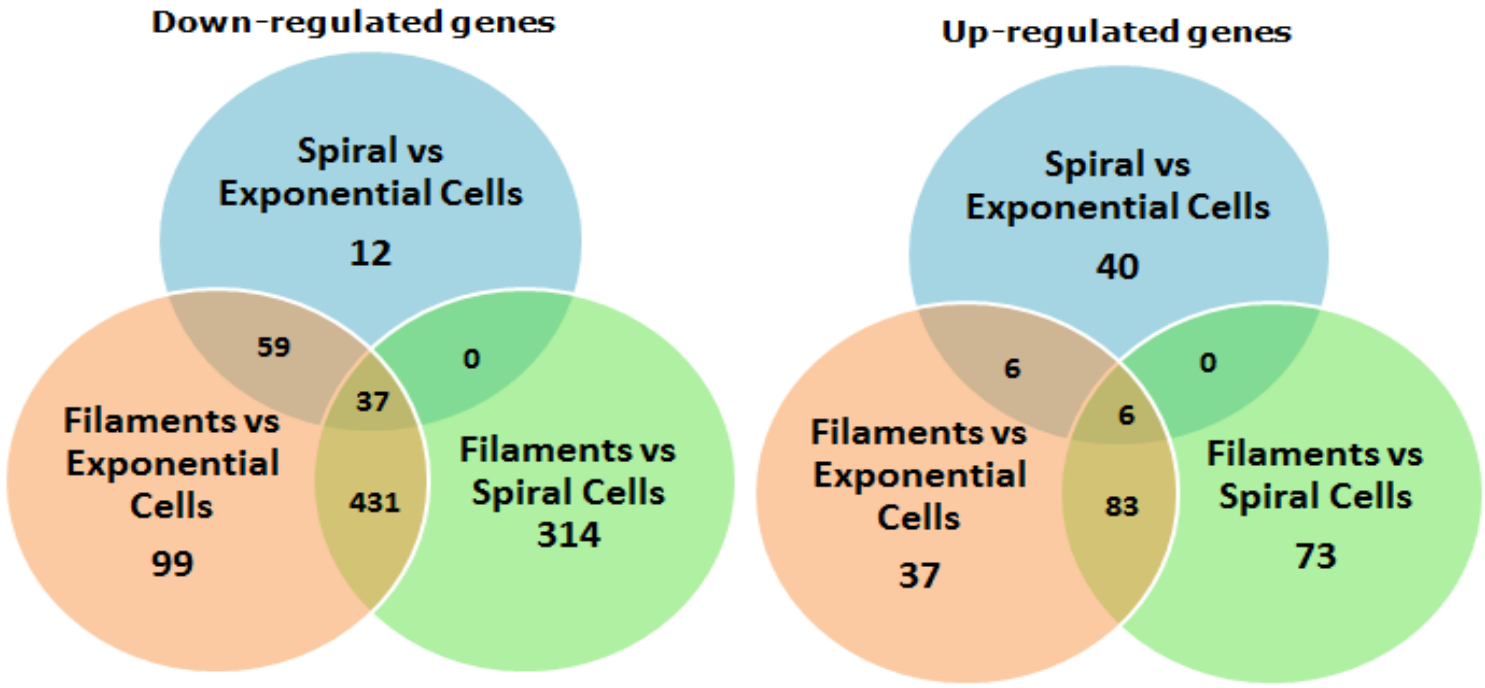


Figure 5-6: Venn diagram showing the distribution of differentially expressed genes in different morphotypes of *C. jejuni* 12661.

The distribution of genes significantly differentially expressed in the two different morphotypes (spirals and filaments) compared to each other and to exponential growth phase cells isolated at 30h and 8 h, respectively.

5.2.3.1.1 Transcriptomic analysis of the spiral morphotype with cells in exponential growth phase from *C. jejuni* 12661

A total of 160 genes were differentially expressed between stationary phase spiral cells and exponential growth phase cells of *C. jejuni* 12661. Of these, 53 genes were up-regulated and functionally categorised according to the Sanger Centre *C. jejuni* genome database into 13 groups, which are primarily associated with the energy metabolism, cell envelope, transport binding proteins, biosynthesis of cofactors, prosthetic groups and carriers and some of unknown function. Whilst 107 down-regulated genes could be placed into 16 functional groups, which corresponded with changes in cell envelope, amino acid biosynthesis, transport and binding proteins, biosynthesis of cofactors, prosthetic groups and carriers, synthesis and modification of macromolecules and a number of conserved and unknown function genes **(Figure 5-7)**. The complete list of up-regulated and down-regulated genes of spirals against exponential cells can be found in **Appendices 1** and **2** respectively.

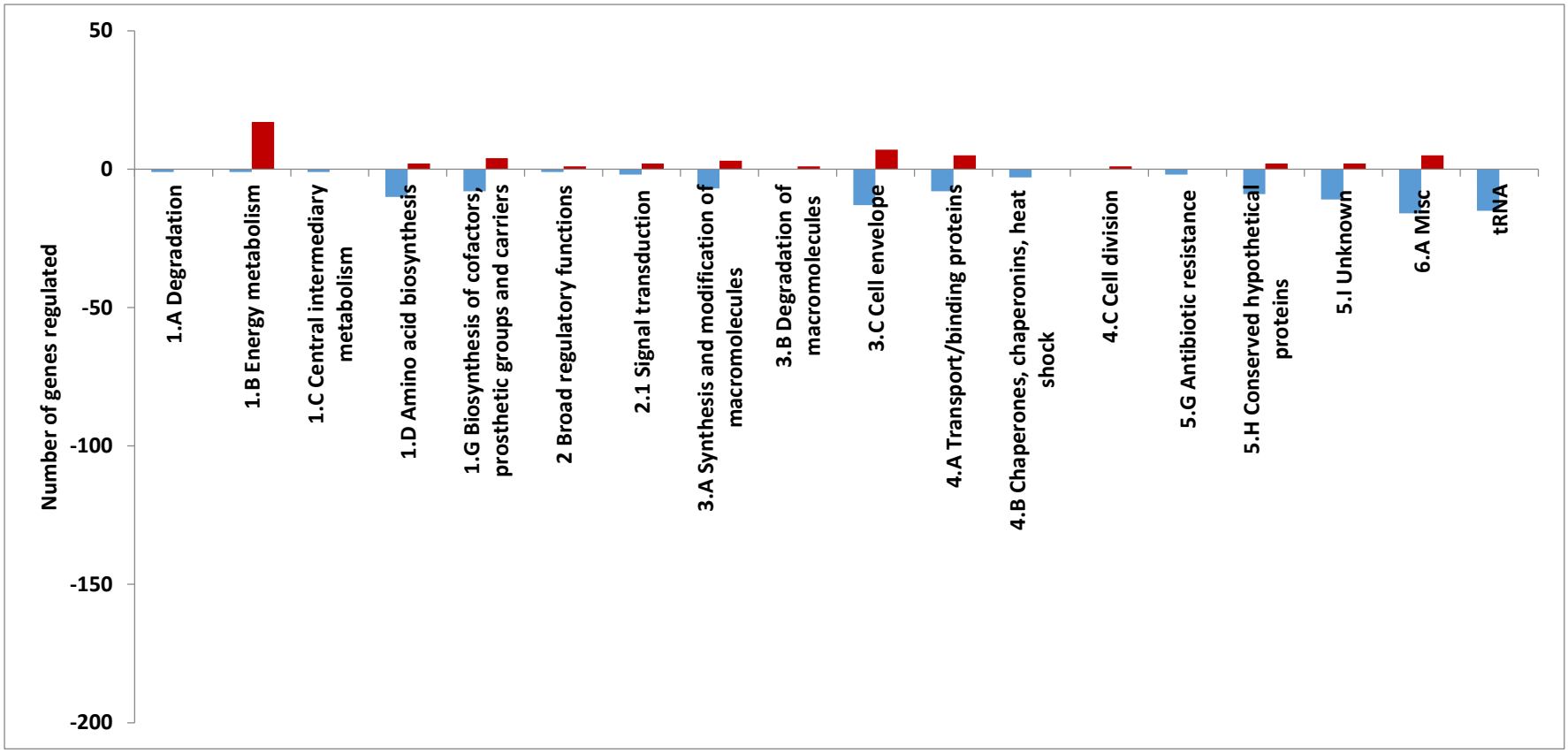


Figure 5-7: Numbers of genes differentially regulated between spiral morphotypes and exponential growing cells of *C. jejuni* 12661 assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene.

(Gundogdu et al. 2007)

5.2.3.1.2 Transcriptomic analysis of the filamentous morphotype with the spiral morphotype from *C. jejuni* 12661

When comparing each of the morphotypes to cells in exponential growth phase*, C. jejuni* 12661 filamentous cells showed differential regulated expression (≥ 1.5-fold, padj<0.05) of 760 genes compared to 160 genes in the spiral morphotype. These values represent 45.6% and 9.6% of the overall gene content, suggesting that the filamentous morphotype showed greater metabolic diversity than the spiral morphotype when compared with exponential phase cells. From the total of 944 differentially regulated genes, 162 genes were up-regulated in the filamentous morphotype comapared with spiral stationary phase cells. These genes could be placed into 20 functional groups related to the synthesis and modification of macromolecules, cell processes affecting the cell envelope, biosynthesis of cofactors, prosthetic groups and carrier transport and binding proteins, energy metabolism and a number of conserved hypothetical proteins. The remaining 782 genes were found to be down-regulated and placed into 25 functional categories largely featuring changes in cell envelope, synthesis and modification of macromolecules, transport and binding proteins, energy metabolism, amino acid biosynthesis, biosynthesis of cofactors, prosthetic groups and carriers, cell division (*maf*, *mreC*, *parB*, *ftsZ*, *ftsW*, *ftsA* and *mreB*) and several conserved genes of unknown function (**Figure 5-8)**. The complete list of up-regulated and down-regulated genes for the filamentous morphotype compared to the spiral morphotype can be found in **Appendices 3** and **4**, respectively.

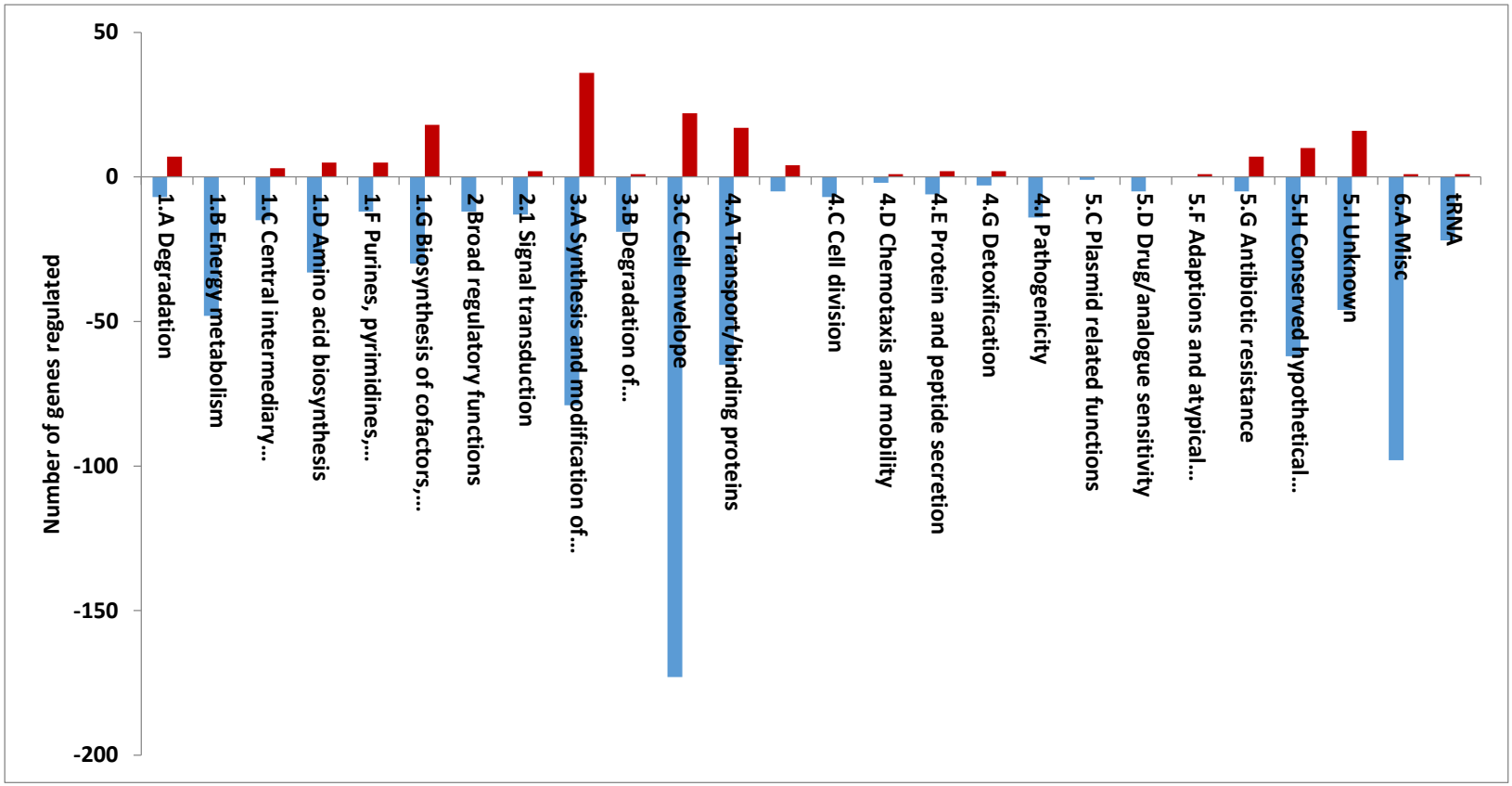


Figure 5-8: Numbers of genes differentially regulated between filaments and spiral growing cells of *C. jejuni 12661* assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene. **(**Gundogdu et al. 2007)

5.2.3.1.3 Transcriptomic analysis of the filamentous morphotype with cells in exponential growth phase from *C. jejuni* 12661

Comparing the filamentous morphotype with exponentially growing cells of *C. jejuni* 12661, revealed that 132 genes that were differentially activated. A substantial number of these were found to be associated with the synthesis and modification of macromolecules, including *aspS*, genes encoding disulphide bond formation proteins, *dnaE*, *gatB*, *gltX*, *greA*, *ssb*, *topA* and *typA*. A large number of genes encoding ribosomal proteins (*rplBCDEFNOPRUWVX* and *rpsBCFHJQRSU*) were also co-regulated.

Eighteen genes involved in energy metabolism were found to be significantly up-regulated in the filamentous morphotype compared to exponentially growing cells, including cytochrome c-type protein TorC and Ferredoxin. In addition, genes involved in central intermediary metabolism (*maeB*, *adk*, *fbp*, *metK* and *ppk*), amino acid biosynthesis (*ilvE*, *lysC*), biosynthesis of cofactors, prosthetic groups and carriers and cell envelope protein were found to be up-regulated in *C. jejuni* 12661 filamentous morphotype cells compared to cells in exponential growth phase. The greatest fold increases in gene expression for filamentous morphotype cells compared to cells in exponential growth phase were: a gene encoding a hypothetical protein UC78_1330, with a 62-fold change; the ferric aerobactin receptor precursor gene *iutA*, with a 30-fold change and the gene encoding catalase *katA* with a 21-fold change. The complete list of up-regulated genes can be found in **Appendix 5**.

Expressions of a group of 628 genes were found to be down-regulated, when comparing the filamentous morphotype with exponentially growing cells of *C. jejuni* 12661. Many of these were found to be associated with the cell envelope, including a number of flagellar biosynthesis proteins and basal body associated proteins. Genes encoding synthesis and modification of macromolecules was also repressed in this group of down regulated genes. In addition genes with functions involved in cell division and septum formation (*maf* and rod shape-determining protein *mreC*), pathogenicity like fibronectin type III domain protein, cytolethal distending toxin A/C family protein and Hemolysin C, chaperones (*clpA*, *dnaJ*) and amino acid biosynthesis (*aroE*) were also down-regulated, along with a large number of hypothetical proteins.

The most highly repressed gene was a tRNA-Leu (UC78_0841) with a 34 fold change, followed by the transport binding protein rubrerythrin-1 (*rbr1*), which was 33 fold lower in the filamentous morphotype compared to the exponential growth phase cells. The complete list of down-regulated genes comparing the filamentous morphotype with exponentially growing cells of *C. jejuni* of 12661 can be found in **Appendix 6.** Up-regulated genes from the filamentous morphotype comparison to exponential growth phase cells were placed into 17 functional categories while the down-regulated genes represented 24 groups **(Figure 5-9).** The highest number of activated genes in this set was related to synthesis and modification of macromolecules whilst the greatest numbers of down-regulated genes were involved in cell envelope functions.

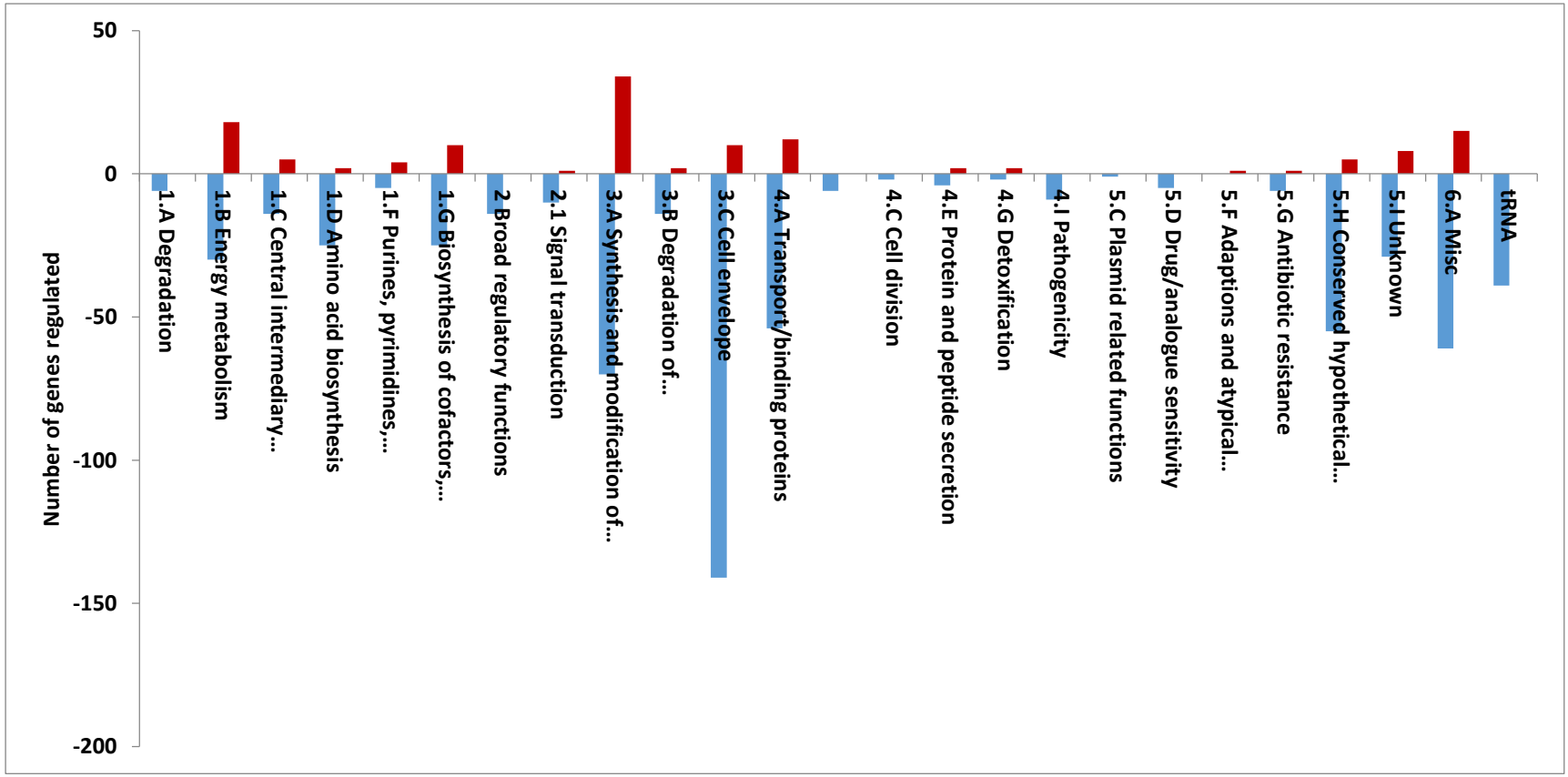


Figure 5-9: Numbers of genes differentially regulated between the filamentous morphotype compared to exponentially growing cells of *C. jejuni 12661* assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene.

(Gundogdu et al. 2007)

5.2.3.2 Transcriptome analysis of *C. jejuni* PT14 and its cell morphotypes

The total number of genes that were significantly changed in their expression levels (≥ 1.5-fold, padj<0.05) in the two different *C. jejuni* PT14 morphotypes compared to cells in exponential growth phase and to each other are summarised in **Table 5-4.**

Table 5-4: Number of differentially regulated genes between *C. jejuni* PT14 morphotypes

Regulation	Number and percentage of differentially regulated genes within the *C. jejuni* PT14 genome		
	Spiral morphotype compared to exponential cells	Filamentous morphotype compared to spiral morphotype	Filamentous morphotype compared to exponential cells
Up-regulated	162 (9.95%)	876 (52.87%)	292 (17.62%)
Down-regulated	1085 (65.47%)	93 (5.61%)	689 (41.58%)

Figure 5-10 provides a summary of the distribution of the regulated genes in the two different *C. jejuni* PT14 morphotypes compared to cells in exponential growth phase and to each other.

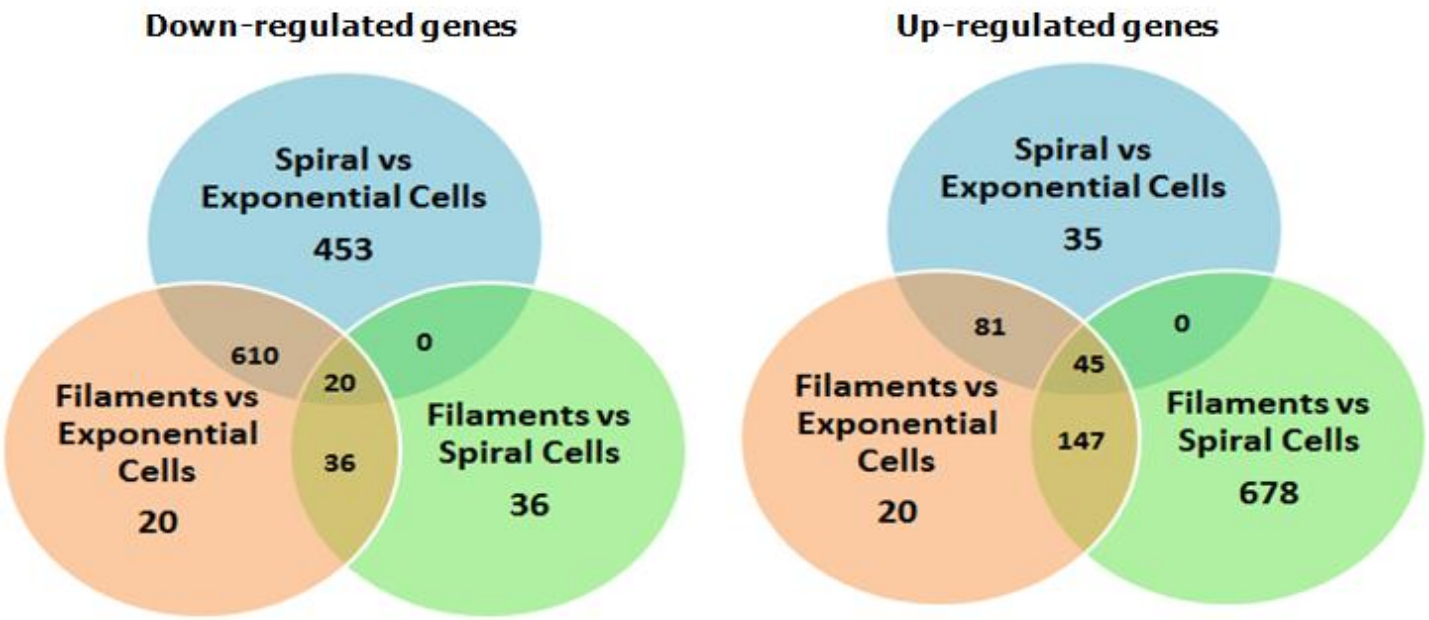


Figure 5-10: Venn diagram showing the distribution of differentially expressed genes in different morphotypes of *C. jejuni* PT14.

The distribution of genes significantly differentially expressed in different morphotypes isolated at 8h (exponential cell) and 30h (filaments and spirals). Among the three cellular forms, there are three groups of common genes that were activated and three sets that were found to be repressed.

5.2.3.2.1 Transcriptomic analysis of the spirals morphotype compared to exponential growth phase cells of *C. jejuni* PT14

A group of 1247 genes were differentially expressed when comparing the spiral morphotype cells to exponential growth phase cells. Of these, 162 genes were up-regulated in the spiral morphotype. These could be functionally categorised in to 18 groups as follows: those that are primarily associated with the synthesis and modification of macromolecules; those involved in the cell envelope; genes for transport and binding proteins; genes involved in energy metabolism and a number of conserved hypothetical, unknown proteins. The remaining 1085 of the differentially expressed genes were down-regulated and could be classified in to 25 functional groups as follows: those which were mainly associated with the cell envelope; those involved in synthesis and modification of macromolecules; genes for transport and /binding proteins; genes involved in energy metabolism; genes involved in biosynthesis of cofactors, prosthetic groups and carriers; genes involved in amino acid biosynthesis and a number of conserved hypothetical, unknown proteins (**Figure 5-11**)**.**

The complete list of up-regulated and down-regulated genes comparing the spiral morphotype with exponential growth phase cells can be found in **Appendices 7** and **8,** respectively.

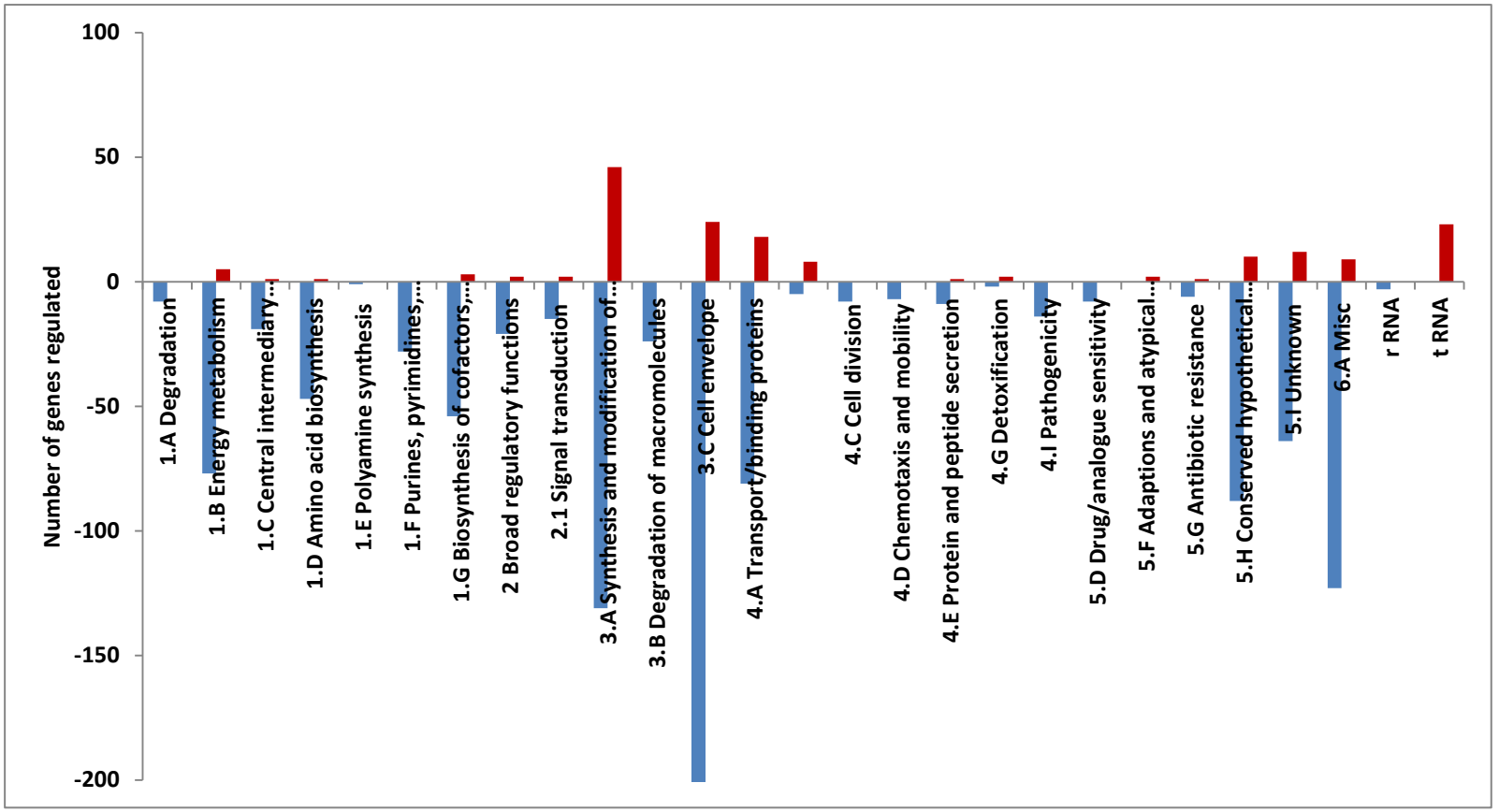


Figure 5-11: Numbers of genes differentially regulated between the spiral morphotype and exponential growth phase cells of *C. jejuni PT14* assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene. (Gundogdu et al. 2007)

When the functional profiles of the differentially expressed genes for the filamentous and spiral morphotypes are compared to the exponentially growing cells, these cell types would be expected to show similarities since both are effectively in stationary phase. It is interesting that the filaments are more metabolically active than the spirals in PT14.

5.2.3.2.2 Transcriptomic analysis of the filamentous morphotype compared to the spirals morphotype of *C. jejuni* PT14

The *C. jejuni* PT14 filamentous morphotype showed activation of 876 genes compared with the spiral morphotype. A large number of these were found to be related to cell envelope structure and function, including a number of flagellar biosynthesis proteins and basal body associated protein (*flhA*, *flhB*, flhF, *fliR*, *fliH*, *fliP, fliL*, *fliG*, *fliM* and *flgA*), synthesis and modification of macromolecules, transport and binding proteins and a number of conserved hypothetical and unknown proteins were also similarly regulated. In addition, 52 genes involved in energy metabolism were found to be significantly up-regulated along with genes involved in central intermediary metabolism, amino acid biosynthesis, biosynthesis of cofactors, prosthetic groups and carriers. Nine genes involved in cell division were also up-regulated including: Maf-like protein, cell division proteins FtsA, FtsZ, FtsW and rod shape-determining proteins MreB and C. The most activated genes for the filamentous morphotype compared to the spiral morphotype cells were associated with transport binding proteins such as exbD/tolR family transport protein (A911_00525; 13.3-fold), putative hemin uptake system permease protein (A911_07775;

13.1-fold), putative hemin uptake system ATP-binding protein (A911_07780; 12.4-fold) and a putative ABC transport system permease (A911_07930; 11.6-fold). The complete list of up-regulated genes for the filamentous morphotype compared to the spiral morphotype in *C. jejuni* PT14 can be found in **Appendix 9.**

Fewer genes were down-regulated in the separated *C. jejuni* PT14 filamentous morphotype compared to the spiral morphotype (93 or 5.61%). Many of these were found to have functions associated with the cell envelope, including a number of flagellar biosynthesis proteins and basal body associated proteins. Genes encoding chaperones (*groES*, *groEL*, *grpE* and *dnaK*), were down-regulated together with a number of hypothetical proteins largely due to the strong expression of the chaperones in stationary phase spiral cells. The most highly repressed genes encoded a hypothetical protein A911_07060 and FspA2, which exhibited changes >5-fold lower in the filamentous morphotype compared to the spiral morphotype. The complete list of down-regulated genes of the filamentous morphotype compared to the spiral morphotype of *C. jejuni* PT14 can be found in **Appendix 10.**

Differentially expressed genes of the filamentous and spiral morphotypes were functionally allocated and appear in **Figure 5-12.** Up-regulated genes were placed into 25 functional categories while the down-regulated genes represented 17 groups. The cell envelope gene category contained the largest group of differentially regulated genes in *C. jejuni* PT14 filaments/spirals, featuring up and down-regulated genes.

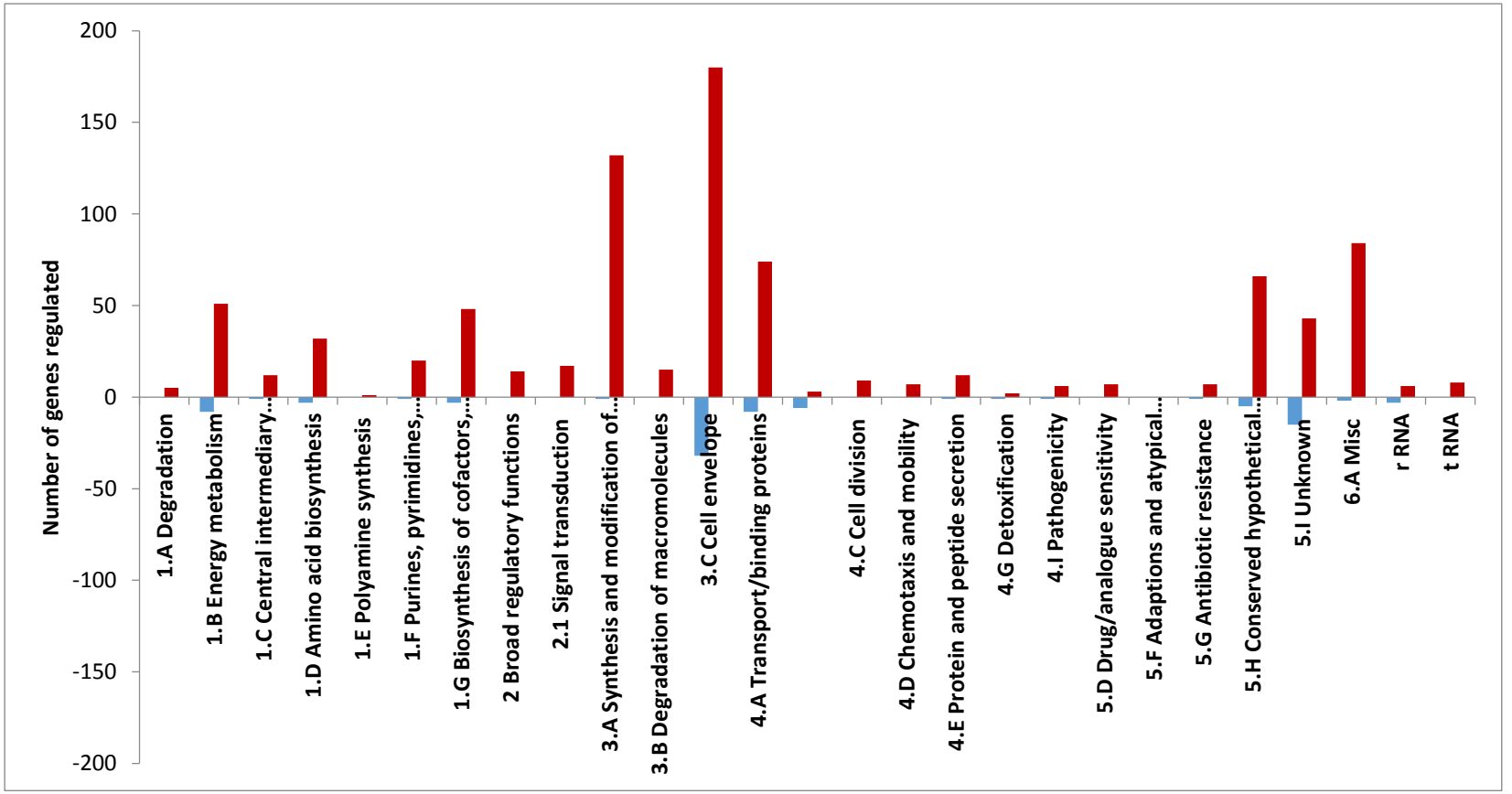


Figure 5-12: Numbers of genes differentially regulated between filamentous morphotypes and spiral morphotypes of *C. jejuni* PT14 assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene.

