Campylobacter in Farm Animals

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ABSTRACT

Campylobacter jejuni and *C. coli* are common causes of acute gastroenteritis in humans that are also associated with Guillain Barré and Miller Fisher syndrome. Poultry and other farm animals are the major sources of these pathogens. In this thesis it was demonstrated that hydrogen has the potential to act as an antioxidant to reduce oxidative stress caused during the growth of *C. jejuni* HPC5 when grown in a gas replacement jar. Growth in the absence of hydrogen in a modular atmosphere controlled system (MACS) was characterised by an intial lag that could be overcome by adding an antioxidant reagent FBP (10% ferrous sulphate, sodium pyruvate and sodium metabisulphite). Transcriptomic studies revealed that growth in the absence of hydrogen resulted in significant increases in the expression of superoxide dismutase, thiol peroxidase and ribosomal proteins.

Transcriptomic studies were performed on the variants of *C. jejuni* HPC5 where bacteriophage predation had provoked intragenomic recombination to create 2nd generation resistant types that are inefficient colonisers of chickens but revert to efficient colonisers and bacteriophage sensitivity when reintroduced into chickens to create 3rd generation variants. The 2nd generation variants were temperature sensitive, exhibited increased expression of prophage Mu genes and low expression of motility associated genes. In contrast 3rd generation variants showed an increase in the expression of the motility genes, an increase in the genes associated with the putative bacteriophage immunity factor CRISPR and reduced expression of Mu genes.

Studies conducted on pigs demonstrated that a single pig can be colonised by campylobacters belonging to multiple genotypes and species. Comparative genomic hybridisation (CGH) of *C. coli* and *C. jejuni* isolated from the intestines of a single pig demonstrated these isolates shared plasmid and chromosomal encoded genes, and therefore may have undergone inter-species gene transfer due to cohabitation of a common intestinal niche.

The aim of this thesis is to genotypically characterise *Campylobacter* strains from chicken and pig in ideal atmospheric conditions. Our hypothesis is that *Campylobacter* can be grown *in vitro* both in gas replacement jar (GRJ) and in MACS and the molecular characterisation by transcriptomic analysis and CGH of the strains will be ideal in an atmospheric condition which is stress free.

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INTRODUCTION

1.1. *CAMPYLOBACTER* TAXONOMY

1.1.1. Discovery and early history of *Campylobacter*

Theodor Escherich first described *Vibrio* like organisms which were non culturable spiral shaped bacteria in the stools of children who died from diarrhoeal disease (Escherich, 1886). There were repeated instances of identifying spiral shaped bacteria from aborted bovine foetuses which were given the name *Vibrio fetus* subsp. *intestinalis* because of their morphology and source (McFadyean and Stockman 1913; Smith and Taylor, 1919). During 1953, Florent found similar shaped bacteria affecting fertility of cows and ewes (Debruyne *et al.*, 2008). The infectious infertility was spread by a symptom free bull during sexual contact and the organism which was the cause of these symptoms was *Vibrio fetus* subsp. *venerealis.* After 1963, *V. fetus* and the related organism *V. bubulus* were transferred to the new genus *Campylobacter* (Sebald and Véron 1963). During 1973, Véron and Chatelain published the taxonomy of *Vibrio* like organisms and included four distinct species in the genus *Campylobacter*: *C. fetus, C. coli, C. jejuni, C. sputorum* subsp. *sputorum* and *C. sputorum* subsp. *bubulus* (Véron and Chatelain, 1973). In the 1970's Butzler and his co-workers applied a filtration method to isolate bacteria from the stools of humans with diarrhoea that resembled *Campylobacter* (Butzler *et al.*, 1973). In the late seventies, Skirrow described the use of a selective supplement to isolate similar organisms from human faeces (Skirrow, 1977). An important milestone in the history of the genus *Campylobacter* occurred when *C. pyloridis* later corrected to *C. pylori* was moved to the new genus *Helicobacter* and was found to be associated with gastric problems in humans. The advent of ribosomal RNA homology studies brought new insight to the classification of *Campylobacter*. During 1987 Romaniuk *et al.*, (1987) and Lau *et al.*, (1987) compared partial 16S sequences of the *Campylobacter* spp. and

presented the first phylogenentic data. Revision of the *Campylobacter* taxonomy was proposed by Goodwin *et al.*, (1989) where he summarised genotypic and phenotypic arguments to exclude the gastric species *C. pylori* and *C. mustelae* from the genera *Campylobacter* and *Wolinella* and proposed a novel genus, *Helicobacter* to accommodate both.

1.1.2. Characteristics of Campylobacteraceae

The family Campylobacteraceae includes the genera *Campylobacter*, *Arcobacter*, *Sulfurospirillum* and *Bacteroides ureolyticus* and forms a family of Gram negative, nonsaccharolytic bacteria, with low GC content and micro aerobic growth requirements (Vandamme, 2000). There are 18 species and subspecies of *Campylobacter* as defined by biochemical methods, rRNA- DNA homology and 16S rRNA sequencing (On and Holmes, 1995, Stern *et al.*, 1992, Vandamme, 2000). The phylogenetic relationship between Campylobacteraceae and the related bacteria based on 16S rRNA gene sequence is given in Fig. 1.1.

Figure 1.1. Phylogenetic tree of the family Campylobacteraceae and related family based on 16S rRNA gene sequence

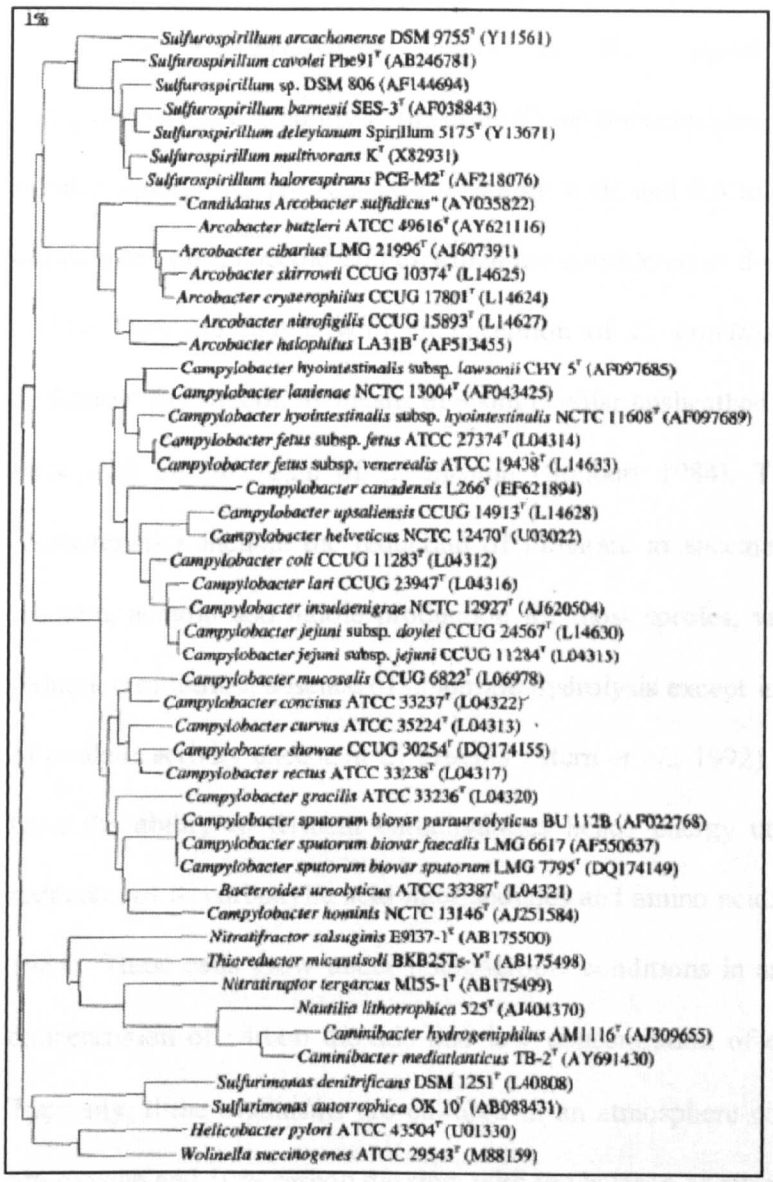


Figure adapted from Debruyne *et al.*, 2008. Phylogenetic tree of the family *Campylobacteraceae* and the related bacteria based on the percentage of 16srRNA gene sequence similarity.

1.1.3. Characteristics of the *Campylobacter* genus

The *Campylobacter* genus is the largest constituent of the Campylobacteraceae family in the order Campylobacteriales. *Campylobacter* cells are slender, spirally curved rods, 0.2 to 0.8μm wide and 0.5 to 5μm long while cells in old cultures form coccoid bodies which are considered as dormant forms (Holt *et al.*, 1994). They are motile with an exception of *C. gracillis*, using a characteristic corkscrew like motion by means of a single polar unsheathed flagellum at one or both ends with an exception of *C. showae* (Smibert 1984). The general biochemical characteristics include the reduction of fumarate to succinate, negative methyl red reaction, acetoin and indole production for most species, variable catalase activity, reduction of nitrate, absence of hippurate hydrolysis except in *C. jejuni*, and presence of oxidase activity except in *C. gracilis* (Stern *et al.*, 1992). Campylobacters do not have the ability to ferment carbohydrates hence energy utilisation is through the reduction of tri carboxylic acid intermediates and amino acids as substrates (Smibert, 1984). These cells grow under microaerobic conditions in an atmosphere with high concentration of carbon dioxide and low concentration of oxygen (Butzler, 2004). Typically, these organisms are cultured in an atmosphere containing approximately 5% oxygen and 10% carbon dioxide, with the balance of nitrogen (Stern *et al.*, 1992). Unlike the closely related bacteria *Helicobacter pylori*, campylobacters do not require significant levels of hydrogen. The optimum temperature for growth is 42 °C. These cells have an inability to grow below 25 °C and hence are called thermophillic campylobacters (Phillips, 1995; Shane, 2000). Lipase or lecithinase activity is absent and the G+C content of the DNA ranges from 29 to 47 mol% (Debruyne *et al.*, 2008).

The major respiratory quinones in *Campylobacter* species include Menaquinone- 6 and methyl substituted Menaquinone- 6 (Debruyne *et al.*, 2008).

The most widely used biochemical assay for identifying pathogenic *Campylobacter* is the hippurate test which identifies *C. jejuni* which possess *hip*O gene whose product (hippuricase) hydrolyses hippurate ($C_6H_5.CO.NH.CH_2.CO_2^-$) to benzoate ($C_6H_5O_2^-$) and glycine ($C_2H_5NO_2$) (Morris *et al.*, 1985).

1.2. BACTERIAL DORMANCY

When subjected to unfavourable conditions *Campylobacter* enters a viable but non culturable state (VNC) (Moore, 2001; Murphy *et al.*, 2006). The cells transform from a motile spiral shape to a coccoid stage when it undergoes dormancy (Rollins and Colwell, 1986). The coccoidal morphology involved the constriction of the cytoplasmic membrane, thus segregating the polar regions of the cell. It occurs due to the lack of nutrients or stress (Pead, 1979). Ng *et al.*, (1985) reported a transient doughnut shaped morphology which may be the intermediate stage for becoming dormant. The VNC forms may be recovered when they pass through the intestine of birds or mammals and are able to cause infection again (Beumer *et al.*, 1992). Hazelger *et al.*, (1995) suggested that this transition was not an active process as there was no alteration in the protein profiles or protein synthesis during transition. It is a degenerative response to toxic oxygen in culture (Moran and Upton, 1987). The detection of VNC is difficult using conventional methods like culturing of the cell but techniques like use of animal infectivity models (Jones *et al.*,1991), viability dyes (Cappelier *et al.*, 1997), flow cytometry (Nebe von Caron *et al.*, 2000) and nucleic acid amplification methods (Churruca *et al.*, 2007) can be used to detect its presence.

1.3. ENVIRONMENTAL DISTRIBUTION

Campylobacters are commonly found in the gastro intestinal tract of poultry especially in the caeca, large intestine and cloaca (Harris *et al.*, 1986; Kramer *et al.*, 2000). They were also found in the intestine of many mammals including the ileum and colon of infected humans (Murphy *et al.*, 2006). The main source of *Campylobacter* infection is thought to be through poultry and cattle meat but infections have also been traced to raw milk, contaminated water and contact with pets and farm animals (Kapperud *et al.*, 1992; Fahey *et al.*, 1995; Gillespie *et al.*, 2002; Olson *et al.*, 2008). As poultry is viewed as a reservoir, efforts have been made to reduce the incidence rate in poultry flocks which include: introduction of competing microbial population into newly hatched chicks, chlorination of poultry drinking water, vaccination or selective breeding of poultry for the resistance against the pathogen (Saleha *et al.*, 1998). Once introduced into a flock the transmission of *Campylobacter* in chicken occurs rapidly. Since chickens are coprophagic, they facilitate this rapid spread and it also occurs due to communal source of drinking (Montrose *et al.*, 1985). Studies have shown that 3 days of contact with artificially inoculated seeder birds was sufficient for the whole brood be colonised with *Campylobacter* (Shanker *et al.*, 1990). The presence of *Campylobacter* has also been reported in cattle, pigs, (Jensen *et al.*, 2006) rodents and birds (Park *et al.*, 1991).

Campylobacters can be isolated from streams (Jones *et al.*, 1999; Duke *et al.*, 1996; Champion *et al.*, 2002; Said *et al.*, 2003), rivers (Bolten *et al.*, 1987) exposed to agricultural runoff and treatment effluents and even in recreational water (Adak *et al.*, 1995). Stanley *et al.*, (1998) provided the first culturable evidence that *Campylobacter* can survive in ground water. The source of contamination was a dairy farm present in the catchment area. Campylobacters can also be isolated from coastal waters and

estuaries (Obri-Danso and Jones, 1999). Isolation occurs more frequently during the summer months which can be due to the occurrence of more outdoor activities and consumption of undercooked meat (Hudson *et al.*, 1999, Louis *et al.*, 2005).

1.4. EPIDEMIOLOGY

Over the past 30 years an enormous increase has occurred in the number of cases of diarrhoea caused by the zoonotic pathogens *C. jejuni* and *C. coli* (Luber and Bartelt, 2006). Data shows that 90% of the cases are attributed with *C. jejuni* and the remaining 10% with *C. coli* and *C. lari* (Lastovica and Skirrow 2000). Vertical transmission from parent flocks (Pearson *et al.*, 1996), carryover from previously positive flocks (Peterson and Wedderkopp, 2001), and horizontal transmission *via* contaminated water (Pearson *et al.*, 1993), domestic and wild animals (Hiett *et al.*, 2002), and the external environment (Newell, 2001) are the different sources of flock colonisation. Humphrey *et al.*, (1993) reported that horizontal transmission is generally considered the most significant cause of broiler flock colonisation. Studies done by Bull *et al.*, (2006) have shown that vertical transmission was not a major source of colonisation for the housed broiler flocks. *Campylobacter* is found in the environment surrounding the broiler houses often in puddles of water which may protect the Campylobacters from desiccation (Bull *et al.*, 2006). Transmission of *Campylobacter via* the air may also be important for spreading the organism between broiler flocks, as *Campylobacter* was detected in the air exiting broiler sheds.

Studies of *Campylobacter* infection in temperate countries have suggested a strong seasonality of infection, with peaks in summer and at the turn of the year (Kapperud and Aeson, 1992; Nylen *et al.*, 2002; Walckier *et al.*, 1989). This is

hypothesised to be due to human lifestyle at these times of the year. Taking this into account, a recent risk assessment indicated that a 2 $\log_{10}$ reduction in *Campylobacter* counts in chickens would lead to a 30 fold reduction in the number of cases of campylobacteriosis (Rosenquist *et al.*, 2002).

1.5. *CAMPYLOBACTER* IN BROILER FLOCKS AND CHICKEN MEAT

The total meat production in the United Kingdom was 3.4 million tonnes in 2007, according to the reports of Livestock Meat Commission (http://www.thecattlesite.com/news/26137/lmc-fall-in-uk-meat-production) which include beef, veal, pork, poultry, mutton and lamb. Studies have shown that 50-70% of the *Campylobacter* infection is due to the consumption of undercooked poultry meat (Tauxe, 1992; Allos, 2001) and also due to the handling of raw poultry meat (Skirrow 1982; Oosterom *et al.*, 1984; Harris *et al.*, 1986). Studies have shown that doses as low as 500 organisms can cause illness in humans (Black *et al.*, 1988). The species of *Campylobacter* that cause human infections include: *C. jejuni, C. coli, C. lari, C. upsaliensis* (Robinson, 1981; Black *et al.*, 1988; Friedman *et al.*, 2000). The extent of *Campylobacter* colonisation in the chicken gut ranges from $\log_{10}$ 6.4 to $\log_{10}$ 7.8 colony forming units (cfu) $g^{-1}$of the cecal content without showing any symptoms (Loc Carrillo *et al.*, 2005). In the United Kingdom up to 90% of the flocks are infected with *Campylobacter* (Evans, 1997). During slaughter and processing, cross-contamination can also occur on previously negative carcasses (Wassenaar *et al.*, 1998). Within the poultry house the organism can spread through aerosols (Berndtson *et al.*, 1996), drinking water (Näther *et al.*, 2009) and vector mediated infection (Jacobs-Reitsma *et al.*, 1995). Several studies have indicated that positive

flocks are generally more common among organic and free-range birds than intensively reared birds, possibly due to greater environmental exposure (Hald *et al.*, 2001; Heuer *et al.*, 2001). Studies have shown that broiler house cleaning procedures can remove the occurrence of *Campylobacter* between successive flocks (Evans and Sayers, 2000); however the same strain can be isolated from subsequent flocks indicating the occurrence of carry over (Petersen and Wedderkop, 2001; Connerton *et al.*, 2004).