(Gundogdu et al. 2007)

5.2.3.2.3 Transcriptomic analysis of the filamentous morphotypes with cells in exponential growth phase from *C. jejuni* PT14

C. jejuni PT14 filamentous morphotypes differentially activated a total of 292 genes and repressed 689 genes compared to exponential growth cells, which is a significantly larger gene set compared to the differential activation of 162 genes recorded for the spiral morphotype compared exponential cells for PT14. These values represent 17.62% and 9.95% of the overall respective gene contents. The number of repressed genes in filaments against exponential of PT14 were 689 (41.58%) compared to 1085 (65.47%) genes in spirals against exponential.

Genes displaying significant differential expression were assigned into functional categories (**Figure 5-13**). The functional classification of differentially up-regulated genes of the filamentous morphotype compared to exponential growth phase cells were placed in to 21 functional clusters. They were mostly genes associated with the cell envelope, synthesis and modification of macromolecules, amino acid biosynthesis, energy metabolism and number of conserved hypothetical, unknown proteins. The down-regulated genes fell in to 24 clusters including: genes associated with synthesis and modification of macromolecules; transport and binding proteins; cell envelope energy metabolism genes; amino acid biosynthesis genes; genes associated with biosynthesis of cofactors, prosthetic groups and carriers, and a number of conserved hypothetical and genes encoding unknown proteins.

The complete list of up-regulated and down-regulated genes for the filamentous morphotype compared to exponential growth phase cells can be found in **Appendices 11** and **12,** respectively.

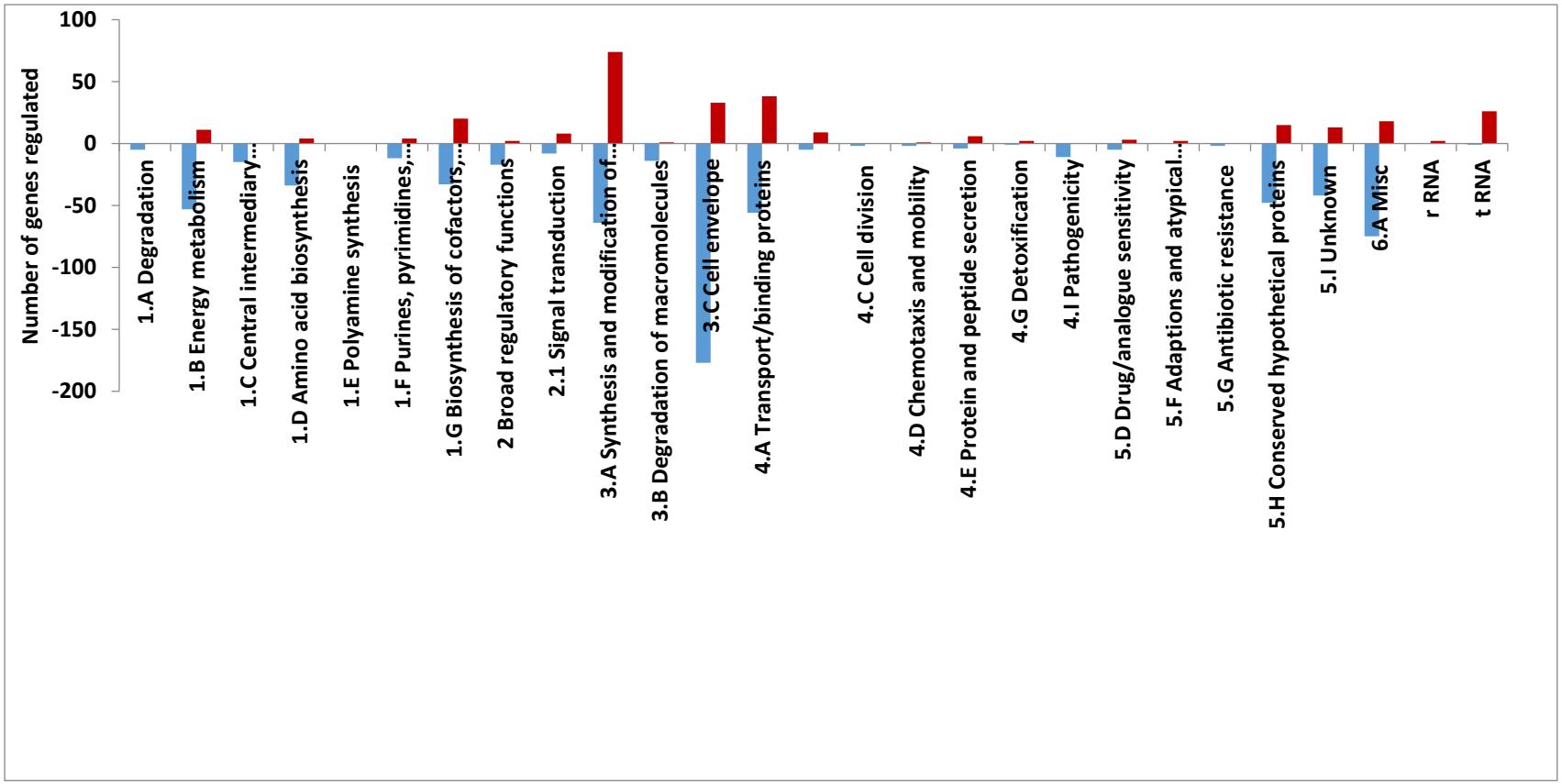


Figure 5-13: Numbers of genes differentially regulated between the filamentous morphotype and exponential growth phase cells of *C. jejuni PT14* assigned into functional groups.

The red bars represent the upregulated groups of gene, while the blue one represents the downregulated groups of gene. (Gundogdu et al. 2007)

5.2.4 Non-coding RNAs (ncRNAs) for *C. jejuni* PT14 and *C. jejuni* 12661

A non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into protein. Generally, the role of ncRNAs is to regulate gene expression at the transcriptional and post-transcriptional level. The ncRNAs can be divided into two main groups; the short ncRNAs less than 30 nucleotides and the long ncRNAs more than 200 nucleotides.

Strand-specific sequencing has been applied to determine ncRNAs and antisense transcription in *C. jejuni* (Chaudhuri et al., 2011, Taveirne et al., 2013, Butcher and Stintzi, 2013). 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). In this study no differentially regulated were found for the majority of the candidate ncRNAs. However, CJnc20 was found to be up-regulated in *C. jejuni* PT14 in the filamentous morphotype and the spiral morphotypes. The number of ncRNAs in both *C. jejuni* PT14 and *C. jejuni* 12661 are listed in **Tables 5.5 and 5.6** using the nomenclature of Dugar et al. (2013).

Table 5-5: sRNA candidates for *C. jejuni* 12661

sRNA	Strand	Start	End	Length	Orientation	LLT	RLT	LRF Description	RRF Description
SRP RNA	+	75544	75651	108	<>>	UC78_0054	UC78_0055	bacteriohemerythrin	putative malate transporter
CJnc10	+	104246	104341	96	>><	UC78_0085	UC78_0086	cytochrome bd-I ubiquinol oxidase subunit 2	aspartate racemase
CJnc20	-	248245	248092	156	<<<	UC78_0252	UC78_0253	putative cytochrome C-type protein (*torC*)	hypothetical protein
TPP riboswitch	+	426301	426436	136	>>>	UC78_0434	UC78_0435	DNA polymerase III subunit epsilon	thiamine biosynthesis protein (*thiC*)
RnpB	-	521382	521069	317	><>	UC78_0534	UC78_0535	hypothetical protein	elongation factor P
CJnc60	-	671428	671148	281	><>	UC78_0692	UC78_0693	putative reductase	DNA polymerase III subunit alpha
CJnc110	+	1118984	1119120	137	>>>	UC78_1150	UC78_1150	S-ribosylhomocystein lyase (*luxS*)	2-oxaglutarate/iron oxygenase
6S/ SsrS RNA	+	1170573	1170758	186	>>>	UC78_1201	UC78_1209	hypothetical protein	phosphoribosylamine-glycine ligase (*purD*)
CJnc140	+	1179686	1179758	73	>>>	UC78_1202	UC78_1210	putative phosphotyrosine protein phosphatase	major outer membrane protein (*porA*)
tmRNA	-	1281828	1281470	359	<<<	UC78_1307	UC78_1309	hypothetical protein	hypothetical protein
crRNA1	+	1428997	1429026	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
crRNA2	+	1429063	1429092	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
crRNA3	+	1429129	1429158	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
crRNA4	+	1429195	1429224	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)

Table 5.5 continued

sRNA	Strand	Start	End	Length	Orientation	LLT	RLT	LRF Description	RRF Description
crRNA4	+	1429195	1429224	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
crRNA5	+	1429261	1429290	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
crRNA6	+	1429327	1429356	30	>>>	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
TracrRNA	+	1429465	1429538	74	>><	UC78_1451	UC78_1452	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated protein 2 (*cas2*)
CJnc170	+	1531383	1531409	27	<><	UC78_1559	UC78_1560	serine transporter (*sdaC*)	hypothetical protein
CJnc180	+	1551864	1552018	155	>><	UC78_1584	UC78_1585	hypothetical protein	methionine aminopeptidase (*map*)
CJnc190	-	1552109	1551893	217	>><	UC78_1584	UC78_1585	hypothetical protein	methionine aminopeptidase (*map*)
CJnc200	+	1557372	1557520	149	>>>	UC78_1591	UC78_1592	hypothetical protein	FTR1 family iron permease
CJnc230	-	1609128	1609030	99	<<<	UC78_1662	UC78_1663	7-cyano-7-deazaguanine reductase (*queF*)	flagellar hook protein (*flgE*)

Table 5-6: sRNA candidates for *C. jejuni* PT14

sRNA	Strand	Start	End	Length	Orientation	LLT	RLT	LRF Description	RRF Description
SRP RNA	+	67233	67340	108	<>>	A911_00220	A911_00225	putative iron-binding protein	C4-dicarboxylate transport protein pseudogene
CJnc10	+	94715	94759	96	>><	A911_00395	A911_00400	hypothetical protein	aspartate racemase
CJnc11	-	237792	237728	65	>><	A911_01250	A911_01255	dihydroorotase (*pyrC*)	putative small hydrophobic protein
CJnc20	-	245179	245024	156	<<<	A911_01280	A911_01285	TMAO cytochrome C-type haem-binding protein	putative integral membrane protein
TPP riboswitch	+	418181	418316	136	>>>	A911_02205	A911_02210	DNA polymerase III subunit epsilon	thiamine biosynthesis protein (*thiC)*
RnpB	-	513252	512928	315	>><	A911_02680	A911_02685	hypothetical protein	elongation factor P
CJnc21	+	542897	543065	169	>>>	A911_02840	A911_02845	aspartate kinase (*lysC*)	hypothetical protein
CJnc60	-	673417	673137	281	>><	A911_03500	A911_03505	ArsC family protein	DNA polymerase III subunit alpha
CJnc110	+	1128382	1128518	137	>>>	A911_05810	A911_05815	S-ribosylhomocysteine lyase	2-oxaglutarate/iron oxygenase
CJnc120	+	1151441	1151537	97	>><	A911_05925	A911_05930	*groES*	sensor histidine kinase (*dccS*)
6S /SsrS RNA	+	1179976	1180161	186	>>>	A911_06070	A911_06075	hypothetical protein	phosphoribosylamine--glycine ligase
CJnc140	+	1189088	1189160	73	>>>	A911_06110	A911_06115	protein tyrosine phosphatase	major outer membrane protein (*porA*)
tmRNA	-	1293125	1292767	359	>><	A911_06605	A911_06610	polyphosphate kinase (*ppk*)	signal transduction histidine kinase
crRNA1	+	1448613	1448642	30	>>>	A911_07320	A911_07325	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated cas2 protein (*cas2*)
crRNA2	+	1448679	1448708	30	>>>	A911_07320	A911_07325	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated cas2 protein (*cas2*)

Table 5.6 continued

sRNA	Strand	Start	End	Length	Orientation	LLT	RLT	LRF Description	RRF Description
crRNA3	+	1448745	1448774	30	>>>	A911_07320	A911_07325	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated cas2 protein (*cas2*)
crRNA4	+	1448699	1448869	171	>>>	A911_07320	A911_07325	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated cas2 protein (*cas2*)
TracrRNA	+	148883	1448956	74	>><	A911_07320	A911_07325	Molybdopterin biosynthesis protein (*moeA2*)	CRISPR-associated cas2 protein (*cas2*)
CJnc150	-	1393825	1393745	81	>><	A911_07005	A911_07020	peptide chain release factor 2	tRNA pseudouridine synthase D
CJnc160	+	1548922	1548948	27	<><	A911_07825	A911_07830	amino acid transporter	hypothetical protein
CJnc170	+	1554056	1554102	47	>><	A911_07865	A911_07870	putative ATP-binding protein	chorismate synthase (*aroC*)
CJnc180	+	1569394	1569492	99	>><	A911_07950	A911_07955	hypothetical protein	methionine aminopeptidase
CJnc190	-	1569637	1569422	216	>><	A911_07950	A911_07955	hypothetical protein	methionine aminopeptidase
CJnc230	-	1631897	1631800	98	<<<	A911_08315	A911_08320	O-acetylhomoserine (thiol)-lyase	flagellar hook protein (*flgE*)

5.2.5 Validation of RNA-Seq results using qRT PCR

To support the reliability of the RNA-Seq data, a series of genes showing a range of differential expression levels were selected for transcriptional analysis by qRT PCR. The expression of 8 genes from *C. jejuni* 12661 and 11 genes from *C. jejuni* PT14 were selected on the basis their broad expression levels observed from RNA-Seq data analysis for confirmation by qPCR analysis. The selected genes and the primers used for their amplification in the qPCR experiments are listed in **Table 2.2**. Total RNAs were extracted from the two morphotypes and from exponential growth phase cells as described in section 2.12.1. The qPCR experiments were performed using the SYBR green methodology described in section 2.13.4. The relative transcription level of each gene in this study was normalized to the internal control genes *rplA* (50s Ribosomal RNA protein L1) and *icd (*isocitrate dehydrogenase) for *C. jejuni* 12661 and *C. jejuni* PT14 respectively, which showed none or minimal changes in gene expression between the two morphotypes or growth phase conditions in the RNA-Seq experiments. Quantitative values were obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method. The transcript level from each RNA sample was assayed three times, and the mean C_T value used for further analysis. The difference in the transcription of each specific gene was calculated by the $2^{-\Delta\Delta CT}$method (2.13.4).

Ct is the intersection value where the amplification curve meets the threshold line, which is a relative measure of the target template sequence content. **Tables 5.7** and **5.8** confirm the trends observed in

the transcriptomic study for the *C. jejuni* 12661 spiral and filamentous morphotypes, respectively, by comparing the RNA-Seq and qPCR fold change values. **Figures 5.14** and **5.15** show the concordance between the RNA-Seq results and the qPCR data of *C. jejuni* 12661 for spiral and filamentous morphotypes.

Table 5-7: Relative comparison of RNA-Seq and qPCR fold changes C. *jejuni* 12661 spiral morphotype

Gene 12661	Δ Ct of gene	Δ Ct of gene -Δ Ct of control	qPCR fold change 2 ^(-ΔΔCt)	log_{10} qPCR	RNA-Seq fold change	log_{10} qPCR RNA-Seq
Catalase	-5.27	-3.25	9.51	0.97	2.61	0.42
***aro*E**	-0.32	-1.33	2.51	0.4	1.8	0.25
Irup	-2.51	-2.28	4.85	0.68	8.32	0.92
FlgG_1	2.43	1.97	0.25	-0.6	0.06	-1.22
Hypothetical protein	4.37	1.3	0.4	-0.39	0.23	-0.64
Cj1491	-2.84	-0.96	1.94	0.28	2.95	0.47
Anrp	-2.01	-1.18	2.26	0.35	3.31	0.52
chaperone	-1.82	-0.37	1.29	0.11	1.59	0.2

The table represents the calculation of fold change in qPCR.

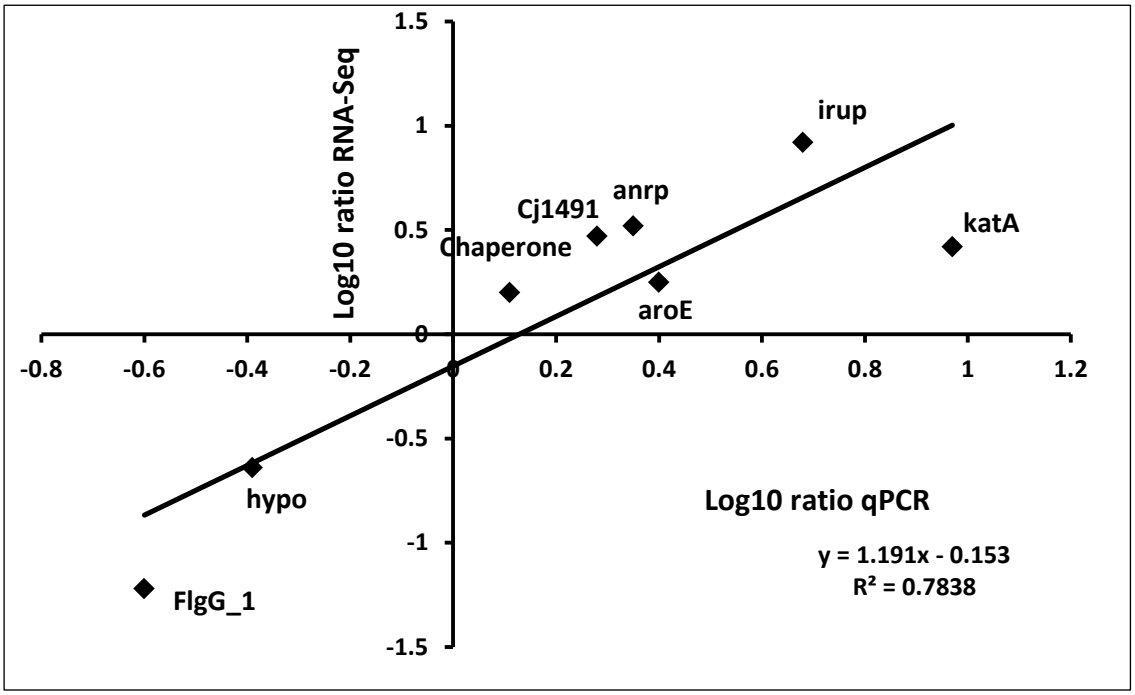


Figure 5-14: Correlation of the gene expression levels measured by RNA-Seq and qPCR of selected genes of *C. jejuni* 12661 for the spiral morphotype compared with exponential growth phase cells.

The X axis represents the log ratio values of the RNA-Seq normalised expression values while the Y axis represents the $\log_{10}$ qPCR ratios. R^2 represents the coefficient of determination.

Table 5-8: Relative comparison of RNA-Seq and qPCR fold changes *C. jejuni* 12661 filamentous compared to exponential growth phase cells

Gene 12661 (Filaments)	Δ Ct of gene	Δ Ct of gene -Δ Ct of control	qPCR fold change 2 ^ (-ΔΔCt)	log10 qPCR	RNA-Seq fold change	log10 qPCR RNA-Seq
Catalase	-5.46	-3.44	10.85	1.03	20.82	1.32
***aro*E**	2.33	1.32	0.4	-0.39	0.27	-0.57
Irup	-2.31	-2.08	4.22	0.62	3.67	0.56
FlgG_1	3.51	3.05	0.12	-0.92	0.042	-1.37
Hypothetical protein	4.1	1.03	0.48	-0.31	0.14	-0.85
Cj1491	-2.04	-0.16	2.23	0.34	0.9	-0.04
Anrp	-5.6	-4.77	27.25	1.43	15.4	1.19
Cu chaperone	0.16	1.99	0.25	-0.6	0.36	-0.44

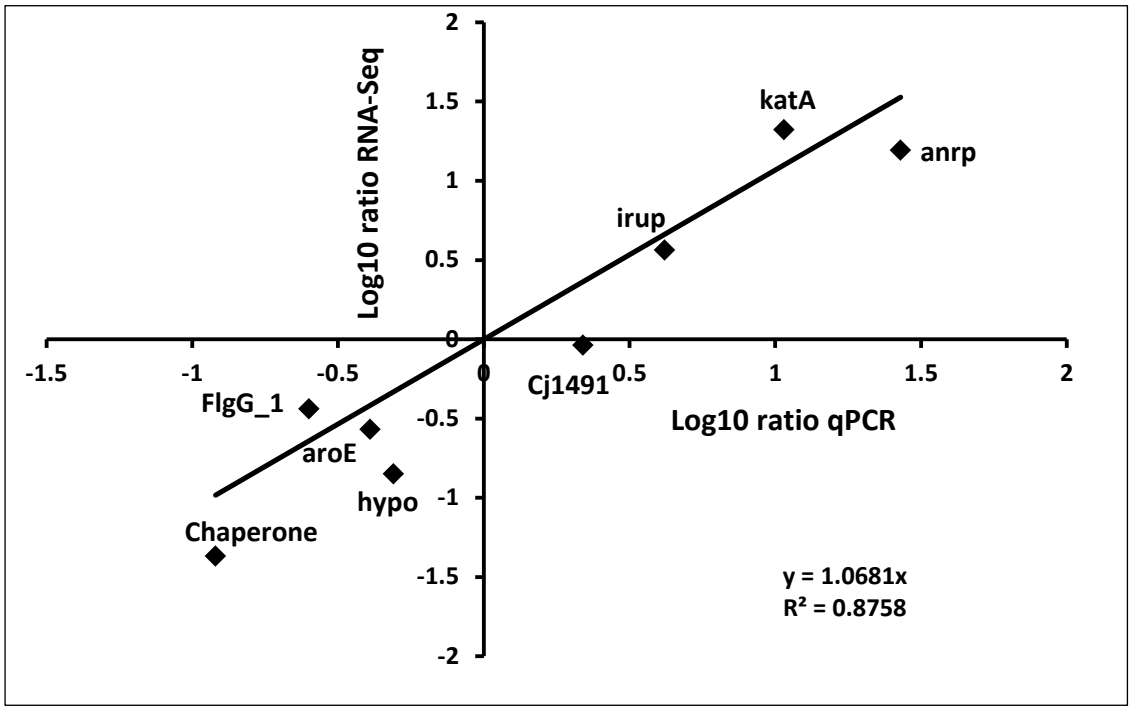


Figure 5-15: Correlation of the gene expression levels measured by RNA-Seq and qPCR of selected genes of *C. jejuni* 12661 for the filamentous morphotype compared to exponential growth phase cells.

The X axis represents the log ratio values of the RNA-Seq normalised expression values while the Y axis represents the $\log_{10}$ qPCR ratios. R^2 represents the coefficient of determination.

Tables 5.9 and **5.10** validate the trends detected in the transcriptomic study of *C. jejuni* PT14 spiral and filamentous morphotypes respectively, by comparing the RNA-Seq and qPCR fold changes. **Fig. 5.16** and **5.17** presents the correlation between the RNA-Seq results and the qPCR data of *C. jejuni* for PT14 spirals and filaments expressed as $\log_{10}$ transformed ratios, showing correlation coefficients of 0.896 and 0.92, respectively.

Table 5-9: Relative comparison of RNA-Seq and qPCR fold changes *C. jejuni* PT14 spiral morphotypes compared to exponential growth phase cells

Genes (PT14)	Δ Ct of gene	Δ Ct of gene -Δ Ct of control	qPCR fold change 2 ^(-ΔΔCt)	log10 qPCR	RNA-Seq fold change	log10 qPCR RNA-Seq
Catalase	-1.81	-2.21	4.62	0.66	4.75	0.68
hump	-4.51	-5.16	35.75	1.55	56.46	1.75
omp	-0.85	-2.67	6.36	0.8	31.3	1.5
***clp*B protease**	-1.47	-1.72	3.29	0.51	11.98	1.08
***gro*ES**	-2.72	-2.28	4.85	0.69	10.38	1.01
***pet*A**	1.09	2.09	0.23	-0.64	0.57	-0.24
***his*A**	1.55	3.13	0.11	-0.95	0.23	-0.64
***fla*G**	-2.22	-1.6	3.03	0.48	2.08	0.32
hypothetical	-3.69	-1.43	2.69	0.42	4.33	0.64
CJ1491	-0.16	1.38	0.38	-0.42	0.34	-0.47
***aro*E**	3.32	3.14	0.11	-0.95	0.051	-1.29

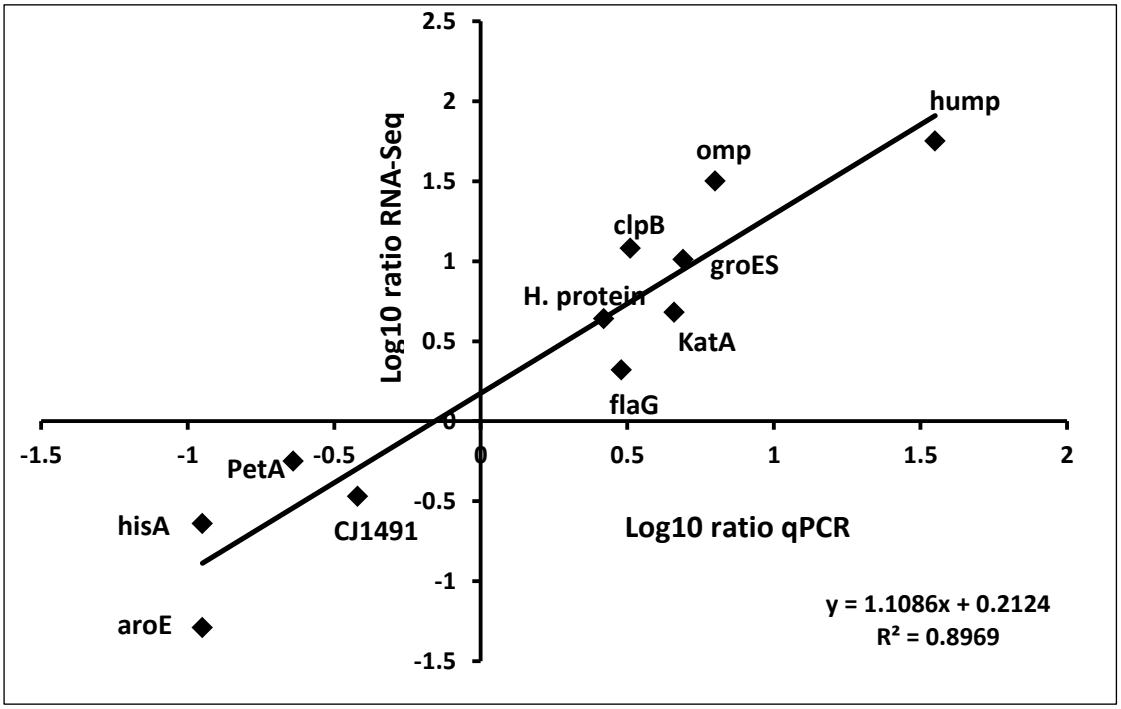


Figure 5-16: Correlation of the gene expression levels measured by RNA-Seq and qPCR of selected genes of *C. jejuni* PT14 spiral morphotypes compared with exponential growth phase cells.

The X axis represents the log ratio values of the RNA-Seq normalised expression values while the Y axis represents the $\log_{10}$ qPCR ratio. R^2 represents the coefficient of determination.

Table 5-10: Relative comparison of RNA-Seq and qPCR fold changes *C. jejuni* PT14 filament vs exponential cells

Genes (PT14)	Δ Ct of gene	Δ Ct of gene-Δ Ct of control	qPCR fold change 2 ^(-ΔΔCt)	$\log_{10}$ qPCR	RNA-Seq fold change	$\log_{10}$ qPCR RNA-Seq
Catalase	-4.04	-4.45	21.8	1.34	15.45	1.18
hump	-6.28	-6.93	121.9	2.08	192.92	2.28
omp	-3.94	-5.76	54.19	1.73	168.42	2.22
***clp*B**	-2.2	-2.45	5.46	0.73	6.51	0.81
***gro*ES**	-2.61	-2.17	4.5	0.65	3.43	0.54
***pet*A**	0.06	1.13	0.45	-0.34	0.64	-0.19
***his*A**	-1	0.58	0.66	-0.18	0.66	-0.18
***fla*G**	1.04	1.66	0.31	-0.5	0.63	-0.2
hypo	-4.06	-1.8	3.48	0.54	1.08	0.03
CJ1491	2.35	3.89	0.067	-1.17	0.62	-0.2
***aro*E**	1.25	1.07	0.47	-0.32	0.23	-0.64

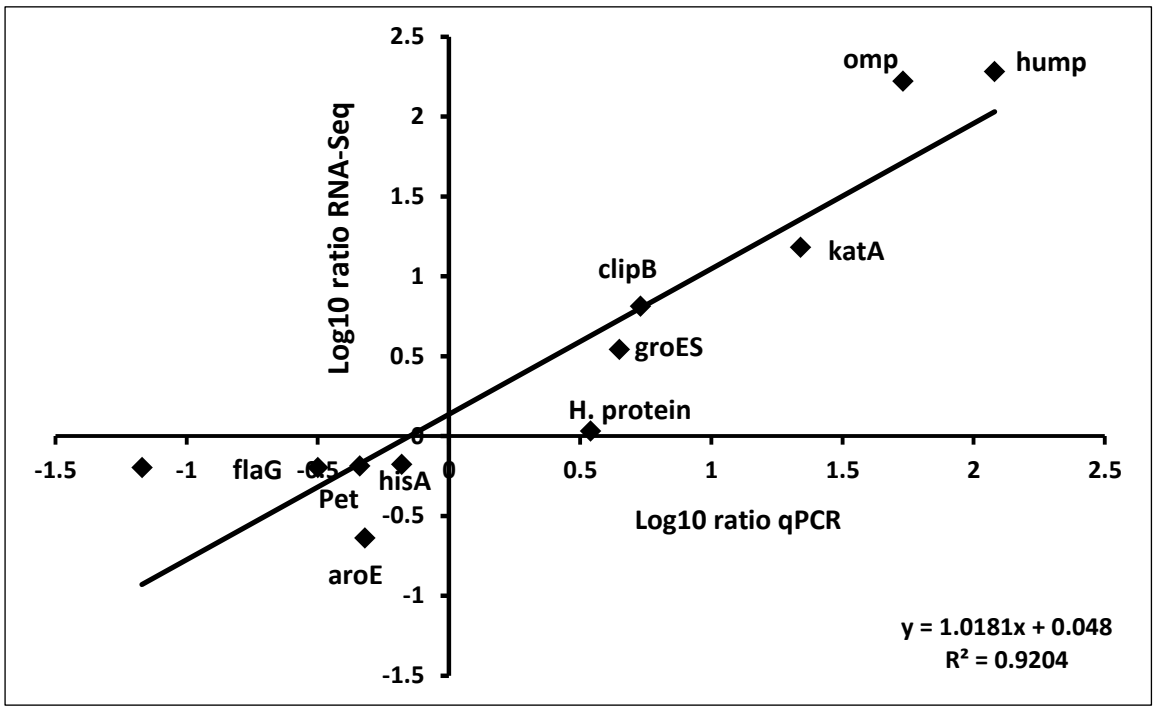


Figure 5-17: Correlation of the gene expression levels measured by RNA-Seq and qPCR of selected genes of *C. jejuni* PT14 filamentous morphotypes compared with exponential growth phase cells.

The X axis represents the log ratio values of the RNA-Seq normalised expression values while the Y axis represents the $\log_{10}$ qPCR ratios. R^2 represents the coefficient of determination.

5.2.6 Motility of different *C. jejuni* morphotypes

Primary analysis of the RNA-Seq data indicated that motility associated functions were significantly down regulated in the spiral and filamentous morphotypes. Motility assays were therefore implemented as described in section 2.4 in order to determine the functional effects of these expression changes. **Figure 5-18** shows motility plates inoculated with cells from exponential growth and from the two morphotypes recovered from the stationary phases of growth for *C. jejuni* 12661 and *C*. *jejuni* PT14. The plates inoculated with cells recovered from the exponential growth phase show that the cultures were motile but there was an obvious difference in the size of the motility zones between the two strains, suggesting that *C. jejuni* 12661 at exponential phase is less motile than *C. jejuni* PT14. Both *C. jejuni* 12661 and C. *jejuni* PT14 were markedly impaired in motility and did not recover within the 24 h incubation period of the experiment.

The zones of motility observed for the two morphotypes of *C. jejuni* 12661 and *C. jejuni* PT14 were measured in triplicate and the average diameter of growth in cm, for each strain and morphological form are presented in **Table 5-11**. The filamentous and spiral morphotypes of *C. jejuni* 12661 showed a reduced spread of growth compared to the same morphotypes for *C. jejuni* PT14 Student's t-Test ($p<0.05$) confirmed that *C. jejuni* 12661 were significantly less motile than *C*. *jejuni* PT14, comparing the corresponding morphotypes and the exponential growth phase conditions.

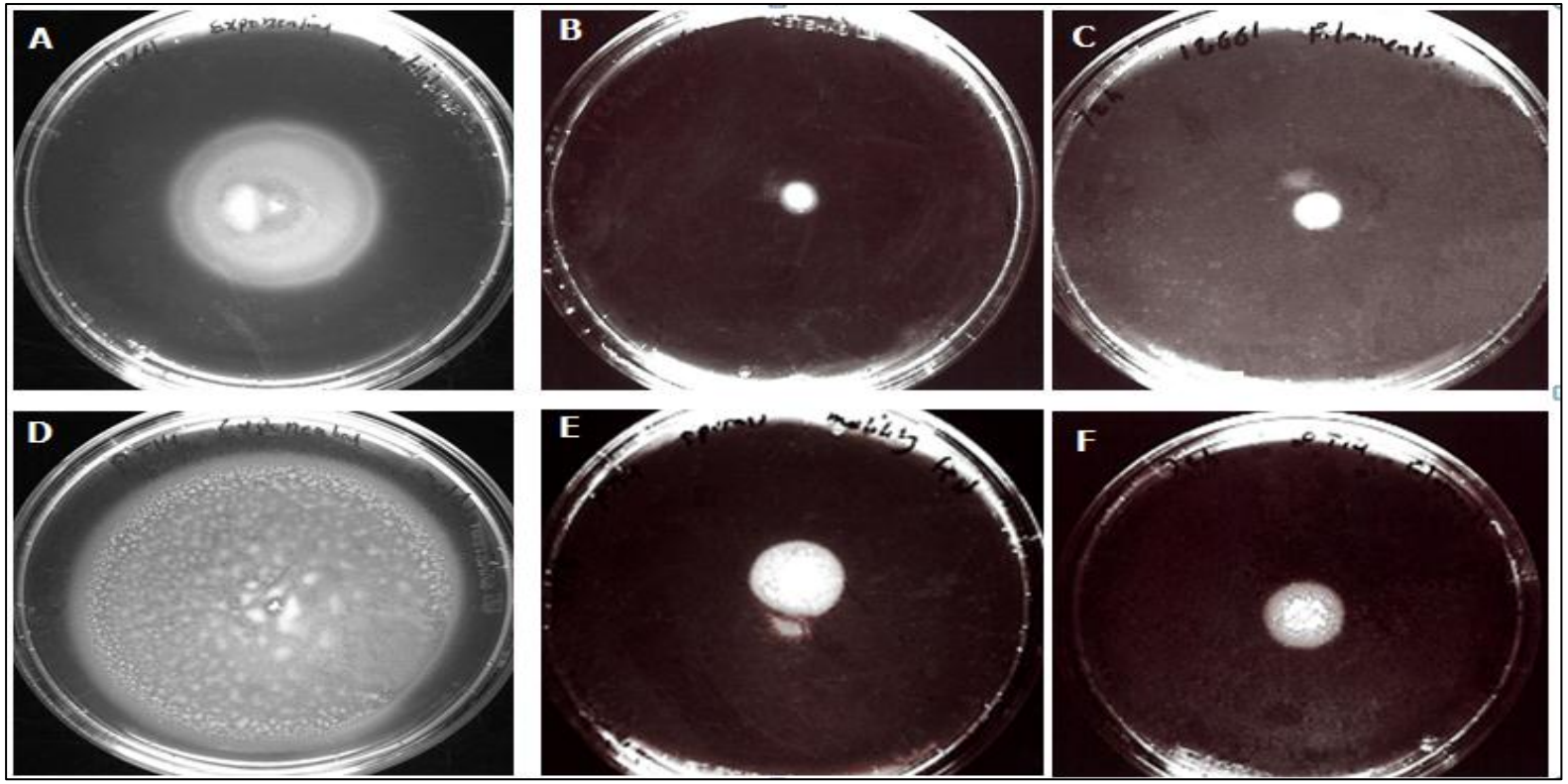


Figure 5-18: Motility assays for different morphotypes of *C. jejuni* 12661 and *C. jejuni* PT14 recovered from cultures growing in MEM with 10mM sodium pyruvate.

Panels A and D are inoculated respectively with cells from the exponential growth phase of *C. jejuni* 12661 and *C. jejuni* PT14, panels B and E are the separated spiral morphotypes after incubation for 72 h in MEM with 10mM sodium pyruvate and panels C. and F are the separated filamentous morphotypes incubated as for B and E. Motility was determined by measuring the diameter of the spread of growth in cm after 24 h.

Table 5-11: Average diameters of motility zones for *C. jejuni* morphotypes in two different strains

Growth phase/ morphotype	Strains	
	***C. jejuni* PT14**	***C. jejuni* 12661**
Exponential	7.83 ± 0.21	4.47 ± 0.11
Spiral	1.70 ± 0.10	0.37 ± 0.12
Filamentous	1.47± 0.15	0.53 ± 0.15

Averages of triplicate measurements are given ± standard deviation.

5.2.7 Transmission electron microscopy (TEM) of different *Campylobacter* morphotypes

Negative stains of cell suspensions were prepared for TEM (2.8) from *C. jejuni* cells grown on MEM with 10mM sodium pyruvate for the two stationary phase morphotypes and exponential growth phase cells. The TEMs confirmed photo microscopy data that spiral, filamentous and coccoid forms occured during the longer period of growth of *C. jejuni.* TEM images for *C. jejuni* PT14 **(Figures 5-19, 5-20** and **5-21)** and *C. jejuni* 12661 **(Figures 5-22, 5-23** and **5-24)** show the spiral and filamentous morphotypes. Cells harvested from the exponential growth phase of either strain could be observed as small, helical, curved, rod-shaped forms with one or more flagella **(Figures 5-19 and 22)**.

Decline phase was accompanied by a further increase in cell length the loss of cellular integrity, and the presence of coccoid forms. Cells incubated for extended periods (72 h) and observed by TEM showed

similar forms to those observed with the syto-9 and propridium iodide LIVE/DEAD stains (3.2.1) having a mixture of spirals cells and filamentous forms. At this point the majority of the spiral and filamentous morphotype cells of *C. jejuni* PT14 had polar flagella at one or both poles (**Figure 5-20)**. However, for *C. jejuni* 12661, some of the spiral morphotype cells lacked their flagella by the time the culture had reached 72h and almost all of the filamentous morphotypes of this strain lacked any flagella (**Figure 5-23).** Coccus shaped cells also appeared after 72 h of growth. The majority of these coccoid cells had lost their flagella; however, there were examples from both strains where the coccoid cells had retained the flagella (**Figures 5-21 and 5-24)**. As changes in cell length occurred, 'bleb' formation was evident on many cells. The appearance of blebs when the expansion of cell membranes is limited may represent an intermediary form before coccoid formation (**Figure 5-25)**.

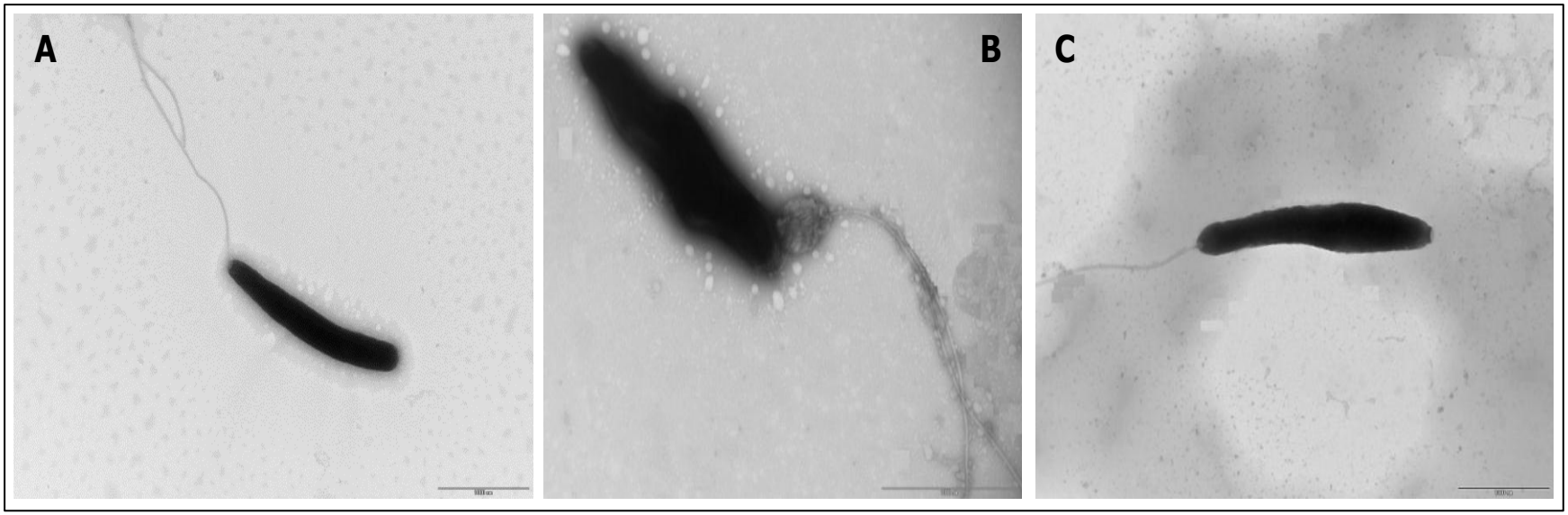


Figure 5-19: Transmission electron micrographs of negative-stained cells of *C. jejuni* PT14 cells, showing spiral morphotype cells with flagella, observed when the samples were taken from exponential growth phase (A-C). Bar represents 1 µm.

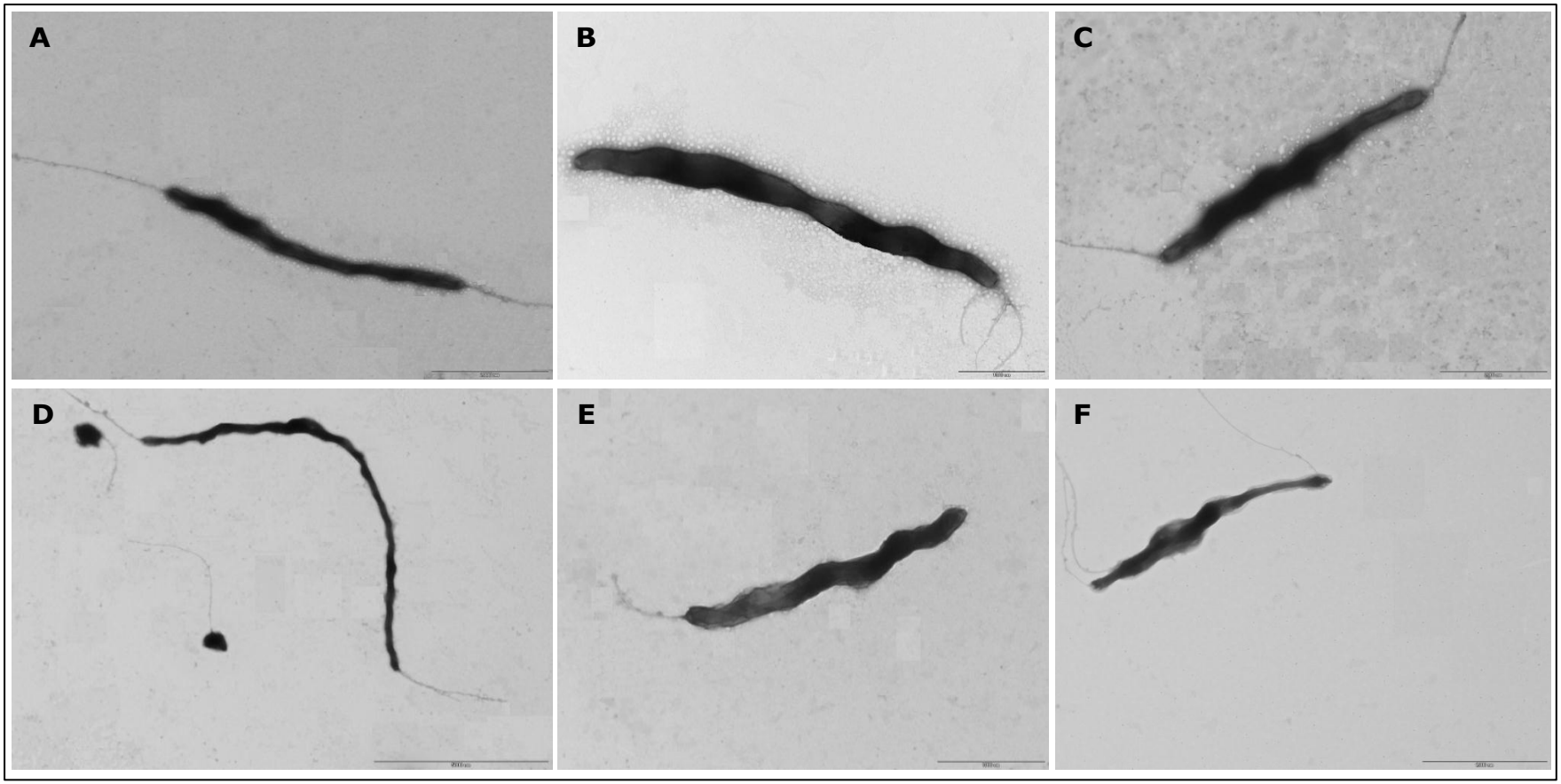


Figure 5-20: Transmission electron micrographs of negative-stained cells of *C. jejuni* PT14 cells, showing filamentous morphotypes with one or more flagella at one or both ends , observed when the samples were taken after 72h of incubation in MEM with sodium pyruvate (A-F), (D) showing coccoid cells with filaments . Bar represents 1 µm (E and B), 2 µm (A, C and F), and 5 µm (D)

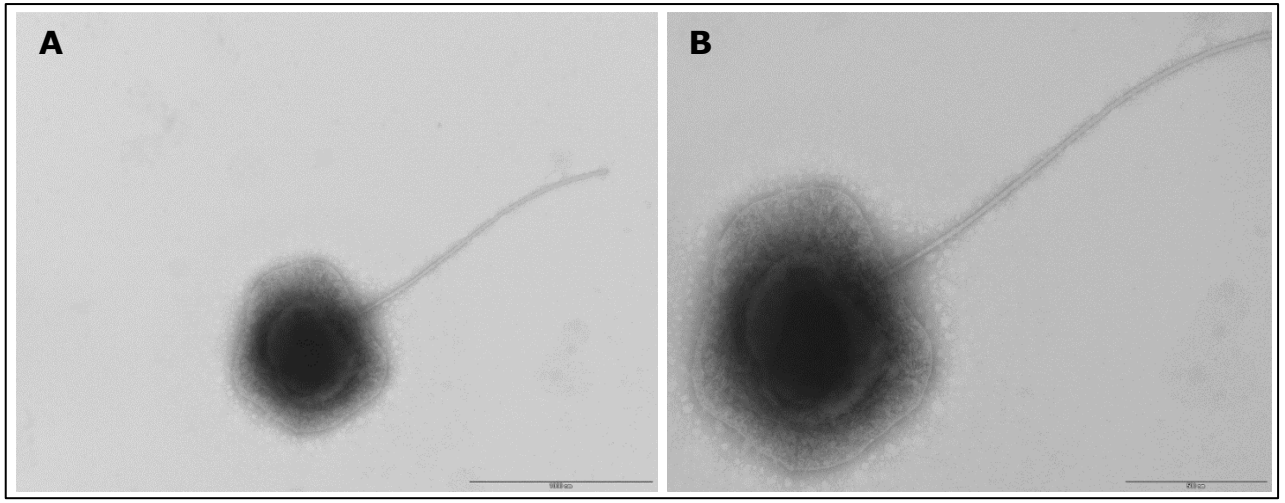


Figure 5-21: Transmission electron micrographs of negative-stained cells of *C. jejuni* PT14 cells, showing coccoid cells with flagella, observed when the samples were taken after 72h of incubation in MEM with sodium pyruvate (A-B). Bar represents 0.5 µm.

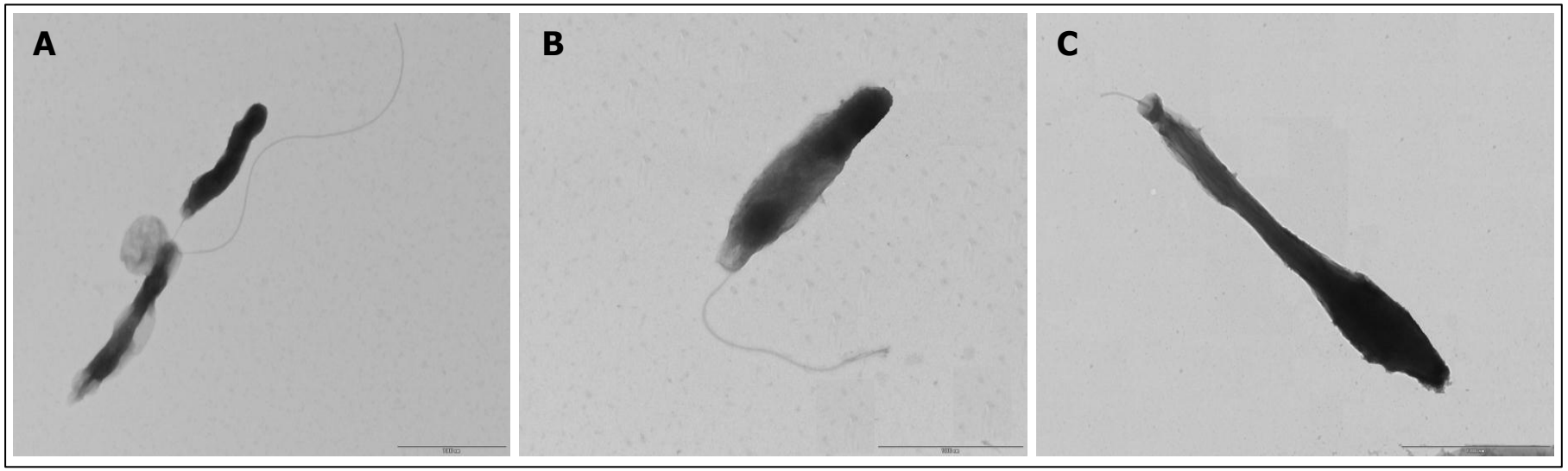


Figure 5-22: Transmission electron micrographs of negative-stained cells of *C. jejuni* 12661 cells, showing spiral morphotypes with flagella, observed when the samples were taken from exponential growth phase (A-C). Bar represents 1 µm.

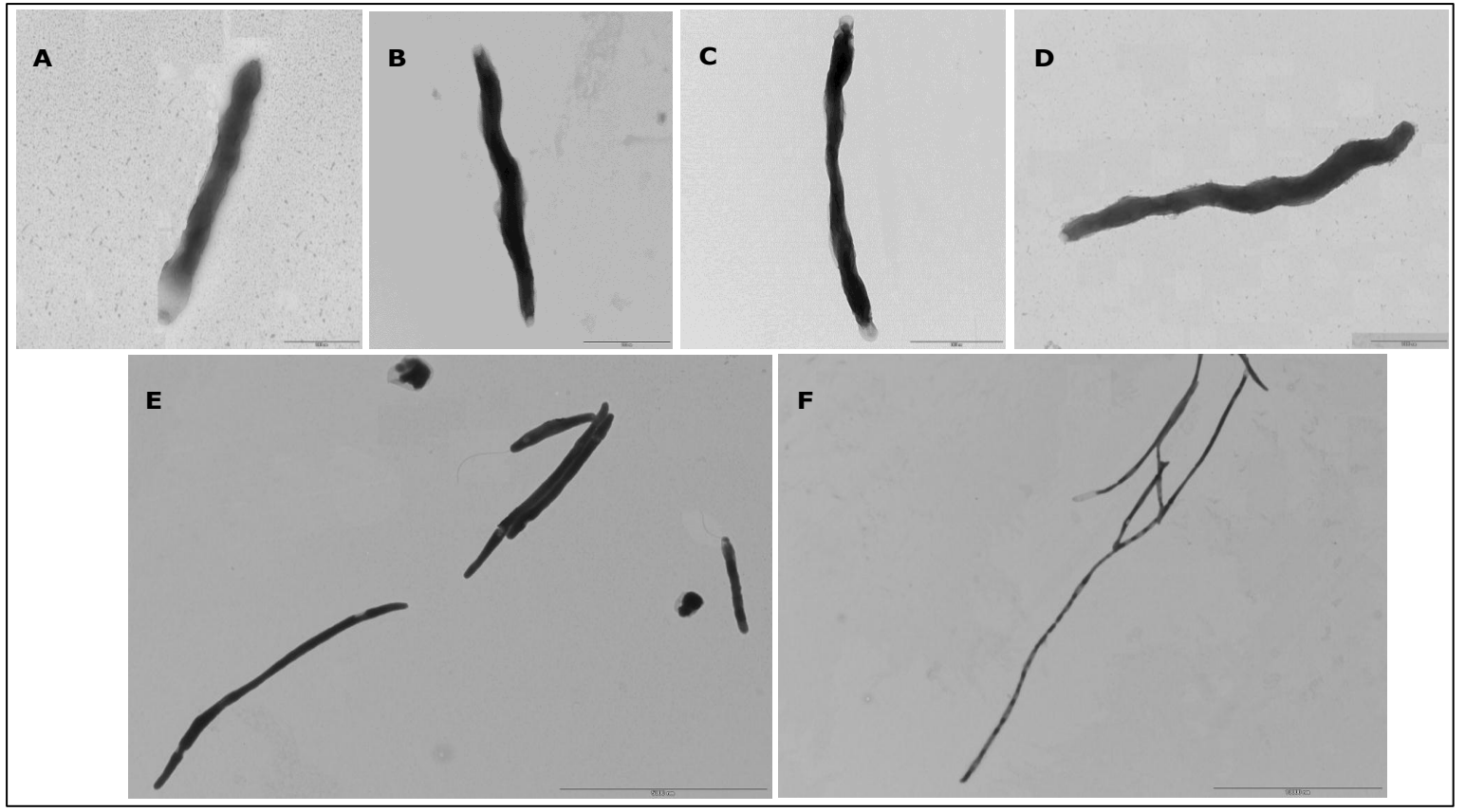


Figure 5-23: Transmission electron micrographs of negative-stained cells of *C. jejuni* 12661 cells, showing filamentous morphotypes with no flagella, observed when the samples were taken after 72 h of incubation in MEM with sodium pyruvate (A-F), (E) showing spiral morphotypes with flagella and filamentous morphotypes that lack their flagella. Bar represents 1 µm (A, B, C and D), 5 µm (E) and10 µm (F)

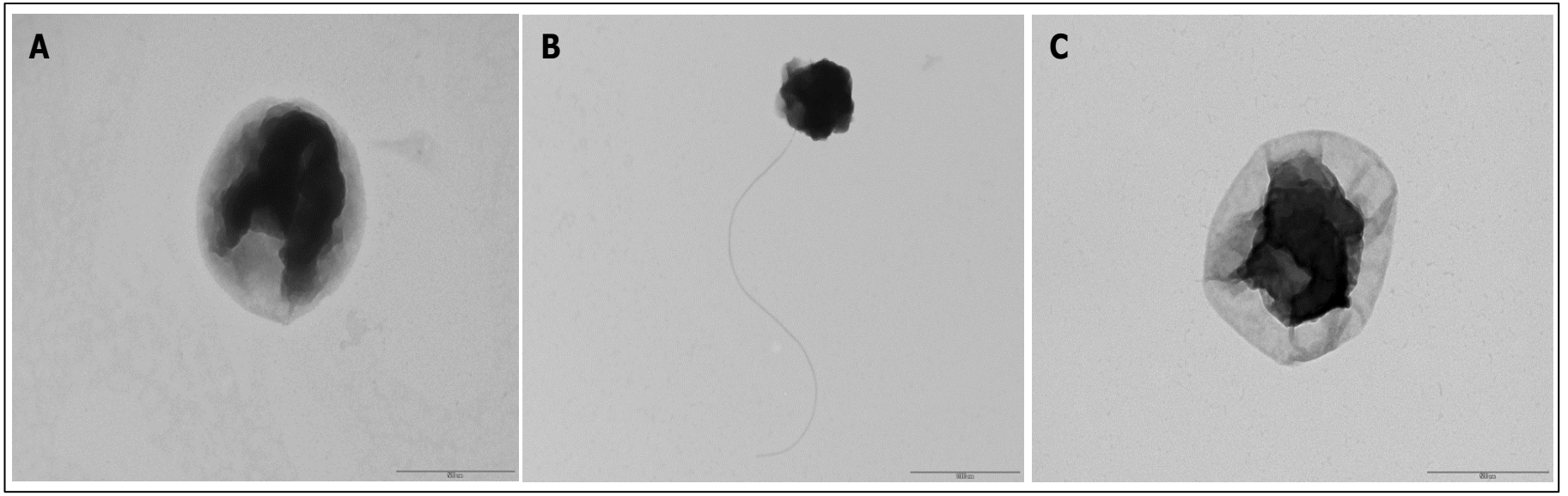


Figure 5-24: Transmission electron micrographs of negative-stained cells of *C. jejuni* PT14 cells, showing coccoid cells. (B) With flagella, A) and (C) show coccoid cells with no flagella each observed when the samples were taken after 72 h of incubation in MEM with sodium pyruvate. Bar represents 0.5 µm (A and C) and 1 µm (B)

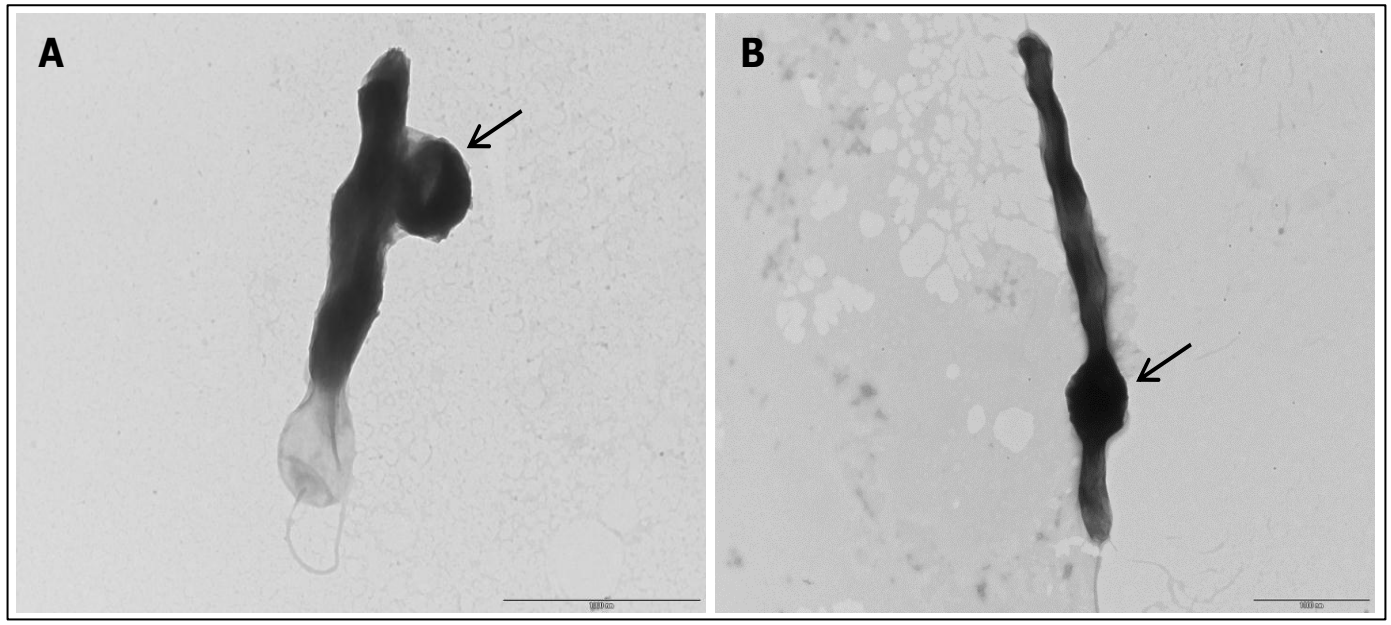


Figure 5-25: Transmission electron micrographs of negative-stained cells of *C. jejuni* PT14 cells, showing localized expansion of the cell membranes with flagella. **Spiral morphotype (A), and filamentous morphotype (B). Observed when the samples were taken after 72 h of incubation in MEM with sodium pyruvate. Bar represents 1 µm (A and B)**

5.3 Discussion

The transcriptional profile of *C. jejuni* PT14 and *C. jejuni* 12661 morphotypes was characterised using strand specific RNA-Seq technology. RPKM values were calculated and provided a measure of gene expression levels between the different *Campylobacter* morphotypes. The transition from the spiral morphotype typical of the exponential phase of growth, to filamentous forms is accompanied by entry in to the stationary phase. Transition to stationary phase is accompanied by substantial changes in gene expression. As nutrients become scarce transcription of genes involved in metabolism are reduced whilst genes involved in maintenance and survival are increased. Thus, identifying genes specifically involved in the change in morphotype from spiral to filamentous cells and distinguishing such changes from those responding to the transition to stationary phase, presents a challenge.

5.3.1 Gene expression in stationary phase *C. jejuni*

Where authors have attempted to identify changes in gene expression associated with stationary phase, they have had to make compromises in order to extract meaningful data. These include a comparison of mid exponential and stationary phase growth *in vitro* (Taveirne et al., 2013), where the authors focussed on genes with greater than 4.5-fold changes in expression (1.5 to 2-fold are more common cut-off boundaries to indicate where a change in the expression levels could result in functional differences). Even with this high cut-off, around 200 genes were found to be differentially expressed. A different approach was adopted by (Wright

et al., 2009). Here the authors examined transcriptional change over shorter time periods, for example comparing the difference between late exponential and early stationary phase, and then early stationary and mid stationary phase, thus identifying sequential changes. These authors identified an acetate switch in late stationary phase whereby *C. jejuni* cease to excrete acetate and begin to assimilate acetate as nutrients become limiting. The pathway is characterised by the constitutive expression of phosphotransacetylase (*pta*) and acetate kinase (*ackA*) that together excrete acetate, and together with acetyl-CoA synthetase (*acs*) contribute to the assimilation. Contrary to the expectation that *pta* and *ackA* should be constitutive the stationary phase spirals and filaments show significant down regulation of 2.8 and 3.3-fold respectively when compared to the exponential phase expression levels for *C. jejuni* PT14. For *C. jejuni* 12661 *ackA* and *pta* are down regulated in the filaments by comparison with exponential phase (4.9- and 3.9-fold respectively) or stationary phase (8.9- and 6.9-fold respectively) spirals. These cultures are supplemented with pyruvate but it is unclear how this could obviate the need to recycle CoA and the production of acetate as a by-product. What is clear is that under these circumstances the production of acetyl-CoA from acetate is unlikely in the filaments since *acs* is significantly down-regulated (3.4-fold) in addition to *ackA* and *pta.* This observation is consistent with the observation noted in chapter 3 that pre-used NB2 can still support growth with rates indistinguishable from those recorded for the fresh culture medium.

To facilitate the analysis of gene expression in stationary phase a consensus was sought for the regulated genes common to the transcriptome data published by Wright et al. (2009) and Taveirne et al. (2013). Comparison of RNA-Seq expression profiles from both the filamentous and spiral morphotypes to exponentially growing cells would be expected to produce similar expression profiles to the data cited above since all represent batch cultures. This comparison revealed some similarities for *C. jejuni* PT14, but also some differences in the numbers of genes matching the consensus profile for stationary phase gene expression (**Table 5-12**). In accordance with the expected stationary phase profiles, both morphotypes showed significant down-regulation of all the genes encoding electron transport functions and up-regulation of the genes encoding chaperones and heat shock proteins. Spiral cells also showed up regulation of a subset of the motility associated genes and down regulation of the genes for nitrate reductase that would be expected for the comparison of stationary phase and exponential phase cells. The filamentous cells also showed up regulation of *aphC* (alkyl hydroperoxide reductase) and down regulation of one of the succinate dehydrogenase encoding genes, both of which would be expected when stationary phase cells are compared to exponential cells. However, none of the ribosomal subunit genes were down regulated as expected but to the contrary all were up regulated, indicating that the translation apparatus remains active in both the spiral shaped cells and the filamentous cells at this stage of growth. Transcriptional differences between the two morphotypes harvested at the same time point were

clearly evident even in the consensus data. This raises the question that the previous datasets may actually represent a composite of the morphotypes present and their respective transcript levels. Changes in morphotype were noted by Wright et al. (2009) but not explored in the course of the experiment.

A similar comparison for *C. jejuni* 12661 revealed fewer similarities between the differential gene expression profiles observed for the two morphotypes and the consensus differences between exponential and stationary phase (**Table 5-13**). However, significant differences between the filamentous and spiral cells highlighted similarities with the consensus profile, implying in this case that the filamentous cells were more similar to the stationary phase response. These data again highlight transcriptional differences between the morphotypes harvested at the same time point in stationary phase and also indicate strain differences within the species.

RelA and/or SpoT are global transcriptional regulators involved in the stringent response, which represents a common flexible adaptation mechanism by which bacteria are able to survive a variety of adverse conditions (Gaca et al., 2015). Nutrient deprivation, for example, produces an excess of uncharged tRNAs at the ribosomal acceptor site due to the limited availability of aminoacylated tRNAs. As a consequence the ribosomes stall, which triggers ribosome-bound RelA or SpoT to catalyse the synthesis of guanosine pentaphosphate (pppGpp). Cellular levels of pppGpp, and the hydrolysis product guanosine tetra-phosphate

(ppGpp) therefore increase in response to stress, and are accordingly referred to as alarmones (Potrykus and Cashel, 2008). The (p)ppGpp molecules are recruited to RNA polymerase with Dksa protein to modify gene expression by affecting transcription initiation and modifying promoter selectivity. Increased cellular (p)ppGpp levels have inhibitory effects on GTP biosynthesis, protein translation and DNA replication(Gaca et al., 2015). These effects collectively down-shift the mobilisation of cellular resources for rapid growth and reallocate them in preparation to survive stressful conditions. Mutation of the *spoT* gene of *C. jejuni* gives rise to aberrant cell morphologies that include early coccoid cell formation in stationary phase cultures (Gaynor et al., 2005). The *spoT* gene appears differentially regulated in all the stationary phase morphotypes recovered in this study compared to exponential growth. Stationary phase spirals and filaments are significantly down-regulated for *spoT* expression by 2.4- and 1.4-fold respectively in *C. jejuni* PT14, whilst the genes encoding two alternative hydrolases that have the potential to produce ppGpp also show significant differential regulation but in opposing directions. The gene *ppx1*/*gppA* (A911_01710) is up-regulated 1.3- and 3.3-fold, whereas *ppx2* (A911_06005) is down-regulated 2.3- and 1.5-fold in stationary phase spiral and filamentous morphotypes respectively, when compared to exponential phase expression. Independent mutation of *ppx1* and *ppx2* enabled the identification of the *ppx1* encoded phospohydrolase as being responsible for controlling ppGpp levels and the stringent response (Malde et al., 2014). The cellular poly-phosphate and ppGpp pools are also controlled

by the opposing activities of polyphosphate kinases 1 and 2. The function of PPK1 is to synthesise poly-phosphate using ATP (Candon et al., 2007), whereas PPK2 degrades poly-phosphate in the production of GTP from GDP (Gangaiah et al., 2010). The *ppk1* and *ppk2* genes are significantly down-regulated in *C. jejuni* PT14: *ppk1* (A911_06605) 2.7- and 1.5-fold for the filamentous and spiral morphotype cells compared to exponential cells; *ppk2* (A911_02950) 1.4-fold, for both filaments and spiral cells compared to exponential phase cells.

Transcription of the *spoT* gene of *C. jejuni* 12661 in stationary phase spirals is up-regulated (1.5-fold) whilst the filamentous cells are down-regulated (1.7-fold). The phosphohydrolase encoding gene *ppx1*/*gppA* (UC78_0341) did not show a difference in regulation but *ppx2* (UC78_1187) is down-regulated 2.1- and 1.3-fold in stationary phase spirals and filaments, when compared to exponential phase expression. The polyphosphate kinase encoding genes *ppk1* and *ppk2* behave differently in *C. jejuni* 12661 showing up-regulation of *ppk1* (UC78_1304) by factors of 1.1- and 3.3-fold for filaments and spiral cells compared to exponential cells, and up-regulation of *ppk2* (UC78_0588) 2.6- and 1.8-fold for filaments and spiral cells compared to exponential phase cells.

An increase in the capacity of cells to synthesise (p) ppGpp, as implied by the up-regulation of *spoT* in the stationary phase spirals of *C. jejuni* 12661, could bring about an early or enhanced stress response. However, a reduction in the capacity of the cells to synthesise (p) ppGpp

could diminish the stress response and maintain a commitment to growth even though members of the population are becoming non-viable in stationary phase. Consistent with this view the ribosomal protein genes are not down-regulated in these stationary phase cultures. Ribosomal RNA transcription is under negative control of (p) ppGpp via interaction with RNA polymerase and the co-regulatory protein DksA. Mutation of the *dksA* gene of *C. jejuni* relieves the repression of stable RNAs under stress conditions and leads to differential expression of genes involved in amino acid metabolism and iron acquisition amongst others (Yun et al., 2008). However, the *dksA* gene is significantly up-regulated in *C. jejuni* PT14 stationary phase spiral (1.7-fold) and filamentous cells (2.3-fold). It has been demonstrated in chapter 3 that pre-used MEM plus pyruvate culture medium can support a second round of exponential growth, which effectively means the cells are not subject to nutrient limitation or toxicity from diffusible molecules under these conditions. The reasons for cell death at stationary phase in these cultures are likely to be independent of external stressors but could be brought about by intra-cellular stress such as the accumulation endogenous reactive oxygen species. An alternative role for DksA has recently been reported for the orthologue in *Salmonella* Typhimurium, whereby the zinc finger motif can act as a thiol-based sensor of redox stress, which via the transcriptional regulator CysB can activate sulphur acquisition and cysteine biosynthesis as an integral component of anti-nitrosative and anti-oxidative defence and repair mechanisms (Crawford et al., 2016).

Notably at variance to the previous reports of stationary phase gene expression in *C. jejuni* were the absence of down-regulation in the ribosomal protein genes and the limited up-regulation of the motility associated functions. Transcriptome analysis of the *Campylobacter* morphotypes highlighted regulatory differences to those reported for the genes that affect flagellar biogenesis and motility in stationary phase (Wright et al., 2009, Taveirne et al., 2013), which prompted investigation into the motility of the stationary phase morphotypes.

Table 5-12: Comparison of the differential gene expression of *C. jejuni* PT14 morphotypes against a consensus subset of genes showing differential expression between exponential and stationary phases of growth.

	Number of up-regulated genes				Number of down-regulated genes			
	M	HC	O	C	R	E	S	N
Stationary to exponential phase consensus profile[1]	12	6	3	2	13	5	3	3
Filamentous cells compared to exponential cells		5	1			5	1	
Spiral cells compared to exponential cells	6	6				5		3
Filamentous cells compared to spiral cells							3	

[1]The consensus subset profile consists of regulated genes in stationary phase compared to exponential phase, in batch cultures, as reported by Wright et al. (2009) and by Taverirne et al. (2013). The up regulated subsets consist of 23 genes as follows: motility associated genes (M; *flaB, flaA, flgB, flgC, flgD, flgH, flgK, fliE, flaG, fliD, flaC, flgL*); heat shock and chaperone encoding genes (HC; *groEL, groES, clpB, grpE, cbpA dnaK*); oxidative stress associated genes (O; *sodB, ahpC, tpx*) and genes associated with cell surface functions (C; *murF, pseB*). The subset of 24 genes down regulated in stationary phase included: ribosomal subunit encoding genes (R; *rplE, rplF, rplN, rplO, rplP, rplR, rplX, rpmI, rpsC, rpsH, rpsN, rpsQ, rpmC*); genes encoding electron transport functions (E; *nuoN, nuoL, nuoJ, nuoI, nuoH*); succinate dehydrogenase encoding genes (S; *sdhA, sdhB, sdhC*) and genes encoding nitrate reductase (N; *napA, napG, napH*).