For the successful colonisation of chickens various genes are required. Studies using isogenic mutants have indicated that the most important genes involved in colonisation are the flagellin genes, *flaA* and *flaB*, and associated motility factors, including the chemotaxis regulatory protein *che*Y, the flagella motor proteins *mot*A and *mot*B and a flagella biosynthesis protein *fli*R (Nachamkin *et al.*, 1993; Hendrixson and Di Rita, 2004). Transcriptional analysis of the strains 11168-O and 11168-GS indicated that several genes involved in flagellar export and motility were expressed to significantly higher levels in the more motile and successful coloniser 11168-O compared to 11168-GS (Gaynor *et al.*, 2004). Whether this was due to motility *per se* or the use of flagella for adherence to the chicken intestinal mucosa is unclear at this time, though it is suggested that the presence of flagella rather than motility is the more important factor (Scott *et al.*, 2007). It has also been shown recently that glycosylation of the flagella is necessary for correct assembly of the flagella filament (Goon *et al.*, 2003) in *Campylobacter*. Mutations in genes involved in biosynthesis of the glycosyl moieties, notably in *Cj*1293 and *ptm*D, resulted in non-motile and non-flagellated *Campylobacter*. Transcriptional analysis of 11168-O and 11168-GS and other *C. jejuni* strains has indicated that a number of key genes involved in the tri-carboxylic acid cycle and electron transport are also important for

successful colonisation, highlighting the importance of the ability to tolerate different oxygen conditions (Gaynor *et al.*, 2004, Woodall *et al.*, 2005). Some other genes which appear to be necessary for colonisation but are unnecessary for *in vitro* growth include: the major adhesion protein, cadF, which is likely involved in adherence to the mucosa of chicken intestines (Ziprin *et al.*, 1999; Ziprin *et al.*, 2001), the heat-shock protein dnaJ (Konkel *et al.*, 1998) and docA, which encodes a cytochrome c peroxidase likely involved in oxygen tolerance (Atack and Kelly, 2007).

1.6. *CAMPYLOBACTER* IN PIGS

Pigs are a natural reservoir of campylobacters and also are an important source for Campylobacteriosis in humans. The excretion level of *Campylobacter* in pigs ranges from 10^2 to 10^7 cfu g^{-1} (Jensen *et al.*, 2006). The majority of the isolates obtained from pigs are *C. coli* (Weijtens, *et al.*, 1999), but *C. jejuni* was also reported in 10- 100 fold lower numbers (Madden *et al.*, 2000). A high percentage of *C. jejuni* was reported from porcine livers (Kramer *et al.*, 2000). According to Brown *et al.*, (2004), the closer contact of pig with the outdoor environment where *C. jejuni* is present in a higher level could result in the higher number to be present in pigs and thereby it can cause food safety concern. Alter *et al.*, (2005) reported that free range pigs from a single farm seemed to be colonised with *Campylobacter* earlier in their life than conventional pigs while according to Mikkelsen *et al.*, (2004) the lower animal density in outdoor reared pigs reduces the infection period and roughage promote gastrointestinal health.

1.7. HUMANS AND CAMPYLOBACTERS

1.7.1. Human diseases caused by *Campylobacter*

Campylobacter spp. is an important cause of human illness (Friedman *et al.*, 2000). Laboratory reports of human *Campylobacter* cases reported to the Health Protection Agency Centre for Infections in England and Wales for the year 2008 was 49880 when compared to 6019 for *Salmonella* and 6827 for Norovirus (www.hpa.org.uk). An incidence rate of 47 per 100,000 of the population per year, of *Campylobacter* caused bacterial gastrointestinal disease, was reported both in the Republic of Ireland and Northern Ireland (Danis *et al.*, 2009). Studies done by Centers for Disease Control and Prevention in US, which began tracking *Campylobacter* infections from 1996, reported *Campylobacter* infection has declined by 30% between 1996 and 2006 yet remains highest among children under 5 years of age (Brunk, 2007). The incidence in the USA in 2006 was reported to be 13 per 100,000 (www.entrepreneur.com/tradejournals/article/173150685.html). With such high incidence rates throughout the world, *Campylobacter* is driving an important public health concern. In addition to the considerable illness and loss of productivity it may be associated with sequelae, such as reactive arthritis and Guillain Barré syndrome (Allos, 1998; Friedman *et al.*, 2004; Wingstrand *et al.*, 2006), which are discussed further in the next section. Risk factors associated with *Campylobacter* infection in developed countries included: consumption and handling of chicken, and in particular undercooked chicken or commercially prepared chicken; unpasteurised milk and dairy products; consumption of untreated water; contact with domestic pets like dogs and cats; contact with farm animals; travel abroad etc (Tenkate and Stafford, 2001; Eberhart-Phillips *et al.*, 1997; Rodrigues *et al.*, 2001; Studahl and Anderson, 2000;

Potter *et al.*, 2003; Schonberg-Norio *et al.*, 2004). In addition, epidemiological studies conducted in the United Kingdom have suggested that there may even be regional differences in the contributing risk factors for infection (Adak *et al.*, 1995).

1.7.2. Symptoms of diseases

Once *Campylobacter* has been ingested the incubation period ranges between 18 h to 8 days. The clinical consequence of infection is thought to depend upon the virulence of the infecting strain, challenge dose and susceptibility of the patient, although data is very limited. The onset of the disease is marked by the presence of one or more symptoms which include acute diarrhoeal disease characterised by diarrhoea, fever and abdominal cramps (Butzler, 2004). In 50% of the population this is followed by a febrile period with malaise, myalgia, abdominal pain and fever. Some patients develop rigors (22%) and the high temperature can cause convulsions in children or delirium in adults (Jones *et al.*, 1981). The prodome symptoms can mislead if abdominal symptoms are absent and the patients develop more illness when compared to others. Stool samples may contain blood, pus, mucus and inflammatory exudates with leucocytes, bile stained and *Campylobacter* upon examination under a microscope. Surveys have shown that at least 50% of the patients attending hospital have 10 or more bowel movements per day (Blaser and Enberg, 2008). Blood appears in the stools of 15% of the patients confirming the presence of infection in the colon and rectum (Blaser and Enberg, 2008). The abdominal pain is sufficiently intense and continuous and it may mimic appendicitis. Other symptoms include fulminating sepsis and death occurs rarely in immunosusceptible hosts. Patients having severe

abdominal pain especially teenagers and adults may develop peritonitis from acute appendicitis (Butzler, 2004) but this is also very rare. After a variable period of 3-4 days the diarrhea begins to ease and the patient's condition improves though the abdominal pain may persist for several more days. Minor relapses have been reported in 15-25% of the patients (Drake *et al.*, 1981). Weight loss of up to 5 kg is reported. Even after clinically recovering from the disease, patients may continue to excrete campylobacters in their feces if not treated with antibiotics. The mean excretion period was found to be 37.6 days (maximum 69 days) (Kapperud *et al.*, 1992). Less than 1% of the patients whose immune system is compromised develop bacteraemia. Some patients may develop reactive arthritis (Butzler, 2004). In rare cases extra intestinal diseases like meningitis osteomyletis and neonatal sepsis are also seen (Vandenberg *et al.*, 2003). The intestinal complications also include genuine appendicitis where campylobacters can be isolated from the inflammed appendixes (Chan *et al.*, 1983), colitis and proctitis (Colgan *et al.*, 1980; Price *et al.*, 1979), toxic mega colon (McKinley *et al.*, 1980), intestinal hemorrhage (Michalak *et al.*, 1980), ileostomy stoma ulceration (Skirrow, 1981) and perirectal abscess (Krajden *et al.*, 1986).

Infection by *Campylobacter* has increasingly been associated with a number of chronic diseases and the most serious of which is Guillain- Barré syndrome (GBS) which results in reduced or absence of tendon reflexes (Nachamkin, 2002; Rantala *et al.*, 1991). Though the incidence ratio is less than 1 / 1000 as infection rates are so high this is a significant number. Symptoms appear after 3 weeks of the onset of *Campylobacter* enteritis although other infections from *Salmonella* may precede GBS. The peripheral nerve cells are destroyed due to the strong similarity between microbial antigens and the self antigens leading to the death of the tissues

(Nachamkin *et al.*, 2000). Plasma exchange and intravenous immunoglobulin transfer are the treatments available with the later treatment being the more safer and economical of the two (Hadden and Gregson, 2001). According to Winer *et al.*, (1988) one third of the GBS patients have returned to normality, one third has difficulty in walking, one sixth can walk but could not run, 7-20% are unable to walk without assistance and 2-18% is dead. Miller- Fisher syndrome, a variant of GBS is also seen which is characterised by ophthalmoplegia, ataxia and moral areflexia (Allos and Blaser, 1995). The fatality rate for *C. jejuni* infection is 0.05 per 1000 infections (Allos, 2001).

1.7.3. Pathogenic mechanisms

Campylobacters preferentially colonise the mucous layer of the gastrointestinal tract of poultry, animals, and humans. The infection interferes with the normal secretory and absorptive capacity of the intestine thereby leading to gastroenteritis. Campylobacters have been reported to show four major virulence properties; motility, adherence, invasion and toxin production (Walker *et al.*, 1986). The importance of the flagella and motility in the colonization of chicken intestines has been demonstrated (Wassenaar *et al.*, 1993a), and both have an important role in pathogenesis. Flagella are not implicated in adherence alone but they probably aid campylobacters to cross the mucous layer covering the epithelium. For successful invasion there are a number of potential adhesins which mediate the organism's attachment to the host cells. Reported adhesins include flagella, outer membrane proteins (Omps) like PorA and P95 and surface polysaccharide moieties (Hu and Kopecko, 2008). The best characterized *C. jejuni* adhesins include the 37 kDa outer membrane protein termed CadF (*Campylobacter* adhesion to fibronectin) (Konkel *et*

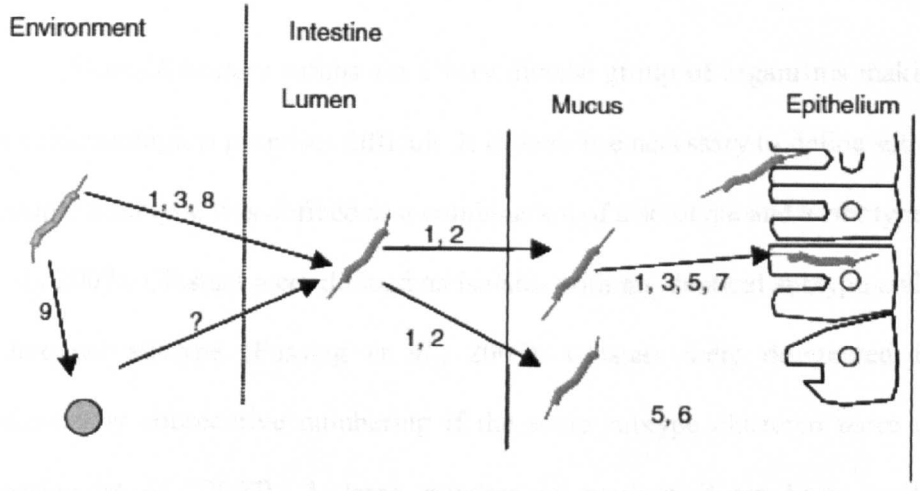
al., 1999), a 42.3 kDa surface exposed lipoprotein termed JlpA (Jin *et al.*, 2001), and a 28 kDa periplasmic membrane-associated protein termed PEB1 (Pei and Blaser, 1993).

Viable *Campylobacter* cells enter the host through food and water ingestion. They penetrate the stomach acid barrier and colonise the mucous layer overlying the epithelium of the ileum and colon (Ketley, 1997). Chemotaxis occurs when they colonize the intestines (Ketley, 1997). *Fla*A mutants are unable to invade human epithelial cells since they lack the structural gene for flagella, thereby motility (Fields and Swerdlow, 1999). According to Yao *et al.*, (1994) *C. jejuni* mutants with decreased motility because of paralysed flagella show reduced adhesion and no invasion indicating that flagella are involved in adherence. Adhesins are involved in subsequent internalisation. Super oxide dismutase (SodB), was the main component of super oxide stress defence and the *sod*B mutants would be having less survival rates in the intestine (Pesci *et al.*, 1994). The internalisation mechanism of *C. jejuni* is due to the combined effect of microfilaments and microtubules of host intestinal epithelium cells (Biswas *et al.*, 2000; Hu and Kopecko, 1999). *Campylobacter jejuni* cannot escape this tubulin compartment and the replication rate is limited inside this. Catalase enzyme is produced to protect against the oxidative stress of lysosome (Forsythe, 2000). The symptoms produced in humans due to *C. jejuni* infection are reported to be partly due to the release of cytolethal distending toxin (CDT), which induces host cell death and the following inflammatory responses (Newell, 2001). CDT triggers cell cycle arrest following the death of the cell and mediates interleukin-8 production and its release from the epithelium (Kopecko *et al.*, 2001).

Flagella are one of the best defined virulence factors, as *fla*A and *fla*B mutants are markedly reduced in virulence. Whether this is due to a direct involvement of the

flagellin, or perhaps an inability to secrete proteins through the flagella export apparatus, is unknown. Studies have shown that motility and the expression of the *fla*A gene is needed for maximum invasion into the host cells (Grant *et al.*, 1993) though its also shown by Guerry *et al.*, (1991) that *C. jejuni fla*A(*fla*B$^+$) strain is more invasive than *C. jejuni fla*A(*fla*B). This is due to the secretion of Cia proteins which occurs more in *C. jejuni fla*A(*fla*B$^+$) strain than in *C. jejuni fla*A(*fla*B) (Grant *et al.*, 1993).

Figure 1.2. The different phases of *Campylobacter* invasion of the host intestine



1- motility; 2- chemotaxis; 3-oxidative stress defence; 5- invasion; 6- toxin production; 7- iron acquisition; 8- temperature stress response; 9- coccoid dormant stage. —- viable *Campylobacter* cell; - coccoid dormant *Campylobacter* cell; - epithelial cell. Adapted from van Vliet, A.H. and Ketley, J.M. (2001) Pathogenesis of enteric *Campylobacter* infection. Symp. Ser. Soc. Appl. Microbiol. 30, 45S–56S and Snelling *et al.*, 2005a.

C. jejuni exports their proteins through the flagellar Type III secretion system (T3SS) which shows the relatedness between flagellum and virulence. Cia proteins which contribute to the invasion are secreted through the flagellum which requires a functional basal body, hook and at least one of the filament proteins (Larson *et al.*,

2008). Mutations that affect the export of flagellar components (*flh*B) and non filament structural components (*flg*B, *flg*C, *flg*E2) show negative *cia*B phenotype (Larson *et al.*, 2008). The Cia proteins are also distinct from the flagellar proteins based on the reasons that the *C. jejuni cia*B mutant is motile (Konkel *et al.*, 2004) and the expression of *cia* genes is regulated in a manner which is distinct from flagellar genes. σ^{28} is responsible for the expression of the *C. jejuni* flagellar class III genes which includes the gene encoding the *fla*A filament proteins (Larson *et al.*, 2008).

1.8. *CAMPYLOBACTER* TYPING METHODS

Campylobacters strains are a very diverse group of organisms making typing for epidemiological purposes difficult. It is therefore necessary to define subtypes. For example a subtype was defined as a combination of a serotype and a ribotype (Fussing *et al.*, 2007). Clusters were defined as isolates with an identical subtype and hence as a frequent subtype (Fussing *et al.*, 2007). Clusters were designated B01–B34, followed by consecutive numbering if the same subtype clustered more than once (Fussing *et al.*, 2007). A large number of methods have been used to type *Campylobacters* and these are described in the following sections.

1.8.1. Ribotyping

Campylobacter has three copies of the rRNA genes coding for 16S and 23S rRNA which are conserved and yet surrounded by variable flanking DNA (Fouts *et al.*, 2005). Restriction endonuclease digested *Campylobacter* genomic DNA can be separated by agarose gel electrophoresis and transferred to nylon membranes by Southern blot which is a commonly used technique. The DNA is digested using a six

cutter enzyme and is hybridised with a common rRNA probe though the genes are located in different genomic fragments. Since there are only three copies of ribosomal genes in *Campylobacter* when compared to other enteric bacteria, this method is considered to be less discriminatory when compared to others (Fayos *et al.*, 1992). However, there are several literature reports that have used the method to type *Campylobacter* isolates (Fayos *et al.*, 1992; Fitzgerald *et al.*, 1996; Gibson *et al.*, 1995; Ge *et al.*, 2006; O' Reilly *et al.*, 2006). The automated ribotyping involves the use of Dupont qualicon riboprinter which undertakes lysis of the cells, DNA isolation, restriction digestion, electrophoresis, Southern blot analysis and hybridisation with chemiluminescently labelled 16S to 23S rRNA primer, detection of patterns and comparison of the pattern with previously isolated ones (On *et al.*, 2008).

1.8.2. Restriction fragment length polymorphisms (RFLP)

Campylobacter coli and *C. jejuni* have two flagellin genes designated as *fla*A and *fla*B of 1.7 kb in size and are separated by an intergenic spacer region of approximately 170 bp. The 5' and 3' regions of these genes are highly conserved with considerable sequence variation in the region between them (Meinersmann *et al.*, 1997) and thus can be used as a basis for typing by restriction fragment length polymorphism. PCR primers are designed for these regions that amplify specific regions of the cluster. The product is digested by using various restriction enzymes and the fragments are separated by electrophoresis in an agarose gel. In essence the technique is fairly simple and applicable to several *Campylobacter* species (Owen *et al.*, 1993). However, there is difficulty in standardisation of the electrophoresis conditions between different labs (Djordjevic *et al.*, 2007; Nachamkin *et al.*, 1996) and this makes comparison of data from different labs difficult. Direct sequencing of

the PCR amplified short variable regions (SVR) overcomes these problems, however studies found that the diversity of SVR is less than the RFLP types (Corcoran *et al.*, 2006).

1.8.3. Random amplified polymorphic DNA (RAPD)

RAPD is a PCR based DNA finger printing method which uses short (10 bp) arbitrarily designed primers in low stringency PCR to differentiate DNA from various sources. As with *fla*A typing there are numerous RAPD schemes (Fayos *et al.*, 1993, Fujimoto *et al.*, 1997, Lam *et al.*, 1995, Mazurier *et al.*, 1992, Nishimura *et al.*, 1997). Giesendorf *et al.*, (1993) modified the procedure by using purified *C. jejuni* DNA and a pair of randomly generated primers to study the heterogeneity of *C. jejuni* DNA which hinders general application of the method. There are also issues related to the reproducibility of the method (Hernandez *et al.*, 1995) and the subjective basis on which the gel profiles are interpreted (Fayos *et al.*, 1993).

1.8.4. Pulsed field gel electrophoresis (PFGE)

PFGE, also known as macro restriction profiling, is a modification of RFLP which is used to type *Campylobacter* (Yan *et al.*, 1991). The cells are lysed *in situ* in chromosomal grade agarose to prevent DNA shearing. The *Campylobacter* genomic DNA is digested using rare cutting enzymes especially *Sma*I so that they are cut into relatively few fragments of larger size and are separated using electrophoresis using pulsed electric fields from different positions in the electrophoresis cell thereby obtaining the macro restriction profiles (MRP). PFGE was originally used to determine the genome size of the various *Campylobacter* strains and also used to construct the genetic map of *C. jejuni* strain. There are several disadvantages

associated with this method which include: it is relatively time consuming to make the DNA agarose plugs, degradation of the DNA samples due to the activation of DNase before electrophoresis, limited number of restriction enzymes that cut the DNA of *Campylobacter* spp. into useful size fragments and differences in the interpretation of the results due to genetic instability.

1.8.5. Amplified fragment length polymorphisms (AFLP)

AFLP is a high resolution genotyping method used to genotype bacteria including *Campylobacter* (Aspinall *et al.*, 1996). It involves the digestion of the chromosomal DNA with two restriction enzymes, one with a 4 bp recognition site and one with a 6 bp recognition site which digest *Campylobacter* genomic DNA completely. The restriction fragments are amplified using appropriate PCR primers so that only those genomic fragments that feature alternative restriction sites at their termini are amplified. The PCR products are separated by agarose gel electrophoresis and the profiles compared. This technique has significant discriminatory power because it reports on a significant portion of the genome (Duim *et al.*, 1999; Kokotovic and On, 1999). Two AFLP methods are used to subtype campylobacters. They use either *Hind*III and *Hha*I or *Bgl*II and *Csp6*I. Studies have shown that AFLP and PFGE provided the same level of discrimination but AFLP needed the use of several restriction enzymes for optimal discrimination (Kokotovic and On, 1999).

1.8.6. Multi locus sequence typing (MLST)

MLST examines nucleotide polymorphisms generated by neutral genetic variation in multiple chromosomal locations. Multilocus sequence typing (MLST) is a

comparative genomics method for characterising isolates of bacterial species using the sequences of internal fragments of the seven house-keeping genes. Using automated DNA sequencing approximately 450-500 bp internal fragments of each gene are accurately sequenced. The allelic profile is determined by numbering the house keeping genes as distinct alleles.In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The great advantage of MLST is that sequence data are unambiguous and the allelic profiles of isolates can easily be compared to those in a public database (Manning *et al.*, 2003). Identification of lateral gene transfer is possible through MLST and it is facilitated by the presence of four common loci ie *atp*A, *gln*A, *gly*A and *tkt* within the MLST typing methods (Miller *et al.*, 2005; Stoddard *et al.*, 2007; van Bergen *et al.*, 2005) but the MLST set of seven genes is < 0.5% of the total no of genes in the average *Campylobacter* genome.

1.8.7. Other typing methods

Other typing methods include serotyping and phage typing which are used to classify campylobacters from different origin. Penner and Hennessy (1980) developed a serotyping scheme based on heat stable antigen using a panel of 42 antisera (Wareing *et al.*, 2002). Passive haemagglutination was used as a detection system so as to eliminate non specific agglutination reactions. The main disadvantages of this system include lack of reproducibility as a result of variation in source, age, concentration and condition of the red blood cells (Fricker *et al.*, 1987) and many isolates agglutinated more than one antiserum particularly serogroups 4, 13, 16 and 50 (Abbott *et al.*, 1980). A modified serotyping method based on direct bacterial agglutination and using absorbed antisera was developed in the Laboratory of Enteric

Pathogen (LEP); (Frost *et al.*, 1998). It defines 44 serotypes of *C. jejuni* and 17 in *C. coli* and 19% of untypable serotypes. *Campylobacter* has two distinct heat stable antigens and it is suggested that the Penner method detects the capsular antigen while the direct agglutination method detected both capsule and lipopolysaccharide (Oza *et al.*, 2002). A phage type is defined as two or more epidemiologically unrelated isolates giving the same reaction pattern (Newell *et al.*, 2000). It is a common method of typing strains of *C. jejuni* and *C. coli* and a total of 76 defined phage types have been recognised (Frost *et al.*, 1999). The predominant phage type in *C. jejuni* is PT1, a type defined on the basis of sensitivity to phages 4 and 12 and that in *C. coli* is PT 2 and PT 7 (Frost *et al.*, 1999). Some other phage type distribution in *C. jejuni* includes PT 14, PT 33, PT 10 and that for *C. coli* is PT 44 and PT 17. Phage typing has enabled the subdivision of each of the 10 serotypes into 6 sub types. According to Frost *et al.*, (1999) to date there are 66 serotypes and 76 defined phage types. All these techniques are now superseded with molecular methods to type *Campylobacter.*

1.9. DIVERSITY OF *CAMPYLOBACTER*

Extensive genetic variations have been revealed among strains of *C. jejuni.* Phenotypic variations have also been observed for traits like invasion, sialylation of lipooligosaccharide, colonisation of chickens, natural transformation, toxin production and serum resistance (Wiesner and DiRita, 2008). The variation can occur through multi gene insertions and deletions that may result in homologous recombination (Taboada *et al.*, 2004). *C. jejuni* has also been shown to undergo intra genomic recombination during colonisation in the chicken gut (Scott *et al.*, 2007, Hanninen *et*

al., 1999). The genetic diversity can occur as a result of local sequence changes due to nucleotide substitutions or small insertions or deletions of one or few nucleotides (Arber, 2000). Another cause of diversity is DNA rearrangement where related sequences in the genome undergo recombination to create novel fusion genes. Diversity can also develop through horizontal acquisition of DNA through mechanisms such as natural transformation, conjugation and transduction (Wiesner and DiRita, 2008).

1.9.1. Horizontal DNA transfer

Campylobacter has been shown to be naturally transformable both in the laboratory (Taylor, 1992; Wassenaar *et al.*, 1995) and under environmental conditions (Avrain *et al.*, 2004). A number of studies have also suggested that horizontal transfer of DNA may be responsible for a large degree of the observable phenotypic diversity in *Campylobacter*, including changes in antibiotic resistance (Wittwer *et al.*, 2005), capsular synthesis (Karlyshev *et al.*, 2005), lipooligosaccharide synthesis (Gilbert *et al.*, 2004) and flagella synthesis (Harrington *et al.*, 1997). In *C. jejuni*, *fla*A is required for motility while *fla*B acts as a donor for recombination into *fla*A and horizontal DNA transfer has frequently been observed in *C. jejuni* flagellin genes *fla*A and *fla*B. Both intra genomic and inter genomic recombination occur at this locus (Alm *et al.*, 1993). Nuijten *et al.*, (2000) observed that intra genomic rearrangement and recombination occurred through cellular division when *fla*A mutants and wild strain 81116 were simultaneously used to colonise chicks. Horizontal gene transfer events were also identified in the studies done by de Boer *et al.*, (2002), Dingle *et al.*, (2001a) and Schouls *et al.*, (2003).

1.9.2. Intra genomic recombination

It has been shown that *Campylobacter* is able to undergo high frequency chromosomal recombination to generate diversity. The two flagellin genes of *C. jejuni*, *fla*A and *fla*B, are located adjacent to each other and share extensive sequence similarity but have different promoters (Guerry *et al.*, 1990; Nuijten *et al.*, 1990). It is found that *fla*B is not required for motility and it is speculated to be a sink for antigenic variation or for motility changes under different environmental conditions (Alm *et al.*, 1993; Wassenaar *et al.*, 1994; Wassenaar *et al.*, 1995). Recombination involving these two genes has been observed *in vitro* (Alm *et al.*, 1993; Wassenaar *et al.*, 1995) and *in vivo* (Nuijten *et al.*, 2000). With the *fla*B product playing an apparently minimal role in flagellin structure and cell motility, the reason for flagellin gene duplication in *Campylobacter* spp. is yet to be established. However, it has been suggested that *fla*B may serve as a gene donor, of which parts could be introduced (through homologous recombination) into the *fla*A gene, either to compensate for deleterious mutations or possibly to increase the immunogenic repertoire of a given *C. jejuni* strain. Homologous recombination has also been noted at the *C. fetus sap* locus, encoding genes for S-layer proteins (SLP). The SLP are encoded by five to nine *sap*A homologues which are tightly clustered on the chromosome (Dworkin *et al.*, 1995; Tu *et al.*, 2001; Tu *et al.*, 2003), and each *sap*A homologue is potentially expressed by a unique *sap*A promoter (Dworkin and Blaser, 1996). The *sap* locus undergoes high frequency recombination to place this promoter in front of different *sap*A homologues, thereby causing SLP phenotypic switching (Dworkin and Blaser 1997, Garcia *et al.*, 1995, Ray *et al.*, 2000). Chromosomal rearrangements have also been implicated as the cause of *in vivo* changes to macro-restriction profiles observed by pulsed field gel electrophoresis (de Boer *et al.*, 2002; Hänninen *et al.*, 1999, Stern *et*

al., 1994, Wassenaar *et al.*, 1998). Similarly, macro-restriction profile instability has been noted in some *C. jejuni* and *C. coli* isolate *in vitro* (Dickins *et al.*, 2002).

1.10. BACTERIOPHAGE

The discovery of bacteriophages was an important milestone in the history of microbiology which happened shortly after Mendel's laws were rediscovered. Bacteriophages are defined as viruses that can infect, multiply and kill susceptible bacteria (Connerton *et al.*, 2008). They are viruses which prey upon bacteria. They were first described in the early 1900s by Frederick Twort (1915) and Felix d'Herelle (1917) and it was d'Herelle who gave them the name bacteriophages which mean bacteria eaters (Ackermann, 2003). Bacteriophages (phage) have an enormous influence in the ecosystem by imparting their role as biological agents for the rapid decay and replenishments of bacterial populations and also in nutrient cycling (Fuhrman, 1999).There are estimated to be ~10^{31} phages in the biosphere, making them the most abundant biological entities on the planet (Rohwer and Edwards, 2002). Phages are ubiquitous, and have been isolated from numerous and extremely diverse environmental conditions. It has been reported that there are approximately 10^{10} phage per liter in surface seawater (Bergh *et al.*, 1989) and 10^{7} to 10^{9} per g in sediment or topsoil. Their presence was also reported from sewage (Salama *et al.*, 1989), soil (Ashelford *et al.*, 2003), food (Atterbury *et al.*, 2003b), hot springs (Børsheim *et al.*, 1990) and animal sources like poultry (Loc Carillo *et al.*, 2005). Bacteriophages have long been exploited as a source of molecular biology tools (Ackerman and Du Bow, 1987) as major conduits of genetic exchange, transducing an

estimated 10-20 kb of DNA. Humanised bacteriophages which were derived from a modification of the M13 bacteriophage were used for gene transfers in mammalian cells (Saggio, 2008). They were also successfully used to treat bacterial infections a decade before penicillin was discovered. Though recently there has been resurgence in interest in the use of bacteriophage as an alternative to antibiotic therapy (Barrow and Soothill, 1997; Matsuzaki *et al.*, 2005). The resurgence in interest has been galvanised by the rise in antibiotic resistance in bacteria (Hanlon, 2007). Other novel applications of bacteriophage include anti-cancer therapies (Agu *et al.*, 2006; Dabrowska *et al.*, 2004) and they are also used as potential vaccine delivery vehicles in two different ways: by directly vaccinating with phages carrying vaccine antigens on their surface or by using the phage particle to deliver a DNA vaccine expression cassette that has been incorporated into the phage genome (Clark and March, 2006).

1.10.1. Characteristics and life cycle

Bacteriophages are obligate intracellular parasites of bacteria that require specific hosts in which to replicate. They consist of either a DNA or an RNA genome contained within a protein coat. They infect bacteria and either incorporate their viral genome into the host genome, replicating as part of the host (lysogeny), or they multiply inside the host cell before releasing phage particles either by budding from the membrane or by actively lysing the cell referred to as the lytic life cycle (Clark and March, 2006). The bacteriophage coat (capsid) may or may not be surrounded by a lipid envelope or have a tail structure. Bacteriophages are currently classified to family and genus by the International Committee on the Taxonomy of Viruses (ICTV) based largely on particle morphologies and genome structure, although antigenic

properties and host range are also considered. According to ICTVdB 2005 there are 72 families, 255 genera and 1433 species (http://www.ncbi.nlm.nih.gov/ICTVdb/) and numerous viruses still awaiting classification.

1.10.2. Bacteriophage of *Campylobacter*

Campylobacter bacteriophages can be isolated from the gut of broiler chickens, chicken meat (Salama *et al.*, 1989; Atterbury *et al.*, 2003a) and also from pig manure, abattoir effluents, sewage, retail poultry meat and poultry manure (Atterbury *et al.*, 2005; El-Shibiny *et al.*, 2005; Grajewski, 1985). Over 170 *Campylobacter* bacteriophage have been reported with the majority belonging to the *Myoviridae* family and a few from the *Siphoviridae* family (Atterbury *et al.*, 2003b, Sails *et al.*, 1998). *Campylobacter* bacteriophage belonging to the *Myoviridae* family generally have double stranded DNA genomes, are lytic and have icosahedral heads and contractile tails (Loc Carillo *et al.*, 2007). It is thought that extracellular polysaccharide and the bacterial flagella serve as receptors for *C. jejuni* phages (Scott, 2006). In addition to the lytic phage, the genome sequence of RM1221 has revealed the presence of Mu like phage sequences in *C. jejuni*. One of these sequence regions, CMLP1, is located upstream of *arg*C (CJE0275) and encodes several proteins with similarity to bacteriophage Mu and other Mu like prophage proteins (Morgan *et al.*, 2002) including putative MuA and MuB transposase homologs. Although this Mu like prophage contains no characterised virulence determinants, it could potentially alter pathogenicity or other phenotypes *via* insertional inactivation (Fouts *et al.*, 2005).

1.10.3. Phage therapy

Bacteriophages have major applications in modern biotechnology and industry. Phage therapy uses an appropriate titre of virulent phages to lyse the pathogenic bacteria. The idea of phage therapy was developed by d'Herelle where therapeutic and prophylactic treatments were used to destroy the pathogenic bacteria (Fruciano, 2007). Even in early 1917, d'Herelle and his collegues isolated phages which could kill pathogenic bacterias such as *Shigella dysenteriae*, *Salmonella Typhi*, *Escherichia coli*, *Pasteurella multocida*, *Vibrio cholerae*, *Yersinia pestis*, *Streptococcus* species, *Pseudomonas aeruginosa* and *Neisseria meningitidis* (Ackermann., 1987). Although phage therapy was used to treat and prevent bacterial infectious diseases in the former Soviet Union and Eastern Europe, it was abandoned by the West in the 1940s with the arrival of the antibiotic era. Phages have been successfully used as biocontrol agents to destroy plant bacterial pathogens like *Pseudomonas, Erwinia amylovora, Agrobacterium* spp. though some reports highlight the fact that bacteriophage may destroy beneficial bacteria like *Pseudomonas fluorescens* which may have protective attributes to the plants (Keel *et al.*, 2002). Studies on humans reported that the filamentous bacteriophages were able to move into the brain and dissolve β-amyloid plaques thereby protecting against the neural degeneration (Fisher and Solomon, 2008). Phage treatments have been successfully carried out in chickens which showed drastic reductions in the population of colonising *Campylobacter* (Wagenaar *et al.*, 2005, Atterbury *et al.*, 2003a; Loc Carillo *et al.*, 2005). Another application involved a cocktail of 8 bacteriophages of which 5 against *Pseudomonas aeruginosa*, 2 against *Staphylococcus aureus* and one against *Escherichia coli* were used to treat infected leg ulcers in Texas, US (Mazra *et al.*, 2006). Bacteriophages are also used as potential vehicles for the delivery of

vaccines by carrying antigens on their surface or to deliver a DNA extension cassette that has been engineered into the bacteriophages genome (Clark and March, 2004).

1.10.4. Obstacles to the acceptance of phage therapy

1.10.4.1. Biological problems

Resistance of bacteria to phages is often caused by changes in the phage receptor molecules in Gram negative bacteria. In phages of Gram negative bacteria, host range mutant phages, which have the ability to adsorb to the host, are easily isolated from the original phage population. Bacterial resistance occurs due to phase variation, point mutation and intra genomic recombination (Scott *et al.*, 2007). Phages may be inactivated by a neutralising antibody as such antibodies have been detected in mouse blood (Matsuzaki *et al.*, 2005). It is also known that phages can incorporate bacterial toxin genes within their genomes which may be transferred to other strains increasing their pathogenicity. This can be overcome by developing phages that do not have generalised or specialised transduction ability (Schoolnik *et al.*, 2004). One of the biggest problems to overcome is the fact that so little is known about the interactions of bacteriophages in the gut and other anaerobic environments.

1.10.4.2. Regulatory issues

The development of bacteriophage therapeutics has been obstructed by issues of intellectual property rights since they were first suggested a century ago. Government regulatory authorities require removing phages used for bio control purposes from the final food product (Connerton *et al.*, 2008).

1.11. ANTIBIOTIC RESISTANCE

The wide spread use of antibiotics both for therapeutic purposes and feed supplements have led to the development of resistance in microorganisms. The use of the same antibiotics used in medicine for treating cattle and poultry has lead to antibiotic resistance in many human pathogens since the animals are the basic source of these pathogens and vehicles for transmission (Aquino *et al.*, 2002). Resistance can also emerge due to the use of antibiotics as growth promoters in domestic animal breeding (Aarestrup and Wegner, 1999). Reports have shown that all *C. jejuni* and *C. coli* strains are intrinsically resistant to rifampin, novobiocin, bacitracin, streptogramin B, cephalothin (Taylor and Courvalin, 1988). Flouroquinolones may be prescribed for adults having *Campylobacter* enteritis, although in most cases treatment with antibiotics does not generally affect the outcome of infection. These interact with DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997; Enberg *et al.*, 2001). Ciprofloxacin for early empirical treatment of adults, especially for travel related disease, and erythromycin for treatment following microbiological confirmation was widely used (Hakanen *et al.*, 2003). The prevalence of fluoroquinolone resistant *C. jejuni* in the USA was 0% in 1990, increasing to 13% in 1997, and to 18% in 1999, following the approval of fluoroquinolones for use in poultry farming in 1995 (Smith *et al.*, 1999a; Nachamkin *et al.*, 2002). It is reported that from 1997 to 2001 in US, ciprofloxacin resistant *Campylobacter* has significantly increased from 13 to 19% (De Moura Oliveira, 2006). Fluoroquinolone resistance has also been reported from Europe (Talsma *et al.*, 1999) Asia (Murphy *et al.*, 1996; Padungtod *et al.*, 2003), and Latin America (Fernadez, 2001).