Table 5-13: Comparison of the differential gene expression of *C. jejuni* 12661 morphotypes against a consensus subset of genes showing differential expression between exponential and stationary phases of growth.

	Number of up-regulated genes				Number of down-regulated genes			
	M	HC	O	C	R	E	S	N
Stationary to exponential phase consensus profile[1]	12	6	3	2	13	5	3	3
Filamentous cells compared to exponential cells			1			2	3	
Spiral cells compared to exponential cells								
Filamentous cells compared to spiral cells	4	3	1			5	1	

[1]The consensus subset profile consists of regulated genes in stationary phase compared to exponential phase, in batch cultures, as reported by Wright et al. (2009) and by Taverirne et al. (2013). The up regulated subsets consist of 23 genes as follows: motility associated genes (M; *flaB, flaA, flgB, flgC, flgD, flgH, flgK, fliE, flaG, fliD, flaC, flgL*); heat shock and chaperone encoding genes (HC; *groEL, groES, clpB, grpE, cbpA dnaK*); oxidative stress associated genes (O; *sodB, ahpC, tpx*) and genes associated with cell surface functions (C; *murF, pseB*). The subset of 24 genes down regulated in stationary phase included: ribosomal subunit encoding genes (R; *rplE, rplF, rplN, rplO, rplP, rplR, rplX, rpmI, rpsC, rpsH, rpsN, rpsQ, rpmC*); genes encoding electron transport functions (E; *nuoN, nuoL, nuoJ, nuoI, nuoH*); succinate dehydrogenase encoding genes (S; *sdhA, sdhB, sdhC*) and genes encoding nitrate reductase (N; *napA, napG, napH*).

5.3.2 Regulation of flagellar biogenesis genes in different *Campylobacter* morphotypes of 12661 and PT14

Polar flagella drive *C. jejuni* as a motile species. This involves over 40 structural and regulatory genes and it includes an O-linked glycosylation system that post translationally modifies the flagellin subunits (Guerry et al., 2006, Hendrixson, 2008). Three sigma factors have been predicted in the *C. jejuni* NCTC 11168 genome: σ70 (encoded by rpoD) which regulates class I genes involved in the assembly of the basal body and the export apparatus, σ54 (encoded by rpoN) regulates class II genes that encode proteins forming part of the hook and basal body and σ28 (encoded by fliA) regulates class III genes that are involved in filament biosynthesis, capping and flagellin post translational modification (Carrillo et al., 2004). Of these *rpoN* is significantly down-regulated in the filamentous morphotype of *C. jejuni* 12661 (3.6-fold) and *C. jejuni* PT14 (1.7-fold) as compared to exponential cells. The expression of rpoN is down-regulated in *C. jejuni* PT14 for the stationary phase spiral cells (3.4-fold). Mutation of the *rpoN* gene leads to a profound loss of motility, and RNA-seq analysis revealed a corresponding down–regulation of the flagellar hook and basal body associated genes (Chaudhuri et al., 2011) . A similar trend is evident in this study upon down-regulation of rpoN and is likely a major contributory factor to the loss of motility in cells recovered from stationary phase. The σ54-RNA polymerase operates in conjunction with FlgR to initiate the transcription of the genes encoding flagellar components. FlgR is partner to FlgS that operate together as a

two-component regulator, in which FlgS histidine kinase autophosphorylates to activate the FlgR response regulator. Transcript levels of the *flgR* and *flgS* genes are also down-regulated 2 to 3-fold in the stationary phase spiral and filamentous cells for *C. jejuni* 12661 and PT14.

The genes encoding the chemotactic response proteins CheV, CheA and CheW (A911_01365 - A911_01375) show significant differential expression in the stationary phase morphotypes of *C. jejuni* PT14 with 6-fold down-regulation in the spiral cells and 2-fold up-regulation in filaments compared to exponential cells. The chemoreceptors (methyl-accepting chemotaxis proteins) of *C. jejuni* PT14 also showed differential transcript levels to exponential cells with Tlp1 (A911_07255) responding to aspartate 1.5-fold down-regulated in spiral cells and 2.1-fold up-regulated in filaments, Tlp6 (A911_02185) cytoplasmic transducing protein 2-fold up-regulated in filaments and Tlp7 (A911_04600) responding to formate 2-fold up in spirals and 3-fold up filaments).

A summary of the transcriptional differences observed in this study for the motility associated genes based on their putative operon organisations are presented for *C. jejuni* 12661 in **Table 5-14** and for PT14 in **Table 5-15**.

Table 5-14: Differential regulation of flagellar associated genes in *C. jejuni* 12661

Operon	Gene symbol	Function	Fold change spirals/ exponential	Fold change Spirals /filaments	Fold change filaments/ exponential
fliL-acpS	*fliL*	polypeptide flagellar basal body-associated protein	-	-4.17	-4.11
fliFGH-UC78_0310-UC78_03011	*fliF*	flagellar MS-ring protein	-	-2.94	-2.68
	fliG	flagellar motor switch protein G	-	-2.25	-
	fliH	flagellar assembly protein H	-	-	-
fliR	*fliR*	flagellar biosynthesis protein	-	-2.35	-6.26
fliI	*fliI*	flagellum-specific ATP synthase	-	-1.53	-1.68
flhF-fliAMN- UC78_0062-UC78_0066-UC78_0067- UC78_0069	*fliY*	flagellar motor switch protein FliY	-		-
	fliM	flagellar motor switch protein FliM	-	-2.17	-3.51
	fliN_1	Flagellar motor switch protein FliN	-	-	-1.89
	fliA	flagellar biosynthesis sigma factor	-	-	-
	flhF	flagellar biosynthesis regulator FlhF	-	-	-
fliQ-murB- UC78_1615	*fliQ*	flagellar biosynthesis protein FliQ	-	-5.5	-2.69
flhB1	*flhB1*	flagellar biosynthesis protein FlhB1	1.78	-1.64	-2.33
flhB2	*flhB1*	flagellar biosynthesis protein FlhB2		-4.79	-2.68
flgH	*flgH*	flagellar basal body L-ring protein	-	-1.77	-
flgE_1	*flgE1*	flagellar hook protein FlgE1	-	-5.09	-
flgE_2	*flgE2*	Flagellar hook protein FlgE2		-2.8	-
flgG_2-UC78_0672	*flgG2*	flagellar basal body rod protein FlgG	-	-2.56	-
FlgL	*flgL*	hook-associated protein FlgL	-	-1.86	-
flaB	*flaB*	Flagellin B	-	-4.38	-
flaA	*flaA*	Flagellin A	-	1.73	-

Table 5-14 continued

Operon	Gene symbol	Function	Fold change spirals/ exponential	Fold change Spirals /filaments	Fold change filaments/ exponential
flaB2	*flaB2*	Flagellar filament 31.3 kDa core protein	-	2.49	-
fliK	*fliK*	flagellar hook-length control protein	-	-3.34	-
FliSD- UC78_0531-UC78_0534	*fliS*	Flagellar protein FliS	-	1.79	-
	fliD	Flagellar hook-associated protein 2	-	1.92	-
	flaG	Flagellar protein	-	-	-
flgI- UC78_1396	*flgI*	flagellar basal body P-ring protein	-	-2.01	-
FliW- UC78_1034-UC78_1035-UC78_1036-UC78_1037	*fliW*	Flagellar assembly factor FliW 2	-	-3.18	-3.03

Table 5-15: Differential regulation of flagellar associated genes in *C. jejuni* PT14

Operon	Gene symbol	Function	Fold change spirals/ exponential	Fold change Spirals /filaments	Fold change filaments/ exponential
fliL-acpS	*fliL*	polypeptide flagellar basal body-associated protein	-3.24	2.36	-2.27
flgBC-fliE-A911_02555	*fliE*	flagellar hook-basal body protein	1.63	-	-
	flgC	flagellar basal body rod protein	-	-1.58	-
	flgB	flagellar basal body rod protein	-	-2.59	-
flgK-A911_07075-A911_07070-A911_07060	*flgK*	flagellar hook-associated protein	1.56	-2.52	-1.61
fliFGH-A911_01555-A911_01550	*fliF*	flagellar MS-ring protein	-3.07	-	-2.05
	fliG	flagellar motor switch protein G	-5.21	2.15	-2.29
	fliH	flagellar assembly protein H	-7.5	3.27	-2.29
fliR	*fliR*	flagellar biosynthesis protein	-2.4	4.1	1.7
fliI	*fliI*	flagellum-specific ATP synthase	-2.23	-	-
fliS-A911_02680-A911_02670-A911_02665	*flag*	flagellar protein FlaG	2.08	-3.32	-1.59
	fliD	flagellar capping protein	1.59	-2.27	-1.95
	fliS	flagellar protein FliS	-	-	-
flhF-fliAM-A911_00280-A911_00295-A911_00300-A911_00310	*fliY*	flagellar motor switch protein FliY	-2.25	-	-1.91
	fliM	flagellar motor switch protein FliM	-3.2	1.81	-1.75
	fliA	flagellar biosynthesis sigma factor	-	-	-
	flhF	flagellar biosynthesis regulator FlhF	-2.64	1.65	-1.6

Table 5-15 continued

Operon	Gene symbol	Function	Fold change spirals/ exponential	Fold change Spirals/ filaments	Fold change filaments/ exponential
flgA-coaD-tmk-hisS-A911_03695-A911_03700-A911_03720	*flgA*	flagellar basal body P-ring biosynthesis protein FlgA	-2.44	1.85	-
fliQ-murB-A911_08065	*fliQ*	flagellar biosynthesis protein FliQ	-3.16	-	-2.89
flhB	*flhB*	flagellar biosynthesis protein FlhB	-	1.96	2.25
flhA-A911_04250-A911_04255-A911_04260-A911_04270	*flhA*	flagellar biosynthesis protein FlhA	-1.59	2.65	1.67
fliP	*flip*	flagellar biosynthesis protein FliP	-	3.04	3.59
flgI-A911_07050	*flgI*	flagellar basal body P-ring protein	-	-3.71	-2.57
flgH	*flgH*	flagellar basal body L-ring protein	-	-3.25	-2.5
hisH-A911_06390-A911_06400	*pseA*	flagellin modification protein	-	-2.21	-2.51
flgE	*flgE*	flagellar hook protein FlgE	-	-1.99	-1.59
A911_05220, A911_05215, A911_05210, A911_05205, A911_05200	*fliW*	flagellar assembly protein FliW	-	-1.65	-2.04
flgG-A911_03400	*flgG*	flagellar basal body rod protein FlgG	1.5	-2.54	-1.75
	A911_03400	flagellar basal-body rod protein	-	-2.39	-
flgL	*flgL*	flagellar hook-associated protein FlgL	1.64	-	-
flaA	*flaA*	flagellin flaA	1.89	-	-
flaB	*flab*	flagellin flaB	-	-2.77	-
911_01705-A911_01700 A911_01695	A911_01700	flagellar motor switch protein	-2.05	1.92	-

Table 5-15 continued

Operon	Gene symbol	Function	Fold change spirals/ exponential	Fold change Spirals/ filaments	Fold change filaments/ exponential
flgD-A911_00210	*flgD*	flagellar basal body rod modification protein	-	-2.86	-
	flgE	flagellar hook protein FlgE	-2.03	-1.93	-2.21
A911_03530, A911_03525, A911_03520, A911_03515, A911_03510	*flaC*	flagellin flaC		-1.57	-
motB-A911_01630- A911_01635	*motA*	flagellar motor protein MotA	-2.53	-	-2.23
	motB	flagellar motor protein MotB	-1.54	-	-
A911_04125- A911_04120- A911_04115	A911_04115	FlhB domain-containing protein	-	-	-2.59
A911_06495-A911_06490 A911_06485-A911_06480 A911_06475-A911_06470, A911_06465-A911_06460, A911_06455-A911_06450, A911_06440-A911_06435	A911_06475	flagellin modification protein A	-	-1.79	-5.41

5.3.2.1 Motility associated genes differentially regulated for spiral morphotypes compared to exponential growth phase cells for *C. jejuni* 12661 and *C. jejuni* PT14

The *C. jejuni* 12661 spiral morphotype cells differentially expressed many genes that modify the cell envelope of the bacteria including flagellar biosynthesis genes when compared to exponential growth phase cells. The *flhB* genes, which encode a flagellar biosynthetic protein, was found to be activated in the spiral morphotype cells by a factor 1.78 compared to exponential growth phase cells. Several genes related to the cell envelope were found to be repressed in the *C. jejuni* 12661 spiral morphotype compared to exponential growth phase cells but no flagellar motility associated genes were differentially regulated **Table 5-14**.

In the *C. jejuni* PT14 spiral morphotype compared to exponential growth phase cells, modification of cell surface structures are implied by the up regulation of genes that include flagellar biosynthesis genes such as *flaG*, *flgL*, *fliE* and *flgK*. However, many motility associated proteins were down-shifted such as, *fliG fliM*, *fliF*, *flhF*, *flgA* and *fliQ* **Table 5-15**. The motility assays of the tested strains showed a significant increase in the motility of the exponential cells when compared to separated spiral morphotypes of both *C. jejuni* 12661 and *C. jejuni* PT14.

5.3.2.2 Motility associated genes differentially regulated in the filamentous morphotype compared to the spiral morphotype of *C. jejuni* 12661 and *C. jejuni* PT14

In the *C. jejuni* 12661 filamentous morphotype, modifications of the cell surface structure are implied by the activation of 22 cell envelope associated genes including three flagellar associated genes when compared to the spiral morphotype. Similarly in the *C. jejuni* PT14 filamentous morphotype compared to the spiral morphotype, modifications of the cell surface structure are implied by the up regulation of 173 cell envelope associated genes including several flagellar modification proteins *flhF*, *fliM*, *flgA*, *flhB*, *fliG*, *fliL*, *flhA*, *fliP* and *fliH*. However, motility assays of the filamentous morphotype and the spiral morphotype indicated that both were impaired in motility with no significant difference between them, for either *C. jejuni* PT14 or *C. jejuni* 12661. RNA-Seq data revealed that for *C. jejuni* 12661, seventeen motility genes were down-regulated including *flaA1* and *flgG,* whilst in *C. jejuni* PT14; eleven genes including the *flaG* flagellar associated genes were also down-regulated. Several genes were repressed in both strains including *flaB*, *flgG*, *flgI*, *flgH* and *flgK* as summarised in **Table 5-15**. These results support the transmission electron microscope findings that show that almost all the *C. jejuni* 12661 filamentous morphotype cells lacked their flagella compared with the *C. jejuni* PT14 filamentous and other morphotypes.

The hook protein FlgK is a key component of flagellar assembly, being associated with the distal rod protein, FlgG and proximal hook associated protein FlgF. In both strains examined the *flgK* gene was down-regulated in

the filamentous morphotype, which would affect hook filament junction production with FlgL. Studies have shown that the deletion of *fliR* reduced transcription of the σ54 dependent genes that are involved in flagellar hook formation and the minor flagellin gene *flaB* (Hendrixson and DiRita, 2003). In the *C. jejuni* PT14 filamentous morphotype *fliR* was up-regulated by a factor of 4.1, possibly due to signals arising due to the cells inability to complete hook-formation.

5.3.2.3 Motility associated genes differentially regulated in the filamentous morphotype compared to exponential growth phase cells of *C. jejuni* 12661 and *C. jejuni* PT14

The transcriptome of *C. jejuni* 12661 did not shown any increase in differential expression of flagellar associated genes in the filamentous morphotype compared to exponential growth phase cells. Whereas in *C. jejuni* PT14, four flagellar biosynthesis genes were up-regulated: *fliP, flhB, fliR* and *flhA*. Ten flagellar biosynthesis genes in *C. jejuni* 12661 were down-regulated compared to twenty one genes in *C. jejuni* PT14. The motility assays showed a significant decrease in the motility of the filamentous morphotype of both strains when compared to exponential growth phase cells.

The *fliD* gene product is involved in filament cap formation and appeared down-regulated in filamentous cells; see **Table 5-14** and **Table 5-15**. However, studies have found that *fliD* is not essential for flagellar motility *in vitro* even though it constitutes part of the flagellum, but might play a role in motility when expressed in other environments (Golden and Acheson, 2002).

5.3.3 Genes involves in cellular process

5.3.3.1 Heat shock proteins

A number of heat shock proteins are expressed when bacteria are exposed to stressful conditions, these proteins fall into two categories, the chaperones and the ATP dependent proteases (Andersen et al., 2005). Several heat shock protein homologues that include the major chaperones have been identified in the genome sequence of *C. jejuni* NCTC 11168, such as *groEL*, *groES*, *grpE*, *dnaK* and *dnaJ* and several ATP dependent proteases (Parkhill et al., 2000).

The heat shock regulons are controlled by HrcA and HspR. The repressor protein HspR controls the heat shock responsive operons, *dnaK* (that includes *hrcA*), *cbpA* (that includes *hspR*) and *clpB*. Relief from spR turns on the heat shock operons, which simultaneously results in a significant down regulation of motility associated genes (Andersen et al., 2005, Holmes et al., 2010). HspR functions by binding target promoters containing HAIR (HspR associated inverted repeat) DNA sequences in complex with the chaperone DnaK, and is derepressed by competition for DnaK binding when unfolded proteins accumulate following heat stress (Bucca et al., 2000). HrcA represses the *groE* operon encoding the chaperones GroES and GroEL, by binding at the DNA sequence element CIRCE (controlling inverted repeat of chaperone expression) within the promoter (Holmes et al., 2010). HrcA is heat sensitive and will unfold upon heat shock, causing depression of the groE operon until sufficient GroEL/ES is available to fold HrcA. The *hspR* gene of *C. jejuni* PT14 is significantly up-regulated in stationary phase spiral

(1.6-fold) and filamentous cells (1.5-fold) compared to exponential phase cells implying the heat shock operons are activated due to stress. As a consequence *hrcA* is up-regulated (6.6- and 2.2-fold for the spiral and filamentous morphotype respectively compared to exponential growth phase cells) but also subject to stress-related unfolding and the derepression of GroES and GroEL (>7-fold and >3-fold for spirals and filaments respectively compared to exponential cells). In contrast the transcript levels of the stress regulators *hrcA* and *hspR* from *C. jejuni* 12661 spiral and filamentous morphotypesshow no significant difference to exponential grow phase cells. However, several ATP dependent protease transcripts were found to be activated in the *C. jejuni* 12661 filamentous morphotype compared to spiral cells: *clpA*, *clpB*, *clpP* and *clpX*, implying damaged proteins are being actively turned over.

The cooperative action of *clpB* with *dnaK*, *dnaJ* and *grpE* helps to reactivate chemical and/or heat denatured substrate proteins (Konieczny and Liberek, 2002). Mutants of *clpB* are defective for aerobic survival, host cell adherence and invasion (Svensson et al., 2009). ClpB is amongst many heat shock proteins that have been identified as being expressed *in vivo,* suggesting that these proteins may play a significant role in bacterial growth within the gastrointestinal tract (Stintzi et al., 2005). The significance of the heat shock response for *Campylobacter* intestinal tract colonization had been demonstrated previously (Van Vliet and Ketley, 2001).

5.3.3.2 Cell division genes

Several cell division associated genes were found to be differentially regulated in the morphotypes of *C. jejuni* 12661 and *C. jejuni* PT14. A comparison of the transcript levels of the spiral morphotype to exponential growth phase cells for *C. jejuni* 12661, revealed three genes encoding cell division proteins were significantly up-regulated: *ftsX* (ABC transporter) by a factor of 3.8, *soj*/*parA* (chromosome segregation) 1.5-fold and *ftsZ* (tubulin-like z-ring formation) 1.4-fold. The *ftsW* gene encoding the lipid II-linked peptidoglycan transporter was down-regulated 2.3-fold. Comparing the filamentous morphotype with the exponential growth phase cells of *C. jejuni* 12661 revealed the cell division genes, *mreC* (rod-shape determining protein) and *maf* (inhibitor of septum formation protein) were down-regulated 4.0 and 3.9-fold respectively. In addition the genes *ftsH*, *ftsH4_1* and *ftsH4_2* annotated as ATP-dependent zinc metallopeptidases were down-regulated 2.3 to 3.4-fold. Differences in the transcriptional responses of the cell division genes between the two morphotypes of *C. jejuni* 12661 were therefore clearly evident. Comparing the two morphotypes to each other revealed significant down-regulation of *maf*, *mreB* (actin-like rod-shape determining protein), *mreC*, *soj*/*parA*, *parB* (encoding a probable chromosome partitioning protein), *ftsA* (actin-like function in cytokinesis), *ftsH*, *ftsW* and *ftsZ*.

In contrast to the above, all nine of the cell division associated genes noted were differentially up-regulated in *C. jejuni* PT14 the filamentous morphotype compared to the spiral morphotype. This difference was essentially due to greater down regulation of the cell division genes in the

stationary phase spiral morphotype compared to exponential growth phase cells. Notably the *maf* gene was down-regulated 8.8-fold, which could support the possibility that the short stationary phase spiral morphotype cells are relieved of the inhibition of septum formation imposed by the *maf* gene product. Nevertheless it appears that the *C. jejuni* PT14 filamentous morphotype maintains transcription of the cell division machinery compared to the spiral morphotype, which could explain why PT14 does not elongate without septation to achieve the length of filaments observed for *C. jejuni* 12661.

The formation of the filamentous cell morphotype by *C. jejuni* 12661 corresponds with reduced differential expression of key cell division genes, and specifically septum synthesis. Incomplete septum formation could prevent the partition of the chromosomes into daughter cells whilst the cell elongation process continued to produce filamentous cells. Incomplete septum formation could also influence the uptake of fluorescent dyes, as was used to examine the cell morphotypes by fluorescent microscopy in chapter 3. If the inner membranes and cell wall remained fused then dye diffusion could occur between cells could be a site of access to otherwise impermeable dyes.

In *E. coli ,* MreB functions as a complex with MreC, MreD and RodZ (Kruse et al., 2005, Shiomi et al., 2008, Bendezu et al., 2009, White et al., 2010), which together interact with the peptidoglycan (PG) cell-wall biosynthetic enzymes to produce lateral PG that results in rod-shaped cell elongation. Structural analyses of MreB have established the protein as a true actin homologue, and have identified the domains that mediate MreB–RodZ

coupling and rotation (van den Ent et al., 2001, van den Ent et al., 2010, Morgenstein et al., 2015). It has also been demonstrated that MreB interacts with FtsZ that forms the z-ring, and that this interaction is required for septum synthesis and cell division(Fenton and Gerdes, 2013). A recent development identified MreB as the target for the redox protein thioredoxin (Arts et al., 2016). The interaction could confer redox-based regulation on MreB and protect MreB protein from endogenous oxidative damage. All the stationary phase cells of *C. jejuni* 12661 and PT14 show down-regulation of the thioredoxin encoding gene *trxA* 1.3-1.6-fold, which could impact on the availability of thioredoxin and the ability of the cell to maintain reduced targets such as MreB, ribonucleotide-diphosphate reductase (NrdA/B) and methionine sulphoxide reductases (MrsA and B). However, the expression of *trxB* encoding thioredoxin reductase was significantly up-regulated 2.1 to 4.6-fold and would have the potential to rapidly recycle reduced thioredoxin using NADPH. Rodrigues et al. (2015) have studied the role of oxidative stress in the regulation of the *mreB* gene in *C. jejuni* and found that oxidative stress changes the cell morphology by reducing the transcription and expression of the rod shape determining protein MreB.

In *E coli* , *Bacillus subtilis* and *Caulobacter crescentus,* inactivation of *mreB* results in the formation of coccoid morphotypes (Figge et al., 2004, Bendezu et al., 2009, Kawai et al., 2009). Transcriptional down-regulation of *Helicobacter hepaticus mreB* and *mreC* genes also induced the formation of spherical cells (Okoli et al., 2010). Balaban and Hendrixson (2011) discovered that *flhG* negatively influences the ability of *ftsZ* to initiate cell

division. Balaban and Hendrixson (2011) suggest that *flhF* and *flhG* are linked in a mechanism that influences cell division in *C. jejuni* because a minicell phenotype caused by deletion of *flhF* could be suppressed. FlhG is proposed to operate as a component of a multipartite mechanism that contributes to control the flagellar number for amphitrichous flagellation and cell division (Gulbronson et al., 2016).

5.3.3.3 Pathogenicity associated genes

Six pathogenicity associated genes were upregulated in the filaments of *C. jejuni* PT14, including a putative hemolysin, putative fibronectin binding protein, methionine sulphoxide reductase A and putative fibronectin domain-containing lipoprotein (Stintzi et al., 2005). However, neither of the other stationary phase morphotypes exhibited any other differential expression of putative virulence factors (Bolton, 2015).

5.3.4 Regulation of oxidative stress genes in morphotypes of *C. jejuni* 12661 and *C. jejuni* PT14

C. jejuni as a microaerophilic bacterium, needs to deal with free oxygen radicals and other reactive molecules produced by normal aerobic metabolism and host defenses against microbial attack (Van Vliet and Ketley, 2001). *C. jejuni* has established a number of defence mechanisms for protection against this type of stress. These genes include sodB (superoxide dismutase), ahpC (alkyl hydroperoxide reductase), tpx (thiol peroxidase), and katA (catalase) (Stintzi et al., 2005).

The *perR* gene product controls the expression of genes that function to mediate against oxidative stress and is significantly up-regulated in the

filamentous morphotype of *C. jejuni* 12661 (1.4-fold) and *C. jejuni* PT14 (2.6-fold) compared to exponential growth phase cells. PerR autoregulates itself and functions to repress the genes encoding the major oxidative stress responsive proteins: AhpC (alkyl hydroperoxide reductase), KatA (catalase) and TrxB (thioredoxin reductase) (Handley et al., 2015). Similarly alkyl hydroperoxide reductase was derepressed in *C. jejuni* 12661 (3.3-fold) and PT14 (1.7-fold) filaments. It would seem that the filamentous cells are preferentially responding to oxidative stress. PerR repression is iron dependent and the stationary phase cultures feature up-regulation of an assortment of genes involved in iron acquisition controlled by the regulator Fur. Since iron participates in the Fenton reaction to generate reactive oxygen species, it is critical that iron acquisition is coordinated with cellular activities that control and detoxify these species.

C. jejuni contains a solitary iron dependent superoxide dismutase that does not feature in the Fur regulon (Butcher et al., 2012). SodB removes superoxides to produce oxygen and hydrogen peroxide, the latter product of which is the preferred substrate of thiol peroxidase (Atack et al., 2008). SodB (superoxide dismutase) and Tpx (thiol peroxidase) show no significant transcriptional differences between the two morphotypes and the exponential growth phase cells. Deficiency in either SodB or Tpx impairs the ability of the strains to survive atmospheric oxygen (Purdy et al., 1999, Atack et al., 2008), which would not be manifest when the cultures were maintained in microaerophilic environments.

Differential expression of AhpC and SodB has been reported previously in *C. jejuni* upon lifting CosR repression, which leads to increased resistance

to the oxidative stressors paraquat and H_2O_2 (Hwang et al., 2011). The *cosR* gene was down-regulated >2-fold in both the filamentous and spiral morphotypes of *C. jejuni* PT14 compared to exponential growth phase cells. In *C. jejuni* 12661 *cosR* was down regulated 9.4-fold but only in the filamentous morphotype compared to exponential growth phase cells. The down-regulation of *cosR* was accompanied by a large increase in the transcription of *katA* in filamentous morphotype, which is contrary to the observations of Hwang et al. (2012). Gene silencing of CosR using antisense technology has been reported to result in an increase in the expression of LuxS by 1.7-fold, and conversly overexpression of CosR resulted in a 2.2-fold down-regulation of LuxS (Hwang et al., 2011). CosR was therefore proposed to negatively regulate the expression of *luxS*, and further CosR binding to a promoter sequence 10 bp upstream of *luxS* was established using electrophoretic mobility shift assays and DNase1 finger printing methods. However, in these experiments the *luxS* genes were significantly down-regulated in thefilamentous morphotype of *C. jejuni* 12661 by a factor of 5.6-fold, and down-regulated in the spiral and filamentous morphotypes of *C. jejuni* PT14 by 2.1- and 4.0-fold respectively when the *cosR* gene was also down-regulated.

The product of the *luxS* gene is the enzyme s-adenosyl-homocysteinase that hydrolyzes s-adenosyl-homocysteine to homocysteine, which functions within the recycling pathway for s-adenosylmethionine (SAM). SAM is critical for the donation of methyl groups for the methyltransferase activities of bacteria that are associated with DNA methylation, chemotaxis, motility and a variety of biosynthetic reactions (Parveen and Cornell, 2011). LuxS is

also responsible for the synthesis of autoinducer-2 (AI-2) since the enzyme can cleave S-ribosylhomocysteine to form homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), which can spontaneously cyclize to form a variety of molecules with varying AI-2 activities (Vendeville et al., 2005). AI-2 molecules harvested from *C. jejuni* can act as luxP ligands when used in bioluminescence assays in combination with a reporter strain of *Vibrio harveyi* (Plummer et al., 2011, Elvers and Park, 2002, Jeon et al., 2003). The role proposed for excreted AI-2 is to facilitate intercellular communication, generally referred to as quorum sensing, but specific activities implicated in the *C. jejuni* response include motility, autoagglutination, cytolethal distending toxin (CDT) expression, flagellar expression, oxidative stress and animal colonization, reviewed in (Plummer, 2012). The possibility that an excreted autoinducer could influence filamentation behaviour was considered in chapter 3, where pre-used MEM plus pyruvate culture medium was demonstrated to support a second round of exponential growth without any changes in the rate of growth or the onset of filamentation. Down-regulation of *luxS* in stationary phase would also militate against the accumulation of the inducer in this phase growth. Consistent with the conclusion that quorum sensing has a limited impact on *C. jejuni* gene expression, Holmes et al. (2009) added exogenous AI-2 to exponentially growing cells without the wholesale changes in transcription reported for other enteric Gram–negative bacteria.

Mutation of the sensor kinase gene *cprS* of the two-component regulator CprRS reduces the tolerance of *C. jejuni* to oxidative stresses and is essential for the colonization of chickens (Svensson et al., 2009). The *cprR*

response regulator gene is essential and dependent on *cprS* to reach the highest transcription leveles in exponential phase but down-regulated in stationary phase (Svensson et al., 2015). Consistent with this observation *cprR* and *cprS* are down-regulated between 2 and 9-fold in the two stationary phase morphotypes of *C. jejuni* PT14 and *C. jejuni* 12661. Despite the dispensability of *cprS*, the CprR protein maintains an essential phosphorylation site that is required for the protein to bind target DNA sequences.

The expression of oxidative stress responsive genes of *C. jejuni* were found to be increased during gastrointestinal growth (Van Vliet and Ketley, 2001). Mutation of *katA* sensitizes *C. jejuni* to hydrogen peroxide and decreases its intracellular survival in macrophages (Van Vliet and Ketley, 2001). Furthermore, a mutation of *C. coli* *sodB* has been reported to inhibit colonization of the chick gut (Purdy et al., 1999). In *Helicobacter pylori*, a functional *katA* has been revealed to be necessary for persistent colonization in a mouse model (Harris et al., 2003).

Rubrerythrin (Rbr) was down-regulated in stationary phase cells of *C. jejuni* PT14 and 12661, and notably highly repressed in the filamentous morphotype compared to exponential cells in *C. jejuni* 12661 by a factor of 33.2-fold. Rbr are considered to be involved in oxidative stress protection of anaerobic microorganisms (Lumppio et al., 2001).

5.3.5 Regulation of nitrosative stress genes in *Campylobacter* morphotypes of 12661 and PT14

The nitrosative stress response activator gene *nssR* was significantly down-regulated in the the two stationary phase morphotypes of *C. jejuni* PT14 and *C. jejuni* 12661. A reduction in the expression of *nssR* results in significant down-regulation of the NssR response genes, which include those encoding the oxygen binding globins *cgb* (single-domain globin) and *ctb* (truncated globin) (Monk et al., 2008).

5.3.6 Genes involved in energy metabolism which were differentially expressed in different morphotypes of *C. jejuni* 12661 and PT14

The *atp* operon and the *atpB* gene encoding subunits of the F_1F_0-ATPase were up-regulated 1.7 to 3.9-fold for the filamentous morphotype compared to the exponential cells of *C. jejuni* PT14, which may be contrasted with significant down-regulation of four of the genes for the stationary phase spiral morphotype compared to exponential cells. An increase in the ATP synthesising capacity of the filamentous morphotype over the spiral morphotype is consistent with the greater cellular ATP content of the filamentous morphotypes measured in chapter 3. However, there are many down-regulated genes encoding components of the electron transport chain in the two stationary phase morphotypes (1.5 to 18.8-fold), which include NADH-ubiquinone oxidoreductase subunits, ferredoxin oxidoreductase, ferredoxin (A911_01610) and Ni^{2+}/Fe^{2+}-hydrogenase 1 cytochrome b subunits. Ferredoxin NADP (H) reductases (FNRs) are flavoenzymes that catalyze electron transfer between NADP(H) and the iron sulphur protein

ferredoxin (Fd; A911_00350 and A911_00355) or Flavin mononucleotide containing flavodoxin (FldA; A911_06715) (Ceccarelli et al., 2004). The exceptions to this were the alternative ferredoxin (A911_01715) and cytochrome d ubiquinol oxidase subunits I and II (A911_00385 and A911_00390), which are significantly up-regulated (1.3 to 3.4-fold).

Similarly there were many down-regulated genes with functions in electron transport for the two *C. jejuni* 12661 stationary phase morphotypes (1.5 to 5.0-fold) with a limited set of 13 genes showing up-regulation (1.5 to 3.5-fold). For the filamentous morphotype compared to exponential growth phase cells, the cytochrome c-type protein (TorC) and ferredoxin (Fdx; UC78_0342) were found to be upregulated by a factor 3.9 and 2.3, respectively. The genes *frdC, A1* and *B1* encoding fumarate reductase components (UC78_0394 to UC78_0396) are up-regulated in *C. jejuni* 12661 filamentous morphotype (1.5 to 3.5-fold) whilst the alternative fumarate reductase annotated genes (UC78_0421 to UC78_0423) were down regulated (3.6 to 4.9-fold). Mutation of *frdA1* in *C. jejuni* resulted in the loss of cellular succinate dehydrogenase activity but retained fumarate reductase activity, and exhibited an impaired ability to colonise chickens, whereas mutation of the orthologue of *frdA2* (*sdhA*) had no effect on succinate dehydrogenase (Weingarten et al., 2009). On this basis the two stationary phase morphotypes of *C. jejuni* 12661 have an enhanced ability to perform succinate: quinone reductase likely through the use of pyruvate via the TCA cycle, and can discriminate against use of the dedicated fumarate reductase enzyme complex.

5.3.7 Differential expression of genes involved in synthesis and modification of macromolecules in *C. jejuni* 12661 and *C. jejuni* PT14

A number of genes involved in modification of macromolecules were significantly regulated in the two different morphotypes of both *C. jejuni* 12661 and PT14. Generally, ribosomal proteins were identified as the largest group that were regulated within this functional group including both 50S ribosomal proteins (Rpl) and 30S ribosomal proteins (Rps). The majority of these were found to be up-regulated in the filamentous morphotype of both strains compared with the spiral morphotype and with exponential growth phase cells. For example, up-regulation of *rplO*, *rplW*, *rplR* and *rpsC*, which assist in the maturation and modification of ribosomes were significantly up-regulated. RplO encodes for the 50S ribosomal protein L15 subunit and RplR codes for 50S ribosomal protein L18. L15 and LI8 are late assembly proteins that help in the formation of active particles during the late stage of assembly of ribosomes. RpsC encodes for 30S ribosomal protein S3 and RplW codes for 50S ribosomal protein L23. Ribosomal protein L23 is one of the proteins from the large ribosomal subunit that binds to a specific region of 23S rRNA.