Antibiotic resistance can be achieved by the horizontal acquisition of resistance genes (carried by plasmids or transposons) or by recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci (Martinez and Baquero, 2000). The *Campylobacter* genus, with its Gram negative cell wall but very low G+C content, has apparently been able to acquire resistance determinants from both Gram positive and Gram negative organisms, although the former seem to be the more common source. Genes such as *tet*O, *aph*A-I, and *aph*A-3 have been able to become integrated into a plasmid, which was probably indigenous to *C. jejuni* and *C. coli*, or occasionally into the chromosome (Trieu- Cout *et al.*, 1985). The mechanisms involved in fluoroquinolone resistance include the modification of DNA gyrase and/ or the topoisomerase IV, active efflux, altered membrane permeability and reduced target enzyme expression (Zhang and Plummer, 2008) and that of the macrolides is by antibiotic modification, target site modification or alteration and drug efflux (Leclercq, 2002). *Campylobacter* resistance to tetracycline involves a ribosomal protection protein called *tet*O which is mainly encoded on transferable plasmids as well as chromosomally encoded in some strains (Manavathu *et al.*, 1988). Resistance to kanamycin in *C. coli* is probably due to *in vivo* acquisition of a gene from a Gram positive coccus, 3′ aminoglycoside phosphotransferases account for the majority of aminoglycoside modifying enzymes reported in *Campylobacter* spp. They are responsible for the phosphorylation of 3′ hydroxyl group of aminoglycosides (Zhang and Plummer, 2008). Resistance to β lactams is by the production of β-lactamases and their presence is documented in some *Campylobacter* strains which thereby show resistance to β- lactam antibiotics (Li *et al.*, 2007). The organism may also develop alterated membrane structures such as the porin proteins and the modifications may reduce the uptake of β- lactam drugs from the environment (Siu,

2002; Page *et al.*, 1989). The chloramphenicol resistance gene occurs on a *C. coli* plasmid and it encodes an acetyltransferase that modifies chloramphenicol and thereby confers resistance to *Campylobacter* (Tracz *et al.*, 2005). The acquired antibiotic resistance of *Campylobacter* sp is given in table 1.1.

Table 1.1. Acquired antibiotic resistance in *Campylobacter* sp.

Antibiotic	MIC (μg/ml)	Gene	Location	Mechanism
Tetracycline Minocycline	⩽1024 ⩽32	*tet*O	Plasmid	Ribosomal protection
Kanamycin	>4000	*aph*A- 3 *aph*A-1	Plasmid Chromosome	3'-Aminoglycoside phosphotransferase 3'-Aminoglycoside phosphotransferase
Chloramphenicol	100	*cat*	Plasmid	Ribosomal protection
Streptomycin Spectinomycin	>4096	*aad*A	Chromosome	6-Aminoglycoside nucleotidyltransferase
Erythromycin	≥1024	*erm*	Chromosome	Ribosomal protection
Ampicillin	≥128	blaOXA-61	Plasmid	β-Lactamase

Table adapted from Taylor and Courvalin, 1988, Anderson, 2005 and Zhang and Plummer, 2008. MIC represents Minimum Inhibitory concentration of the corresponding antibiotic.

To reduce the resistance rates Fernandez (2001) recommended surveillance, monitoring of resistance, prudent use of antibiotics, education, research and antimicrobial treatment using vaccines.

1.12. THE *CAMPYLOBACTER* GENOME

To date ten fully sequenced and annotated *Campylobacter* genomes have been published of which five were *C. jejuni* (http://www.ncbi.nlm.nih.gov/sites/entrez). The genomes of the three human clinical strains share 1,474 core genes, with 35 genes unique to 81-176, 38 genes unique to CG8486 and 8 genes unique to NCTC 11168

(Champion *et al.*, 2008). Their chromosomal loci are hot spots for intra and inter genomic combinations which include *Cj*0564 and *Cj*0570 which are unique to NCTC 11168 and RM1221. The genome of *C. jejuni* is characterised by a low G+C content that ranges between 29.5 in *C. lari* (Fouts *et al.*, 2005) to 44.5% in *C. curvus* (Miller, 2008). The size of the genome also ranges wildly between 1.5 Mb for *C. lari* (Miller *et al.*, 2008) to 2.1Mb for *C. concisus* (Matsheka *et al.*, 2001) and there are relatively few open reading frames between 1425 and 1931 ORFs (Duong and Konkel, 2009) .

The genome of *C. jejuni* NCTC 11168 is 1, 641, 481 base pairs (bp) in length (Parkhill *et al.*, 2000) and there are 1643 coding sequences (Gundogdu *et al.*, 2007). The average gene length is 948 bp, and 94.3% of the genome codes for proteins, making it the densest bacterial genome sequenced to date (Parkill *et al.*, 2000). Functional information could be deduced for 77.8% of the 1,643 Coding sequences (CDSs), whereas 13.5% matched genes of unknown function in the database and 8.7% had no database match. The *C. jejuni* genome almost completely lacks repetitive DNA sequences. There are only four repeated sequences within the entire genome; three copies of the ribosomal RNA operon (6 kilobases (kb)) and three duplicated or triplicated coding sequences (CDS) (Parkhill *et al.*, 2000). *C. jejuni* NCTC 11168 unlike *C. jejuni* RM1221 and 81-176 does not have any plasmids insertion sequences, prophages or pathogenicity islands (Poly *et al.*, 2007). *C. jejuni* RM1221 though shows some synteny with NCTC 11168 is disrupted with four large integrated elements (Fouts *et al.*, 2005). A *Campylobacter* Mu like phage (CMLP1) is also present in this strain as described in section 1.9.2. Fouts *et al.*, (2005) has reported that the presence of CMLP1 influences the phenotypes by insertional inactivation though it does not have any virulence determinants. The *Campylobacter* genome is not organised into operons or clusters except for the two ribosomal protein operons

and gene clusters involved in Lipo-oligosaccharide (LOS) biosynthesis, Exo polysaccharide (EP) biosynthesis and flagellar modification. The differential expression is mainly due to the presence of phase variable genes (Karlyshev *et al.*, 2005).

The *Campylobacter* genome contains only three predicted sigma factors (*rpo*D, *rpo*N and *fli*A) and 34 regulators which belong to specific transcription factors (Wösten *et al.*, 2008). The *rpo*D is the main essential sigma factor regulating almost all *C. jejuni* promoters. The protein belongs to the σ^{70} family which comprises the primary or housekeeping sigma factors (Wösten *et al.*, 2008). The *fli*A gene which encodes an alternative sigma factor belongs to the σ^{28} family of sigma factors (Wösten *et al.*, 2008). It regulates 10 promoters that direct the transcription of 14 different genes which include the assembly of the flagellar apparatus, proteins involved in glycosylation of the major flagellin subunits (Logan *et al.*, 2002) and virulence associated proteins secreted through the flagellum like *fsp*A and Cj0977 (Goon *et al.*, 2006). The activity of *fli*A is tightly regulated and is kept inactive by *flg*M (Wösten *et al.*, 2004). It only becomes active once the protein has been secreted through the basal body hook structure that forms the platform of filament formation. The *rpo*N gene encodes a sigma factor which belongs to the σ^{54} family of the sigma factors (Wösten *et al.*, 2008) and is conserved throughout the bacterial kingdom (Studholme and Buck, 2000). The 17 identified *rpo*N promoters of *C. jejuni* control the transcription of 23 genes of which 15 encode flagellar assembly proteins (Wösten *et al.*, 2008). In addition *rpo*N regulates components of the basal body, flagellar hook protein, the filament subunit flagellin B and the putative anti sigma factor *flg*M (Carillo *et al.*, 2004; Wösten *et al.*, 2004). It also regulates a number of hypothetical genes (Cj0243, Cj0428, Cj1242, Cj1650) and a UDP-GlcNAc C6 dehydratase

(*Cj*1293; Creuzenet, 2004). Transcription factors other than the sigma factors are classified based on the presence of conserved sequence motifs and homology with related protein species. The largest proportion of regulatory genes consists of members of the two-component regulator family. The genome encodes five major iron acquisition systems, which are mostly organised in operons under the control of the Fur protein (Parkhill *et al.*, 2000). Studies have shown that the activation of fur protein influences the transcription of 44 genes involved in iron acquisition and oxidative stress defense (Holmes *et al.*, 2005; Palyada *et al.*, 2004)

Campylobacters are unable to digest carbohydrates but they utilise amino acids, so unsurprisingly the genomes revealed genes encoding enzymes to digest these substrates. However *C. jejuni* appear to have all the genes necessary for gluconeogenesis, though orthologues of the glucokinase and 6-phosphofructokinase genes were not found. These functions may well be supplied by non orthologous genes (Debruyne *et al.*, 2008).

Sequence diversity between housekeeping genes from different *C. jejuni* strains is minimal (Dingle *et al.*, 2001b; Suerbaum *et al.*, 2001). The first *C. jejuni* microarray, or the clone array which used vectors as a by product from the sequencing project, was constructed in 2001 (Dorrell *et al.*, 2001). These comparisons revealed a great genetic diversity between strains; though they shared a set of core genes and some accessory genes present in one or more strains in a species (Champion *et al.*, 2008).

Optimum clones for all putative coding sequences (CDS) were selected, and PCR products were amplified from these clones using a single pair of vector primers. These PCR products were spotted onto glass slides to produce a low cost *C.*

jejuni whole genome microarray. Although only 34.5% of the PCR products were gene specific, this type of microarray is suitable for performing genomotyping, where the data generated are qualitative rather than quantitative.

1.13. SURVIVAL OF *CAMPYLOBACTER* IN EUKARYOTIC CELLS

Microbial pathogens have evolved their own secure replication and survival mechanisms when they are in association with their host cells. This involves a group of bacterial determinants that interact with the host molecules at the cell interfaces to modulate the cell interface and the cellular process for the benefit of the pathogen (Watson and Galán, 2008). Campylobacters show a high level of interaction with vertebrate gastrointestinal cells and consequently they have evolved specific adaptations to modulate cellular functions of these cells.

1.13.1. *C. jejuni* internalisation into non-phagocytic intestinal epithelial cells

Campylobacters have a high level of association with avian, animal and human gastrointestinal cells. On entering the gastrointestinal system *Campylobacter* colonises and attaches to the mucous layer and crypts of the intestinal mucosa of the small and large intestine (Russel *et al.*, 1993; Yao *et al.*, 1997). Studies have shown the role of motility in the invasion of *C. jejuni* (Carillo *et al.*, 2004; Grant *et al.*, 1993). Once within epithelial cells, *C. jejuni* can multiply intracellularly and can gain

access to the underlying tissues and reach different cellular receptors (Konkel *et al.*, 1992; Watson and Galan, 2008). Studies have also shown that the non flagellar proteins like the *Campylobacter* invasion antigens (Cia proteins) secreted through the flagellar apparatus trigger internalisation (Konkel *et al.*, 2004). The attachment of the bacteria to the epithelial cells is an important step in the invasion process which is mediated by flagella and the capsular polysaccharide of the pathogen. Mutations affecting protein glycosylation or capsular synthesis show a measurable decrease in the rate of invasion of tissue culture cells due to reduction in motility (Bacon *et al.*, 2001; Vijayakumar *et al.*, 2006). Oelschlaeger *et al.*, (1993) reported that drugs that inhibit microtubule dynamics block bacterial internalisation.

1.13.1.1. Intracellular survival and trafficking of *C. jejuni*

Campylobacter is able to remain viable during its intracellular replication in contrast with other bacterial pathogens (Candon *et al.*, 2007; Watson and Galan, 2008). Once inside the host cell the bacteria has to overcome a variety of unfavourable conditions triggered by the host's killing mechanisms such as, oxidative products, nutrient limitation, acidic pH and other stimuli (Pogačar *et al.*, 2009). Following internalisation *C. jejuni* resides within a membrane bound compartment called *C. jejuni* containing vacuole (CCV) (Russel and Blake, 1994). This compartment is functionally distinct from lysosomes that digest engulfed bacteria. When *C. jejuni* is taken into macrophages it quickly loses its viability due to the activity of the lysosome (Wassenaar *et al.*, 1997).

1.13.1.2. Stimulation of innate immune responses in intestinal epithelial cells

The intestinal epithelial cells produce innate immune responses upon detection of microbial pathogens (Dann and Eckmann, 2007). Bacterial secreted proteins are required for the internalisation of *C. jejuni* into cultured mammalian cells. A strong dependence of both microtubules and microfilaments are required for invasion. *Campylobacter* containing vacuole (CCV) or caveolae markers at early time points during infection and the function of caveolin-1 is required for efficient *Campylobacter* entry. *Campylobacter* remains within the endosome during the passage through the host cell. CCV has close association with golgi apparatus which is dependent on microtubules and motor protein dynein (Watson and Gálon, 2008). Pathogen associated molecular patterns (PAMP) serve as elicitors of responses sense by Toll like Receptors (TLRs) or a family of intracellular receptors belonging to the nucleotide binding oligomerisation domain (Nod) family of proteins. Activation of NF-κB and mitogen activated protein kinase occurs leads to the production of proinflammatory cytokines in eukaryotic cells (Mellits *et al.*, 2002).

1.13.2. Survival of *Campylobacter* in protozoa

A strong interaction has been found between protozoa in the broiler drinking water and bacteria in the broiler house (Altekruse *et al.*, 1998). Campylobacters can easily enter the drinking water systems through contaminated faecal matter (Altekruse *et al.*, 1998). Once in the water supply *Campylobacter* can survive through a variety of mechanisms including survival within protozoa. Studies have shown that *Campylobacter* can be protected from environmental stress

conditions within the vacuoles of *Tetrahymena pyriformis, Acathamoeba castellanii* and *Acathamoeba pyriformis* (Snelling *et al.*, 2005b) and is found to be fifty times more resistant to free chlorine in this condition. Internalised bacteria were also resistant to iodine based disinfectant (Snelling *et al.*, 2008). This might explain why disinfection treatments have no effects on *Campylobacter* colonisation in poultry. However, co-culture studies between *Campylobacter* and the protozoan to evaluate the viability of *Campylobacter* has shown that the number of recovered viable bacteria decreased drastically during the initial hours because the protozoa were feeding at a high rate after being starved. Using *Tetrahymena pyriformis* the overall viability was significantly higher for an extra 36 h from 7.75 days when *Campylobacter* was incubated with it (Snelling *et al.*, 2005b).

Campylobacters released from the vacuoles of protozoa are more resistant to environmental stress since they have adapted to the nutrient depleted conditions. This may increase their potential to infect poultry (Brown and Barker, 1999). It was also suggested by Snelling *et al.*, (2005b) that when campylobacters were inside the food vacuoles in protozoa, the signalling molecules, auto inducer-2 produced by *lux*S would be at a higher concentration when compared to the free living ones and it could potentially result in higher stress resistance. AI-2 produced by *lux*S is responsible for the late exponential phase peak of expression of VirB, a virulence protein required for invasion (Elvers and Park, 2002). *Lux*S mutants were sensitive to oxidative stress, hydrogen peroxide and have got limited chance of invading host cells. For *E. coli* O157 strain the *lux*S mutants were also having reduced motility (Elvers and Park, 2002).

1.13.3. Survival of *Campylobacter* in human monocytic cells

C. jejuni can invade the intestinal sub mucosal layer causing intense infiltration of mononuclear cells (Kiehlbauch *et al.*, 1985) and can remain viable for extended periods. It can readily be internalised by macrophages and monocytes (Duffy *et al.*, 1980). Studies done by Kiehlbauch *et al.*, (1985) state that viable campylobacters could be recovered consistently up to 7 days from a human monocyte line 28SC, infected with 81-176 at the multiplicity of infection 100:1. Campylobacters were recovered to the highest from 48h cultures which represent an approximate 3 log unit increase in bacterial counts. Using the live/dead staining it was found that *C. jejuni* were localised within the vacuoles either as single or multiple and they were found to be motile (Hickey *et al.*, 2005).

1.13.4. Survival of *Campylobacter* in the environment

Campylobacter spp. thought of as fragile organisms once expelled from their host have to survive various different hostile conditions including: exposure to oxygen, exposure to temperatures below their minimum growth temperature, desiccation and other stress factors before eventually colonising a new host. The two-component regulators (TCS) help the bacteria to overcome the changing environmental conditions by regulating a set of genes and thereby enhancing it's survival. It has been found that biofilm formation where cells are enclosed in extracellular polymeric substance significantly increases the ability of the bacteria to survive in extreme conditions. *Campylobacter* can form biofilms in aquatic environments or even on stainless steel or glass (Gunther and Chen, 2009).

Campylobacter in biofilms lack adaptive stress resistant mechanisms and they are found to enter a viable but non culturable state where they require fewer nutrients and are in microaerobic conditions (Buswell *et al.*, 1998; Murphy *et al.*, 2006). During stationary phase the cell membrane of *Campylobacter* spp. undergo changes in the fatty acid composition resulting in changes in the integrity and pressure resistance of the cell membrane (Martínez Rodriguez *et al.*, 2004). In *C. jejuni* poly phosphate produced by poly phosphate kinase 1 increases in abundance which helps in low nutrient survival (Candon *et al.*, 2007). The prolonged ageing of the stationary phase cells result in resistance to aeration, peroxide challenge and heat (Martínez Rodriguez *et al.*, 2004).

1.13.4.1. Two component regulator systems (TCS)

TCS consists of a sensory histidine kinase in the cell cytoplasm and a response regulator in the cytoplasmic membrane (Murphy *et al.*, 2006). The sensor proteins provide external sensing and the response regulators function as effectors within the intricate regulatory network of the bacteria. Parkhill *et al.*, (2000) reported that there are nine putative response regulators, six putative hisitdine kinases and five putative TCS in the genome of *C. jejuni*. The sensor genes include: the *rac*R-*rac*S system which is responsive to temperature and colonisation (Bras *et al.*, 1999); the *cbr*R system which modulates deoxycholate resistance and colonisation (Raphael *et al.*, 2005); the *dcc*RS system for optimal colonisation (MacKichan *et al.*, 2004), *Cj*0889c and *Cj*0890c which are involved in oxidative stress resistance in *C. jejuni* (Garosi *et al.*, 2003); and the two genes *flg*A and *flg*R which are involved in the regulation of the *fla* regulon in *C. jejuni* (Wösten *et al.*, 2004). When compared to

other enteric bacteria *Campylobacter* has very limited gene response regulation (Park, 2002).

1.13.4.2. Heat Stress

The thermal stress response of the *Campylobacter* is mediated by the heat shock proteins (HSPs) which are some of the most conserved coding sequences (Murphy *et al.*, 2006). The genes that encode these include *groES*/L, *clp*B, *dna*K, *grp*E, *dna*J, *hrc*A and *htr*A/*deg*P (Svensson *et al.*, 2008) and they are expressed more during heat and aerobic stress (Takata *et al.*, 1995) and in alkaline conditions (Wu *et al.*, 1994). Konkel *et al.*, (1998) proved that the product of *dna*J was required for both heat shock survival and chick colonisation. A whole genome array study by Stintzi, (2003) has identified *groES*L, *grp*E, *dna*K, *dna*J, *clp*B, *lon* and *hsl*U to be upregulated at 42 °C versus 37 °C. The *htr*A gene encodes a periplasmic protease that is required for growth above 44 °C having a role in misfolded protein response (Svensson *et al.*, 2008). It is also required for aerobic survival, host cell adherence and invasion highlighting its role in virulence and transmission. The product of the *Gro*EL gene prevents misfolding and aggregation of partially denatured proteins through an ATP dependent process (Thies *et al.*, 1999) and this aids survival because of its increased adaptation to higher temperature.

1.13.4.3. Cold Stress

Campylobacter maintain their metabolic activity even at low temperatures like 4 °C though they lack the cold shock genes like *csp*A. Studies have shown that

Campylobacter survive better at 4 °C in various biological conditions than in 25 °C. Moen *et al.*, (2005) showed that a number of genes were up regulated for *Campylobacter* grown at 5 °C compared to 25 °C which shows the greater need for energy at lower temperatures. The products of the *sod*B and *kat*A genes have been shown to help *Campylobacter* to survive freeze thawing (Garénaux *et al.*, 2009). Oxidative damage is a mechanism that contributes to freeze thaw injury since it is predicted that an oxidative burst occurs upon thawing (Park *et al.*, 1998). Stead and Park, (2000) described how oxidative stress plays a role in freeze thaw induced killing of *C. coli* by using mutants deficient in key antioxidant functions.

1.13.4.4. UV stress

Butler *et al.*, (1987) showed that *C. jejuni* is more sensitive to UV stress than *E. coli* and it survives more in a climate where there is less temperature and sunlight for a few hours per day. A high expression of *rec*A was noticed in strains that were more resistant to UV (Murphy *et al.*, 2006). Campylobacters in river water have a high tolerance to UV due to the modification in the physiological properties (Murphy *et al.*, 2006).

1.13.4.5. Acid stress

C. jejuni is acid sensitive (Doyle and Roman, 1981) and a pH lower than 5.5 and above 8.0 will result in drastic reduction in its population. Gastric fluid is therefore a great barrier to campylobacters but this may be overcome by introducing

the cells to sub lethal stress which induces the production of an adaptive tolerance response (ATR) (Goodson and Rowbury, 1989). The ATR was shown to include up regulation of general stress proteins that were involved in protein protection or degradation which include the up regulation of the universal chaperones *dna*K and *gro*EL.

1.13.4.6. Aerobic Stress

Campylobacter spp. can adapt to aerobic growth (Jones *et al.*, 1993) or can grow aerobically in 10% CO_2 in moist air (Fraser *et al.*, 1992). The respiratory metabolism in *C. jejuni* is a branched electron transport chain based on the use of oxygen as a terminal electron acceptor; but alternative terminal electron acceptors can also be used (Sellars *et al.*, 2002). Microbial respiration leads to the production of partial reactive oxygen species (ROI) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which is toxic to the cells. The iron containing *sod*B provides the first line defence for *C. jejuni* when exposed to atmospheric oxygen and reduce superoxide to hydrogen peroxide and oxygen (Pesci *et al.*, 1994; Purdy and Park 1994). The hydrogen peroxide is degraded to water and oxygen in the presence of catalase (*kat*A) (Grant and Park, 1995) which is a stress defence protein. *Ahp*C (alkyl hydroperoxide) reductase, confers increased resistance to cumene hydroperoxide and aerobic stress (Baillon *et al.*, 1999). The putative DNA binding protein, *dps* has been isolated from starved cells which are important for hydrogen peroxide resistance in the presence of iron (Ishikawa *et al.*, 2003). Components, such as blood, charcoal, $FeSO_4$, metabisulfite and pyruvate which are thought to act as quenchers can protect

C. jejuni against oxygen stress and even enable them to grow fully aerobically (Fraser *et al.*, 1992; Hodge and Krieg, 1994).

1.13.4.7. Dessication survival

Campylobacter spp. are highly sensitive to desiccation. Doyle and Roman (1982) suggested that the factors responsible for desiccation tolerance include: the type of strain, temperature, humidity and the medium used to suspend the cells. Oosterom *et al.*, (1985) suggested that drying of pig carcasses eliminated *Campylobacter* from pork by ventilation of the cooling rooms. This treatment is not possible for poultry meat due to shorter processing cooling time and the different texture of the skin.

1.14. AIMS AND OBJECTIVES OF THIS STUDY

It is generally noted that *Campylobacter* sp. showed quantitative and qualitative differences when grown in MACS despite the fact that oxygen levels were comparable. The only difference was in the presence of hydrogen which was present in the gas replacement jar. Investigatins were carried out to compare the growth of a low passage chicken *C. jejuni* isolate in both the conditions and the accompanying physiological and transcriptional differences were noted.

Chickens and pigs constitute a main component of farm animals which harbour *Campylobacter* sp. in their intestine. *C. jejuni* HPC5 and its variants isolated from chicken strains, described by Scott *et al.*, 2007 showed the occurrence of

intramolecular recombination in their genome and the changes in the bacteriophage sensitivity and colonisation potential. Studies were conducted to find the genes that showed changes in the regulation pattern due to the recombination effect in *C. jejuni* HPC5 and its revertants and also the possible mechanisms that were responsible for the generation of resistance in *C. jejuni* R14 and R20.

Pigs are considered to be colonised both by *C. coli* and a 100 fold lower *C. jejuni*. The co- colonisation of both *C. coli* and *C. jejuni* in pigs' intestine led to the investigation of genetic exchange occurring between those strains. Studies were also conducted to find the proportion of *C. jejuni* and *C. coli* present in pigs and also the localisation of these species in different parts of the intestine.

The aim of this thesis is the molecular characterisation of Campylobacters isolated from chickens and pig in an ideal condition which is stress free.

CHAPTER TWO

MATERIALS AND METHODS

2.1. GROWTH AND STORAGE MEDIA

All growth and storage media were made in reverse osmosis (RO water) and were autoclaved at 121 °C and 15 psi for 20 min in a liquid cycle. The media were cooled to 50 °C before adding selective supplements and antibiotics. For agar plates the media were poured into Petri dishes using sterile conditions. Once set the plates were stored at 4 °C, and for a maximum of 4 weeks before use.

2.1.1. *Campylobacter* blood free selective agar (mCCDA)

Campylobacter blood free agar (Oxoid, UK) was prepared according to manufacturer's instructions but with additional agar to a 2% (w/v) g to prevent swarming. *Campylobacter* selective supplement (Pro Labs, UK) @ 42 mg L^{-1} was added after cooling to 50 °C when the plates were used for primary isolation studies.

2.1.2. New Zealand Casamino Yeast Medium (NZCYM) basal agar

NZCYM broth (Difco, Oxford, UK), supplemented with bacteriological agar No. 1 (Oxoid, Basingstoke, UK) to a final concentration of 1.2% w/v, was prepared according to manufacturer's instructions.

2.1.3. NZCYM overlay agar

NZCYM broth was supplemented with bacteriological agar to give a final concentration of 0.6% and 6 ml aliquots were dispensed into universals and stored at room temperature. The agar was melted in a microwave and cooled to 50 °C before use.

2.1.4. Blood agar (BA)

Blood agar base No. 2 (Oxoid, Basingstoke, UK) was prepared according to manufacturer's instructions. Following sterilisation, defibrinated horse blood (TCS Basingstoke, UK) was added to a final concentration of 5% v/v before dispensing into Petri dishes.

2.1.5. Maximum recovery diluent (MRD)

MRD (Oxoid) was made according to manufacturer's instructions, sterilised and stored at room temperature.

2.1.6. Phosphate saline buffer (PBS)

PBS (Oxoid, Basingstoke, UK) was made according to the manufacturer's instructions and was sterilised at 120 °C for 15 min and stored at room temperature.

2.1.7. *Campylobacter* motility agar

Mueller Hinton (MH) broth (Oxoid), supplemented with bacteriological agar No. 1 to a final concentration of 0.4% w/v, was prepared according to manufacturer's instructions.

2.1.8. Nutrient broth No. 2 (NB2)

Nutrient broth No. 2 (Oxoid) was prepared according to manufacturer's instructions. Following sterilisation, NB2 was stored at room temperature for a maximum of 8 weeks.

2.1.9. Mueller Hinton (MH) Broth and agar

Mueller Hinton broth was prepared according to manufacturer's instructions. Following sterilisation, MH broth was stored at room temperature in the dark for a maximum of 8 weeks. For agar plates 1.2 % bacteriological agar was added and plates were made.

2.1.9.1. Mueller Hinton Blood agar

MH agar was supplemented with defibrinated horse blood (TCS Basingstoke, UK) to a final concentration of 5% v/v before dispensing into Petri dishes.

2.1.9.2. Mueller Hinton deoxycholate agar (MHD)

MH agar supplemented with 0.1% sodium deoxycholate (Sigma Aldrich).

2.1.10. Salt Magnesium Buffer (SM)

Salt Magnesium buffer was prepared by adding 0.1 M NaCl, 10 mM of $MgSO_4 .7H_2O$, 0.1% gelatine and 50 ml of 1.0 M Tris-Cl to 1 litre (L) RO water. The pH of the solution was adjusted to 7.5 with concentrated hydrochloric acid. This was sterilised at 120 °C at 15 psi for 20 min and stored at room temperature (20 °C).

2.1.11. Bacteriological Storage Medium

Bacteriological storage medium consisted of NB No: 2 (2.1.8) supplemented with 20% v/v glycerol. This solution was dispensed in to universals (15 ml) and sterilised before storage at 4 °C.

2.1.12. Reagents and Buffers

All chemicals were dissolved in RO water and sterilised by autoclaving at 120 °C and 15 psi for 20 min. They were stored at room temperature with appropriate labels and hazard symbols. These chemicals were obtained from Sigma Aldrich unless otherwise stated.

2.1.13. FBP Reagent

It consists of Ferrous sulphate – 10% v/v, Sodium pyruvate – 10% w/v and Sodium metabisulphite – 10% w/v which is filter sterilised and dispensed 3 ml in bijoux and stored at -20 °C.

2.1.14. Antibiotics

Selective antibiotic stock solutions were made by dissolving them in appropriate solvents, dispensed in eppendorf tubes and stored at -20 °C. The information on stock and the working solutions used for the respective antibiotics are given in Table 2.1

Table 2.1. Solvent and final concentrations of antibiotics used with CCDA for isolating *Campylobacter*

Antibiotic	Solvent	Stock concentration	Working concentration
Polymyxin	Water	0.03g/10 ml	0.2 ml/l
Trimetroprim	Ethanol	0.025g/10 ml	0.2 ml/l
Vancomycin	Water	0.5g/10 ml	2 ml/l

Antibiotics used in CCDA with their working concentration.

2.2. *CAMPYLOBACTER* SPP.

2.2.1. Growth Conditions

Campylobacter were subcultured on BA (2.1.4) and the plates were incubated in 3.5 L jars (Oxoid, Basingstoke, UK) or in 7 L anaerobic jars (BD Diagnostics, Maryland, USA). Before incubation the pressure was reduced to -22 psi and atmospheric pressure was restored with a gas mixture consisting of 85% v/v nitrogen, 10% v/v carbon dioxide and 5% v/v hydrogen (Air Products, Crewe, UK). This resulted in a microaerobic atmosphere containing approximately 5.6% v/v oxygen, 3.6% v/v hydrogen, 7.3% v/v carbon dioxide and 83% v/v nitrogen providing conditions for *Campylobacter* growth. Campylobacters were also grown in a Modular Atmospheric Controlled System (MACS) (Don Whitely Scientific) having a gas mixture of 85% v/v Nitrogen, 10% v/v carbon dioxide and 5% v/v oxygen. The plates were incubated at 42 °C for 18 h unless otherwise stated.

2.2.2. Storage

Campylobacter strains for long term storage were inoculated in bacteriological storage medium (2.1.11) and kept at -80 °C. Resuscitation of long term stocks was performed by thawing the frozen aliquot, transferring 50 µl to a blood agar plate and incubating at 42 °C, under microaerobic conditions for 48 h.

2.3. STRAINS AND STORAGE

For short term storage cultures were stored at 4 °C under microaerobic conditions for approximately 1 month. This provided a ready source of cells for further growth without the need for continuous thawing of long term stocks.

Table 2.2. List of *Campylobacter* strains used in this thesis

Campylobacter species	Strain Name	Source
C. jejuni	NCTC 11168	NCTC strain
C. jejuni	RM1221	Fouts *et al.*, 2005
C. coli	RM2228	Fouts *et al.*, 2005
C. jejuni	HPC5	Scott, 2006
C. jejuni	R14	Scott, 2006
C. jejuni	R20	Scott, 2006
C. jejuni	R14A	Scott, 2006
C. jejuni	R14B	Scott, 2006
C. jejuni	R20A	Scott, 2006
C. jejuni	R20B	Scott, 2006
C. jejuni	R20C	Scott, 2006
C. coli	C101	This thesis
C. jejuni	U101	This thesis
C. coli	L101	This thesis
C. jejuni	OR1	El Shibiny *et al.*, 2007
C. coli	OR12	El Shibiny *et al.*, 2007
C. jejuni	PT14	Laboratory stock

Source of *C. jejuni* and *C. coli* isolates used in this thesis.

2.4. ENUMERATION OF *CAMPYLOBACTER*

Campylobacter enumeration was carried out using a modification of the technique described by Miles and Misra (1938). A cell suspension to be enumerated was serially diluted in ten fold steps using MRD (2.1.5) to a dilution factor of 10^{-8}. Three 20 µl aliquots of each dilution were spotted onto the surface of a CCDA plate (2.1.1), allowed to dry near a Bunsen burner and incubated at 42 °C under microaerobic conditions for 24 - 48 h. The number of colonies in each spot of that dilution was counted where 3-30 colonies per spot were present and the average colony count per 20 µl spot was calculated. This was multiplied by the inverse of the dilution factor and finally by 50 to yield cfu ml^{-1}.

2.5. CONFIRMATORY TEST FOR *CAMPYLOBACTER*

2.5.1. Gram Stain

A loop full of fresh culture was taken from colonies to be tested for Gram's reaction. The bacteria were emulsified using PBS, smeared on a glass slide and heat fixed. Slides were immersed in crystal violet solution for 1 min and the excess was rinsed off using water. The slide was immersed in Lugol's iodine for 30 s and the excess rinsed using gentle stream of RO water. Decolourisation was performed by immersing the slides in methylated spirit for 1 min with gentle agitation and the excess methylated spirit removed by rinsing with RO water. Slides were finally immersed in carbol fuchsin - the counter stain, for 30 s and the excess carbol fuchsin was removed by rinsing with RO water. Slides were air dried and examined using a 100x, oil immersion objective of a light microscope. Small, Gram negative, pink coloured, curved cells indicating that the isolate was likely to be *Campylobacter.*

2.5.2. Oxidase test

Oxidase sticks (Oxoid, Basingstoke, UK) were swabbed on the cultures to be tested. Oxidase positive *Campylobacter* developed a deep blue colour within 1 min. Oxidase negative bacteria were light blue or colourless.

2.5.3. Hippurate test

Sodium hippurate was prepared as a 5% w/v solution which was filter sterilised and dispensed in 500 µl aliquots and stored at -20 °C until required. After thawing these were heavily inoculated with a fresh culture of *Campylobacter* and incubated aerobically at 37 °C for 2 h. Hippuricase activity was detected by adding 200 µl of Ninhydrin (1,2,3-triketohydrindene monohydrate) (BioMérieux) to the

Campylobacter suspension before incubation at room temperature for 10 min. A deep violet colour indicated the presence of hippurate positive *Campylobacter*.

2.6. MOTILITY ASSAY

A colony of the *Campylobacter* to be tested was resuspended in PBS and it was spotted into the centre of a motility agar plate, and the agar plate was incubated at 42 °C under microaerobic conditions for 48 h, at which time the radius of the halo of motility was measured.

2.7. *IN VITRO* GROWTH CURVES

Campylobacter were cultured on blood agar 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 OD_{600} reading was taken. Approximate *Campylobacter* counts were calculated using a formula, derived from a standard *Campylobacter* dilution series:

Campylobacter titre in cfu ml^{-1} = $(OD_{600} * 2 \times 10^9) - 6 \times 10^6$

The remaining sample was diluted with PBS to give a count of approximately $\log_{10}$ 7 cfu ml^{-1}. A 1 ml aliquot of this was added to 100 ml of NB2 (2.1.8) 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. 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 2.4. In growth curves with oxygen radical scavengers, the FBP reagent (2.1.13) was added after sterilising the media.

Growth curves of *C. jejuni* HPC5 also performed, using gas generating packs CN35 (CampyGen™; Oxoid, UK) and BR0060 (Gas generating kits; Oxoid, UK) in

stackable air tight containers with a volume of 6 l. The CN35 sachet produces a residual concentration of 5% O_2, 10% CO_2 and 85% N_2 in 30 min while BR0060 produces a residual concentration of 6% O_2, 10% CO_2, 25% H_2 and 59% N_2 in 30 min.

2.8. ISOLATION OF *CAMPYLOBACTER* AND BACTERIOPHAGES FROM PIG FAECAL SAMPLES

2.8.1. Isolation of *Campylobacter*

Samples were intestinal contents of the caecum, upper intestine or lower intestine from free range pigs aged 10 - 12 months old from different farms. An appropriate dilution of the intestinal contents was prepared (2.4) and this was plated on mCCDA (2.1.1). The plates were incubated at 42 °C for 24 h under microaerobic conditions.