C. jejuni contains *nrdA* and *nrdB* (*nrdF*) orthologues encoding an oxygen-dependent class I ribonucleotide-diphosphate reductase and therefore require oxygen for DNA synthesis. Sellars et al. (2002) demonstrated that cell filamentation occurred when DNA synthesis was inhibited using hydroxyurea, and also under anaerobic conditions in the presence of the alternative electron acceptors, fumarate or nitrate. Transcription of the *nrdA*

ribonucleotide reductase gene in *C. jejuni* PT14 (A911_00115) was up-regulated in the filamentous morphotype and down-regulated in the spiral morphotype compared to exponentially growth phase cells. Transcription of the *nrdB* gene (A911_01115) in *C. jejuni* PT14 was also significantly down-regulated 1.5-fold in the spiral morphotype compared to exponential growth phase cells. Reducing the availability of ribonucleotide-diphosphate reductase and the capacity to produce deoxyribonucleotides could impact on DNA replication in the spiral morphotype compared to the filamentous morphotype that continue to divide and elongate.

5.3.8 Iron responsive transcriptome of the morphotypes of *C. jejuni* 12661 and *C. jejuni* PT14

Iron-uptake systems of *C. jejuni* usually consist of an outer-membrane receptor protein, which specifically binds to and transports ferric iron sources through the outer-membrane 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, which consequently sends it to the cytoplasmic membrane ABC transporter system, which comprises of a permease and an ATP-binding protein. Iron has been regularly identified as a critical micronutrient for *C. jejuni* growth and colonization (Field et al., 1986, Van Vliet et al., 1998). The genes for iron uptake are regulated by Fur. The $Fe2^{+}$-bound form of Fur or Holo-Fur acts as repressor of the iron uptake systems but the non-iron bound form is also reported to bind DNA and has regulate a diverse set of genes involved in the transport of alternative divalent cations, flagellar and ribosome biosynthesis (Butcher et al., 2012). The *fur* gene is down-regulated in the

filamentous morphotype of *C. jejuni* 12661 and *C. jejuni* PT14. The major iron transporters are strongly derepressed in the stationary phase cells of the study strains, which includes the TonB-dependent energy transduction system exbB-exbD-tonB, the ferric aerobactin receptor precursor and hemin transport system permease. Three iron transport systems were up-regulated *in vivo* compared to *in vitro* cultures, which led to the conclusion that the chicken cecum is a very iron-limiting environment (Taveirne et al., 2013). The *fur* mutant of *C. jejuni* is compromised in it's ability to the colonise the gastrointestinal tract of chicks, as was the case for all of ferric receptor (CfrA), enterochelin ABC transporter, substrate-binding protein (CeuE) and TonB-dependent receptor (Yun et al., 2008).

In a transcriptome study using microarrays probed with cDNAs derived from RNA recovered from chickens, increased expression of the heme transport genes *chuA* and *chuB* were recorded for *C. jejuni* (Lin et al., 2002). A subsequent RNA-Seq study confirmed the increased abundance of *chuA* and *chuB* transcripts along with *chuC* and *chuD* of the heme transport operon (Stintzi et al., 2005). The significance of iron metabolism for effective host colonization has been recognized for most pathogens (Ratledge and Dover, 2000) and should also be an important factor for *C. jejuni* colonisation in the intestine (Stintzi et al., 2005).

6 . General discussion

6.1 General discussion

C. jejuni is a major reason of human bacterial gastroenteritis worldwide. Consumption of contaminated poultry meat is considered to be one of the main sources of infection. Poultry frequently harbour *Campylobacter*s in large numbers as a part of their intestinal flora. There are multiple environmental routes of exposure by which the bacteria can find their way in to chicken flocks and proliferate. However, *Campylobacter*s are microaerophiles and generally become inactivated in atmospheric oxygen at ambient temperatures. The ability to survive sub-optimal conditions and the stresses imposed by transition of the extra-intestinal environment is therefore an important attribute for success of intestinal *Campylobacter* species.

Campylobacter cells are usually slender, spiral shaped rods, however, like many other microorganisms, filamentous forms have been detected under particular conditions (Griffiths, 1993, Thomas et al., 1999, Apel et al., 2012, Cameron et al., 2012). In many different bacteria filamentation has been recognised and is thought to occur through inhibition of cell division, metabolic changes or DNA damage, which includes the SOS response resulting in the inhibition of septum formation whilst the chromosome is repaired (Justice et al., 2008). Filamentation has commonly been linked to the stress and starvation conditions during which it may confer survival advantages (Justice et al., 2008). However, filamentation may simply occur through an inadvertent loss of control of the normal cell division process.

To achieve an understanding of the mechanisms involved in filament formation and how these contribute to the survival of extra-intestinal environmental stresses are key elements to our understanding of the dissemination and transmission of *Campylobacter* species.

The main objective of this study was to therefore examine the cues that give rise to filamentation of *C. jejuni* growing in model broth cultures. This was achieved by investigating the transcription of different *C. jejuni* morphotypes taken from exponential and the late stationary phase. Due to the longer filament formation observed for *Campylobacter* strain 12661 compared with other *C. jejuni* strains; this strain was selected for this study beside *C. jejuni* PT14 as a reference strain.

Growth curves and microscopic examination of broth grown cultures confirmed that filamentous morphotypes were formed upon entry to stationary phase. These studies suggested vital staining may not be suitable for distinguishing live *Campylobacter* cells from dead ones because of variable responses to staining and where viable motile cells could efficiently take up propridium iodide. The staining patterns suggest that the growth and septa formation can occur within the filaments leading to differential dye permeability and preventing dye migration between cells. Furthermore, the interspersed pattern would suggest cell division may be proceeding at the predefined cell poles within the filament signifying at least some of the component cells were viable.

The presence of starch in the culture media did not impact on the timing or degree of filamentation, which suggests that the accumulation of reactive

oxygen species (ROS) in the growth medium is not a predisposing factor to the appearance of the filamentation morphotypes. Without a carbon source *Campylobacters* were unable to increase in viable count in MEM, but some increase in cell size was detected. The addition of pyruvate to the MEM allowed growth but with the early onset of filamentation as compared with growth in nutrient rich medium. All the *C. jejuni* tested showed relatively rapid filamentation in MEM with pyruvate. However, the *C. coli* strain FB1 did not form long filaments but neither did the strain increase in viable count in the MEM plus pyruvate culture medium, so it remains unclear whether this strain lacks the capacity to form filaments or is just impaired in growth under these conditions.

Filter sterilization and re-use of the NB2 medium did not result in slower growth or alter the timing for the formation of filaments, suggesting that the nutrients remain in excess and that excretion of a diffusible signal molecule, such as those operating in quorum sensing, could not explain the morphotype transition. It was notable that *luxS* encoding autoinducer 2 synthetic ability was expressed in exponential phase but significantly down-regulated in stationary phase. Within a well-mixed broth culture the response of *Campylobacter* to nutrient limitation is not uniform, in most cultures filamentous types arise among spirals that continue to divide. This suggested that when the growth rate has become limited due to nutritional depletion, then the development of the filaments is either a stochastic process or developmentally controlled to produce a subpopulation that are more able to survive nutritional reduction and/or environmental stresses. *C. jejuni* cultured in MEM with pyruvate reveal an ability to retain a viable

subpopulation through decline phase at 37◦C under microaerobic conditions despite a fall in viable count from >8 $\log_{10}$ cfu/ml at the end of exponential phase (24 h in Figure 3.5) to 3 $\log_{10}$ cfu/ml at 168 h. The ATP contents of the cell morphotypes showed significant increases in the cellular ATP contents of the filamentous types as compared with spiral forms. These could be due to the filaments consist of multiple metabolically active cells. The *C. jejuni* strain 12661 produces obviously longer filaments and has a higher ATP content than *C. jejuni* PT14. However, ATP content estimates of coccal cells were either very low or non-detectable, indicating they are not viable as concluded in previous reports (Moran and Upton, 1986, Boucher et al., 1994). The filamentous morphotypes have the ability to survive longer in water compared to the short spiral cells. The presence of multicellular filaments may also lead to underestimates of viable cell numbers in cultures, since a single filament can form a single colony despite a multicellular origin. The relative transcription the *atp* operon encoding subunits of the F1F0-ATPase is greater in stationary phase filaments than spiral cells of *C. jejuni* PT14, suggesting an increase in the ATP synthesising capacity of the filamentous cells over spirals that is consistent with the greater cellular ATP content of filaments.

The genome sequence of *C. jejuni* 12661 was characterised and examined for possible reasons to explain the ability of this strain to be morphologically different from other strains based on its genome content and arrangement. However, despite that the 12661 forms longer filaments compared to other *C. jejuni* strains, the genome analysis identified no obvious gene content that could explain this phenomenon. Genomic characterization of this strain

identified many variable loci along the genome, including the characterization of several features related to bacterial capsule, lipooligosaccharide, flagellar modification genes, putative anaerobic DMSO reductase and the TonB-ExbB-ExbD complex. Genes encoding these functions were found to be variable by comparing their presence or absence across the corresponding regions of other *C. jejuni* strains. Furthermore, this strain has multiple R-M systems and 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. BLAST searches of the spacer regions of the 12661CRISPR array found no obvious matches to the chromosomal DNA, but instead identified matches to the chromosome and sequences of other *C. jejuni* strains.

Several studies have suggested that *C. jejuni* shows a dynamic stationary phase, due to switches in motility, the production and utilization of substrates with associated by concurrent changes in gene expression. These studies have been extended to compare *in vitro* cultures with *Campylobacter*s recovered from animals. Taveirne et al. (2013) examined the complete transcriptome of *C. jejuni during* colonization of the chicken caecum and compared these with transcription in two different *in vitro* growth phases using strand-specific RNA-Seq identified that 135 genes that were differentially up-regulated and 14 genes that were repressed in chick samples compared to mid-log phase cultures. The transcriptome study reported by Wright et al. (2009) identified that a large number of genes showed significant changes between late exponential phase and late

stationary phase. The majority of changes were found during stationary phase, in which 133 genes were upregulated and 151 genes were downregulated (Wright et al., 2009). In a rabbit ileal loop study 185 genes were found to be induced and 199 genes were repressed *in vivo* when the samples were recovered from rabbits 48 h post infection (Stintzi et al., 2005).

Salmonella typhimurium and *E. coli* are reported to show increased levels of acetate production (Wilson et al., 2003) or carbon starvation responses (Sezonov et al., 2007) upon the cessation of growth in stationary phase. The transition to stationary phase for *Campylobacter* has been reported to feature a switch from acetate production to utilization together with a peak in motility and numerous gene expression changes (Wright et al., 2009). The acetate switch has been proposed to occur in late stationary phase when *C. jejuni* cease to excrete acetate and begin to assimilate acetate as nutrients become limiting. Filament formation in MEM plus pyruvate occurs before the carbon source becomes limiting, so the switch could occur in late stationary phase when coccoid cells become evident but is not operating during filament formation. Acetate excretion occurs due to the constitutive expression of phosphotransacetylase (*pta*) and acetate kinase (*ackA*) that together excrete acetate. Contrary to this the *pta* and *ackA* genes show significant down regulation in stationary phase spiral and filamentous cells. Moreover acetyl-CoA synthetase (*acs*) that contributes to the assimilation of acetate with pta and ackA is also down regulated. Under the growth conditions used in these experiments acetate production and assimilation will be limited.

In this study no differentially regulated were found for the majority of the candidate ncRNAs. However, CJnc20 was found to be up-regulated in PT14 in stationary phase morphotypes. However, strand specific nucleotide sequencing in conjunction with TEX (5'P-dependent terminator exonuclease) offer the prospect of identifying, mapping and monitoring the non-coding RNAs and their effects on transcription. Short ncRNAs, including microRNAs (miRNA), function to bind to a specific target mRNA with a complementary sequence to induce cleavage, or degradation or block translation (Collins et al., 2011, Cui et al., 2009).The function of short interfering RNAs (siRNA) is similar to miRNAs which mediate post-transcriptional gene silencing (PTGS) as a result of mRNA degradation (Carthew and Sontheimer, 2009). Other type of short ncRNAs is Piwi-interacting RNAs (piRNA), these RNA molecules involves chromatin regulation and suppression of transposon activity in germline and somatic cells (Kaikkonen et al., 2011, Brennecke et al., 2007). Long ncRNAs (lncRNAs), many of them can complex with chromatin-modifying proteins and apply their catalytic activity to particular sites in the genome, consequently modifying chromatin states and influencing gene expression (Mercer and Mattick, 2013). The majority of non-coding RNA transcripts belong to the group lncRNAs. Long ncRNAs function in chromatin remodelling, transcriptional regulation, post-transcriptional regulation, and as precursors for siRNAs (Kaikkonen et al., 2011).

Polar flagella drive *C. jejuni* as a motile species The flagella are essential for invasion *in vitro* and for adhesion, invasion and colonization *in vivo* (Black et al., 1988). Flagella expression involves over 40 structural and regulatory genes, and includes an O-linked glycosylation system that post

translationally modifies the flagellin subunits (Guerry et al., 2006, Hendrixson, 2008). Three sigma factors have been predicted in the *C. jejuni* NCTC 11168 genome: σ70 (encoded by rpoD), σ54 (encoded by rpoN) and σ28 (encoded by fliA) (Carrillo et al., 2004).

Of these *rpoN* is significantly down-regulated in the stationary phase cells of *C. jejuni* 12661 and *C. jejuni* PT14 as compared to exponential cells, and is likely a major contributory factor to the loss of motility in cells harvested from stationary phase. FlgR is partner to FlgS that operate together as a two-component regulator, in which FlgS histidine kinase autophosphorylates to activate the FlgR response regulator. Transcript levels of the *flgR* and *flgS* genes are also down-regulated in stationary phase morphotypes for *C. jejuni* 12661 and PT14. The genes encoding the chemotactic response proteins CheV, CheA and CheW show significantly expressed in the stationary phase morphotypes of *C. jejuni* PT14 compared to exponential cells. The chemoreceptors (methyl-accepting chemotaxis proteins) of *C. jejuni* PT14 also showed differential transcript levels to exponential cells with Tlp1 responding to aspartate 1.5-fold down-regulated in spiral cells and 2.1-fold up-regulated in filaments, Tlp6 cytoplasmic transducing protein up-regulated in filaments and Tlp7 responding to formate stimulated in spirals and filaments.

A number of heat shock proteins express when bacteria are exposed to stressful conditions, these proteins fall into two categories, the chaperones and the ATP dependent proteases (Andersen et al., 2005). Several heat shock protein homologues that include the major chaperones have been

identified in genome sequence of *C. jejuni* NCTC 11168, such as *groEL*, *groES*, *grpE*, *dnaK* and *dnaJ*, and several ATP dependent proteases (Parkhill et al., 2000).The heat shock regulons are controlled by HrcA (Heat-inducible transcription repressor) and HspR (global negative regulator of heat shock gene expression).

*Campylobacter*s are reported not to exhibit enhanced stress resistance in stationary phase, unlike many other bacteria, which is consistent with the absence of RpoS homologues (Kelly et al., 2001). However, it is evident from this study and of others (Wright et al., 2009, Taveirne et al., 2013) that a variety of *Campylobacter* strains appear to respond to stress in stationary phase with the up-regulation of the autoregulatory genes *hspR* and *hcaA* with the genes encoding chaperones and co-chaperones. In contrast the transcript levels of the stress regulators *hrcA* and *hspR* from *C. jejuni* 12661 stationary phase morphotypes show no significant difference to exponential growing cells. However, several ATP dependent protease transcripts were found to be activated in *C. jejuni* 12661 filaments compared to spiral cells: *clpA*, *clpB*, *clpP* and *clpX*, implying damaged proteins are being actively turned over. The cooperative action of *clpB* with *dnaK*, *dnaJ* and *grpE* helps to the reactivate chemical and/or heat denatured substrate proteins (Konieczny and Liberek, 2002).

*Campylobacter*s retain a stringent response that assists survival in the stationary phase of many bacterial species. Mutation of the *spoT/relA* gene results in aberrant cell morphologies and early coccoid cell formation in stationary phase cultures (Gaynor et al., 2005). This gene was found to be

down-regulated in stationary phase *C. jejuni* PT14 cells. Reducing the ability to produce (p) ppGpp would diminish the stress response and failure to down-shift some aspects of metabolism. The ribosomal protein genes are not down-regulated in stationary phase cultures. However, much of the amino acid synthetic capacity in these cells is down-regulated in stationary phase. Control of the cellular poly-phosphate and ppGpp pools is complex with growth phase related expression of alternative genes for phosphohydrolases and polyphosphate kinases.

Several cell division associated genes were found to be differentially regulated between the morphotypes of *C. jejuni* 12661 and PT14. Nine cell division associated proteins in *C. jejuni* PT14 filaments were up-regulated compared to spirals indicating that *C. jejuni* PT14 filaments continue transcription of the cell division process, but do not elongate to the same extent to achieve the length of filaments observed for *C. jejuni* 12661. Rodrigues et al. (2015) found that the oxidative stress changes the cell morphology of *C. jejuni* by decreasing the expression of the rod shape determining protein MreB. Critically this study highlighted significant differential expression for the genes *mreB*, *mreC* and *maf* in filaments compared to spiral and exponential cells. Inactivation of *mreB* results in the formation of coccoid and spherical morphotypes in *E coli* , *Bacillus subtilis*, *Caulobacter crescentus and Helicobacter hepaticus* (Figge et al., 2004, Bendezu et al., 2009, Kawai et al., 2009, Okoli et al., 2010).

It is suggested that *flhF* and *flhG* are linked in a mechanism that influences cell division in *C. jejuni* because a minicell could be suppressed by deletion

of *flhF* (Balaban and Hendrixson, 2011). *flhF* was found to be repressed in stationary phase morphotypes of *C. jejuni* PT14.

As a microaerophilic bacterium, *C. jejuni* has established a number of defence mechanisms for protection against oxygen radicals and other reactive molecules. The genes involved in this process are *sodB*, *ahpC*, *tpx*, and *katA* (Stintzi et al., 2005). PerR autoregulates itself and functions to negatively regulate the major oxidative stress responsive proteins AhpC and KatA. The *perR* gene was significantly up-regulated in filamentous cells of *C. jejuni* 12661 and PT14 compared to exponential phase cells. The genes encoding catalase and alkyl hydroperoxide reductase were found to be activated in filamentous cells of *C. jejuni* 12661 and PT14. It would appear that the filamentous cells are responding better to oxidative stress. PerR suppression is iron dependent, which correlates with the ability of ironto produce reactive oxygen species. The *fur* gene regulates iron uptake genes and is down-regulated in the filamentous cells of *C. jejuni* 12661 and PT14, which corresponds with strong derepression of the major iron transporters. Yun et al. (2008) reported that fur, CfrA, enterochelin ABC transporter, substrate-binding protein (CeuE) and TonB-dependent receptor mutants of *C. jejuni* are compromised in their capacity to the colonise the gastrointestinal tract of chicks. The stationary phase morphotypes would be primed for iron acquisition and would not present a barrier to colonisation but would definitely have to re-regulate to ensure motility.

In conclusion, this thesis has provided valuable insights into some fundamental aspects of the physiology and transcriptome of *C. jejuni in*

response to stationary phase and in the differentiation of morphotypes. The selection and use of minimal medium to increase filamentation enabled reliable comparisons between strains and established that morphological change was wide spread amongst *C. jejuni* strains tested. The formation of filamentous cells did not appear to be due to the release of diffusible molecules, toxic metabolites, or be in response to oxidative stress in the medium. In addition the data provided evidence that separated filaments from stationary phase exhibited greater intracellular ATP contents than spiral forms and showed enhanced survival in water at 4 and 37°C compared to spiral cells. These observations support the conclusion that the filaments are adapted to survive extra-intestinal environments.

Transcriptomic analysis of different morphotypes from stationary phase and exponential phase provided a better understanding of *C. jejuni filamentation*. A number of flagella associated genes were down-regulated which is supported by phenotypic loss of motility cell division associated proteins were differentially up-regulated, which include the bacterial actin homologue MreB. Differential expression of of oxidative stress genes in filamentous cells of *C. jejuni* 12661 and PT14 indating that these cells are preferentially responding to oxidative stress.

6.2 Future work

The time limitation on this study makes it impossible to test all the hypotheses emerging from the transcriptome studies of this thesis.

The ability of the filamentous morphotype to survive in water compared to the short spiral form suggests that further research is necessary to assess the influence this may have on the transmission of the *Campylobacter*s from the environment to farm animals and on the safety of post-process foods.

Mutational studies could also be carried out to translate the role of selected genes which were differentially expressed in this study and their role in different morphotypes, beside to the proteomic analysis of the gene products. This should provide more comprehensive information to broaden our knowledge regarding the mechanisms employed by *Campylobacter*s to survive and change morphology during the stress conditions occurring in stationary phase compared with exponential phase.

Studies may also be carried out on the genes recognized in this study as significant in the process of chicken colonization for different morphotypes of *C. jejuni.*

A combination of transcriptional, proteomic and mutational studies should identify the genes critical for filamentation and differentiate them from those that do not contribute to the changes in morphology.

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APPENDIX 1

***C. jejuni* 12661 genes up-regulated of spirals vs exponential cells**

No.	Feature ID	products	fold change	p_{adj}[a]
1	UC78_1330	hypothetical protein	8.22	2E-02
2	pcaB	3-carboxy-cis,cis-muconate cycloisomerase	4.10	4E-02
3	UC78_1595	FtsX-like permease family protein	3.82	1E-02
4	tpd	34 kDa membrane antigen precursor	3.65	0E+00
5	UC78_1562	colicin uptake protein TolQ	3.16	3E-09
6	UC78_0815	hypothetical protein	3.04	2E-02
7	mprA_1	Response regulator MprA	2.95	1E-02
8	UC78_0059	hypothetical protein	2.93	4E-02
9	zraS_2	Sensor protein ZraS	2.93	2E-02
10	UC78_1327	hypothetical protein	2.89	4E-02
11	UC78_1407	hypothetical protein	2.87	1E-03
12	UC78_1543	hypothetical protein	2.75	1E-06
13	arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	2.68	3E-02
14	fumC	Fumarate hydratase class II	2.63	5E-04
15	frdB_1	Fumarate reductase iron-sulfur subunit	2.44	6E-03

Appendix 1 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	UC78_0813	Putative F0F1-ATPase subunit	2.21	3E-02
17	trxB	Thioredoxin reductase	2.11	0E+00
18	sucC	Succinyl-CoA ligase [ADP-forming] subunit beta	2.08	4E-03
19	slyD	FKBP-type peptidyl-prolyl cis-trans isomerase SlyD	2.08	5E-03
20	atpA	ATP synthase subunit alpha	2.04	5E-03
21	porA	Major outer membrane protein precursor	2.04	2E-02
22	mprA_2	Response regulator MprA	2.00	3E-02
23	glnQ_3	Glutamine transport ATP-binding protein GlnQ	1.96	4E-02
24	def	Peptide deformylase	1.95	6E-04
25	moaE	Molybdopterin synthase catalytic subunit	1.92	3E-02
26	exbB	Biopolymer transport protein ExbB	1.91	5E-03
27	clpP	ATP-dependent Clp protease proteolytic subunit	1.87	3E-02
28	atpC	ATP synthase epsilon chain	1.85	6E-04
29	eno	Enolase	1.84	4E-02
30	sucD	Succinyl-CoA ligase [ADP-forming] subunit alpha	1.80	3E-03

Appendix 1 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	flhB_2	Flagellar biosynthetic protein FlhB	1.78	4E-03
32	korA	2-oxoglutarate oxidoreductase subunit KorA	1.77	3E-04
33	frdA_1	Fumarate reductase flavoprotein subunit	1.77	2E-02
34	caiE	Carnitine operon protein CaiE	1.75	3E-02
35	korB	2-oxoglutarate oxidoreductase subunit KorB	1.73	2E-02
36	torZ	Trimethylamine-N-oxide reductase 2 precursor	1.68	1E-02
37	atpD_2	ATP synthase subunit beta	1.68	3E-02
38	ttcA	tRNA 2-thiocytidine biosynthesis protein TtcA	1.64	2E-02
39	mog	Molybdopterin adenylyltransferase	1.62	6E-08
40	UC78_1145	Phosphate transporter family protein	1.61	1E-05
41	UC78_1215	hydrogenase 2 maturation endopeptidase	1.60	4E-07
42	mdh	Malate dehydrogenase	1.60	4E-02
43	UC78_0865	SCO1/SenC	1.59	3E-04
44	hisD	Histidinol dehydrogenase	1.57	3E-02
45	UC78_0519	2-oxoglutarate-acceptor oxidoreductase subunit OorD	1.57	2E-04

Appendix 1 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	dnaG	DNA primase	1.57	3E-03
47	hisB	Histidine biosynthesis bifunctional protein HisB	1.55	2E-02
48	hydC	Quinone-reactive Ni/Fe-hydrogenase B-type cytochrome subunit	1.54	4E-07
49	soj	Sporulation initiation inhibitor protein Soj	1.54	4E-02
50	UC78_0853	HIT-like protein	1.52	3E-02
51	hydB	Quinone-reactive Ni/Fe-hydrogenase large chain	1.51	0E+00
52	relA	Bifunctional (p)ppGpp synthase/hydrolase RelA	1.50	2E-02
53	nifF	Flavodoxin-2	1.50	3E-02

APPENDIX 2

***C. jejuni* 12661 genes down-regulated of spirals vs exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	UC78_0841	tRNA-Leu	-55.78	3E-02
2	UC78_1300	tRNA-Ser(gct)	-17.57	2E-18
3	UC78_0053	hypothetical protein	-15.89	2E-05
4	UC78_0554	tRNA-Met	-14.49	4E-13
5	UC78_0095	hypothetical protein	-10.39	3E-33
6	UC78_0723	hypothetical protein	-9.09	3E-02
7	legG	GDP/UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing)	-8.91	2E-05
8	UC78_0231	hypothetical protein	-6.82	3E-15
9	uxaA	Altronate dehydratase	-6.78	5E-02
10	hisH1	Imidazole glycerol phosphate synthase subunit HisH 1	-6.77	1E-07
11	rhaM	L-rhamnose mutarotase	-6.62	7E-16
12	panB	3-methyl-2-oxobutanoate hydroxymethyltransferase	-6.43	4E-02
13	UC78_1400	hypothetical protein	-5.78	1E-05
14	trpB	Tryptophan synthase beta chain	-5.46	9E-25

Appendix 2 continued

No.	Feature ID	products	fold change	p_{adj}[a]
15	UC78_1273	hypothetical protein	-5.35	2E-08
16	UC78_08923 (aas)	Bifunctional protein Aas	-5.24	1E-08
17	UC78_0208	hypothetical protein	-4.92	1E-02
18	trpC_1	Tryptophan biosynthesis protein TrpCF	-4.80	7E-23
19	dnaJ	Chaperone protein DnaJ	-4.48	1E-03
20	UC78_0207	hypothetical protein	-4.39	3E-03
21	aldA	Lactaldehyde dehydrogenase	-4.32	1E-04
22	dtpT_1	Di-/tripeptide transporter	-4.21	4E-16
23	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	-4.09	2E-03
24	dapA_1	4-hydroxy-tetrahydrodipicolinate synthase	-4.01	3E-03
25	UC78_0891	tRNA-Leu	-3.90	5E-03
26	UC78_0829	hypothetical protein	-3.90	2E-02
27	trpA	Tryptophan synthase alpha chain	-3.88	3E-17
28	murI	Glutamate racemase	-3.82	3E-02
29	nhaA_2	Na(+)/H(+) antiporter NhaA	-3.73	3E-04

Appendix 2 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
30	UC78_0454	tRNA-Gly	-3.70	1E-02
31	UC78_0991	adenylosuccinate lyase	-3.70	4E-04
32	UC78_0043	hypothetical protein	-3.63	1E-02
33	UC78_1653	tRNA-Ala	-3.57	9E-05
34	hisF_1	Imidazole glycerol phosphate synthase subunit HisF	-3.52	1E-02
35	UC78_1193	hypothetical protein	-3.49	1E-03
36	arsR	Arsenical resistance operon repressor	-3.49	3E-02
37	UC78_1648	hypothetical protein	-3.34	5E-04
38	arsC	Protein ArsC	-3.34	1E-04
39	cobB	NAD-dependent protein deacylase	-3.33	8E-08
40	panD	Aspartate 1-decarboxylase precursor	-3.32	3E-03
41	ribA	GTP cyclohydrolase-2	-3.26	4E-03
42	miaA	tRNA dimethylallyltransferase	-3.24	3E-12
43	zraS_1	Sensor protein ZraS	-3.22	1E-19
44	queF	NADPH-dependent 7-cyano-7-deazaguanine reductase	-3.15	4E-11

Appendix 2 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
45	UC78_0453	tRNA-Tyr	-3.07	2E-02
46	trpGD	Bifunctional protein TrpGD	-3.02	1E-03
47	UC78_1011	phosphoglycerol transferase I	-2.91	3E-02
48	bla	Beta-lactamase OXA-10 precursor	-2.88	3E-02
49	UC78_1279	hypothetical protein	-2.87	2E-04
50	UC78_0206	hypothetical protein	-2.85	2E-15
51	glnM_2	putative glutamine ABC transporter permease protein GlnM	-2.76	1E-02
52	UC78_0789	hypothetical protein	-2.71	2E-05
53	UC78_1173	Bacteriohemerythrin	-2.69	4E-03
54	trpE	Anthranilate synthase component 1	-2.69	1E-02
55	UC78_0452	tRNA-Thr	-2.63	4E-04
56	ntrC	Nitrogen assimilation regulatory protein	-2.62	2E-04
57	dapE	Succinyl-diaminopimelate desuccinylase	-2.61	2E-05
58	UC78_0928	hypothetical protein	-2.61	3E-07
59	moeZ	putative adenylyltransferase/sulfurtransferase MoeZ	-2.57	1E-04

Appendix 2 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
60	cusC	Cation efflux system protein CusC precursor	-2.56	1E-05
61	UC78_1267	hypothetical protein	-2.48	2E-18
62	mepS	Murein DD-endopeptidase MepS/Murein LD-carboxypeptidase precursor	-2.46	1E-03
63	cas2	CRISPR-associated endoribonuclease Cas2	-2.43	1E-02
64	UC78_1654	tRNA-Val	-2.42	7E-03
65	UC78_1247	UC78_1247	-2.39	9E-14
66	eptA	Phosphoethanolamine transferase EptA	-2.32	2E-02
67	leuA	2-isopropylmalate synthase	-2.32	2E-08
68	xecE	2-(S)-hydroxypropyl-CoM dehydrogenase	-2.29	4E-02
69	dapD	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	-2.27	3E-02
70	exoA	Exodeoxyribonuclease	-2.26	1E-05
71	cas1	CRISPR-associated endonuclease Cas1	-2.25	4E-02
72	pseH	UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine N-acetyltransferase	-2.22	3E-03
73	cbpA	Curved DNA-binding protein	-2.22	4E-03
74	obg	GTPase Obg	-2.20	3E-02

Appendix 2 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
75	UC78_0438	hypothetical protein	-2.18	2E-02
76	ribD	Riboflavin biosynthesis protein RibD	-2.18	3E-02
77	yigZ	IMPACT family member YigZ	-2.16	5E-05
78	UC78_0611	DNA polymerase III subunit delta	-2.14	2E-04
79	UC78_0537	hypothetical protein	-2.13	4E-04
80	UC78_0897	tRNA-Val	-2.12	6E-11
81	UC78_1459	hypothetical protein	-2.10	9E-05
82	hddC_1	D-glycero-alpha-D-manno-heptose 1-phosphate guanylyltransferase	-2.10	2E-12
83	UC78_0902	tRNA-Lys	-2.08	2E-02
84	gmhA1	Phosphoheptose isomerase 1	-2.07	4E-23
85	murE_1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase	-1.98	2E-03
86	trmA	tRNA/tmRNA (uracil-C(5))-methyltransferase	-1.90	3E-02
87	UC78_1602	tRNA-Leu	-1.88	2E-06
88	UC78_0901	tRNA-Val	-1.84	2E-03
89	asd	Aspartate-semialdehyde dehydrogenase	-1.84	5E-09

Appendix 2 continued

No.	Feature ID	Products	**fold change**	p_{adj}[a]
90	UC78_1536	antiporter inner membrane protein	-1.81	4E-02
91	UC78_1162	putative FAD-linked oxidoreductase	-1.80	4E-04
92	UC78_0455	tRNA-Thr	-1.80	4E-02
93	rnhA	Ribonuclease HI	-1.76	1E-03
94	rpoD	RNA polymerase sigma factor RpoD	-1.73	3E-04
95	UC78_1259	hypothetical protein	-1.71	1E-05
96	UC78_0538	hypothetical protein	-1.69	1E-03
97	UC78_0986	hypothetical protein	-1.68	7E-04
98	UC78_0900	tRNA-Asp	-1.64	2E-02
99	UC78_0338	hypothetical protein	-1.63	2E-02
100	ycaD	putative MFS-type transporter YcaD	-1.63	6E-07
101	tdcB	L-threonine dehydratase catabolic TdcB	-1.63	2E-02
102	czcB	Cobalt-zinc-cadmium resistance protein CzcB	-1.62	2E-03
103	prfB	Peptide chain release factor 2	-1.59	3E-07
104	map	Methionine aminopeptidase 1	-1.58	6E-03

Appendix 2 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
105	rfaF	ADP-heptose--LPS heptosyltransferase 2	-1.57	5E-04
106	UC78_1277	hypothetical protein	-1.56	2E-03
107	UC78_0086	Aspartate racemase	-1.54	1E-04

APPENDIX 3

***C. jejuni* 12661 genes up-regulated of filament against spirals cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	UC78_08923 (aas)	Bifunctional protein Aas	7.99	0E+00
2	UC78_1066 (aat)	Leucyl/phenylalanyl-tRNA--protein transferase	7.56	0E+00
3	UC78_0427 (accA)	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	7.52	2E-11
4	UC78_1243 (accB)	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	6.16	1E-07
5	UC78_1242 (accC)	Biotin carboxylase	5.80	2E-04
6	UC78_0129 (accD)	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	5.61	1E-05
7	UC78_0665 (ackA)	Acetate kinase	5.53	2E-10
8	UC78_0796(acnB)	Aconitate hydratase 2	5.43	3E-08
9	UC78_0425 (acpP)	Acyl carrier protein	5.42	3E-03
10	UC78_1351 (acpS)	Holo-[acyl-carrier-protein] synthase	5.27	7E-04
11	UC78_0354 (acrE)	Multidrug export protein AcrE precursor	5.04	4E-04
12	UC78_1464 (acsA)	Acetyl-coenzyme A synthetase	4.93	3E-11
13	UC78_1413 (addA)	ATP-dependent helicase/nuclease subunit A	4.87	8E-10
14	UC78_1475 (adhA)	putative formaldehyde dehydrogenase AdhA	4.65	9E-05
15	UC78_1491 (adhR)	HTH-type transcriptional regulator AdhR	4.57	1E-05

Appendix 3 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	UC78_0620)(adk)	Adenylate kinase	4.41	9E-04
17	aer_1	Aerotaxis receptor	4.30	1E-02
18	aer_2	Aerotaxis receptor	4.28	8E-11
19	aguA	Agmatine deiminase	4.22	3E-08
20	alaC	Glutamate-pyruvate aminotransferase AlaC	4.09	9E-15
21	alaS	Alanine--tRNA ligase	3.85	2E-04
22	aldA	Lactaldehyde dehydrogenase	3.80	3E-07
23	algC	Phosphomannomutase/phosphoglucomutase	3.61	6E-06
24	alr	Alanine racemase	3.54	8E-03
25	alsT	Amino-acid carrier protein AlsT	3.45	2E-04
26	amiA	N-acetylmuramoyl-L-alanine amidase AmiA precursor	3.43	2E-04
27	ansZ	L-asparaginase 2 precursor	3.41	1E-02
28	appA	Oligopeptide-binding protein AppA precursor	3.37	2E-05
29	apt	Adenine phosphoribosyltransferase	3.35	2E-11
30	argB	Acetylglutamate kinase	3.26	2E-05

Appendix 3 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	argC	N-acetyl-gamma-glutamyl-phosphate reductase	3.26	1E-04
32	argD	Acetylornithine aminotransferase	3.26	4E-05
33	argF	Ornithine carbamoyltransferase	3.21	6E-04
34	argG	Argininosuccinate synthase	3.19	1E-12
35	argH1	Argininosuccinate lyase 1	3.16	9E-15
36	argS	Arginine--tRNA ligase	3.14	3E-08
37	arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase	3.06	2E-05
38	aroA	3-phosphoshikimate 1-carboxyvinyltransferase	3.04	1E-03
39	aroB	3-dehydroquinate synthase	2.99	9E-06
40	aroC	Chorismate synthase	2.98	1E-02
41	aroE	Shikimate dehydrogenase	2.92	2E-04
42	aroQ	3-dehydroquinate dehydratase	2.87	2E-07
43	arsB	Arsenical pump membrane protein	2.86	8E-06
44	arsC	Protein ArsC	2.81	9E-03
45	arsR	Arsenical resistance operon repressor	2.80	2E-04

Appendix 3 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	aspA	Aspartate ammonia-lyase	2.79	5E-05
47	aspS	Aspartate--tRNA ligase	2.76	4E-04
48	assT_1	Arylsulfate sulfotransferase AssT	2.71	2E-03
49	assT_2	Arylsulfate sulfotransferase AssT precursor	2.67	1E-03
50	atpA	ATP synthase subunit alpha	2.65	2E-03
51	atpB	ATP synthase subunit a	2.63	1E-04
52	atpC	ATP synthase epsilon chain	2.56	0E+00
53	atpD_1	ATP synthase subunit delta	2.50	2E-02
54	atpD_2	ATP synthase subunit beta	2.49	2E-03
55	atpF_1	ATP synthase subunit b	2.49	2E-05
56	atpF_2	ATP synthase subunit b	2.47	4E-02
57	atpG	ATP synthase gamma chain	2.47	1E-04
58	atpH	ATP synthase subunit c	2.46	6E-09
59	bamD	Outer membrane protein assembly factor BamD	2.44	6E-04
60	basS	Sensor protein BasS	2.43	1E-02

Appendix 3 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	bcp	Putative peroxiredoxin	2.38	9E-06
62	bcr	Bicyclomycin resistance protein	2.36	6E-03
63	bdlA	Biofilm dispersion protein BdlA	2.36	6E-03
64	bepE	Efflux pump membrane transporter BepE	2.35	2E-02
65	bioB	Biotin synthase	2.34	1E-02
66	bioD	ATP-dependent dethiobiotin synthetase BioD	2.34	1E-02
67	bioF	8-amino-7-oxononanoate synthase	2.33	9E-03
68	birA	Bifunctional ligase/repressor BirA	2.32	3E-02
69	bla	Beta-lactamase OXA-10 precursor	2.31	2E-02
70	bsdB	Phenolic acid decarboxylase subunit B	2.30	2E-06
71	btuC	Vitamin B12 import system permease protein BtuC	2.29	8E-06
72	btuF	Vitamin B12-binding protein precursor	2.27	4E-02
73	caiE	Carnitine operon protein CaiE	2.27	4E-03
74	carA	Carbamoyl-phosphate synthase small chain	2.26	2E-04
75	carB	Carbamoyl-phosphate synthase large chain	2.26	6E-04

Appendix 3 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	cas1	CRISPR-associated endonuclease Cas1	2.25	4E-02
77	cas2	CRISPR-associated endoribonuclease Cas2	2.25	2E-02
78	cas9	CRISPR-associated endonuclease Cas9	2.25	2E-03
79	cbf2	Putative peptidyl-prolyl cis-trans isomerase Cbf2 precursor	2.24	5E-03
80	cbpA	Curved DNA-binding protein	2.23	9E-03
81	cca	Multifunctional CCA protein	2.23	2E-02
82	ccp	Cytochrome c551 peroxidase precursor	2.23	4E-03
83	ccpA	Cytochrome c551 peroxidase precursor	2.21	1E-03
84	ccsA_2	Cytochrome c biogenesis protein CcsA	2.20	2E-03
85	cdsA	Phosphatidate cytidylyltransferase	2.20	1E-03
86	cdtA	Cytolethal distending toxin subunit A precursor	2.19	4E-06
87	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	2.18	5E-04
88	cfiB	2-oxoglutarate carboxylase small subunit	2.15	2E-10
89	cheA	Chemotaxis protein CheA	2.15	2E-02
90	cheB	Chemotaxis response regulator protein-glutamate methylesterase	2.14	2E-03

Appendix 3 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	cheR	Chemotaxis protein methyltransferase	2.13	1E-02
92	cheV	Chemotaxis protein CheV	2.12	1E-07
93	cheW	Chemotaxis protein CheW	2.12	3E-02
94	cirA	Colicin I receptor precursor	2.11	3E-02
95	citN	Citrate transporter	2.10	1E-02
96	clpA	ATP-dependent Clp protease ATP-binding subunit ClpA	2.09	0E+00
97	clpB	Chaperone protein ClpB	2.09	3E-02
98	clpP	ATP-dependent Clp protease proteolytic subunit	2.07	4E-03
99	clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	2.06	7E-03
100	cmoA	tRNA (cmo5U34)-methyltransferase	2.06	1E-03
101	coaD	Phosphopantetheine adenylyltransferase	2.03	2E-07
102	coaE	Dephospho-CoA kinase	2.02	2E-03
103	cocE	Cocaine esterase	2.01	3E-03
104	comEA	ComE operon protein 1	2.01	5E-04
105	comM	Competence protein ComM	2.00	1E-03

APPENDIX 4

***C. jejuni* 12661 down-regulated genes of filament against spirals cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	zupT	Zinc transporter ZupT	-21.48	4E-54
2	zraS_2	Sensor protein ZraS	-18.80	5E-05
3	zraS_1	Sensor protein ZraS	-15.27	3E-02
4	znuA	High-affinity zinc uptake system protein ZnuA precursor	-15.20	2E-02
5	yycB	putative transporter YycB	-14.91	1E-04
6	yxeP_2	putative hydrolase YxeP	-12.88	1E-103
7	yxeP_1	putative hydrolase YxeP	-12.37	2E-02
8	ywlF	Putative sugar phosphate isomerase YwlF	-11.84	6E-04
9	yqgF	Putative Holliday junction resolvase	-11.23	2E-02
10	yqaA	Inner membrane protein YqaA	-11.08	6E-05
11	ypeA	Acetyltransferase YpeA	-10.86	1E-03
12	yojI	ABC transporter ATP-binding protein YojI	-10.36	4E-03
13	yohD_2	Inner membrane protein YohD	-10.27	3E-03
14	yohD_1	Inner membrane protein YohD	-10.25	2E-03
15	ynaI	Low conductance mechanosensitive channel YnaI	-10.23	4E-06

Appendix 4 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	ymfD	Bacillibactin exporter	-10.21	2E-03
17	ylxH	Flagellum site-determining protein YlxH	-9.51	1E-08
18	ykkD	Multidrug resistance protein YkkD	-9.51	1E-08
19	ykkC	Multidrug resistance protein YkkC	-9.49	1E-04
20	yjjP	Inner membrane protein YjjP	-9.31	3E-03
21	yihN_2	Inner membrane protein YihN	-9.30	3E-02
22	yihN_1	Inner membrane protein YihN	-9.17	6E-03
23	yigZ	IMPACT family member YigZ	-9.15	2E-02
24	yidD	Putative membrane protein insertion efficiency factor	-9.05	5E-04
25	yidC	Membrane protein insertase YidC	-8.99	2E-03
26	yicO	Putative permease YicO	-8.92	6E-03
27	yicL	putative inner membrane transporter YicL	-8.89	5E-04
28	yheS	putative ABC transporter ATP-binding protein YheS	-8.83	2E-11
29	yhbU	putative protease YhbU precursor	-8.79	4E-04
30	ygiC	Putative acid--amine ligase YgiC	-8.71	9E-07

Appendix 4 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	yflS	Putative malate transporter YflS	-8.70	3E-06
32	yfkO	Putative NAD(P)H nitroreductase YfkO	-8.63	6E-08
33	yfkJ	Low molecular weight protein-tyrosine-phosphatase YfkJ	-8.48	8E-04
34	yfiC	tRNA1(Val) (adenine(37)-N6)-methyltransferase	-8.48	4E-12
35	yedZ	Sulfoxide reductase heme-binding subunit YedZ	-8.47	4E-05
36	yedY	Sulfoxide reductase catalytic subunit YedY precursor	-8.44	4E-07
37	yecS	Inner membrane amino-acid ABC transporter permease protein YecS	-8.40	7E-04
38	ydhP_2	Inner membrane transport protein YdhP	-8.27	1E-03
39	ydhP_1	Inner membrane transport protein YdhP	-8.21	6E-18
40	ydfG	NADP-dependent 3-hydroxy acid dehydrogenase YdfG	-8.15	7E-05
41	yclQ	putative ABC transporter solute-binding protein YclQ precursor	-8.03	2E-04
42	ychF	Ribosome-binding ATPase YchF	-7.97	8E-04
43	ycfH	putative deoxyribonuclease YcfH	-7.92	1E-05
44	yceI	Protein YceI	-7.85	2E-13
45	yccM	Putative electron transport protein YccM	-7.78	3E-03

Appendix 4 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	yccA	Modulator of FtsH protease YccA	-7.57	1E-06
47	ycaD	putative MFS-type transporter YcaD	-7.56	3E-05
48	ybgC	Acyl-CoA thioester hydrolase YbgC	-7.47	3E-27
49	ybeY	Endoribonuclease YbeY	-7.42	2E-15
50	yajR	Inner membrane transport protein YajR	-7.42	1E-32
51	yajL	Chaperone protein YajL	-7.39	2E-03
52	yagU	Inner membrane protein YagU	-7.32	4E-03
53	yafP	putative N-acetyltransferase YafP	-7.20	4E-05
54	yabJ_2	Enamine/imine deaminase	-7.16	3E-29
55	yabJ_1	Enamine/imine deaminase	-7.01	2E-03
56	xseA	Exodeoxyribonuclease 7 large subunit	-6.97	1E-09
57	xpsD	Type II secretion system protein D precursor	-6.86	4E-04
58	xerD	Tyrosine recombinase XerD	-6.83	2E-03
59	xecE	2-(S)-hydroxypropyl-CoM dehydrogenase	-6.80	2E-05
60	wbpE	UDP-2-acetamido-2-deoxy-3-oxo-D-glucuronate aminotransferase	-6.74	1E-07

Appendix 4 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	waaA	3-deoxy-D-manno-octulosonic acid transferase	-6.68	6E-42
62	vhb	Bacterial hemoglobin	-6.67	8E-04
63	valS	Valine--tRNA ligase	-6.66	4E-09
64	uxaA	Altronate dehydratase	-6.62	1E-04
65	uvrD	DNA helicase II	-6.62	1E-06
66	uvrC	UvrABC system protein C	-6.60	3E-04
67	uvrB	UvrABC system protein B	-6.59	4E-12
68	uvrA	UvrABC system protein A	-6.59	9E-13
69	uup	ABC transporter ATP-binding protein uup	-6.51	3E-05
70	uppS	Isoprenyl transferase	-6.49	1E-14
71	uppP	Undecaprenyl-diphosphatase	-6.47	9E-03
72	upp	Uracil phosphoribosyltransferase	-6.47	1E-03
73	ung	Uracil-DNA glycosylase	-6.45	1E-02
74	UC78_1667	rRNA-5S ribosomal RNA	-6.42	1E-03
75	UC78_1666	rRNA-5S ribosomal RNA	-6.25	5E-17

Appendix 4 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	UC78_1665	rRNA-5S ribosomal RNA	-6.23	4E-06
77	UC78_1661	hypothetical protein	-6.22	2E-06
78	UC78_1660	hypothetical protein	-6.20	2E-27
79	UC78_1655	Acetyltransferase (GNAT) family protein	-6.07	8E-12
80	UC78_1654	tRNA-Val	-6.06	5E-03
81	UC78_1652	tRNA-Ser	-6.04	6E-16
82	UC78_1651	tRNA-Met	-6.04	1E-18
83	UC78_1650	hypothetical protein	-6.04	9E-12
84	UC78_1648	hypothetical protein	-6.02	4E-04
85	UC78_1646	Ribonuclease J 1	-6.02	8E-03
86	UC78_1621	Major Facilitator Superfamily protein	-5.96	6E-06
87	UC78_1618	Sodium/hydrogen exchanger family protein	-5.95	7E-09
88	UC78_1615	hypothetical protein	-5.93	3E-05
89	UC78_1609	hypothetical protein	-5.91	2E-05
90	UC78_1608	hypothetical protein	-5.90	4E-06

Appendix 4 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	UC78_1606	tRNA-Pro	-5.81	9E-04
92	UC78_1605	tRNA-His	-5.79	6E-04
93	UC78_1604	tRNA-Arg	-5.77	4E-03
94	UC78_1603	tRNA-Arg	-5.72	2E-05
95	UC78_1602	tRNA-Leu	-5.72	3E-16
96	UC78_1601	hypothetical protein	-5.69	5E-10
97	UC78_1600	hypothetical protein	-5.67	2E-03
98	UC78_1599	hypothetical protein	-5.63	2E-13
99	UC78_1596	FtsX-like permease family protein	-5.60	2E-60
100	UC78_1595	FtsX-like permease family protein	-5.59	4E-04
101	UC78_1594	hypothetical protein	-5.58	2E-03
102	UC78_1591	hypothetical protein	-5.57	8E-03
103	UC78_1590	hypothetical protein	-5.51	2E-04
104	UC78_1584	hypothetical protein	-5.50	3E-08
105	UC78_1583	hypothetical protein	-5.48	2E-02

APPENDIX 5

***C. jejuni* 12661 genes up-regulated of of filament vs exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	UC78_1330	hypothetical protein	62.18	0E+00
2	iutA	Ferric aerobactin receptor precursor	29.59	4E-14
3	katA	Catalase	20.82	0E+00
4	cirA	Colicin I receptor precursor	17.39	0E+00
5	UC78_1332	Ankyrin repeats (3 copies)	15.40	0E+00
6	UC78_1329	hypothetical protein	14.08	0E+00
7	tpd	34 kDa membrane antigen precursor	12.23	0E+00
8	UC78_1562	colicin uptake protein TolQ	6.72	5E-05
9	hmuU	Hemin transport system permease protein HmuU	6.26	3E-12
10	UC78_1543	hypothetical protein	4.98	0E+00
11	hmuV	Hemin import ATP-binding protein HmuV	4.69	9E-10
12	trxB	Thioredoxin reductase	4.62	2E-11
13	UC78_0400	Gluconate 2-dehydrogenase subunit 3 precursor	4.44	2E-13
14	nifS	Cysteine desulfurase	4.03	1E-07
15	btuF	Vitamin B12-binding protein precursor	3.94	0E+00

Appendix 5 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	torC	Cytochrome c-type protein TorC	3.90	4E-04
17	dnaE	DNA polymerase III subunit alpha	3.83	2E-07
18	futA1	Iron uptake protein A1 precursor	3.67	8E-09
19	torZ	Trimethylamine-N-oxide reductase 2 precursor	3.57	3E-15
20	fumC	Fumarate hydratase class II	3.52	0E+00
21	tsaA	putative peroxiredoxin	3.36	0E+00
22	ppk	Polyphosphate kinase	3.33	0E+00
23	iscU	NifU-like protein	3.15	2E-05
24	UC78_1563	biopolymer transport protein ExbD	3.02	2E-02
25	hemL	Glutamate-1-semialdehyde 2,1-aminomutase	2.86	2E-05
26	rplC	50S ribosomal protein L3	2.83	0E+00
27	greA	Transcription elongation factor GreA	2.80	0E+00
28	UC78_0033-	hypothetical protein	2.71	4E-02
29	rpmA	50S ribosomal protein L27	2.68	4E-04
30	guaA	GMP synthase-glutamine-hydrolyzing	2.67	7E-10

Appendix 5 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	tcyP	L-cystine uptake protein TcyP	2.65	3E-06
32	rplW	50S ribosomal protein L23	2.64	0E+00
33	ssrA	transfer-messenger RNA, SsrA	2.63	1E-10
34	lutA_1	Lactate utilization protein A	2.62	1E-03
35	UC78_0401	Gluconate 2-dehydrogenase flavoprotein precursor	2.61	5E-04
36	rplD	50S ribosomal protein L4	2.61	2E-03
37	coaE	Dephospho-CoA kinase	2.54	9E-03
38	UC78_0016	Disulfide bond formation protein DsbB	2.46	1E-03
39	UC78_0017	hypothetical protein	2.46	3E-02
40	yclQ	putative ABC transporter solute-binding protein YclQ precursor	2.43	3E-04
41	UC78_1250	Methyltransferase domain protein	2.40	2E-02
42	fdx	Ferredoxin	2.38	7E-13
43	UC78_0074	Bacteriohemerythrin	2.36	4E-03
44	rplU	50S ribosomal protein L21	2.33	1E-03
45	rplY	50S ribosomal protein L25	2.33	8E-03

Appendix 5 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	rpsJ	30S ribosomal protein S10	2.32	0E+00
47	UC78_1446	hypothetical protein	2.29	2E-02
48	UC78_1467	PBP superfamily domain protein	2.28	2E-05
49	rpsC	30S ribosomal protein S3	2.21	0E+00
50	typA	GTP-binding protein TypA/BipA	2.15	5E-03
51	rplB	50S ribosomal protein L2	2.13	2E-08
52	dps	DNA protection during starvation protein	2.13	6E-12
53	UC78_0699	hypothetical protein	2.13	2E-03
54	UC78_1174	hypothetical protein	2.12	0E+00
55	nifJ	Pyruvate-flavodoxin oxidoreductase	2.10	0E+00
56	purB	Adenylosuccinate lyase	2.09	1E-02
57	UC78_1173	Bacteriohemerythrin	2.08	3E-03
58	rpsS	30S ribosomal protein S19	2.08	0E+00
59	UC78_1445	hypothetical protein	2.06	4E-03
60	rplV	50S ribosomal protein L22	2.06	0E+00

Appendix 5 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	murA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	2.04	2E-05
62	UC78_0759	Alpha/beta hydrolase family protein	2.03	7E-03
63	pepA	Cytosol aminopeptidase	2.03	2E-06
64	aspS	Aspartate--tRNA ligase	2.02	7E-04
65	lutB	Lactate utilization protein B	2.01	2E-03
66	UC78_1655	Acetyltransferase (GNAT) family protein	2.01	0E+00
67	apt	Adenine phosphoribosyltransferase	2.00	9E-05
68	yagU	Inner membrane protein YagU	2.00	4E-02
69	fbp	Fructose-1,6-bisphosphatase class 1	2.00	5E-03
70	rpmC	50S ribosomal protein L29	1.97	0E+00
71	topA	DNA topoisomerase 1	1.95	1E-02
72	metK	S-adenosylmethionine synthase	1.94	5E-06
73	UC78_0606	hydrogenase 2 accessory protein HypG	1.94	4E-02
74	UC78_1243 (accB)	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	1.93	0E+00
75	rpsB	30S ribosomal protein S2	1.93	7E-03

Appendix 5 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	hldD	ADP-L-glycero-D-manno-heptose-6-epimerase	1.92	3E-04
77	UC78_0092	hypothetical protein	1.91	3E-03
78	ilvE	Branched-chain-amino-acid aminotransferase	1.91	1E-02
79	lutC	Lactate utilization protein C	1.90	2E-06
80	UC78_0557	Yqey-like protein	1.88	5E-05
81	UC78_0519	2-oxoglutarate-acceptor oxidoreductase subunit OorD	1.88	8E-03
82	rpsQ	30S ribosomal protein S17	1.88	0E+00
83	UC78_0760	hypothetical protein	1.87	1E-02
84	hydA_1	Quinone-reactive Ni/Fe-hydrogenase small chain precursor	1.87	3E-05
85	ywlF	Putative sugar phosphate isomerase YwlF	1.86	0E+00
86	UC78_0543	Ferredoxin--NADP reductase	1.85	2E-03
87	UC78_0800	hypothetical protein	1.84	4E-04
88	nudK	GDP-mannose pyrophosphatase NudK	1.83	4E-06
89	fabF	3-oxoacyl-[acyl-carrier-protein] synthase 2	1.82	8E-04
90	UC78_0588	polyphosphate kinase 2 (PPK2)	1.80	4E-06

Appendix 5 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	rplR	50S ribosomal protein L18	1.80	0E+00
92	UC78_0603	hypothetical protein	1.79	4E-03
93	UC78_0402	SprA-related family protein	1.79	2E-05
94	rplP	50S ribosomal protein L16	1.79	0E+00
95	UC78_0880	hypothetical protein	1.78	2E-02
96	rplN	50S ribosomal protein L14	1.77	0E+00
97	UC78_1129	hypothetical protein	1.76	5E-03
98	gatB	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	1.75	1E-04
99	frdC	Fumarate reductase cytochrome b subunit	1.73	5E-04
100	rpsF	30S ribosomal protein S6	1.72	6E-09
101	gltX	Glutamate--tRNA ligase	1.72	3E-06
102	yedY	Sulfoxide reductase catalytic subunit YedY precursor	1.71	4E-02
103	ribBA	Riboflavin biosynthesis protein RibBA	1.70	2E-05
104	UC78_0523	hypothetical protein	1.70	5E-04
105	zupT	Zinc transporter ZupT	1.69	1E-09

APPENDIX 6

***C. jejuni* 12661 genes down-regulated of filament vs exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	UC78_0841	tRNA-Leu	-33.99	1E-02
2	rbr1	Rubrerythrin-1	-33.26	2E-02
3	UC78_0322	hypothetical protein	-29.58	2E-10
4	UC78_1300	tRNA-Ser(gct)	-25.19	4E-20
5	UC78_0723	hypothetical protein	-20.31	5E-03
6	UC78_1648	hypothetical protein	-20.21	2E-15
7	UC78_1552	tRNA-Gly	-20.18	2E-03
8	UC78_0053	hypothetical protein	-19.38	3E-06
9	UC78_1193	hypothetical protein	-18.59	3E-07
10	UC78_0454	tRNA-Gly	-18.02	1E-05
11	UC78_0231	hypothetical protein	-17.99	5E-22
12	uxaA	Altronate dehydratase	-17.64	6E-03
13	trpE	Anthranilate synthase component 1	-17.54	5E-07
14	UC78_1566	hypothetical protein	-16.30	3E-02
15	UC78_1119	hypothetical protein	-16.22	5E-05

Appendix 6 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	oppD	Oligopeptide transport ATP-binding protein OppD	-15.13	4E-02
17	UC78_1400	hypothetical protein	-14.79	3E-08
18	UC78_0041	hypothetical protein	-14.71	3E-02
19	UC78_0095	hypothetical protein	-14.53	2E-37
20	UC78_0468	hypothetical protein	-14.25	2E-02
21	UC78_0452	tRNA-Thr	-14.11	2E-10
22	UC78_1273	hypothetical protein	-13.74	3E-12
23	lvr	Levodione reductase	-13.43	1E-02
24	UC78_1571	tetratricopeptide repeat protein	-12.38	4E-57
25	UC78_1584	hypothetical protein	-12.04	5E-02
26	trpGD	Bifunctional protein TrpGD	-11.66	1E-07
27	UC78_0469	hypothetical protein	-10.80	7E-03
28	rnhA	Ribonuclease HI	-10.65	2E-24
29	pglB	Undecaprenyl-diphosphooligosaccharide--protein glycotransferase	-10.64	7E-03
30	aroC	Chorismate synthase	-10.57	2E-02

Appendix 6 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	UC78_0755	Archaea bacterial proteins of unknown function	-10.53	1E-02
32	UC78_0344	Dihydroneopterin aldolase	-10.45	2E-02
33	UC78_1196	hypothetical protein	-10.37	6E-32
34	UC78_1565	hypothetical protein	-9.99	2E-02
35	UC78_1279	hypothetical protein	-9.88	3E-09
36	trpC_1	Tryptophan biosynthesis protein TrpCF	-9.69	9E-68
37	dapA_1	4-hydroxy-tetrahydrodipicolinate synthase	-9.67	8E-06
38	legG	GDP/UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing)	-9.62	2E-06
39	fucP	L-fucose-proton symporter	-9.57	1E-02
40	cusR	Transcriptional regulatory protein CusR	-9.45	2E-15
41	UC78_0724	hypothetical protein	-9.44	3E-03
42	UC78_0142	5-methylcytosine-specific restriction enzyme subunit McrC	-9.44	2E-02
43	UC78_0158	16S ribosomal RNA methyltransferase RsmE	-9.14	4E-02
44	UC78_1037	hypothetical protein	-9.12	2E-02
45	lldP	L-lactate permease	-9.12	1E-02

Appendix 6 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	macB	Macrolide export ATP-binding/permease protein MacB	-9.08	2E-57
47	UC78_1621	Major Facilitator Superfamily protein	-9.01	3E-09
48	UC78_0475	Amidohydrolase	-8.77	2E-02
49	kdgR	Pectin degradation repressor protein KdgR	-8.75	6E-03
50	legI	N,N'-diacetyllegionaminic acid synthase	-8.71	2E-02
51	fieF	Ferrous-iron efflux pump FieF	-8.64	2E-04
52	nikE	Nickel import ATP-binding protein NikE	-8.61	3E-02
53	pglI	GalNAc(5)-diNAcBac-PP-undecaprenol beta-1,3-glucosyltransferase	-8.58	7E-03
54	UC78_1022	Putative NAD(P)H nitroreductase	-8.28	1E-06
55	UC78_0411	hypothetical protein	-8.19	4E-02
56	UC78_0455	tRNA-Thr	-8.12	2E-13
57	UC78_1247	UC78_1247	-8.03	2E-41
58	UC78_1197	hypothetical protein	-8.00	7E-13
59	aroK	Shikimate kinase	-7.97	6E-03
60	UC78_0810	hypothetical protein	-7.96	4E-02

Appendix 6 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	UC78_0968	tRNA-Arg	-7.96	3E-07
62	recG	ATP-dependent DNA helicase RecG	-7.91	4E-03
63	UC78_1606	tRNA-Pro	-7.90	3E-02
64	dtpT_1	Di-/tripeptide transporter	-7.89	2E-23
65	trpB	Tryptophan synthase beta chain	-7.83	1E-41
66	UC78_0902	tRNA-Lys	-7.81	7E-75
67	epsJ_2	putative glycosyltransferase EpsJ	-7.77	5E-02
68	hepA	Heterocyst differentiation ATP-binding protein HepA	-7.77	3E-02
69	UC78_1191	hypothetical protein	-7.65	4E-02
70	UC78_0899	tRNA-Lys	-7.58	5E-68
71	UC78_0584	D-cysteine desulfhydrase	-7.53	1E-05
72	UC78_0554	tRNA-Met	-7.52	2E-09
73	UC78_1582	mce related protein	-7.50	1E-02
74	UC78_1581	putative ABC transporter ATP-binding protein	-7.32	2E-02
75	rluD_2	Ribosomal large subunit pseudouridine synthase D	-7.31	3E-92

Appendix 6 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	UC78_0545	hypothetical protein	-7.23	8E-03
77	yfkO	Putative NAD(P)H nitroreductase YfkO	-7.20	1E-06
78	UC78_0897	tRNA-Val	-7.13	5E-28
79	engB	putative GTP-binding protein EngB	-7.12	2E-11
80	miaA	tRNA dimethylallyltransferase	-7.08	1E-40
81	UC78_1414	PD-(D/E)XK nuclease superfamily protein	-7.06	7E-04
82	UC78_1413 (addA)	ATP-dependent helicase/nuclease subunit A	-7.04	4E-02
83	UC78_0453	tRNA-Tyr	-7.00	3E-08
84	UC78_0207	hypothetical protein	-6.99	9E-05
85	hddC_1	D-glycero-alpha-D-manno-heptose 1-phosphate guanylyltransferase	-6.97	1E-42
86	pglJ	N-acetylgalactosamine-N,N'-diacetylbacillosaminyl-diphospho-undecaprenol 4-alpha-N-acetylgalactosaminyltransferase	-6.96	6E-03
87	rbfA	Ribosome-binding factor A	-6.90	4E-02
88	UC78_1385	hypothetical protein	-6.88	1E-14
89	truC	tRNA pseudouridine synthase C	-6.84	2E-06
90	sbmA	Peptide antibiotic transporter SbmA	-6.77	3E-78

Appendix 6 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	UC78_0716	tRNA-Ile	-6.77	9E-03
92	UC78_0901	tRNA-Val	-6.70	3E-14
93	UC78_0161	6-pyruvoyl tetrahydropterin synthase	-6.68	2E-34
94	trpA	Tryptophan synthase alpha chain	-6.66	6E-65
95	cpxA	Sensor protein CpxA	-6.66	3E-06
96	UC78_0031	tRNA-Ile	-6.65	9E-03
97	cas1	CRISPR-associated endonuclease Cas1	-6.64	4E-05
98	pglH	GalNAc-alpha-(1->4)-GalNAc-alpha-(1->3)-diNAcBac-PP-undecaprenol alpha-1,4-N-acetyl-D-galactosaminyltransferase	-6.58	1E-02
99	UC78_0122	hypothetical protein	-6.57	5E-08
100	uvrC	UvrABC system protein C	-6.56	1E-05
101	UC78_0414	tRNA-Ile	-6.55	1E-02
102	UC78_1144	hypothetical protein	-6.53	3E-70
103	UC78_1161	hypothetical protein	-6.49	1E-02
104	UC78_0634	Aminopeptidase	-6.44	4E-05
105	UC78_1186	hypothetical protein	-6.41	2E-07

APPENDIX 7

***C. jejuni* PT14 genes up-regulated of spirals against exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_06725	hypothetical protein	101.22	5E-03
2	A911_07770	hemin uptake system outer membrane receptor	56.46	2E-05
3	A911_03660	ferric enterobactin uptake receptor	31.30	0E+00
4	A911_03670	heat shock protein GrpE	21.50	0E+00
5	dnaK	molecular chaperone DnaK	17.23	0E+00
6	A911_03590	periplasmic protein	12.62	4E-06
7	A911_00855	TonB-dependent colicin lipoprotein, putative	12.05	7E-03
8	A911_02485	ATP-dependent Clp protease ATP-binding subunit	11.98	0E+00
9	A911_05665	hypothetical protein	10.96	0E+00
10	groES	co-chaperonin GroES	10.38	8E-09
11	A911_05670	hypothetical protein	9.92	0E+00
12	A911_06720	hypothetical protein	8.98	9E-06
13	A911_t08380	tRNA-Met	8.02	2E-10
14	A911_07840	exbB/tolQ family transport protein	7.15	4E-03
15	groEL	chaperonin GroEL	7.05	6E-08

Appendix 7 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_03665	heat-inducible transcription repressor	6.67	0E+00
17	A911_07780	putative hemin uptake system ATP-binding protein	6.63	1E-08
18	rplC	50S ribosomal protein L3	5.77	0E+00
19	A911_00845	iron ABC transporter periplasmic iron-binding protein	5.74	1E-04
20	A911_03655	TonB transport protein	5.19	3E-08
21	rpsJ	30S ribosomal protein S10	5.04	0E+00
22	A911_00850	lipoprotein	4.87	2E-02
23	A911_06730	catalase	4.75	2E-06
24	A911_07495	membrane protein	4.72	7E-11
25	A911_t08382	tRNA-Phe	4.64	7E-06
26	A911_03650	transposase	4.41	1E-04
27	rplW	50S ribosomal protein L23	4.41	4E-13
28	A911_07055	hypothetical protein	4.33	7E-12
29	A911_04525	F0F1 ATP synthase subunit C	4.23	0E+00
30	A911_04170	FspA2	4.14	4E-04

Appendix 7 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	rplD	50S ribosomal protein L4	4.05	0E+00
32	A911_06735	ankyrin repeat protein	4.01	5E-10
33	A911_07845	biopolymer transport exbD protein	4.00	2E-04
34	A911_00235	hypothetical protein	3.97	5E-03
35	A911_07060	hypothetical protein	3.81	0E+00
36	rpmA	50S ribosomal protein L27	3.79	0E+00
37	A911_07765	heme iron utilization protein	3.73	5E-03
38	A911_t08366	tRNA-Leu	3.70	5E-03
39	A911_08185	50S ribosomal protein L29	3.70	1E-09
40	rpsS	30S ribosomal protein S19	3.57	1E-12
41	A911_08260	small hydrophobic protein	3.54	0E+00
42	rplP	50S ribosomal protein L16	3.53	2E-09
43	A911_00865	TonB system transport protein ExbB	3.42	4E-06
44	rplV	50S ribosomal protein L22	3.41	1E-08
45	A911_03680	hypothetical protein	3.40	0E+00

Appendix 7 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	rpsC	30S ribosomal protein S3	3.38	3E-08
47	rplB	50S ribosomal protein L2	3.30	4E-14
48	rpmI	50S ribosomal protein L35	3.21	0E+00
49	A911_00145	hypothetical protein	3.11	2E-04
50	rplN	50S ribosomal protein L14	3.11	6E-08
51	rpsI	30S ribosomal protein S9	3.09	6E-04
52	rpsQ	30S ribosomal protein S17	2.97	6E-08
53	rplM	50S ribosomal protein L13	2.95	1E-03
54	A911_00240	hypothetical protein	2.92	5E-03
55	rplX	50S ribosomal protein L24	2.90	5E-08
56	rpsF	30S ribosomal protein S6	2.83	0E+00
57	rpsN	30S ribosomal protein S14	2.80	1E-06
58	rplE	50S ribosomal protein L5	2.77	9E-07
59	A911_05930	sensor histidine kinase	2.76	2E-08
60	A911_01165	putative iron-binding protein	2.70	1E-09

Appendix 7 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	A911_r08420	rRNA-16S ribosomal RNA	2.70	3E-02
62	A911_00870	biopolymer transport ExbD protein	2.65	2E-04
63	A911_t08356	tRNA-Gln	2.63	5E-03
64	A911_07775	putative hemin uptake system permease protein	2.62	4E-07
65	A911_r08418	rRNA-5S ribosomal RNA	2.60	8E-09
66	A911_r08414	rRNA-5S ribosomal RNA	2.59	7E-09
67	A911_07855	hypothetical protein	2.58	4E-04
68	A911_r08416	rRNA-5S ribosomal RNA	2.58	7E-09
69	A911_05180	single-stranded DNA-binding protein	2.57	0E+00
70	rplT	50S ribosomal protein L20	2.54	0E+00
71	rplS	50S ribosomal protein L19	2.53	0E+00
72	rpsR	30S ribosomal protein S18	2.53	0E+00
73	A911_03685	hypothetical protein	2.51	0E+00
74	A911_00745	tetrapyrrole methylase family protein	2.51	3E-02
75	A911_07810	riboflavin-specific deaminase/reductase	2.47	5E-09

Appendix 7 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	A911_04745	Surface-exposed lipoprotein JlpA	2.47	0E+00
77	A911_03965	putative lipoprotein	2.46	1E-02
78	rpsO	30S ribosomal protein S15	2.40	0E+00
79	A911_04380	hypothetical protein	2.39	5E-16
80	A911_08080	lipoprotein	2.35	0E+00
81	A911_r08422	rRNA-16S ribosomal RNA	2.34	3E-02
82	A911_05815	2OG-Fe(II) oxygenase	2.34	2E-05
83	A911_02795	putative GatB/Yqey family protein	2.33	0E+00
84	rpmE	50S ribosomal protein L31	2.32	8E-04
85	A911_02790	bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein	2.32	0E+00
86	rpsT	30S ribosomal protein S20	2.32	5E-03
87	A911_t08386	tRNA-Sec	2.32	4E-07
88	A911_r08424	rRNA-16S ribosomal RNA	2.30	2E-02
89	rplU	50S ribosomal protein L21	2.30	0E+00
90	rplF	50S ribosomal protein L6	2.27	2E-04

Appendix 7 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	rplO	50S ribosomal protein L15	2.26	8E-05
92	A911_06585	enterochelin ABC transporter, periplasmic enterochelin-binding protein	2.26	0E+00
93	A911_00585	hypothetical protein	2.20	1E-02
94	rpsB	30S ribosomal protein S2	2.20	0E+00
95	secG	preprotein translocase subunit SecG	2.17	0E+00
96	rplR	50S ribosomal protein L18	2.17	5E-04
97	rpsE	30S ribosomal protein S5	2.12	4E-04
98	A911_07665	30S ribosomal protein S11	2.11	6E-09
99	rpsH	30S ribosomal protein S8	2.10	8E-04
100	A911_t08354	tRNA-Met	2.10	4E-02
101	A911_02665	flagellar protein FlaG	2.08	7E-09
102	A911_07990	putative FTR1 family iron permease	2.08	2E-04
103	A911_02785	transcriptional regulator	2.06	9E-04
104	A911_02030	hypothetical protein	2.06	4E-04
105	A911_08035	hypothetical protein	2.05	1E-08