The samples were collected from farms and slaughter houses in the East Midlands region of the UK including swine reared in both outdoor reared and indoor environments. A total of eight herds were sampled with 2- 12 pigs from each. The first two herds comprised of outdoor reared pigs and the remaining five comprised of indoor pigs. The intestines from the freshly slaughtered pigs were collected from abattoirs and transported to the laboratory where further dissection was carried out. Intestinal contents were taken from the caecum, upper intestine and lower intestine of each pig. Samples were kept on ice after collection before being processed on the same day to prevent reductions in the viable count.

2.8.2. Isolation of Bacteriophage

Cells of host strain *C. jejuni* PT 14 were grown as described in 2.1.1 and collected into 10 mM $MgSO_4$ using a cotton tipped swab at an approximate cell count of log_{10} 8 cfu ml^{-1}. From this, 500 µl was added to melted and cooled NZCYM overlay agar, poured over NZCYM agar plates and was allowed to set near a Bunsen burner. The intestinal contents or faeces were prepared as a 10 % suspension (w/v) in SM buffer and then stored at 4 °C overnight. These were centrifuged and the supernatant passed through a 0.2 µl filter. The filtrates were then spotted onto the surface of the NZCYM overlay agar (2.1.3) as 10 µl aliquots in triplicate. These were allowed to dry near a Bunsen burner and incubated overnight at 42 °C under microaerobic conditions

2.9. ENUMERATION OF BACTERIOPHAGE

Bacteriophages were enumerated using the host strain from which they were propagated. Overnight blood agar plates of the host strain were swabbed into 10 mM $MgSO_4$ and adjusted with 10 mM $MgSO_4$ to log_{10} 8 cfu ml^{-1} as determined by OD_{600} value. Aliquots of NZCYM overlay agar were melted in a microwave, cooled to 55 °C in a water bath and 500 µl of *C. jejuni* were transferred to the melted NZCYM overlay agar. This was poured onto the surface of NZCYM agar plates and allowed to set near a Bunsen burner. Bacteriophage to be enumerated were serially diluted in tenfold steps using SM buffer to a dilution of 10^{-8} and three 10 µl aliquots of each dilution were spotted onto the surface of the *C. jejuni* containing NZCYM plates. These were allowed to dry near a bunsen burner and incubated overnight at 42 °C under microaerobic conditions. Where dilutions gave rise to 3 – 30 plaques, the number of plaques in each spot of that dilution was counted from which the average

plaque count per 10 µl spot was calculated. This was multiplied by the inverse of the dilution factor and finally by 100 to yield PFU ml^{-1}.

2.10. ISOLATION OF *CAMPYLOBACTER* GENOMIC DNA

2.10.1. Isolation of the *Campylobacter* DNA using GES

Campylobacter cells of 10^8 cfu ml^{-1} were harvested in 1.5 ml PBS at the end of their exponential growth phase and were collected by centrifugation at 13000 *g* (*g* is acceleration due to gravity) for 2 min. The cells were resuspended in 1 ml TE buffer (10 mM tris-HCl [pH 8.0], 1 mM disodium ethylenediaminetetraacetic acid). This was centrifuged for 2 min and the supernatant was removed. The cell pellet was re suspended in 100 µl cold TE buffer and was incubated for 30 min at 37 °C. The cells were vortexed briefly after adding 0.5 ml guanidium thiocynate (GES) and incubated at room temperature for 5-10 min and then transferred to ice for 2 min. Two hundred and fifty ml of cold 7.5 M ammonium acetate were then added and the suspension was incubated 10 min on ice. Five hundred ml of (24:1) $CHCl_3$: isoamylalcohol mix was added and was mixed thoroughly and then it was centrifuged at 13000 *g* for 10 min. The aqueous upper layer was transferred to a clean eppendorff tube and exactly 0.54 volumes of cold isopropanol was added and it was mixed by inverting the tube several times for 1 min. The DNA was collected by centrifugating it at 13000 *g* for 20 s. The pellet was washed three times by centrifugation with 70% ethanol and the residual ethanol removed. The pellet was vacuum dried for 5 min and was stored at 4 °C. It was resuspended in 50 µl of TE or sterile RO water before use and was stored at 4 °C.

2.10.2. Isolation of *Campylobacter* DNA using commercial kits

Genomic DNA was isolated using Qiagen genomic extraction kit (using genomic DNA buffer set Cat no: 19060 and Genomic tip20/G Cat No: 10223) and Wizard genomic DNA purification kit (Cat No: A1120) according to the manufacturer's instruction.

2.11. POLYMERASE CHAIN REACTION

2.11.1. Short Range PCR

PCR reactions were performed in thin walled, flat top 0.5 ml PCR tubes (Fisher) in 50 µl volumes containing 1 unit (U) *Taq* DNA polymerase, each dNTP at 200 µM (Promega, USA), forward and reverse primers at 200 nM each, 50-100 ng of genomic DNA as template, 5 µl of *Taq* DNA polymerase in 10× buffer (500 mM tris-HCl [pH 9.2], 140 mM ammonium sulphate, 17.5 mM magnesium chloride). *Taq* DNA polymerase was added immediately before starting the Techne Progene thermal cycler (Techne, Duxford, UK). Initial denaturation of the template DNA was at 95 °C for 2 min, followed by the main amplification steps consisting of 30 repeats of denaturation at 95 °C for 1 min, primer annealing at 44 - 55 °C for 1 min and extension at 72 °C for 1 - 3 min. The final extension step was held at 72 °C for an additional 3 min and the PCR product stored at 4 °C for analysis.

Table 2.3. List of Primers used in this thesis

Target Gene	Forward Primer (5` -3`)	Reverse Primer (5`-3`)	Annealing Temp. (°C)	Design	Amplicon size (bp)
clp B	AGCGAATCATTGCTTTTTCATC	CTATAGGAAGCTTTTTATTTTTAG	57	This thesis	150
fla G	ATTCAAACCGATAGAAGTCAAG	ATATCAGCCAACTTTTCACTCA	57	This thesis	150
CJE1302	AATCAACTCTTCATTTTGTCCTA	ATTTTATACTTTTTACTGCACTTG	57	This thesis	150
CJE1466	CAGGTTTTAAGGTAGCCATTG	ATGTCACTTGCATCAGTTCC	57	This thesis	150
peb 3	ATCCAAG CATCGGCTTG ATC	AATATCGTGGCCTTTGTTCCA	57	This thesis	150
grp E	GAATGCCAAGATGAAATTAGC	AGCCACTCCATGTTTTTCAAG	57	This thesis	150
his A	ACCATGCAAGGAGTAAATGTAA	CCTACGATAACCCCACTACAA	57	This thesis	150
CJE0265	ACAAAAGGTTGTAATGCACTG	GTGACTTTAGCTTGTAATGAG	57	This thesis	150
CJE0253	ATTAGCCAATCCAAGATTAGAG	GCCTTTTCCTTGCTTTCTTTG	57.8	This thesis	150
grp E	GAATGCCAAGATGAAATTAGC	AGCCACTCCATGTTTTTCAAG	57	This thesis	150
his A	ACCATGCAAGGAGTAAATGTAA	CCTACGATAACCCCACTACAA	57	This thesis	150
mre B	AAGCTTGATATGAGTATAGTTAA	GTTGTATTGCTGAACCTATAG	57	This thesis	150
bio F	ATAAATTGCGTCCAAGCTACA	AAATTTTTCATCAACAAGGCTA	57	This thesis	150
CJE0183	AGCGATAGCCAAATCAAAATG	GAACGCATCAAAGCCACAC	57	This thesis	150
CJE1755	ACCCTAAAAGTGTGAAAATCAG	AGGAGTTGTAAGCAAGGTGG	57	This thesis	150
Cj1006c	TCACTATAACCATTAGCTCCC	TGTATTATTCCTAGCGTGAGG	58	This thesis	150
rps P	TCGTGATGGTGGTTGGATAG	CGCTAAGTTTTGCACCAACGC	58	This thesis	150
grp E	CAGCTGTTAATGTTGAATGCC	ATCTTTGATAAGAGCCACTCC	58	This thesis	150
fli D	AGCTAGTGGAGGAGAGATTG	ATCGCACTGTCTTCGCCTG	58	This thesis	150
lux S	ACCTATGGGTTGTCGCACG	TTTTGCTTTGATCGCTTACGC	58	This thesis	150
Cj1491c	AATCTCTCTTGCCATATCCAG	AATCGGAGCTCAAAATGGAG	58.7	This thesis	150
rho	AGGCGATATCGTTACAGGAC	TAAAGGCAGATAATTGATCGC	58	This thesis	150
pet A	AGCTCTCCATCTTGCATTCC	CAAGTGTCAAAGCTGCAGG	58	This thesis	150
gap A	ATGCGCTCCTGTGCACTC	AGGCAGTGTTGATAGTGAAG	58	This thesis	150
hip O	GAAGAGGGTTTGGGTGGTG	AGCTAGCTTCGCATAATAACTTG	46	Scott *et al*., 2007	735
16srRNA	TTATGGAGAGTTTGATCCTG	TTACCTTGTTACGACTTCAC	56	Scott *et al*., 2007	1400
ASKCC18	GGTATGATTTCTACAAAGCGA	ATAAAGACTATCGTCGCGTG	54	Scott *et al*., 2007	500

Primers used in this thesis along with their annealing temperature and Amplicon size.

2.11.1.1. Identification of *C. jejuni* genomic DNA from whole intestinal contents

Template DNA was prepared as described in 2.10.1. The primer pair HIP400F and HIP1134R was used to perform the PCR and was it carried out as described in 2.11.1 (Scott, 2006).

2.11.1.2. Identification of *C. coli* genomic DNA from whole intestinal contents

Template DNA was prepared by 2.10.1. The PCR reaction was carried out as described in section 2.11.1 with the primer pair ASK CC18F and ASK CC519 (Scott, 2006). In addition a PCR reaction was also carried out using the 16S ribosomal RNA primers (Scott, 2006). They were designed using NCTC 11168 genomic sequence and

the product was sequenced. This was to confirm the presence of *C. coli* in the whole population.

2.11.2. DIG-PCR

For colony hybridisation, PCR amplification using digoxigenin labelled dUTP was performed to create short probes. The reaction conditions were similar to short range PCR (2.11.1) except that there was a replacement of 200 µM dTTP with dTTP at 184 µM and DIG-11-dUTP at 16 µM (Roche, Indianapolis, USA) with all other reaction conditions similar to that of short range PCR.

2.12. DNA PURIFICATION BY GEL ELECTROPHORESIS

2.12.1. Agarose gel electrophoresis

A solution containing 0.8% w/v of Agarose (Invitrogen) was made in 1× TAE buffer (40 mM tris-acetate adjusted with glacial acetic acid to pH 8.3, 1 mM disodium ethylenediaminetetraacetic acid). The agarose was melted in a microwave and the solution was cooled to approximately 70 °C. After cooling ethidium bromide (~1 µl) was added to a final concentration of 0.4 µg ml^{-1}, this solution was poured into casting moulds and allowed to set for 30 min at room temperature. The gel was placed in an electrophoresis tank containing TAE buffer. DNA samples were mixed with 0.2 volumes of 6× blue / orange loading dye and were loaded in to the wells of the gel. The samples were electrophoresed for 1- 2 h at 80 V before visualisation under UV using Gel Doc (BioRad) apparatus and Quantity one software (BioRad).

2.12.2. Pulsed Field Gel Electrophoresis (PFGE)

2.12.2.1. Preparation of DNA plugs

Campylobacter genomic DNA plugs were made by swabbing overnight whole plate growth from a blood agar (2.1.4) plate into 5 ml of PBS (2.1.6). A 400µl aliquot of this suspension with 25 µl of 20 mg^{-1} proteinase K was mixed with an equal volume of molten 1% w/v ultra pure PFGE agarose (Bio-Rad) in TAE buffer and dispensed to plug moulds (Bio-Rad) to set. The DNA plugs were incubated in freshly prepared cell lysis buffer (50mM tris-HCl [pH 8.0], 50mM disodium ethylenediaminetetraacetic acid, 1% w/v N-lauroyl sarcosine, 0.1 mg ml^{-1} proteinase K) at 55 °C for 20 min. The genomic DNA plugs were then incubated at 55 °C in RO water for 20 min, followed by three incubations in TE buffer (10 mM tris-HCl [pH 8.0], 1 mM disodium ethylenediaminetetraacetic acid) at 55 °C for 15 min each. DNA plugs were stored at 4 °C in TE buffer until use.

2.12.2.2. Electrophoresis of Genomic DNA digested with *Sma*I restriction enzyme

Campylobacter genomic DNA was digested with *Sma*I to produce fragments of 20- 1000 kb size. The *Campylobacter* genomic DNA plugs were cut into 2 mm slices using a sterile scalpel and separately placed into an eppendorfs containing 100 µl of freshly prepared SuRE / cut buffer A and equilibrated at room temperature for 15 min. This was replaced with buffer containing *Sma*I (Roche) which had a final concentration of 0.4 U μl^{-1} diluted in the same buffer. This was incubated for 2 h at 30 °C, after which the buffer / *Sma*I mix was removed and replaced by 100 µl of TE buffer. The PFGE agarose gel was made by melting a solution of 1% w/v ultra pure PFGE agarose in TAE (40 mM tris-acetate adjusted with glacial acetic acid to pH 8.3, 1 mM disodium ethylenediaminetetraacetic acid), pouring this to the PFGE casting

tray (Bio-Rad) and letting it set for 1 h. The comb and one end of the casting tray were removed and *Sma*I digested *Campylobacter* genomic DNA plugs were placed in the appropriate wells. The end of the casting tray was replaced and the plugs were sealed in place using 3 ml of molten 1% w/v ultra pure PFGE agarose in TAE. The gels were calibrated using 50- 1000 kb DNA marker (Sigma Aldrich) consisting of λ DNA concatamers of 48.5 kb. Electrophoresis was performed for 18 h at 14 °C using a CHEF-DRII (Bio-Rad) in TAE buffer with an initial switch time of 6.8 s and a final switch time of 38.4 s (gradient of 6 V/cm and an included angle of 120°). Following electrophoresis the gel was immersed in a 50 μg ml^{-1} ethidium bromide solution for 30 min, followed by two washes in RO water for 30 min each and visualised under UV illumination.

2.13. COLONY BLOT HYBRIDISATION

2.13.1. Preparation of Colony Blot

Colony blots were performed on *Campylobacter* colonies to identify specific genes present in them. Hybond N+ nylon membranes (Amersham, UK) were placed on the surface of agar plates with 30- 300 colonies and left for 3 min. The membranes were removed and marked at three sides to orient them. The agar plates were returned to the jar and incubated overnight until the colonies had grown to sufficient size to be picked. The membranes were placed, colony side up, on Whatman 3 MM paper soaked in 10% w/v sodium dodecyl sulphate and incubated at room temperature for 5 min. This was repeated using i) denaturation solution (0.5 M sodium hydroxide, 1.5 M sodium chloride) ii) neutralisation solution (0.5 M tris-HCl [pH 7.5], 3 M sodium chloride) and iii) 2× SSC (30 mM sodium citrate [pH 7], 300 mM sodium chloride) respectively for 5 min each on 3MM Whatman filter paper colony side up. The

cellular debris was removed from the membranes by washing them in 2× SSC. The membranes were dried at room temperature for 30 min before the DNA was fixed to the membranes by exposure to UV for 3 min. Membranes were stored at 4 °C in TE buffer until needed.

2.13.2. Colony Hybridisation

Specific DIG labelled PCR product (2.11.2) was added to Hyb solution (Roche, UK) to achieve a concentration of 15- 25 ng ml^{-1} in 20 ml DIG easy Hyb solution. This solution was boiled for 15 min to denature the probe to make the probe solution. Membranes were incubated for 2 h at 42 °C without shaking in DIG easy Hyb solution and then incubated overnight at 42 °C without shaking in the probe solution. The probe solution was poured off and retained for future use by storage at -20 °C and the membranes incubated twice in 2× wash buffer (30 mM sodium citrate [pH 7], 300 mM sodium chloride, 0.1% w/v sodium dodecyl sulphate) for 5 min each time at room temperature with shaking of 60 cycles min^{-1}. The membranes were then incubated twice in 0.5× wash buffer (7.5 mM sodium citrate [pH 7], 75 mM sodium chloride, 0. 1% w/v sodium dodecyl sulphate) for 15 min each time at 68 °C without shaking, followed by incubation at room temperature for 30 minutes with shaking of 60 cycles min^{-1} in blocking solution (0.1 M maleic acid [pH 7.5], 0.15 M sodium chloride, 1% w/v blocking reagent). Detection of the DIG labelled probe was accomplished by incubation of the membranes in antibody solution (0.1 M maleic acid [pH 7.5], 0.15 M sodium chloride, 1% w/v blocking reagent, 150 mU ml^{-1} anti DIG AP antibodies) for 30 min at room temperature with shaking of 60 cycles min^{-1}. The membranes were then incubated twice in wash buffer (0.1 M maleic acid [pH 7.5], 0.15 M sodium chloride, 0.3% v/v TWEEN-20) for 15 min each time with

shaking of 60 cycles min^{-1} and finally soaked in detection buffer (100 mM tris-HCl [pH 9.5], 100 mM sodium chloride) for 2 min. Colour was developed by incubating the membranes in freshly prepared colour solution (100 mM tris-HCl [pH 9.5], 100 mM sodium chloride, 0.45 mg ml^{-1} nitro-blue tetrazolium chloride, 0.175 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate, 4 toluidine salt) at room temperature without shaking in the dark. Development of a deep purple colour indicated a positive result. Colour development was halted by replacing the colour solution with TE buffer.

2.14. DNA SEQUENCING

2.14.1. Sample preparation

The PCR product (2.11.1) to be sequenced was aliquoted to an eppendorf tube and mixed with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. This was held at -20 °C for at least 2 h before being centrifuged at 13,000 *g* for 45 min at 4 °C in a refrigerated centrifuge. The supernatant was carefully removed and the DNA pellet was washed with 750 µl of 70% v/v ethanol by centrifugation at 13,000 *g* for 15 min at 4 °C in a refrigerated centrifuge. The pellet was washed again using 750 µl of 70% ethanol. Following removal of the supernatant, the DNA pellet was allowed to air dry, before being sequenced by MWG Biotech AG (Ebersberg, Germany) using their Value Read service.