APPENDIX 8

***C. jejuni* PT14 genes Down-regulated of spirals against exponential cells**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_07930	putative ABC transport system permease	-19.80	7E-23
2	aroE	shikimate 5-dehydrogenase	-19.50	1E-148
3	A911_07535	NADH-quinone oxidoreductase, M subunit	-18.86	1E-188
4	A911_07530	NADH-quinone oxidoreductase, N subunit	-16.83	3E-100
5	A911_05465	general glycosylation pathway protein	-13.35	2E-38
6	A911_02980	periplasmic protein	-12.77	1E-44
7	A911_00570	putative pyrazinamidase/nicotinamidase	-12.64	4E-77
8	A911_00810	hypothetical protein	-12.47	1E-85
9	A911_06895	class I glutamine amidotransferase, putative	-11.67	7E-151
10	A911_00155	Putative integral membrane protein	-11.53	1E-18
11	A911_05325	hypothetical protein	-11.34	2E-45
12	A911_07750	putative phosphatidylglycerophosphatase	-11.32	1E-63
13	A911_07620	peptide ABC transporter, periplasmic peptide-binding protein	-11.29	3E-14
14	A911_05470	Alpha-1,4-N-acetylgalactosamine transferase PglH	-11.29	5E-59
15	A911_06460	putative sugar-phosphate nucleotide transferase	-11.24	1E-48

Appendix 8 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_06330	3-oxoacyl-(acyl carrier protein) synthase III	-11.20	2E-38
17	A911_00555	malonyl CoA-acyl carrier protein transacylase	-10.71	2E-66
18	A911_00575	recombination protein RecO	-10.66	3E-52
19	A911_01850	orotidine 5'-phosphate decarboxylase	-10.42	8E-67
20	A911_05365	ATP-dependent Clp protease, ATP-binding subunit ClpA	-10.32	7E-127
21	A911_07615	peptide ABC transporter permease	-10.31	2E-10
22	A911_06925	putative CDP glycerol glycerophosphotransferase	-10.23	3E-60
23	A911_00715	hypothetical protein	-10.21	7E-18
24	A911_06480	PseD protein	-10.16	2E-51
25	A911_00720	homoserine dehydrogenase	-10.00	2E-47
26	A911_07540	NADH-ubiquinone oxidoreductase subunit K	-9.81	2E-114
27	A911_05950	two-component sensor (histidine kinase)	-9.38	1E-66
28	A911_06918	hypothetical protein	-9.35	3E-47
29	A911_01610	ferredoxin, 4Fe-4S	-9.21	1E-206
30	A911_02975	hypothetical protein	-9.17	9E-24

Appendix 8 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	A911_05330	ATP-dependent DNA helicase, UvrD/REP family protein	-9.15	1E-15
32	A911_00695	Methyl-accepting chemotaxis signal transduction protein	-9.05	0E+00
33	A911_01675	anthranilate synthase component II	-8.96	3E-104
34	A911_06930	hypothetical protein	-8.90	2E-34
35	A911_04900	high affinity branched-chain amino acid ABC transporter ATP-binding protein	-8.84	7E-53
36	A911_02475	Maf-like protein	-8.81	5E-16
37	A911_07135	recombination protein RecB	-8.75	3E-17
38	A911_01240	diacylglycerol kinase	-8.72	3E-43
39	A911_00055	rubrerythrin	-8.68	2E-154
40	A911_00665	hypothetical protein	-8.64	1E-17
41	A911_07925	putative ABC transport system permease	-8.59	4E-63
42	A911_06455	UDP-N-acetylglucosamine 2-epimerase	-8.41	2E-114
43	A911_01680	N-(5'phosphoribosyl)anthranilate isomerase	-8.39	1E-129
44	A911_06090	polynucleotide phosphorylase/polyadenylase	-8.38	8E-101
45	trpA	tryptophan synthase subunit alpha	-8.18	3E-183

Appendix 8 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	A911_06920	putative sugar transferase	-8.15	7E-66
47	A911_01685	tryptophan synthase subunit beta	-8.10	2E-187
48	A911_06415	DegT family aminotransferase	-8.06	2E-43
49	A911_06900	hypothetical protein	-8.04	3E-230
50	A911_08235	putative ribosomal pseudouridine synthase	-8.00	6E-22
51	A911_05105	putative integral membrane protein	-7.95	6E-29
52	A911_04325	3-phosphoshikimate 1-carboxyvinyltransferase	-7.75	4E-63
53	A911_00755	16S ribosomal RNA methyltransferase RsmE	-7.70	2E-16
54	A911_01965	membrane protein	-7.69	4E-275
55	A911_06511	Motility accessory factor (missing A911_06510)	-7.64	6E-38
56	fliH	flagellar assembly protein H	-7.50	3E-35
57	A911_02985	poly(beta-D-mannuronate) O-acetylase	-7.49	8E-66
58	A911_05455	oligosaccharide transferase	-7.27	2E-47
59	A911_07710	hypothetical protein	-7.18	1E-33
60	A911_00525	exbD/tolR family transport protein	-7.18	9E-71

Appendix 8 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	A911_04895	high affinity branched-chain amino acid ABC transporter ATP-binding protein	-7.14	2E-62
62	A911_04780	Iron-sulfur cluster-binding domain protein	-7.09	2E-45
63	A911_05450	GalNAc transferase	-7.09	1E-37
64	purH	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-7.00	5E-16
65	A911_07745	sulfate adenylyltransferase	-6.97	2E-94
66	A911_04500	argininosuccinate lyase	-6.92	5E-118
67	A911_02290	amino-acid ABC transporter ATP-binding protein	-6.92	3E-66
68	A911_01960	hypothetical protein	-6.90	5E-157
69	A911_03745	ABC transporter permease	-6.88	5E-118
70	A911_6510	Motility accessory factor	-6.86	2E-40
71	A911_05660	putative integral membrane protein (dedA-like protein)	-6.83	6E-202
72	A911_06685	hypothetical protein	-6.76	1E-34
73	A911_00345	hypothetical protein	-6.75	3E-225
74	A911_06465	hypothetical protein	-6.74	4E-69
75	A911_06935	putative glycerol-3-phosphate cytidylyltransferase	-6.73	2E-103

Appendix 8 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	A911_01550	1-deoxy-D-xylulose-5-phosphate synthase	-6.70	5E-31
77	A911_00730	Putative periplasmic protein	-6.63	9E-37
78	A911_04890	putative cytochrome C biogenesis protein	-6.59	2E-99
79	A911_01245	putative bacterial regulatory protein, ArsR family	-6.55	3E-17
80	A911_07105	transformation system protein	-6.52	2E-08
81	A911_07560	NADH:ubiquinone oxidoreductase subunit H	-6.51	1E-106
82	A911_05320	oligoendopeptidase F	-6.46	3E-52
83	A911_02340	DNA-directed RNA polymerase subunit beta'	-6.45	1E-136
84	A911_02005	GTP-binding protein	-6.45	5E-16
85	A911_06655	phosphoribosyltransferase	-6.44	8E-95
86	A911_01470	hypothetical protein	-6.42	5E-04
87	A911_05155	Oxygen-insensitive NAD(P)H nitroreductase / Dihydropteridine reductase	-6.40	3E-94
88	A911_00725	aspartate aminotransferase	-6.38	2E-97
89	A911_05500	putative two-domain glucosyltransferase	-6.36	3E-20
90	A911_06650	xanthine/uracil permease family protein	-6.35	4E-143

Appendix 8 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	ispH	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	-6.24	1E-56
92	rbfA	ribosome-binding factor A	-6.23	5E-30
93	A911_00160	hypothetical protein	-6.23	1E-18
94	A911_00300	ParaA family ATPase	-6.18	3E-61
95	A911_00560	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	-6.16	2E-216
96	A911_05995	Peptidase, M23/M37 family protein	-6.15	6E-42
97	A911_05435	hypothetical protein	-6.10	9E-43
98	A911_05475	ABC transporter permease	-6.08	3E-22
99	A911_04175	para-aminobenzoate synthase glutamine amidotransferase, component II	-6.08	1E-03
100	A911_06909	Capsular polysaccharide biosynthesis protein	-6.06	9E-61
101	A911_06095	G:T/U mismatch-specific uracil/thymine DNA-glycosylase	-6.04	4E-46
102	A911_04905	high affinity branched-chain amino acid ABC transporter permease	-6.03	3E-55
103	A911_01955	serine hydroxymethyltransferase	-6.02	5E-285
104	A911_08045	Putative periplasmic protein	-6.02	5E-92
105	ddl	D-alanyl-alanine synthetase A	-6.00	7E-30

APPENDIX 9

***C. jejuni* PT14 genes up-regulated of filament against spirals cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_00525	exbD/tolR family transport protein	13.37	0E+00
2	A911_07775	putative hemin uptake system permease protein	13.14	2E-04
3	A911_07780	putative hemin uptake system ATP-binding protein	12.48	4E-04
4	A911_07930	putative ABC transport system permease	11.60	3E-12
5	A911_00570	putative pyrazinamidase/nicotinamidase	11.05	0E+00
6	A911_06930	hypothetical protein	9.21	0E+00
7	A911_00575	recombination protein RecO	8.54	0E+00
8	A911_00870	biopolymer transport ExbD protein	8.21	2E-04
9	A911_06720	hypothetical protein	7.98	2E-03
10	A911_00875	TonB-dependent colicin receptor protein	7.81	1E-05
11	A911_00715	hypothetical protein	7.77	2E-11
12	A911_02980	periplasmic protein	7.42	6E-13
13	A911_08010	integral membrane protein	7.32	2E-05
14	A911_07095	putative ATP/GTP-binding protein	7.13	2E-13
15	A911_00520	ExbB/tolQ family transport protein	6.99	0E+00

Appendix 9 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_02540	hypothetical protein	6.99	0E+00
17	A911_06735	ankyrin repeat protein	6.80	1E-03
18	A911_00530	periplasmic protein	6.65	0E+00
19	A911_02450	ammonium transporter	6.46	1E-03
20	A911_06925	putative CDP glycerol glycerophosphotransferase	6.44	0E+00
21	A911_t08338	tRNA-Ala	6.44	6E-10
22	A911_01290	Putative integral membrane protein	6.34	0E+00
23	A911_01850	orotidine 5'-phosphate decarboxylase	6.29	0E+00
24	A911_t08332	tRNA-Ala	6.20	1E-10
25	A911_05980	hypothetical protein	6.20	1E-11
26	A911_04515	putative sodium:amino-acid symporter family protein	6.16	2E-04
27	A911_07105	transformation system protein	6.16	2E-08
28	A911_t08354	tRNA-Met	5.97	0E+00
29	A911_07100	General secretory pathway protein D	5.91	0E+00
30	A911_t08358	tRNA-Ala	5.90	1E-10

Appendix 9 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	A911_04325	3-phosphoshikimate 1-carboxyvinyltransferase	5.90	0E+00
32	A911_01010	D12 class N6 adenine-specific DNA methyltransferase	5.89	0E+00
33	A911_00730	Putative periplasmic protein	5.88	2E-12
34	A911_00720	homoserine dehydrogenase	5.83	3E-10
35	A911_07535	NADH-quinone oxidoreductase, M subunit	5.76	0E+00
36	hemH	ferrochelatase	5.74	2E-11
37	A911_07090	hypothetical protein	5.71	5E-09
38	A911_t08340	tRNA-Ile	5.71	0E+00
39	A911_05325	hypothetical protein	5.66	4E-13
40	A911_t08360	tRNA-Ile	5.65	0E+00
41	A911_07785	putative hemin uptake system periplasmic hemin-binding protein	5.61	2E-04
42	A911_00505	F0F1 ATP synthase subunit gamma	5.51	2E-08
43	A911_03660	ferric enterobactin uptake receptor	5.38	4E-04
44	A911_06935	putative glycerol-3-phosphate cytidylyltransferase	5.17	3E-14
45	A911_07085	type II protein secretion system E protein	5.12	3E-04

Appendix 9 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	A911_03420	peptidase, U32 family protein	5.11	0E+00
47	A911_00865	TonB system transport protein ExbB	5.10	3E-04
48	A911_04310	hypothetical protein	5.09	4E-11
49	A911_07135	recombination protein RecB	5.09	3E-08
50	A911_07295	Tat pathway signal sequence domain-containing protein	5.03	0E+00
51	A911_07845	biopolymer transport exbD protein	5.03	2E-03
52	A911_06085	hypothetical protein	5.02	0E+00
53	A911_00155	Putative integral membrane protein	4.96	3E-06
54	A911_02855	dihydropteroate synthase	4.94	2E-14
55	A911_t08334	tRNA-Ile	4.93	0E+00
56	A911_07530	NADH-quinone oxidoreductase, N subunit	4.92	0E+00
57	A911_00085	disulfide bond formation protein	4.90	2E-13
58	A911_08235	putative ribosomal pseudouridine synthase	4.88	0E+00
59	A911_02985	poly(beta-D-mannuronate) O-acetylase	4.87	1E-06
60	A911_07925	putative ABC transport system permease	4.86	0E+00

ppendix 9 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	A911_00035	glutamate synthase, large subunit	4.86	2E-04
62	A911_06920	putative sugar transferase	4.85	9E-14
63	A911_03430	hypothetical protein	4.80	2E-15
64	A911_06090	polynucleotide phosphorylase/polyadenylase	4.78	0E+00
65	A911_07710	hypothetical protein	4.76	0E+00
66	A911_03965	putative lipoprotein	4.75	4E-04
67	A911_08240	metallo-beta-lactamase family protein	4.72	0E+00
68	A911_00555	malonyl CoA-acyl carrier protein transacylase	4.72	0E+00
69	A911_01165	putative iron-binding protein	4.71	0E+00
70	purH	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	4.68	8E-09
71	A911_01845	hypothetical protein	4.68	2E-05
72	infC	translation initiation factor IF-3	4.63	0E+00
73	A911_02975	hypothetical protein	4.61	5E-09
74	A911_03475	16S rRNA processing protein RimM	4.60	2E-10
75	mnmC	bifunctional tRNA (mnm(5)s(2)U34)-methyltransferase/FAD-dependent cmnm(5)s(2)U34 oxidoreductase	4.56	1E-07

Appendix 9 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	A911_06520	Type II secretion envelope pseudopilin protein, PulG	4.54	4E-08
77	A911_01470	hypothetical protein	4.53	1E-02
78	A911_03470	hypothetical protein	4.53	7E-07
79	A911_03035	[NiFe] hydrogenase maturation protein HypF	4.48	0E+00
80	A911_01970 (aroE)	shikimate 5-dehydrogenase	4.46	0E+00
81	A911_02005	GTP-binding protein	4.46	1E-09
82	A911_07080	general secretory pathway protein F	4.46	5E-03
83	A911_02475	Maf-like protein	4.46	3E-06
84	A911_04175	para-aminobenzoate synthase glutamine amidotransferase, component II	4.45	2E-02
85	A911_00835	iron ABC transporter ATP binding subunit	4.44	8E-04
86	A911_05985	putative HAD-superfamily hydrolase	4.43	2E-15
87	A911_02460	Gfo/Idh/MocA family oxidoreductase	4.39	0E+00
88	A911_01560	hypothetical protein	4.38	3E-03
89	A911_06515	hypothetical protein	4.36	2E-09
90	A911_02515	periplasmic protein	4.35	2E-10

Appendix 9 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	A911_04900	high affinity branched-chain amino acid ABC transporter ATP-binding protein	4.34	0E+00
92	A911_t08336	tRNA-Glu	4.34	8E-05
93	secY	preprotein translocase subunit SecY	4.29	0E+00
94	A911_07140	hypothetical protein	4.25	0E+00
95	A911_t08342	tRNA-Thr	4.25	1E-09
96	A911_05270	hypothetical protein	4.24	2E-15
97	A911_02520	1-acyl-sn-glycerol-3-phosphate acyltransferase	4.23	0E+00
98	A911_03440	hypothetical protein	4.21	0E+00
99	A911_05330	ATP-dependent DNA helicase, UvrD/REP family protein	4.20	2E-05
100	A911_00580	metal-binding heat shock protein	4.18	2E-07
101	A911_08005	ABC transporter permease	4.17	1E-04
102	A911_03165	hypothetical protein	4.17	0E+00
103	A911_01355	transaldolase	4.14	0E+00
104	A911_05995	Peptidase, M23/M37 family protein	4.13	0E+00
105	ksgA	16S ribosomal RNA methyltransferase KsgA/Dim1 family protein	4.11	0E+00

APPENDIX 10

***C. jejuni* PT14 genes Down-regulated of filament against spirals cells**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_07060	hypothetical protein	-5.76	7E-58
2	A911_04170	FspA2	-5.56	2E-04
3	A911_07055	hypothetical protein	-4.68	5E-10
4	A911_06470	Legionaminic acid cytidylyltransferase	-4.02	1E-06
5	A911_00220	Hemerythrin-like iron-binding protein	-3.82	5E-13
6	flgI	flagellar basal body P-ring protein	-3.71	3E-05
7	A911_02025	hypothetical protein	-3.65	9E-05
8	A911_07855	hypothetical protein	-3.56	5E-05
9	A911_06970	putative ATP/GTP-binding protein	-3.53	3E-08
10	A911_01900	hypothetical protein	-3.51	3E-05
11	A911_02030	hypothetical protein	-3.47	2E-06
12	A911_07050	hypothetical protein	-3.41	1E-06
13	A911_02665	flagellar protein FlaG	-3.32	1E-08
14	A911_07860	hypothetical protein	-3.29	8E-04
15	flgH	flagellar basal body L-ring protein	-3.25	2E-04

Appendix 10 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_03740	NLPA family lipoprotein	-3.24	4E-06
17	A911_03590	periplasmic protein	-3.15	1E-03
18	A911_06030	hypothetical protein	-3.05	4E-06
19	groES	co-chaperonin GroES	-3.02	3E-05
20	A911_03665	heat-inducible transcription repressor	-3.00	8E-29
21	A911_07980	hypothetical protein	-2.91	4E-04
22	flgD	flagellar basal body rod modification protein	-2.86	4E-06
23	A911_06505	flagellin	-2.77	1E-10
24	A911_05815	2OG-Fe(II) oxygenase	-2.77	4E-04
25	A911_00265	hypothetical protein	-2.75	1E-09
26	A911_06280	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	-2.63	2E-07
27	flgB	flagellar basal body rod protein FlgB	-2.59	1E-03
28	flgG	flagellar basal body rod protein FlgG	-2.54	4E-06
29	flgK	flagellar hook-associated protein FlgK	-2.52	4E-09
30	A911_08280	3-isopropylmalate dehydrogenase	-2.45	7E-03

Appendix 10 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	A911_04285	lagellar hook-associated protein FlgL	-2.44	4E-03
32	A911_03400	flagellar basal-body rod protein	-2.39	7E-04
33	A911_06975	dUTPase	-2.33	6E-10
34	A911_05820	putative NLPA family lipoprotein	-2.30	3E-03
35	A911_04450	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein	-2.28	4E-11
36	A911_02670	flagellar capping protein	-2.27	2E-06
37	A911_04715	hypothetical protein	-2.22	9E-03
38	A911_06400	flagellin modification protein, PseA	-2.21	2E-07
39	A911_03735	NLPA family lipoprotein	-2.16	1E-03
40	A911_08285	2-isopropylmalate synthase	-2.16	6E-03
41	A911_00585	hypothetical protein	-2.15	3E-02
42	A911_04740	Putative amino-acid transporter periplasmic solute-binding protein	-2.13	1E-25
43	groEL	chaperonin GroEL	-2.09	1E-03
44	A911_00260	hypothetical protein	-2.07	1E-05
45	A911_00970	hypothetical protein	-2.07	2E-05

Appendix 10 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	A911_04950	hypothetical protein	-2.07	1E-06
47	A911_03670	heat shock protein GrpE	-2.05	9E-08
48	A911_04955	hypothetical protein	-2.05	3E-06
49	flgE	flagellar hook protein FlgE	-1.99	1E-06
50	A911_06285	C4 aminotransferase	-1.98	3E-04
51	A911_00195	hypothetical protein	-1.98	1E-02
52	A911_06795	ferrous iron transport protein A	-1.98	3E-07
53	A911_01460	molybdate transport system substrate-binding protein	-1.95	4E-07
54	A911_02135	succinate dehydrogenase, iron-sulfur protein subunit	-1.95	2E-04
55	A911_00180	cytochrome C	-1.92	1E-15
56	A911_06490	motility accessory factor	-1.92	1E-02
57	A911_00815	superoxide dismutase	-1.91	2E-10
58	A911_07495	membrane protein	-1.90	3E-04
59	A911_05810	S-ribosylhomocysteinase	-1.90	8E-03
60	A911_r08418	rRNA-5S ribosomal RNA	-1.84	1E-02

Appendix 10 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	A911_r08416	rRNA-5S ribosomal RNA	-1.84	1E-02
62	A911_r08414	rRNA-5S ribosomal RNA	-1.84	1E-02
63	A911_02485	ATP-dependent Clp protease ATP-binding subunit	-1.84	6E-15
64	A911_06475	flagellin modification protein A	-1.79	5E-03
65	A911_02225	hypothetical protein	-1.78	5E-05
66	A911_02065	putative acidic periplasmic protein	-1.72	6E-47
67	A911_07795	alpha-ketoglutarate permease	-1.71	9E-25
68	A911_02140	succinate dehydrogenase subunit C	-1.71	1E-02
69	A911_04445	ABC-type amino-acid transporter permease	-1.69	1E-05
70	A911_02130	succinate dehydrogenase flavoprotein subunit	-1.66	8E-05
71	dnaK	molecular chaperone DnaK	-1.65	2E-04
72	A911_07400	acetyl-CoA synthetase	-1.65	5E-07
73	A911_05200	flagellar assembly protein FliW	-1.65	9E-11
74	A911_08030	hypothetical protein	-1.63	5E-03
75	A911_02070	periplasmic protein	-1.62	9E-33

Appendix 10 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	A911_00200	hypothetical protein	-1.60	5E-02
77	A911_04140	glutamate-1-semialdehyde aminotransferase	-1.60	6E-15
78	A911_01780	RND efflux system, membrane fusion protein CmeA	-1.60	4E-03
79	A911_04800	delta-aminolevulinic acid dehydratase	-1.59	4E-02
80	flgC	flagellar basal body rod protein FlgC	-1.58	2E-03
81	A911_05700	Sec-independent protein translocase (TatA/E-like protein)	-1.58	2E-05
82	A911_03515	flagellin	-1.57	8E-07
83	rplI	50S ribosomal protein L9	-1.57	4E-03
84	A911_00360	L-lactate permease	-1.56	7E-43
85	aspA	aspartate ammonia-lyase	-1.56	1E-02
86	A911_04240	hypothetical protein	-1.55	4E-04
87	A911_00165	periplasmic protein	-1.55	2E-06
88	A911_02085	hypothetical protein	-1.55	8E-03
89	A911_05355	periplasmic thioredoxin	-1.54	3E-02
90	sucC	succinyl-CoA synthetase subunit beta	-1.51	4E-04

Appendix 10 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	A911_07265	formate dehydrogenase accessory protein	-1.51	3E-05
92	A911_03240	argininosuccinate synthase	-1.50	1E-02
93	A911_05855	putative lipoprotein thioredoxin	-1.50	1E-04

APPENDIX 11

***C. jejuni* PT14 genes up-regulated of filament against exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_06725	hypothetical protein	289.97	2E-05
2	A911_07770	hemin uptake system outer membrane receptor	192.92	1E-05
3	A911_03660	ferric enterobactin uptake receptor	168.42	1E-05
4	A911_07780	putative hemin uptake system ATP-binding protein	82.69	1E-04
5	A911_06720	hypothetical protein	71.61	4E-04
6	A911_07775	putative hemin uptake system permease protein	34.35	7E-05
7	A911_05670	hypothetical protein	31.41	1E-07
8	A911_06735	ankyrin repeat protein	27.24	3E-04
9	A911_05665	hypothetical protein	25.13	7E-10
10	A911_07840	exbB/tolQ family transport protein	22.67	4E-05
11	A911_00870	biopolymer transport ExbD protein	21.80	5E-05
12	A911_07845	biopolymer transport exbD protein	20.09	1E-04
13	A911_00865	TonB system transport protein ExbB	17.46	2E-05
14	A911_00855	TonB-dependent colicin lipoprotein, putative	16.65	4E-07
15	A911_06730	catalase	15.45	9E-05

Appendix 11 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_07765	heme iron utilization protein	13.93	3E-05
17	A911_01165	putative iron-binding protein	12.75	0E+00
18	A911_t08354	tRNA-Met	12.52	0E+00
19	A911_03965	putative lipoprotein	11.70	2E-05
20	A911_03670	heat shock protein GrpE	10.46	0E+00
21	dnaK	molecular chaperone DnaK	10.44	0E+00
22	A911_00085	disulfide bond formation protein	9.74	0E+00
23	A911_03655	TonB transport protein	9.33	2E-05
24	A911_t08406	tRNA-Met	8.59	1E-03
25	A911_t08382	tRNA-Phe	8.35	1E-03
26	A911_07785	putative hemin uptake system periplasmic hemin-binding protein	8.28	4E-05
27	A911_00080	DsbB family disulfide bond formation protein	7.46	2E-12
28	rplM	50S ribosomal protein L13	7.44	0E+00
29	A911_t08366	tRNA-Leu	7.37	0E+00
30	rpsI	30S ribosomal protein S9	7.30	5E-14

Appendix 11 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	A911_t08380	tRNA-Met	6.61	2E-11
32	A911_02485	ATP-dependent Clp protease ATP-binding subunit	6.51	0E+00
33	A911_00845	iron ABC transporter periplasmic iron-binding protein	6.45	7E-06
34	A911_03970	small hydrophobic protein	6.39	7E-04
35	rpmB	50S ribosomal protein L28	6.38	0E+00
36	A911_08260	small hydrophobic protein	6.33	0E+00
37	A911_00860	TonB-dependent colicin receptor protein	5.83	5E-05
38	A911_07850	putative TonB transport protein	5.83	1E-04
39	A911_t08358	tRNA-Ala	5.78	8E-11
40	A911_t08338	tRNA-Ala	5.75	8E-10
41	A911_t08332	tRNA-Ala	5.68	1E-10
42	rplW	50S ribosomal protein L23	5.67	0E+00
43	rplS	50S ribosomal protein L19	5.67	0E+00
44	rplD	50S ribosomal protein L4	5.52	0E+00
45	rplC	50S ribosomal protein L3	5.32	0E+00

Appendix 11 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	A911_03645	ISCco1, transposase orfB	5.14	4E-03
47	A911_03650	transposase	5.08	0E+00
48	rplB	50S ribosomal protein L2	5.07	0E+00
49	A911_t08336	tRNA-Glu	5.06	7E-05
50	A911_t08356	tRNA-Gln	5.03	5E-04
51	A911_01715	Ferredoxin	4.88	8E-13
52	A911_06570	enterochelin ABC transporter, permease protein	4.88	8E-05
53	A911_03680	hypothetical protein	4.85	0E+00
54	rplV	50S ribosomal protein L22	4.39	0E+00
55	rpsC	30S ribosomal protein S3	4.37	0E+00
56	rpsS	30S ribosomal protein S19	4.36	0E+00
57	A911_06025	transporter	4.32	0E+00
58	rpsJ	30S ribosomal protein S10	4.31	0E+00
59	rpsQ	30S ribosomal protein S17	4.25	0E+00
60	A911_00850	lipoprotein	4.20	2E-05

Appendix 11 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	rpmE	50S ribosomal protein L31	4.12	0E+00
62	A911_08185	50S ribosomal protein L29	4.06	0E+00
63	rplP	50S ribosomal protein L16	4.04	0E+00
64	A911_00145	hypothetical protein	4.02	0E+00
65	A911_04745	Surface-exposed lipoprotein JlpA	4.01	0E+00
66	A911_03590	periplasmic protein	4.00	1E-06
67	A911_02450	ammonium transporter	3.98	4E-03
68	A911_00495	F0F1 ATP synthase subunit delta	3.96	9E-09
69	rpmJ	50S ribosomal protein L36	3.96	3E-07
70	infA	translation initiation factor IF-1	3.96	0E+00
71	rplO	50S ribosomal protein L15	3.95	0E+00
72	A911_07810	riboflavin-specific deaminase/reductase	3.91	2E-14
73	A911_05765	Signal transduction protein CetB	3.88	1E-08
74	rplE	50S ribosomal protein L5	3.88	0E+00
75	A911_00240	hypothetical protein	3.87	5E-05

Appendix 11 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	rplN	50S ribosomal protein L14	3.86	0E+00
77	A911_00235	hypothetical protein	3.83	8E-03
78	coaE	dephospho-CoA kinase	3.81	0E+00
79	rpmA	50S ribosomal protein L27	3.79	0E+00
80	A911_01270	zinc transporter ZupT	3.78	0E+00
81	rplR	50S ribosomal protein L18	3.73	0E+00
82	prfA	peptide chain release factor 1	3.70	0E+00
83	A911_02785	transcriptional regulator	3.63	7E-13
84	rplX	50S ribosomal protein L24	3.62	0E+00
85	fliP	flagellar biosynthesis protein FliP	3.59	1E-04
86	rpsE	30S ribosomal protein S5	3.58	0E+00
87	rplF	50S ribosomal protein L6	3.48	0E+00
88	A911_03685	hypothetical protein	3.47	0E+00
89	A911_07665	30S ribosomal protein S11	3.45	7E-11
90	groES	co-chaperonin GroES	3.43	0E+00

Appendix 11 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	rpsM	30S ribosomal protein S13	3.43	1E-10
92	A911_06230	putative K+ uptake protein	3.42	0E+00
93	A911_00490	F0F1 ATP synthase subunit B	3.41	1E-08
94	A911_04525	F0F1 ATP synthase subunit C	3.39	0E+00
95	A911_00745	tetrapyrrole methylase family protein	3.39	0E+00
96	rpsT	30S ribosomal protein S20	3.39	0E+00
97	groEL	chaperonin GroEL	3.37	0E+00
98	rpsN	30S ribosomal protein S14	3.36	0E+00
99	A911_01710	Ppx/GppA family phosphatase	3.35	0E+00
100	A911_t08344	tRNA-Tyr	3.28	0E+00
101	A911_04600	methyl-accepting chemotaxis protein	3.26	5E-15
102	A911_05835	hypothetical protein	3.22	0E+00
103	A911_t08340	tRNA-Ile	3.20	4E-15
104	A911_t08360	tRNA-Ile	3.20	4E-14
105	tsf	elongation factor Ts	3.19	0E+00

APPENDIX 12

***C. jejuni* PT14 genes Down-regulated of filament against exponential cells (Top 100 genes)**

No.	Feature ID	products	fold change	p_{adj}[a]
1	A911_00055	rubrerythrin	-15.42	6E-141
2	A911_00810	hypothetical protein	-13.80	1E-87
3	trpA	tryptophan synthase subunit alpha	-7.08	3E-242
4	A911_06480	PseD protein	-6.43	4E-43
5	A911_00770	6-pyruvoyl tetrahydrobiopterin synthase	-6.42	7E-149
6	A911_00695	Methyl-accepting chemotaxis signal transduction protein	-6.09	7E-199
7	A911_01685	tryptophan synthase subunit beta	-6.05	2E-181
8	A911_00350	iron-sulfur cluster binding protein	-5.78	8E-191
9	A911_02060	hypothetical protein	-5.73	3E-31
10	A911_02065	putative acidic periplasmic protein	-5.60	2E-17
11	A911_00355	putative oxidoreductase iron-sulfur subunit	-5.47	3E-125
12	A911_00050	competence protein ComEA	-5.44	5E-136
13	A911_06475	flagellin modification protein A	-5.41	9E-69
14	A911_01610	ferredoxin, 4Fe-4S	-5.38	2E-14
15	A911_07620	peptide ABC transporter, periplasmic peptide-binding protein	-5.21	5E-11

Appendix 12 continued

No.	Feature ID	products	fold change	p_{adj}[a]
16	A911_00345	hypothetical protein	-5.10	8E-219
17	A911_03910	dihydrodipicolinate synthase	-4.92	5E-214
18	A911_02995	phosphate ABC transporter substrate-binding protein	-4.84	1E-11
19	A911_06465	hypothetical protein	-4.78	4E-37
20	A911_03770	major antigenic peptide PEB2	-4.70	2E-163
21	A911_05365	ATP-dependent Clp protease, ATP-binding subunit ClpA	-4.65	5E-73
22	A911_02070	periplasmic protein	-4.52	2E-27
23	smpB	SsrA-binding protein	-4.44	1E-115
24	A911_04730	aminoacyl-histidine dipeptidase	-4.44	2E-78
25	A911_00815	superoxide dismutase	-4.38	1E-77
26	aroE	shikimate 5-dehydrogenase	-4.37	1E-82
27	A911_05470	Alpha-1,4-N-acetylgalactosamine transferase PglH	-4.31	5E-38
28	A911_04595	lipoprotein	-4.25	2E-96
29	A911_06975	dUTPase	-4.25	5E-52
30	A911_07615	peptide ABC transporter permease	-4.13	8E-07

Appendix 12 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
31	A911_06470	Legionaminic acid cytidylyltransferase	-4.10	2E-23
32	A911_05250	transcription-repair coupling factor	-4.08	2E-49
33	A911_01105	hexapeptide repeat-containing transferase	-4.07	2E-77
34	A911_06895	class I glutamine amidotransferase, putative	-4.04	2E-91
35	A911_03190	M24 family peptidase	-4.03	3E-89
36	A911_01680	N-(5'phosphoribosyl)anthranilate isomerase	-4.02	3E-94
37	A911_05810	S-ribosylhomocysteinase	-4.01	4E-53
38	A911_01675	anthranilate synthase component II	-3.94	3E-64
39	A911_04820	hypothetical protein	-3.84	9E-19
40	A911_03850	hypothetical protein	-3.84	5E-26
41	A911_05510	putative glycosyltransferase	-3.83	2E-11
42	A911_03355	acetate kinase A/propionate kinase 2	-3.83	2E-123
43	A911_05360	ATP-dependent Clp protease adaptor protein	-3.80	3E-41
44	A911_07980	hypothetical protein	-3.79	6E-05
45	A911_01905	pyruvate kinase	-3.79	2E-219

Appendix 12 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
46	rnhA	ribonuclease H	-3.78	1E-17
47	A911_07455	putative NADP-dependent alcohol dehydrogenase	-3.78	3E-142
48	A911_04590	peptidyl-arginine deiminase family protein	-3.76	4E-30
49	A911_01960	hypothetical protein	-3.76	4E-118
50	A911_06495	PseE protein	-3.74	2E-18
51	A911_05355	periplasmic thioredoxin	-3.71	5E-48
52	dnaG	DNA primase	-3.70	4E-37
53	A911_07885	hypothetical protein	-3.70	3E-47
54	A911_06610	hypothetical protein	-3.68	7E-76
55	A911_01955	serine hydroxymethyltransferase	-3.65	0E+00
56	A911_07745	sulfate adenylyltransferase	-3.65	2E-61
57	A911_06906	SAM-dependent methyltransferase	-3.65	4E-230
58	A911_03915	7-alpha-hydroxysteroid dehydrogenase	-3.64	3E-185
59	A911_05465	general glycosylation pathway protein	-3.64	2E-20
60	A911_05955	DNA-binding response regulator	-3.57	1E-147

Appendix 12 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
61	A911_07605	peptide ABC transporter ATP-binding protein	-3.54	1E-08
62	A911_01975	putative lipoprotein	-3.51	2E-71
63	A911_06490	motility accessory factor	-3.50	6E-13
64	A911_01965	membrane protein	-3.48	4E-297
65	A911_03955	hypothetical protein	-3.44	1E-17
66	A911_04755	hippurate hydrolase	-3.44	1E-37
67	A911_00180	cytochrome C	-3.43	1E-27
68	A911_07530	NADH-quinone oxidoreductase, N subunit	-3.42	3E-47
69	A911_01755	hypothetical protein	-3.42	2E-85
70	A911_06310	acyl carrier protein	-3.41	3E-62
71	A911_02735	hypothetical protein	-3.38	6E-50
72	A911_06460	putative sugar-phosphate nucleotide transferase	-3.37	5E-17
73	A911_00105	RNA pseudouridylate synthase	-3.36	3E-08
74	A911_07235	putative sodium/proline permease	-3.35	2E-41
75	A911_05150	Oxygen-insensitive NAD(P)H nitroreductase / Dihydropteridine reductase	-3.34	7E-77

Appendix 12 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
76	A911_03350	phosphate acetyltransferase	-3.34	2E-117
77	A911_07750	putative phosphatidylglycerophosphatase	-3.33	4E-32
78	A911_00020	hypothetical protein	-3.33	1E-66
79	A911_04415	CiaB protein	-3.31	1E-44
80	A911_05480	UDP-glucose 4-epimerase	-3.28	1E-167
81	A911_07535	NADH-quinone oxidoreductase, M subunit	-3.28	1E-82
82	A911_03950	hypothetical protein	-3.27	6E-23
83	A911_06040	radical SAM domain-containing protein	-3.26	3E-30
84	A911_00765	heme-binding lipoprotein	-3.25	7E-32
85	A911_05170	hypothetical protein	-3.24	3E-15
86	A911_05475	ABC transporter permease	-3.24	6E-14
87	A911_07795	alpha-ketoglutarate permease	-3.23	1E-23
88	rnc	ribonuclease III	-3.22	3E-24
89	A911_00360	L-lactate permease	-3.22	0E+00
90	A911_05455	oligosaccharide transferase	-3.20	9E-27

Appendix 12 continued

No.	Feature ID	Products	fold change	p_{adj}[a]
91	A911_06290	hypothetical protein	-3.19	5E-10
92	A911_07305	putative periplasmic oxidoreductase	-3.19	9E-93
93	A911_04785	coproporphyrinogen III oxidase	-3.19	9E-44
94	A911_03740	NLPA family lipoprotein	-3.19	8E-10
95	fumC	fumarate hydratase	-3.17	0E+00
96	A911_04790	hypothetical protein	-3.16	4E-34
97	A911_06795	ferrous iron transport protein A	-3.16	1E-25
98	A911_06420	transferase	-3.16	1E-12
99	A911_04410	DNA-binding protein HU-like protein	-3.13	2E-98
100	A911_06280	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	-3.12	6E-16
101	A911_06485	Motility accessory factor	-3.11	8E-32
102	A911_00665	hypothetical protein	-3.11	7E-10
103	A911_07400	acetyl-CoA synthetase	-3.10	3E-51
104	A911_06705	hypothetical protein	-3.04	0E+00
105	A911_03945	3-deoxy-manno-octulosonate cytidylyltransferase	-3.04	4E-53

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

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Filamentation of *Campylobacter* in broth cultures

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The transition from rod to filamentous cell morphology has been identified as a response to stressful conditions in many bacterial species and has been ascribed to confer certain survival advantages. Filamentation of *Campylobacter jejuni* was demonstrated to occur spontaneously on entry in to stationary phase distinguishing it from many other bacteria where a reduction in size is more common. The aim of this study was to investigate the cues that give rise to filamentation of *C. jejuni* and *C. coli* and gain insights into the process. Using minimal medium, augmentation of filamentation occurred and it was observed that this morphological change was wide spread amongst *C. jejuni* strains tested but was not universal in *C. coli* strains. Filamentation did not appear to be due to release of diffusible molecules, toxic metabolites, or be in response to oxidative stress in the medium. Separated filaments exhibited greater intracellular ATP contents (2.66 to 17.4 fg) than spiral forms (0.99 to 1.7 fg) and showed enhanced survival in water at 4 and 37°C compared to spiral cells. These observations support the conclusion that the filaments are adapted to survive extra-intestinal environments. Differences in cell morphology and physiology need to be considered in the context of the design of experimental studies and the methods adopted for the isolation of campylobacters from food, clinical, and environmental sources.