2.14.2. Sequence Analysis

DNA sequencing results were received as chromatograms. These were analysed using Chromas v1.45 (Conor McCarthy, Griffith University, Australia) and the raw sequence data exported to ClustalW (Brown and Callan, 2004) sequence alignment tool for further analysis. Sequences were compared to database sequences

using the BLAST suite of tools (Altschul *et al.*, 1997; http://www.ncbi.nlm.nih.gov/blast/).

2.15. SURVIVAL ASSAY AT 50 °C ON *C. JEJUNI* HPC5 AND ITS DERIVATIVES

C. jejuni HPC5 and its variants were grown overnight on BA plates (2) and they were added to 50 ml prewarmed NB (2.1.8) at 50 °C to reach an OD_{600} of 0.05. The flasks were incubated at 50 °C in a shaking incubator at approximately 100 rpm. Plating of cells was performed at 10 min intervals for 1 h and the strains were grown on MH agar plates for 24 h under microaerobic conditions. Percentage survival of the strain was calculated relative to the zero time point.

2.16. MICROARRAYS

2.16.1. Preparation of RNase free working conditions

All equipment unless specified were washed in a solution containing 200 mM NaOH and 0.2% (w/v) SDS, and then thoroughly rinsed with Diethyl Pyrocarbonate (DEPC) treated water. DEPC treated water was prepared by adding 1 µl of DEPC into 11 ml of RO water. The solution was shaken vigorously before being incubated at room temperature in the fume hood overnight and autoclaved twice. All solutions were prepared in DEPC treated water and RNase free tips and eppendorfs were used throughout.

2.16.2. Preparation of Microarray slides

The slides used for microarray were γ APS coated, A^+ bar coded SCHOTT Nexterion® slides (Item number 1064875). The slides were printed according to campy dense array gal by Microgrid II GOD robot using TAS software version V2.4.03. They had 2500 pins with a 26 × 26 pin grid configuration. The spacing between the spots was 0.165mm and the slides were prepared at 60% humidity. The slides represent the genomes of NCTC 11168, RM1221, and RM2228 in duplicates.

2.16.3. Extraction, purification of genomic DNA

2.16.3.1. Extraction of genomic DNA

Genomic DNA was extracted by using Qiagen genomic extraction kit (using genomic DNA buffer set Cat No: 19060 and Genomic tip 20/G Cat No: 10223).

2.16.3.2. Clean up

Mini Elute reaction clean up (Qiagen 28204) was used according to the manufacturer's instructions to clean up the reaction mixture. To the enzymatic reaction mix, 300 µl of buffer ERC was added and mixed. If the colour of the mixture was orange or violet, 10 µl of 3 M sodium acetate at pH 5 was added and mixed. The colour of the mixture would become yellow. The sample was added to the Mini Elute column to bind DNA. To obtain maximum recovery, the column was centrifuged for 13,000 *g* for 1 min. The flow through was discarded, then washed using 750 µl of buffer PE and it was again centrifuged for 13000 *g* for 1 min. The column was then further centrifuged for an additional one min at 13000 *g*. The Mini Elute column was then placed in a clean 1.5 ml micro centrifuge tube. To elute the DNA, 10 µl of buffer EB (10 mM Tris-Cl, pH 8.5) or water was added to the centre of the membrane. The

column was allowed to stand for one min and it was again centrifuged at 13,000 *g* for 1 min. The samples were required to have 200 ng μl^{-1} and 2.5 pmol ml^{-1} dCTT each for efficient hybridisation when quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific). The probes were then mixed together and ethanol precipitated (2.16.3.3).

2.16.3.3. Purification of DNA by ethanol precipitation

To the DNA samples, 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol were added, following mixing they were transferred to -20 °C and stored overnight with light excluded. The samples were warmed to room temperature and were centrifuged at 13,000 *g* at 4 °C for 45 min. The supernatants were removed and one ml of 70% ethanol was added to each. The samples were centrifuged at 13000 *g* for 15 min. The supernatant was removed and the above step was repeated. The DNA was air dried after removing the supernatant.

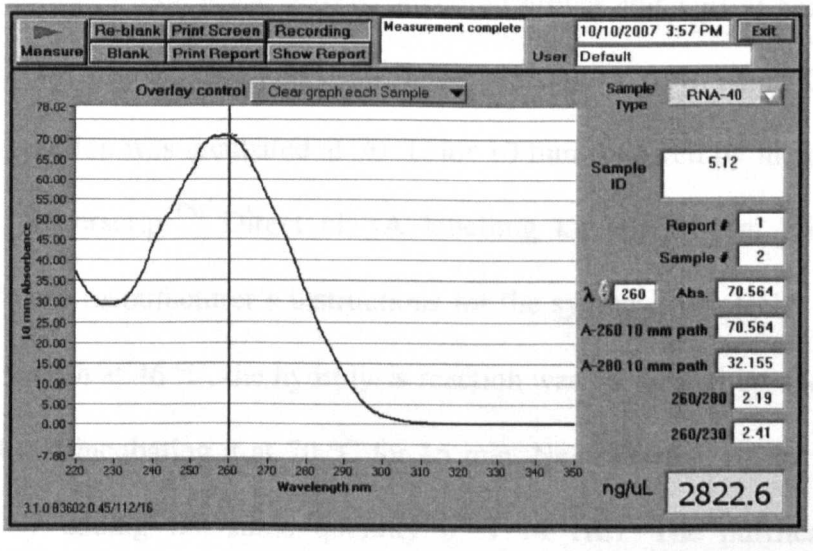
2.16.4. Extraction of total RNA and preparation of cDNA

2.16.4.1. Extraction of total RNA

Campylobacter cells for RNA extraction were grown in 500 ml of NB2 (2.1.8) in a 2 l conical flask shaking in microaerobic conditions. The cells were harvested by centrifuging the entire 500 ml broth culture at 6000 *g* at 4 °C for 15 min and the supernatant was discarded. The cells were mixed with 200 µl of Maximum Bacterial Enhancement Reagent (Invitrogen, UK) which was preheated to 95 °C and incubated at 95 °C for 4 min. One millilitre of Trizol (Invitrogen, UK) was then added and incubated for 5 min at room temperature followed by the addition of 200 µl of ice cold chloroform. After 3 min the suspension was centrifuged at 13000 *g* at 4 °C for 15

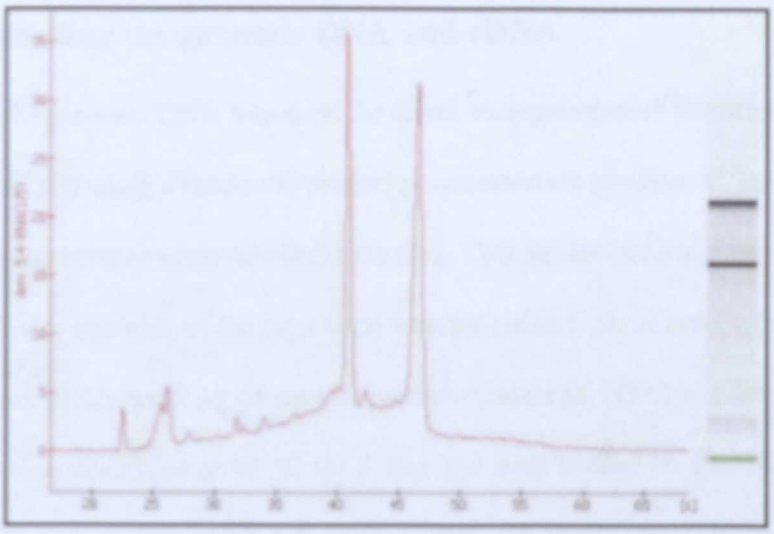
min and the top phase (~200 µl) removed to a clean tube. This was followed by the addition of 700 µl of absolute ethanol. To further purify the RNA, the samples were subjected to Qiagen RNeasy RNA clean up kit following the manufacturer's instructions. DNase treatment was performed using on column DNase (Qiagen) according to manufacturer's instructions. The purified RNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer and the quality was checked using Agilent gels (Agilent BioAnalyzer 2100) and Fig. 2.1 and Fig. 2.2 represent the quantity and quality of HPC5 RNA measured using NanoDrop® ND-1000 UV-Vis Spectrophotometer and Agilent Bioanalyser respectively.

Figure. 2.1. Quantity of HPC5 RNA measured using NanoDrop® ND-1000 UV-Vis Spectrophotometer



Screenshot from NanoDrop® ND-1000 UV-Vis Spectrophotometer showing the absorbance$_{260}$, A_{260}/A_{280} ratio and the quantity of RNA isolated in ng/µl. This was a screen shot obtained from the instrument.

Figure. 2.2. Quality of the extracted HPC5 RNA using Agilent Bioanalyser



The peak represent 23S and 16S rRNA. x axis represent time in 's' and y axis is 'height threshold'.

2.16.4.2. Preparation and purification of cDNA

To 20 µg of total RNA, 2 µl of anchored primer and 1 µl of hexamer primer were added (Invitrogen, UK) and the volume adjusted to 20 µl using DEPC treated water. The reaction was incubated at 70 ºC for 10 min followed by incubation on ice for 1 min. Superscript™ Direct cDNA labelling kit (Invitrogen, UK) was used according to the manufacturer's instructions for the synthesis of cDNA following an incubation for 3 h at 46 ºC, the hydrolysis reaction was performed by adding 15 µl of 1 M NaOH and incubating it at 70 ºC for 15 min. Neutralisation of the reaction was performed by adding the same quantity of 1 M HCl. The purification of the synthesised cDNA was performed by using QIA PCR purification kit (Qiagen, UK) according to the manufacturer's instructions. The eluted cDNA was precipitated with ethanol and was stored at -20 ºC prior to labelling.

2.16.5. Labelling the genomic DNA and cDNA

Whole genomic DNA was used for direct incorporation of fluorescent dyes (Cy 3 and Cy5 dCTP) using a randomly primed polymerisation reaction. *C. jejuni* RM1221 was used as the control strain labelled with Cy5. Test strains isolated from the caecum, upper and lower intestine of the pigs were labelled using Cy3. A reaction mixture of 3 µg of genomic DNA and 9 µg of random primers (Promega, USA) in a reaction volume of 41.5 µl were denatured at 95 °C for 5 min and snap cooled on ice. Five µl of 10× Klenow buffer (Fermentas, UK) 1.5 µl of Cy dye (GE health care, UK) 1 µl of dNTP mixture (5 mM each of dATP, dGTP, dTTP and 2 mM of dCTP) and 1 µl (10U) of Klenow fragment of DNA polymerase 1 (Fermentas, UK) were added to the reaction mixture before incubating at 37 °C for 1.5 h.

To 5 µl of the cDNA, 3 µl of 2.9 M of sodium bicarbonate (Sigma Aldrich, UK) was added and Alexa Fluor (Fisher, UK) dyes were added by dissolving in 2 µl of dimethyl sulfoxide (DMSO; Sigma Aldrich, UK). This was incubated for 1 h in the dark, and the labelled cDNA was purified by using the QIA PCR Purification kit according to the manufacturer's instructions. It was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer after ethanol precipitation as shown in Fig. 2.3.

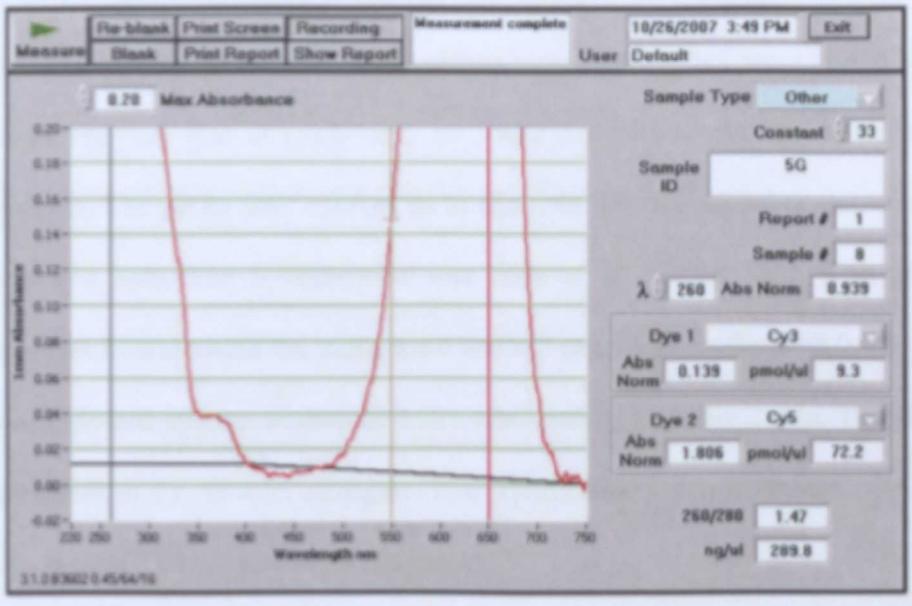
Frequency of incorporation (FOI), is a measure to track labelled samples which is defined as the number of labelled nucleotides incorporated per 1000 nucleotides of cDNA.

FOI = (pmol of dye incorporated ×324.5)/ nucleic acid concentration

Readings leading to Frequency of Incorporation (FOI) calculations greater than 20 represented adequately labelled probe suitable for array hybridisation. The probes with FOI values between 15 and 20 also could be used for hybridisation

though there was a chance of some signal issues in the final array data. Probes with FOI values less than 15 should not be used for hybridisation.

Figure. 2.3. Absorption norm of AF555 labelled HPC5 cDNA using NanoDrop® ND-1000 UV-Vis Spectrophotometer



NanoDrop® ND-1000 UV-Vis Spectrophotometer screen shot showing A_{260}/A_{280} ratio and the quantity of labelled DNA in ng/µl.

2.16.6. Probe Preparation with Fragmentation

A sample of each labelled probe (one Cy3, one Cy5) for each slide was taken to be hybridized which contained 500 ng of labelled DNA and was collected on a 0.5 ml tube. The volume was adjusted to 4.5 µl total volume by adding nuclease free water. Hundred µl of Schott 1× Hybridization Buffer which was pre warmed to 50 °C and added to the probe carefully to avoid introducing any air bubbles. The total volume was 107.5 µl (This work was carried out by Colin Nicholson at QMC, University of Nottingham).

2.16.7. DNA Microarray Hybridisations

The DNA microarray slides were blocked before hybridisation by using blocking solution (5× SSC, 0.2% SDS, 1% BSA). After blocking, the slides were washed in ultra pure water and then in 100% ethanol. The prepared slides were kept in the slide holder and were inserted into the TECAN pro Hybridisation station 4800 chamber. Approximately 5 min before the probes were inserted they were heated to 95 °C for 2 min and then spun at 15,000 rpm for 5 min in a micro centrifuge to pellet up any impurities. The probe was injected in to the hybridisation chamber through the probe insertion hole. The hybridisation was done at 45 °C for 16 h with gentle agitation. After hybridisation the slides were washed in 1× SSC buffer with 0.6% SDS for 2 min, followed by two washes in 0.06× SSC buffer, each for 2 min. The slides were then dried at 23 °C with nitrogen (This hybridisation was perfomed by Colin Nicholson at QMC, University of Nottingham).

2.16.8. Data Acquisition and Analysis

Slides were scanned using Axon Genepix 4200AL scanner (Molecular Devices Corporation, Sunnyvale, CA) at 532 nm (Cy3) and 635 nm (Cy5) excitation wavelengths with 5 μm resolution. The slides were scanned using the auto PMT function with the threshold for saturation set at 0.05%. Fluorescent spot intensities were quantified using Genepix Pro 6.0 software. Spots were excluded from further analysis if they contain anomalous spot morphology or were within regions of non specific fluorescence. The data was further analysed by genespring GX 7.3. The genepix pix result file and the *Campylobacter* array gal files (GPL9022 in Gene Expression Omnibus) were uploaded in the software to provide a link with the annotated NCTC 11168 *Campylobacter jejuni* genome. Loess Normalisation was

carried out in the experimental data set up where the regression curve was developed by considering continuous sections of data and applied in continuity to the rest of the data. The data is then analysed using two tailed t test with unequal variance to identify oligonucleotides that gave more than 2 fold change with a corrected probability $p<0.05$ using Benjamini and Hochberg False Discovery Rate (FDR) multiple test correction. (Genespring analysis were carried out with Dr. Neil Graham, Nottingham Arabidopsis Stock Centre, University of Nottingham). The genes corresponding with these hybridizing oligonucleotides were selected for further study.

2.16.9. Experimental designs used in Chapters 3, 4 and 5

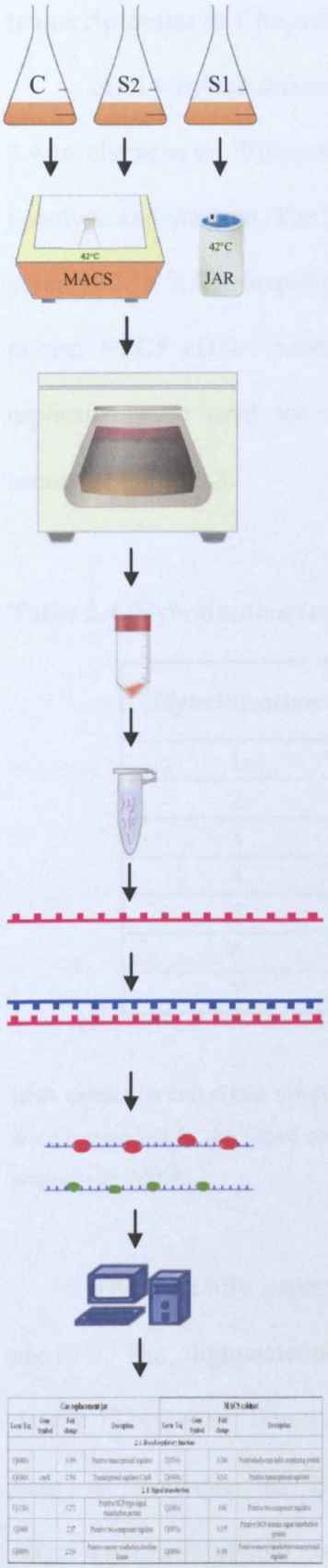
2.16.9.1. Growth conditions of control and sample *C. jejuni* HPC5 used for Transcriptomics in Chapter 3

Seven independent cultures of *C. jejuni* HPC5 were grown in NB No. 2 by incubating them in MACS with shaking. Cells were harvested at 12 h in the exponential phase of growth before independent RNA extraction and labelling with AF555 to create the hybridisation control.