Keywords: Campylobacter, filamentation, morphological changes, morphotypes, survival, intracellular ATP

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Introduction

Campylobacter is frequently responsible for foodborne bacterial gastroenteritis worldwide (World Health Organization [WHO], 2013). *Campylobacter* cells are usually slender, spiral shaped rods measuring 0.2–0.8 μm wide and 0.5–5 μm in length (Vandamme, 2000) but like many other microorganisms, filamentous forms have been observed under certain circumstances (Griffiths, 1993; Thomas et al., 1999; Apel et al., 2012; Cameron et al., 2012). Filamentation has been identified in many different bacteria and is thought to occur through inhibition of cell division, metabolic changes, or DNA damage which includes the SOS response resulting in the inhibition of septum formation whilst the chromosome is repaired (Justice et al., 2008). It has frequently been associated with stress and starvation conditions during which it may confer survival advantages (Justice et al., 2008). Moreover, it has been suggested that filamentation could represent a programmed response to unfavorable environments that aids the bacterium's survival (Justice et al., 2008) and may enhance virulence (Mulvey et al., 2001; Stackhouse et al., 2012). Alternatively, filamentation may simply occur through an inadvertent loss of control of the normal cell division process. Whichever scenario is true may depend on the species of bacteria and the type of environmental stress encountered. Examples of environmental signals that have been identified to induce filamentation include: starvation, exposure to oxidative stress,

reduced water activity, the presence of quorum sensing molecules, antibiotics, or host immune effectors (Allison et al., 1992; Jones et al., 1996; Janion, 2001; Miller et al., 2004). Filamentation as a response to sublethal stress has been observed in a number of foodborne bacteria, which have led to concerns that these bacteria may rapidly divide once the growth conditions become permissive to cause spoilage or disease (Jones et al., 2013).

Filamentation in *Campylobacter* has also been observed in response to mutation of the response regulator RacR and its sensor RacS, which are involved in the heat shock response (Apel et al., 2012), in response to treatment with certain antibiotics such as sitafloxacin (Yabe et al., 2010) and as a general response to hyperosmotic stress (Cameron et al., 2012). Importantly, for *Campylobacter jejuni* and *Helicobacter pylori* broth cultures, grown in a microaerobic atmosphere, filamentation occurred spontaneously on entry in to stationary phase (Griffiths, 1993; Fawcett et al., 1999; Thomas et al., 1999; Wright et al., 2009). Here nutrients may become depleted, potentially leading to starvation stress or there may be a buildup of metabolites present in the spent medium but the lack of a specific stress trigger distinguishes filamentation of these two related bacterial genera from other bacteria where stationary phase cells are generally reduced in size (Nyström, 2004). Moreover, elongated cells can be readily identified in scanning electron micrographs of *Campylobacter* biofilms (for examples see Kalmokoff et al., 2006; Brown et al., 2014) indicating filamentation may occur naturally in situations where biofilms form.

The aim of this study was to investigate the cues that give rise to filamentation of *C. jejuni* growing in broth cultures. We also aimed to investigate the viability of the individual component cells of the filament using vital staining, determine any strain dependency and any possible differences in the ability of the two different morphotypes to survive unfavorable conditions.

Materials and Methods

Bacterial Strains

Campylobacter strains that were used for this study included: HPC5, HF5, (*C. jejuni* poultry isolates); NCTC11168, NCTC12661 (35925B2), 81-176, PT14, 81116 (*C. jejuni* reference strains isolated from humans); OR4451C, OR5482C (*C. coli* poultry isolates), and FB1 (*C. coli* human isolate). All strains were stored at −80°C in Microbank vials (Pro-Lab Diagnostics, Wirral, UK).

Growth in Liquid Cultures

Nutrient Broth Number 2 (NB2; Oxoid, Basingstoke, UK) and Mueller Hinton Broth (MH; Oxoid) were prepared according to manufacturer's instructions. MEM (minimum essential medium) without glutamine and phenol red (Catalog number 51200-038; Life Technologies Ltd, Paisley, UK), with and without addition of 10 mM sodium pyruvate (Sigma Aldrich, Gillingham, UK), as an energy source were also tested. The *Campylobacter* inoculum was prepared by making a suspension containing approximately 10^8 CFU/ml from an overnight culture grown on blood agar (BA; Oxoid) containing 5% (v/v) of defibrinated horse blood (TCS, Buckingham, UK) at 37°C incubated under microaerobic conditions (approximately 7% O_2, v/v) obtained by the evacuation/replacement technique (Bolton and Coates, 1983). The replacement gas mix contained (5% v/v H_2, 10% v/v CO_2, and 85% N_2). To prepare growth curves, 0.1 ml of the *Campylobacter* inoculum was added to three individual 250 ml conical flasks containing 50 ml of medium and the flasks placed in anaerobic jars (Oxoid) under microaeobic conditions generated as described above. The jars were placed in an orbital shaker and shaken at 100 rpm at 37°C with sampling at appropriate intervals. For each time point, an aliquot was serially diluted in maximal recovery diluent (MRD; Oxoid) and the microaerobic atmosphere re-generated. Enumeration of campylobacters was carried out by the Miles and Misra method on *Campylobacter* blood-free selective agar plates without supplement (CCDA; Oxoid) and incubated microaerobically at 42°C for 48 h. The morphology of the cells was examined microscopically over 10 independent fields using bright field, epifluorescence and Gram-stain for each time point. Pre-used NB2 was prepared by carrying out the above procedure with incubation for 48 h and confirmation of the formation of filamentous cells. The cell growth was removed by centrifugation at 13,000 *g* for 15 min, and the supernatant filtered through a 0.2 µm filter (cellulose acetate, Sartorius Stedim Biotech, Epsom, UK). The pre-used filter sterilized medium was inoculated as described above to prepare growth curves. To reduce the potential accumulation of free radicals in MEM with pyruvate, the medium was supplemented with 0.15% w/v starch (Sigma Aldrich) prior to sterilization. Statistical differences were assessed by ANOVA from the Excel Data Analysis package (Microsoft Corporation, Redmond, WA, USA).

Fluorescent Cell Staining (Syto9/Propidium Iodide)

Bacterial suspensions (1 ml) were mixed with 1.5 µl of Syto 9 (absorption/emission 485/498 nm) and 1.5 µl propidium iodide (absorption/emission 535/617 nm) from LIVE/DEAD® BacLight™ Bacterial Viability Kit (Life Technologies) and incubated in the dark for 20 min at room temperature. A 5 µl aliquot was applied to the center of a clean glass microscope slide. An 18 mm^2 coverslip was placed over the suspension. The slides were examined over 10 min at a magnification of 1,250 (100×, plan Apo) with an epifluorescence microscope (Labophot; Nikon, Tokyo, Japan) and images captured from 10 independent fields using a vertical mounted digital camera.

Separation of Short Spiral and Filamentous Morphotypes

In order to separate the short spiral and filamentous forms, three independent biological replicates of *C. jejuni* 12661 or PT14 were inoculated into 500 ml of sterile MEM with sodium pyruvate, to a final density of approximately 10^5 CFU/ml. The flasks were incubated in a shaking incubator at 100 rpm, at 37°C under microaerobic conditions for 30 h. The short spiral cells were separated from filamentous cells by collecting the filtrate from the culture that was passed through a 0.8 µm sterile nitrocellulose

membrane (Sigma–Aldrich). The filtration was repeated with a fresh filter. The filamentous forms were obtained by flushing the first filter with fresh MEM (with pyruvate) and collecting the eluate. The suspensions were examined microscopically and diluted to contain approximately 5×10^6 CFU/ml in either sterile water (reverse osmosis) or NB2 to examine their survival characteristics.

Comparison of the Survival of the Short Spiral and Filamentous Morphotypes

Suspensions of the separated morphotypes in either water or NB2 (prepared as described above) were either incubated at 37 or 4°C for 96 h under microaerobic conditions. Viable counts were performed as described above at 24 h intervals.

Determination of Intracellular ATP

The ATP concentrations were measured by luciferase luminescence using a commercial kit according to the manufacturer's instructions (Promega, Southampton, UK). Either suspensions of selected morphotypes (prepared as above) or culture suspensions collected at various time points were centrifuged at 13,0000 *g* for 15 min and the cell pellets washed in 1ml of TA buffer (20 mM Tris-acetate buffer pH 7.75) before re-pelleting. The cell suspensions were lysed with 1% (w/v) trichloroacetic acid in TA buffer. The ATP assays were performed by adding 10 µl of the cell extract to a polypropylene tube containing 100 µl of recombinant luciferase/luciferin reagent, followed by gentle mixing and immediate reading in a pre-blanked luminometer (Turner Designs TD 20e, Promega). The signal was integrated over 10 s with a 2 s delay, and reported in relative light units (RLU) that could be converted to ATP concentrations using a pre-prepared standard curve. ATP contents were normalized according to either viable counts or microscopic cell counts or protein content (Bradford reagent; Pierce).

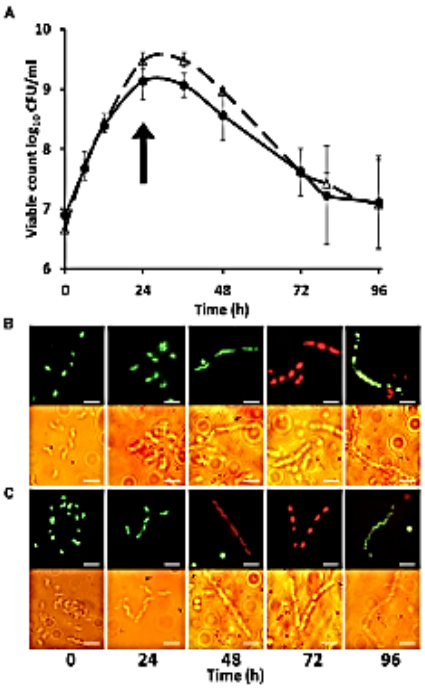


FIGURE 1 | Growth curves (A) of *Campylobacter jejuni* strains HPC5 (Δ) and HF5 (●) and morphological changes during growth of *C. jejuni* HPC5 (B), and HF5 (C) in NB2. Arrow in (A) indicates the time point at which the filamentous cells were first observed (on entry to stationary phase). Error bars are ±SD for $n = 3$. In (B) and (C) the upper row shows cells stained with fluorescent stains whilst the lower row shows bright field microscopy image of the same cells. Scale bar, 5 µm.

Results

Confirmation of Filamentation on Entry to Stationary Phase and the Imaging of Filament Component Cells by Vital Staining

Growth curves and microscopic images of *C. jejuni* strains HPC5 and HF5 growing in NB2 over 48 h are shown in **Figures 1A–C**. These data confirm previous observations that on entry to stationary phase, after 24 h, progressively longer filaments are formed (Griffiths, 1993). The morphological changes coincided with different phases of growth. Exponentially growing cells showed typical short spiral forms (1.5–2.5 µm), while mid-stationary phase cells had become elongated. Cell populations in decline phase featured long filaments and the appearance of coccal forms. Vital staining was carried out using a combination of Syto 9, which stains intact cells green and propidium iodide, which only penetrates membrane damaged cells, staining them red. This did not produce the expected result, with many motile and therefore essentially live red-stained cells, appearing in the exponentially growing population. It appears that propidium iodide was able to enter live cells and was not a good indicator of viability for *Campylobacter*. Vital staining of the stationary phase cells showing filamentation was variable, with the cell filaments, either predominantly red (staining with PI) or predominantly green (stained with Syto-9) with relatively few cells containing both fluors (integrated images colored yellow). Interestingly comparison of the bright field and fluorescent microscope images presented in **Figures 1B,C**, clearly showed that the filaments contained cells that were unstained between the stained cells, and remarkably these often appeared in a regular interspersed pattern as demonstrated at the 72 h time points for *C. jejuni* strains HPC5 and HF5 in **Figure 1**.

Growth of *Campylobacter* in Pre-Used Medium to Investigate the Potential Role of Quorum Sensing, Depletion of Nutrients

The exponential growth rates of *C. jejuni* HF5 and HPC5 in the pre-used NB2 medium ($k = 0.14$ /h for both strains), were marginally reduced compared to fresh medium ($k = 0.18$ /h

for HF5 and $k = 0.25$ /h for HPC5; **Figure 2**). This difference was significant for HPC5 ($p = 0.03$; ANOVA) but not significant for HF5 ($p = 0.4$; ANOVA). Although not optimum, the pre-used medium was able support exponential growth, suggesting that essential nutrients had not become significantly depleted in the course of achieving stationary phase in the previous culture. Moreover the growth period required for filamentation to occur were similar for fresh and pre-used NB2, suggesting that the campylobacters were not responding to the release of soluble signal molecules as observed for auto inducer molecules involved in quorum sensing.

To investigate further the role of nutritional limitation as a possible cause of filamentation, growth curves were prepared using MEM for *C. jejuni* HF5 and HPC5. Under these conditions filamentation was observed as early as 2 h (**Figure 2**; indicated by arrows), but with no increase in viable count and no filaments greater than 10 μm in length (**Figures 3A–C**). Some individual cells were stained both red and green (**Figure 3A**). Addition of sodium pyruvate, as an energy source, produced an increase in the viable count and filamentation was delayed such that filaments appeared after 4–6 h of incubation. Longer incubation periods produced filaments greater than 10 μm in length, which stained with both Syto 9 and PI. This dual staining was particularly evident in a third strain investigated, *C. jejuni* 12661 (**Figures 3D,E**).

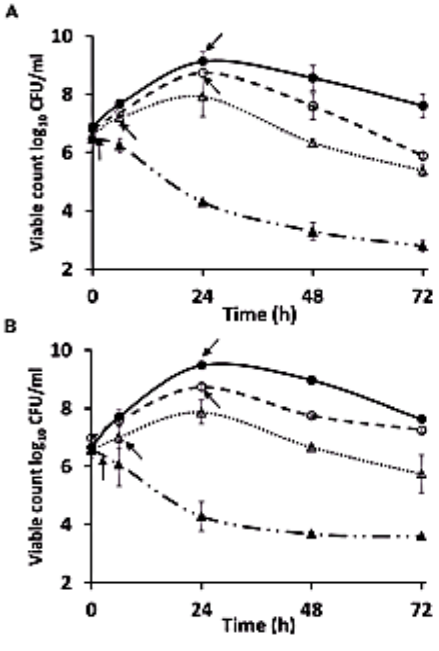


FIGURE 2 | Comparison of the growth of *C. jejuni* strains (A) HPC5 and (B) HF5 in: NB2 (●), pre-used NB2 (○), minimum essential medium (MEM; ▲), and MEM supplemented with 10 mM sodium pyruvate (Δ). Arrows indicate the time points at which filamentous cells were first observed. Error bars are ±SD for $n = 3$.

Growth of *Campylobacter* in Starch-Containing Media to Reduce Oxidative Stress

To assess if the provision of starch as an antioxidant, in the culture media could prevent or delay the onset of filamentation, we cultured campylobacters in either Mueller Hinton broth or MEM (with pyruvate) containing starch. Neither of these alternative growth media altered the time of entry to stationary phase or the subsequent observation of filamentous morphotypes (results not shown). The accumulation of growth related oxidative stressors in the culture medium was probably not a causal effect of filamentation.

Filamentation in Different Strains of *C. jejuni* and *C. coli*

To determine if filamention was widespread amongst *C. jejuni* and *C. coli* a larger group of strains was examined. These were grown in MEM with sodium pyruvate and morphological changes compared in terms of the time taken for filamentation to be first observed (**Table 1**). Differences were observed, but filamentation was a feature of all the strains tested (cells > 5 μm) apart from *C. coli* strain (FB1), which did not form filaments, the viable count did not increase, and this strain produced coccal forms as early as 6 h under these conditions. One strain, *C. jejuni* 12661, produced particularly long filaments and was therefore chosen for further experiments involving separation of filaments from other morphotypes.

Filamentous Forms Show Increased Survival in Water

In order to compare the characteristics of the filamentous and spiral cell forms coexisting in stationary phase cultures of *C. jejuni* 12661 (exaggerated filamentation phenotype) were separated using membrane filtration. A 0.8 μm filter prevented the passage of long filamentous forms and did not hinder the passage of short spiral forms present after 30 h incubation in MEM with sodium pyruvate at 37°C under microaerobic conditions. The survival characteristics of the separated morphotypes were examined in both NB2 and in water. There was no significant difference ($p > 0.5$; ANOVA) in the ability of the two morphotypes to survive in NB2 at either 37 or 4°C (**Figures 4A,B**). However, marked differences were observed in the survival of the two morphotypes incubated in water at either temperature (**Figures 4C,D**). The viable count of the short spiral forms fell below the limit of detection after 72 h at 37°C and 96 h at 4°C, whilst the filamentous forms remained detectable at 37°C and experienced only a modest fall in viability of 1.5 $\log_{10}$ over 96 h at 4°C.

ATP Contents of Spiral, Filamentous, and Coccal Cell Types

In order to study the ATP contents of spiral, filamentous and coccal cell types, *C. jejuni* 12661 (exaggerated filamentation phenotype) and *C. jejuni* PT14 (a strain that showed a typical

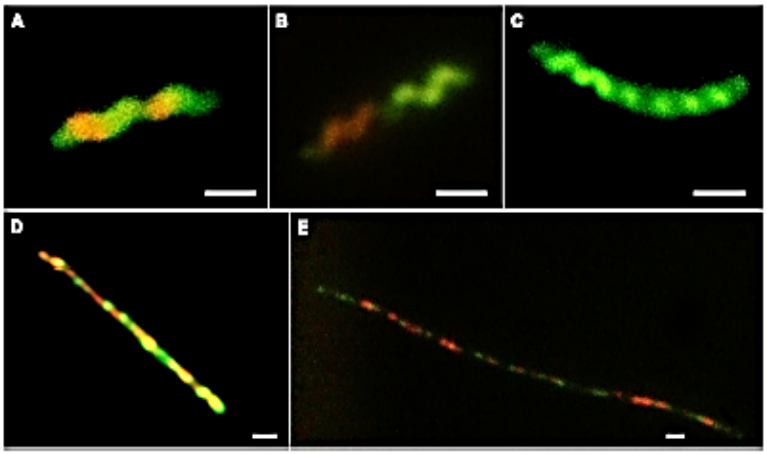


FIGURE 3 | Examples of variation in vital staining observed during growth in MEM and in MEM supplemented with 10 mM sodium pyruvate; (A) *C. jejuni* HPC5 after 2 h incubation in MEM, (B) *C. jejuni* HPC5 after 4 h incubation in MEM, (C) *C. jejuni* HPC5 after 6 h incubation in MEM; (D) *C. jejuni* 12661 after 48 h incubation in MEM supplemented with 10 mM sodium pyruvate; and (E) *C. jejuni* 12661 after 72 h incubation in MEM supplemented with 10 mM sodium pyruvate. Scale bar 1 μm.

filamentation phenotype) were selected. Viable and microscopic counts of microaerobic cultures at 37°C of *C. jejuni* 12661 and PT14 in MEM with sodium pyruvate were collected over 216 h to encompass exponential, stationary and decline phases of growth (Figure 5). Spiral cells harvested in mid-exponential phase at 14 h were serially diluted and the ATP contents of these cells estimated from luciferase/luciferin luminescence after lysis (Table 2). The ATP contents of the exponential phase spiral cells (0.99 and 1.7 fg ATP per CFU) were similar to earlier estimates for *C. jejuni* (Ng et al., 1985). Recovery and separation of filamentous and spiral forms in decline phase at 96 h enabled determination of the ATP contents of these cell populations before the appearance of coccal forms. Beyond 168 h the majority of the cells appeared coccal in these cultures, and estimates of viability required the plating of 0.2 ml culture volumes on multiple blood agar plates and/or recovery at endpoint dilution in broth cultures. Microscopic evaluation of 216 h cultures revealed that 61% of the *C. jejuni* 12661 cells had become coccal compared with 58% for *C. jejuni* PT14. After correction for the ATP contents of decline phase spiral and filamentous cells present in these cultures (Figure 5), estimates of the ATP contents of the dominant coccal cells were calculated (Table 2). The low or undetectable ATP contents of the coccal cells would support the conclusion that they are no longer viable, and that the viable campylobacters recovered from these cultures represent the remaining spiral and filamentous forms.

TABLE 1 | Viability and filamentation of *Campylobacter* strains growing in different media.

Media	Strain	Time to achieve stationary phase (h)*	Time to first filaments (h)†	Viable count at first filamentation (CFU/ml)
NB2	HPC5	30	24	3.0×10^9
	HF5	30	24	1.3×10^8
Pre-used filtered NB2	HPC5	24	24	2.5×10^9
	HF5	24	24	8.3×10^8
Minimum essential medium (MEM; (with sodium pyruvate)	HPC5	24	6	1.0×10^7
	HF5	24	6	1.6×10^7
	FB1[§]	24	24‡	4.0×10^6
	OR4451C[§]	24	6	3.32×10^7
	OR5482C[§]	24	6	6.0×10^7
	81116	24	6	6.2×10^7
	11168	24	6	1.7×10^7
	12661	24	6	2.1×10^7
	81-176	24	6	1.3×10^7
	PT14	24	6	5.2×10^7
MEM (without sodium pyruvate)	HPC5	–	2	2.6×10^6
	HF5	–	2	1.6×10^6

*Determined from growth curve inoculated with ~10^6 CFU.
†Determined microscopically, ‡Double length cells, §Campylobacter coli.

Discussion

Growth curves and microscopic examination of broth grown cultures confirmed that filamentous morphotypes were formed upon entry to stationary phase. Vital staining revealed interesting structural features of the filamentous cells but also that this method may not be suitable for distinguishing live *Campylobacter* cells from dead ones. The staining patterns observed suggest growth and septa formation occur within the filaments, and that this leads to differential dye permeability preventing dye migration between cells. Moreover, the interspersed pattern

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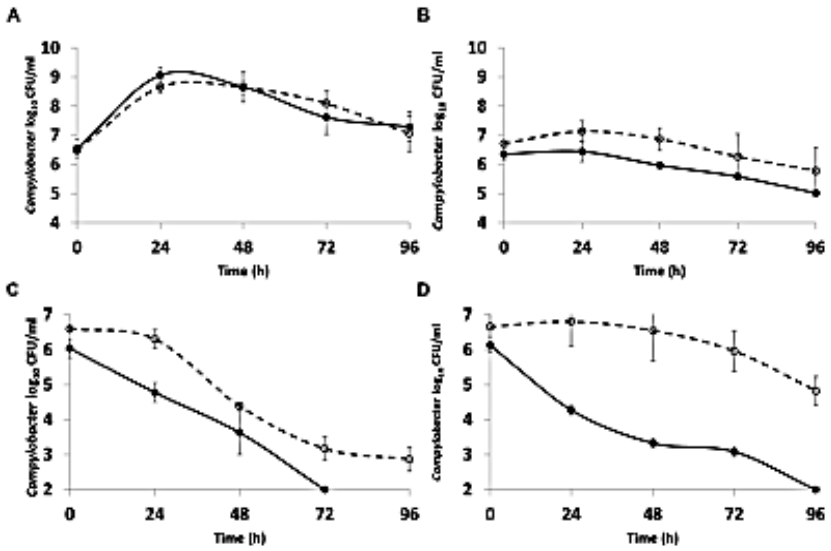


FIGURE 4 | Comparison of the survival of *C. jejuni* 12661 morphotypes incubated microaerobically at: (A) 37°C in NB2; (B) 4°C in NB2; (C) 37°C in water; (D) 4°C in water. Short spiral forms (●), filamentous forms (○). Error bars are ±SD for $n = 3$.

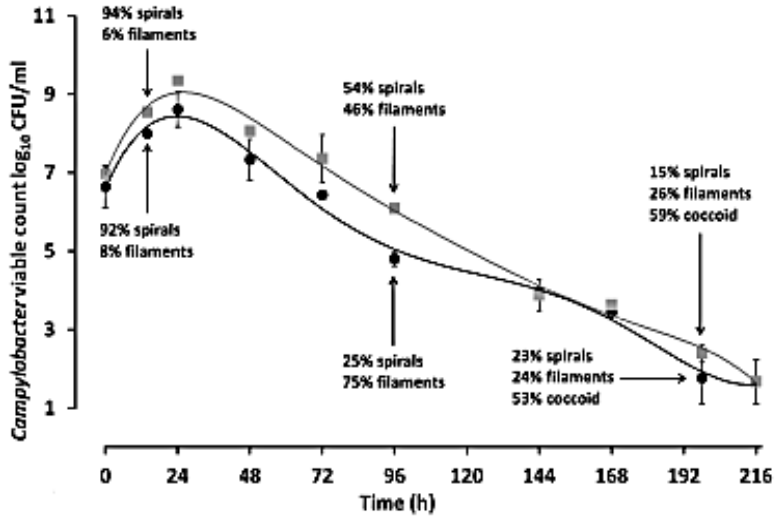


FIGURE 5 | Viable counts and microscopic enumeration of the cell morphotypes observed during microaerobic growth of *C. jejuni* PT14 and 12661. *C. jejuni* PT14 (●) and 12661 (□) were cultured in MEM supplemented with 10 mM sodium pyruvate under microaerobic conditions at 37°C, from which samples were taken for microscopic examination and estimates of cell bound ATP. Error bars are ±SD for $n = 3$.

would suggest cell division may be taking place at the predefined cell poles within the filament suggesting at least some of the component cells were viable. Cameron et al. (2012) observed irregular patterns of cells remaining unstained by PI within *Campylobacter* filaments formed in response hyperosmotic stress. This prompted the authors to investigate septa formation using Vanco-FL stain that binds D-Ala-D-Ala moieties of peptidoglycan indicative of septa and/or sites of new peptidoglycan synthesis. The Vanco-FL stain produced a punctuated staining pattern in hyperosmotic-induced filaments that did not co-localize with PI. This observation is also indicative of internal septa formation, and as the authors

TABLE 2 | Intracellular ATP content of *C. jejuni* morphotypes at different phases of growth.

Cell Forms*	*C. jejuni* 12661 ATP (fg/cell)†	*C. jejuni* PT14 ATP (fg/cell)†
Exponential phase spirals (14 h)	1.7 ± 0.35	0.99 ± 0.12
Decline phase spirals (96 h)	1.19 ± 0.20	0.84 ± 0.14
Decline phase filaments (96 h)	17.4 ± 2.2	2.66 ± 0.54
Decline phase coccoid (196 h)	0.01 ± 0.005	ND
Decline phase coccoid (216 h)	0.01 ± 0.004	ND

**Cell morphotypes observed at different time points (h) in microaerobic culture of MEM with pyruvate. †±SD n = 3. ND, not detectable.*

conclude phenotypic differences between cells that compose the filament.

Quorum sensing bacteria produce and release auto inducers that increase in concentration as a function of cell density leading to an alteration in gene expression (Miller and Bassler, 2001) and in some cases filamentation (Allison et al., 1992). As no change in the time taken to form filaments occurred when pre-used medium was inoculated with a fresh culture, it seems unlikely that quorum sensing was involved in the process. The relatively proficient growth of campylobacters in the pre-used medium was unexpected, as nutrient depletion on entry to stationary phase was suggested as a possible trigger for filamentation. This raises questions as to what actually triggers entry to stationary and decline phases in *Campylobacter* broth cultures. This is not well understood in other bacteria and probably depends on a combination of factors including the species of bacteria and growth medium. For *Salmonella typhimurium* and *Escherichia coli* it has been shown that increased levels of acetate (Wilson et al., 2003) or carbon starvation (Sezonov et al., 2007) are associated with the cessation of growth. The transition to stationary phase for *Campylobacter* is highly dynamic with a switch from acetate production to utilization together with a peak in motility and numerous gene expression changes (Wright et al., 2009). In addition, the bacteria do not appear to exhibit enhanced stress resistance in stationary phase, unlike many other bacteria, which is consistent with the absence of RpoS homologues (Kelly et al., 2001). Despite the absence of RpoS, campylobacters retain a stringent response that assists survival in the stationary phase of many bacterial species. Mutation of the *spoT* gene results in aberrant cell morphologies and early coccoid cell formation in stationary phase cultures (Gaynor et al., 2005).

As a microaerophile, *Campylobacter* is particularly sensitive to the presence of free radicals and may suffer oxidative stress when grown in broth media despite being supplied with a reduced oxygen atmosphere (John et al., 2011). Strictly anaerobic conditions in the presence of alternative electron acceptors nitrate or fumarate have also been shown to induce filamentation in *Campylobacter* indicating an oxygen requirement for DNA synthesis (Sellars et al., 2002). The presence of starch in *Campylobacter* culture media acts as an antioxidant and affords a degree of protection against oxidative stress caused by free radicles that can accumulate during exponential growth (Mehlman and Romero, 1982). The provision of starch in the culture media did not influence the timing or degree of filamentation, which suggests that the accumulation of reactive oxygen species (ROS) in the growth medium is not a predisposing factor to the appearance of the filamentation morphotype. However, this does not rule out a role for oxidative stress at a cellular level. Endogenous ROS produced as a consequence of cellular metabolism have been suggested to play a role as signaling molecules and effectors in the development of microbial multi-cellularity, including programed cell death (Čáp et al., 2012).

Campylobacters were unable increase in viable count in MEM, without a carbon source but some increase in cell size was observed. The addition of pyruvate to the MEM allowed growth but with the early onset of filamentation as compared with growth in nutrient rich medium. Nutritional differences clearly impact on the observed morphological changes but since pyruvate remained in excess during the growth period as a carbon and energy source, it is unlikely that filamentation is a response to carbon starvation in these experiments. Bacteria also require sufficient iron, phosphorous, sulfur, nitrogen, and other trace elements for growth and it is possible that one, or a combination of these, become quickly exhausted in minimal medium resulting in filamentation, compared to rich media. However, the response to nutrient limitation even within a well-mixed *Campylobacter* broth culture is not uniform, in most cultures filamentous types arise among spirals that continue to divide. This implies that once the growth rate has become limited due to nutritional availability, then the formation of the filaments is either a stochastic process or developmentally controlled to generate a subpopulation that are more able to survive nutritional depletion and/or environmental stresses. In the wider environment other limiting physiological factors may also trigger the filamentation response.

Campylobacter jejuni cultured in MEM with pyruvate exhibit an ability to retain a viable subpopulation through decline phase at 37°C under microaerobic conditions despite a fall in viable count from >8 $\log_{10}$ CFU/ml at the end of exponential phase (24 h in **Figure 5**) to 3 $\log_{10}$ CFU/ml at 168 h. We have measured the ATP contents of cell morphotypes recovered from the decline phases of these cultures to demonstrate significant increases in the cellular ATP contents of the filamentous types as compared with spiral forms, sampled in either exponential phase, or separated from filaments in decline phase. These increases may be accounted for because the filaments appear to consist of multiple cells, joined in an ordered conglomerate. Consistent with this view, is the observation that the *C. jejuni* strain 12661 produces notably longer filaments and has a higher ATP content (17.4 fg ATP/CFU) than *C. jejuni* PT14 (2.66 fg ATP/CFU). However, we have noted interspersed staining patterns and cell division within filaments, which could represent cells with greater metabolic activity within a single filament. In the later stages of these cultures (>168 h) the majority of the cells become coccoid. ATP content estimates of coccal cells were either extremely low or non-detectable, suggesting they are not viable as concluded in previous reports (Moran and Upton, 1986; Boucher et al., 1994).

Differences in the ability of *Campylobacter* isolates to survive in microcosm waters have been documented but without reference to the formation of filaments (Buswell et al., 1998). The increased ability of the filamentous morphotype to survive in water compared to the short spiral form suggests that further research is necessary to assess the impact this may have on the transmission of the campylobacters from the environment to farm animals and on the safety of post-process foods. Based on these observations caution is advised when applying mathematical models that predict the survival *Campylobacter*, but do not take into account that changes in cell morphology and physiology that can increase their probability of survival. The presence of multicellular filaments may also lead to underestimates of viable cell numbers in cultures, since a single filament can form a single colony despite a multicellular origin, and filaments can exhibit differing refraction properties to non-dispersed cell suspensions that can make the interpretation of optical density measurements problematic (Wright et al., 2009).

Rapid filamentation was observed in MEM with pyruvate, in all the *C. jejuni* tested, and in all but one of the *C. coli* strains tested regardless of the strains being of either poultry or human origin. The response appears widespread amongst the two species. The minimal medium employed in these experiments did not support any observable growth of the single *C. coli* strain that did not form filaments, it could therefore not be concluded that this strain lacked the capacity to form filaments.

Laboratory based experiments of protein expression and metabolism of *Campylobacter* demand the use of broth grown cultures to control the phase of growth. Cultures are often harvested in late exponential phase to maximize cell yields, which can contain filamentous forms with potentially different characteristics to exponentially grown short spiral forms. Whilst it has long been recognized that growth *in vitro* cannot necessarily be compared with growth *in vivo*, the observation that *in vitro* grown cells harvested late in the growth cycle show such radically different morphology and survival ability, to cells in exponential phase, requires consideration in the context of experimental design and the methods to be adopted for environmental surveys. The filamentation of *Campylobacter* strains in laboratory broth culture appears to be a common occurrence. Whether filamentation occurs in all environments inhabited by campylobacters, for example in the intestines of animals and birds, is not known but the presence of filaments in biofilms indicates that this should be further investigated. Similarly there are no reports of filamentous forms observed from environments such as water courses, or from chilled meat, where campylobacters survive but have limited growth potential.

Author Contributions

NG performed the experimental analyses. NG, PC, and IC designed the experiments, interpreted the data, and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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