The hybridisations were performed as described in section 2.16.7. Each hybridisation was performed with three independent biological replicates, where the cultures were grown to the late exponential growth phase (determined from growth curves) and culture samples were collected to extract total RNA at 8 h with a culture count of $\log_{10}$ 7.9 cfu ml^{-1} (SD ± 0.07) from the jar and 12 h from the MACS cabinet when $\log_{10}$ 7.2 cfu ml^{-1} (SD ± 0.06) was reached. All total RNA samples were reverse transcribed and fluorescently labelled with AF645 as previously described in 2.16.5. The experimental samples were competitively hybridised against the pooled HPC5 cDNA control labelled with AF555. Each hybridisation was repeated to yield two

technical and three biological replicates for each sample. The microarray images were analysed using the Genespring software GX 7.3 (Agilent Technologies) and the data normalised against the Cy5 labelled controls, merged, and expressed in $\log_2$ values. A detailed description of the analysis is documented in section to 2.16.8. Differentially expressed genes gave a *p* value below 0.05 and a minimum of a two fold change in transcript abundance in all independent biological replicates. Fold changes were calculated as the ratio of the normalised log Cy5/Cy3 values of MACS and jar which inturn gave $Cy3_{Jar}/Cy3_{MACS}$. The genes which gave a 2 fold upregulation were differentially expressed in gas replacement jar while those which showed a 2 fold down regulation were differentially expressed in MACS. Figure 2.4 represents the flow chart which shows the experimental design of transcriptomic analysis. Microarray data is available in the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/. The accession number is GSE17801.

Figure. 2.4. Flow chart representing the experimental design of the Transcriptomic analysis



Control (C): Seven flasks containing 500 ml Nutrient Broth (NB) each was inoculated with *C. jejuni* HPC5
Sample 1 (S1): Three flasks containing 500 ml NB each was inoculated with *C. jejuni* HPC5
Sample 2 (S2): Three flasks containing 500 ml NB each was inoculated with *C. jejuni* HPC5

↓

Control: *C. jejuni* HPC5 was grown in MACS at 42 °C for 12h.
Sample 1: *C. jejuni* HPC5 grown in Glass Replacement Jar (JAR) at 42 °C for 8h.
Sample 2: *C. jejuni* HPC5 grown in MACS at 42 °C for 12h.

↓

Spin on pre chilled tubes @ 6000x g for 15 min in Beckman centrifuge

↓

The cells were collected and the tubes were kept on ice. It was then treated with Maximum Bacterial Enhancement Reagent (Invitrogen, UK)

↓

The cells were treated with Trizol (Invitrogen, UK) which releases RNA

↓

Purified and concentrated RNA was collected out independently using RNeasy clean up kit (Qiagen, UK)

↓

cDNA synthesis was carried out independently using Superscript™ Direct cDNA labelling kit (Invitrogen, UK)

↓

Sample 1 and 2: cDNA were labelled with AF645.
Control: 7 independent cDNA labelled with AF555 were mixed together.

↓

Analysis using Genepix Pro6- Quantification of fluorescent signals. Genespring GX7.3- Loess Normalisation, Benjamini and Hochberg False Discovery Rate (FDR) multiple test correction, two tailed t test.

↓

Oligonucleotides were selected with $p<0.05$ and fold change > 2

2.16.9.2. Growth conditions of control and sample *C. jejuni* HPC5 used for transcriptomics in Chapter 4

cDNA hybridisations were performed according to the combinations in table 2.4 to characterise differences in the transcript profiles between the original HPC5 genotype and variants. The hybridisations were performed according to the procedure given in 2.16.7. The experimental samples were competitively hybridised against the pooled HPC5 cDNA control labelled with AF555. Three independent biological replicates were used for each hybridisation pair. The analysis was undertaken according to 2.16.8.

Table 2.4. Hybridisation combination pattern of *C. jejuni* HPC5 variants

Hybridisation	Control (AF555)	Sample (AF647)
1	HPC5	HPC5-R14
2	HPC5	HPC5-R20
3	HPC5	HPC5-R14A
4	HPC5	HPC5-R14B
5	HPC5	HPC5-R20A
6	HPC5	HPC5-R20B
7	HPC5	HPC5-R20C

RNA extraction and cDNA synthesis were carried out each for the respective strains and hybridisations were carried out in the above combination after labelling the control strains with AF555 and sample strains with AF645.

Differentially expressed genes gave a p value below 0.05 and fold change above 2. The oligonucleotides that gave a FC>2 were upregulated and those which gave a FC< 2 were down regulated when compared to *C. jejuni* HPC5. Microarray data is available in the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/. The accession number is GSE17799.

2.16.9.3. Comparative genomic hybridisation and experimental analysis of *C. jejuni* and *C. coli* isolated from different regions of the same pig (Chapter 5)

Whole genomic DNA was used for direct incorporation of fluorescent dyes (Cy3 and Cy5; GE chemicals, UK) by a randomly primed polymerisation reaction. *C. jejuni* RM1221 was used as the control strain and labelled using Cy5 whilst the *C. jejuni* and *C. coli* isolated from the caecum, upper and lower intestine of the same pig were labelled using Cy3. The labelling was performed according to 2.16.5.

Prior to the initiation of this study test microarray slides were quality controlled for each print run by UV scanning for the deposition of the oligonucleotides, and hybridised with labelled genomes of *C. jejuni* subsp. *jejuni* NCTC 11168, *C. jejuni* subsp. *jejuni* RM1221 and *C. coli* RM2228 in order to assess if all the spots printed on the array could be hybridised. For the competitive genomic hybridisation experiments equal concentrations of Cy5 and Cy3 labelled DNAs were introduced on to the slides and the hybridisations were performed according to 2.16.7. Three biological replicates were performed for each experiment.

Slides were scanned using an Axon Genepix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA) at 532nm (Cy3) and 635nm (Cy5) excitation wavelengths with 10 μm resolution. The analysis was performed using Genespring GX 7.3 from Agilent Technologies based on the ratio of Cy5 to Cy3 (2.16.8). The oligonucleotides were grouped into classes based on the following parameters.

a) Group 1: Cy5 to Cy3 ratio <0.1 implies greater sequence similarity between the oligonucleotide and the sample DNA but not to control DNA RM1221,

and therefore identifies a gene carried by the sample genome that is absent in *C. jejuni* RM1221.

b) Group 2: Cy5 to Cy3 ratio between 0.8 and 1.2 implies hybridisation to the oligonucleotide by both sample and RM1221, and therefore identifies shared genes.

c) Group 3: Cy5 to Cy3 ratio > 20 implies sequence similarity between the microarray oligonucleotide and RM1221 DNA but not the sample DNA, and therefore identifies a gene carried by *C. jejuni* RM1221 that is absent in the sample genome.

d) Group 4: These are oligonucletides that do not hybridise to either control or sample DNAs, and are therefore absent in sample and control genomes.

Microarray data for this study is available in the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/. The accession number is GSE17701.

2.17. QUANTITATIVE REAL TIME PCR (QRT PCR)

2.17.1. RNA Extraction and cDNA synthesis for real time PCR

Cultures were grown to mid exponential phase and the RNA was extracted using Trizol along with DNA digestion using DNase (Qiagen, UK) at 37 °C and RNA clean up was employed to minimise the genomic DNA contamination (2.16.3.2). Total RNA was converted to cDNA using Omnisript cDNA synthesis kit (Qiagen, UK) according to the manufacturer's protocol.

2.17.2. Relative quantification of gene expression using SYBR Green Real Time PCR

Specific Primers for real time PCR were designed with an average length of 18-22 bp and melting temperature ranging between 58-60 °C. 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 monitored the purity of the master mix. An optical 46 well micro titre plate (Applied Biosystems) was used with a 20 µl reaction volume consisting of 2 × SYBR Green master mixes (Applied Biosystems), 50 nm gene specific primers and the template. An ABI Prism 7000 sequence detector (Applied Biosystems) was programmed for an initial set up of 30 s at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min 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 specificities of qRT PCR reaction for each primer pair. Cycle threshold (C_t) values were determined using Step one software version 2.0 (Applied Biosystems). The comparative threshold cycle (C_t) method was used to calculate change (*n* fold) where samples were normalized to the control gene, since it was not found to be differentially expressed by microarray analysis. Reactions were performed in triplicate. The fold changes were calculated using the method 2^ΔΔCt (Ritz *et al.*, 2009).

2.18. PROTEIN ANALYSIS USING SDS PAGE AND ^{35}S METHIONINE LABELLING

Campylobacter was grown for 18 h in MH deoxycholate agar (2.1.9.1) plates and was resuspended in MEM. The pellet was harvested by centrifuging the minimum essential medium (MEM; Sigma, Aldrich) without methionine at 6000 *g*. The pellet was washed twice in MEM. For metabolic labelling, approximately 5×10^8 cfu was suspended in 1.5 ml MEM without methionine for labelling with ^{35}S methionine at a concentration of 50 µCi $3ml^{-1}$. This was incubated for 30 min at 37 °C under microaerobic conditions to allow incorporation of ^{35}S methionine (MP Biomedicals, Cat No: 64040). Chloramphenicol (Cm) was added to a final concentration of 128 µg ml^{-1} to immediately halt protein synthesis which is judged by the absence of ^{35}S methionine incorporation. The flasks were incubated for 30 min at 37 °C under microaerobic conditions. The suspensions were incubated with 1% fetal bovine serum (FBS; Sigma Aldrich, UK) for 30 min at 37 °C. The albumin was removed from FBS by using swell gel blue albumin removal kit (Pierce, Cat No 89845) following additional 30 min incubation at 37 °C the supernatant was harvested. The supernatant was then concentrated using Amicon concentrators.

The secreted proteins were visualised and compared to the whole cell lysates using SDS PAGE. Protein samples were prepared by mixing 0.25 volumes of 5× loading buffer (250 mM tris-HCl [pH 6.8], 10% w/v sodium dodecyl sulphate, 0.5% w/v bromophenol blue, 50% v/v glycerol, 5% v/v β-mercaptoethanol) with the samples and boiling for 5 min. Around 10- 20 µl of samples were loaded into the wells. Prestained protein markers were also included and mixed with loading buffer. SDS-PAGE gels were prepared according to protocols described by Sambrook *et al.*,

(1989) using a mini Protean III kit (Bio-Rad) with 1.5 mm gels. Separating gels were prepared in 10 ml volumes by the addition and thorough mixing of 4.17 ml of 30% acrylamide, 0.5 ml of 2% bis-acrylamide, 3.75 ml of 1M tris-HCl [pH 8.8], 100 µl of 10% sodium dodecyl sulphate, 1.5 ml of sterile RO water, 8.35 µl of N,N,N',N'-tetramethyl ethylene diamine and 33.35 µl of 10% ammonium persulphate, with the N,N,N',N'-tetramethyl ethylene diamine and ammonium persulphate added immediately before pouring the gel. The gel was allowed to polymerise under a layer of butanol saturated water at room temperature for 15 - 30 min, after which the butanol was poured off and the gel washed repeatedly with RO water then dried using Whatman 3 MM paper. The stacking gels were prepared using 5 ml volumes of 5% acrylamide by the addition and thorough mixing of 0.85 ml of 30% acrylamide, 0.35 ml of 2% bis-acrylamide, 630 µl of 1M tris-HCl [pH 6.8], 50 µl of 10% sodium dodecyl sulphate, 3.15 ml of sterile RO water, 10 µl of N,N,N',N'-tetramethyl ethylene diamine and 30 µl of 10% ammonium persulphate,with the N,N,N',N'-tetramethyl ethylene diamine and ammonium persulphate added immediately before pouring the gel. A 1.5 mm comb was inserted and this was left to polymerise for 15 - 30 minutes. Following polymerisation of the stacking gel, the comb was carefully removed and each of the wells will be washed repeatedly with RO water to remove any un polymerised acrylamide.

The electrophoresis chamber was assembled as per manufacturer's instructions and flooded with running buffer (25 mM tris-HCl [pH 8.3], 250 mM glycine, 0.1% w/v sodium dodecyl sulphate). Electrophoresis were performed at 150- 200 V for 1- 2 h until adequate separation of the markers had occurred. The gel was fixed by treating it with 40% methanol and 10% acetic acid for more than 30 min. After fixation it was soaked twice in RO water for 15 min then in 1-2% glycerol which helped in

preventing cracking. The gel was dried by placing it face down on plastic wrap. A piece of wet 3 MM paper was placed on the gel and a porous sheet on top. The gel was exposed to phosphorimaging screens to detect secreted proteins and whole cell lysates proteins. The screens were read using Bio Rad scanner.

2.19. GENTAMYCIN ASSAY ON HD11 CELLS USING *CAMPYLOBACTER* STRAINS

2.19.1. Preparing bacteria for the assay

A loopful of *Campylobacter* culture was inoculated into 10 ml of Mueller Hinton Broth (2.1.9) and culture was grown for 2- 3 days at 37 °C in a microaerobic chamber. Hundred µl of the overnight culture was added to 10 ml RPMI (Fisher, UK) without antibiotics and it was incubated for 12 h at 37 °C in a microaerobic chamber. Inoculate 2.5 ml of this 12 h old culture to fresh 10 ml fresh RPMI to set the bacterial count to approximately 10^8 cfu ml^{-1}. Incubate for 2 h at 37 °C with 5% CO_2 in a tissue culture incubator. An approximate of 10^8 cfu ml^{-1} log phase of *Campylobacter* is obtained with MOI = 1:100. This is the 0 h culture.

2.19.2. Preparation of HD11 cells

HD11 cells were seeded at 4×10^5 cell ml^{-1} of RPMI media in 24 well tissue culture plates and it was incubated for 24 h at 37 °C and 5% CO_2. The wells were washed with prewarmed PBS (2.1.6) for 3 times after removing the RPMI media and the wells were now ready for inoculation.

2.19.3. Inoculation and sampling of *Campylobacter* cells

One ml of the prepared bacteria was added to each well and it was incubated for 1 h at 37 °C and 5% CO_2. Serial dilution and plating was performed using 20 µl of the supernatant on MH agar (2.1.9) and it gave the outer 1h bacterial count. After discarding the rest of the supernatant, 1 ml RPMI medium containing gentamycin (100 µg/ ml; Sigma Aldrich, UK) was added to each well. The plates were incubated for 3 h at the same condition. Before determining the 4 h inner bacterial count, the wells were washed three times with prewarmed PBS and cells were lysed using 1 ml 1% Triton X-100 (Aldrich, UK) which was diluted in PBS. The solution was allowed to stand in the well for 30 min at 37° C and 5% CO_2. Serial dilution of the lysate was prepared after 30 min on MH agar which gave the 4 h inner bacterial count. After 4 h the next 3 wells were washed three times with PBS and lysis was performed using 1 ml 1% Triton X-100. Serial dilution was prepared after following the same procedure which gave 8 h inner bacterial count. The above procedure was repeated on the subsequent wells to determine 12 h and 24 h inner bacterial count.

CHAPTER THREE

COMPARATIVE GROWTH AND TRANSCRIPTION OF *C. JEJUNI* HPC5 IN MICROAEROBIC ENVIRONMENTS GENERATED IN JARS BY GAS REPLACEMENT OR IN A MODULAR ATMOSPHERE CONTROLLED SYSTEM

3.1. INTRODUCTION

In the 2nd edition of Bergey's Manual® of Systematic Bacteriology it is reported that several species of campylobacters require fumarate in the presence of formate or hydrogen for microaerobic growth. Campylobacters are microaerophilic and capnophilic in nature with a respiratory type of metabolism supported by oxygen concentrations between 5% and 10% v/v and carbon dioxide concentrations in the range of 1 to 10% v/v (Bolton and Coates, 1983). To achieve this condition a number of methods have been used over the years including: candle jars, gas tight jars with replacement gas, gas jars with gas packs that chemically modify the atmosphere and more recently computer controlled variable atmosphere cabinets (Myers and Kelly, 2005; Goossens *et al.*, 1983; Simmons, 1977). The microaerophilic nature, complex nutritional requirements and relative difficulty of culturing *C. jejuni* have all contributed to the limited understanding of metabolism (Kelly, 2005; Sellers *et al.*, 2002).

Campylobacters are cultured in nutritionally complex media which are supplemented to enable growth and reduce oxidative damage. Some mutants of *Campylobacter* lack superoxide dismutase which assists in the removal of superoxide radicals (O_2^-) and they were extremely sensitive to oxidative and freeze thaw stress (Garénaux *et al.*, 2009; Pesci *et al.*, 1994). It has been noted that *Campylobacter* growth media act more as a detoxifying agents by virtue of their antioxidant properties rather than any enrichment (Bolton *et al.*, 1984). Ferrous sulphate, sodium pyruvate, blood and charcoal are frequently used as supplements in *Campylobacter* media to prevent the accumulation of photochemically generated toxic oxygen derivatives to allow growth of *Campylobacter* spp. (Bolton *et al.*, 1984). Horse blood is reported as the most active supplement in its ability to neutralise hydrogen

peroxide, while charcoal and sodium pyruvate are less active but better than ferrous sulphate and sodium metabisulphite (Bolton *et al.*, 1984). *Campylobacter* enrichment broth (CEB) is therefore supplemented with 5% v/v of lysed horse blood to provide optimum growth conditions for the recovery of campylobacters growing under low oxygen conditions (Baylis *et al.*, 2000).

Members of the *Campylobacter* genus obtain their energy by oxidising amino acids or intermediates of the tricarboxylic acid cycle rather than from carbohydrates by fermentation (Vandamme, 2000; Kelly, 2008). Campylobacters exhibit chemo organotrophic metabolism that ultimately relies upon oxidative phosphorylation to meet their energy demands. *C. jejuni* can utilise multiple electron donors and acceptors through a branched electron transport chain, where respiratory substrates include hydrogen, formate, succinate, malate, lactate, sulphite, and α-ketoglutarate (Weerakoon and Olson, 2008). The electron donor enzymes hydrogenase, formate dehydrogenase and 2- oxoglutarate acceptor oxidoreductase have been demonstrated to operate in *C. jejuni*, and are reported as critical for the efficient colonisation of chickens (Weerakoon *et al.*, 2009).

Since researchers do not use a single standard method to produce a suitable atmosphere it is often difficult to compare studies that are otherwise similar. The cost of the equipment, the scale and the type of experiments to be performed are factors that influence the selection of the particular method. In our laboratory a variety of methods were utilised for producing the correct atmosphere and it was noted that quantitative and qualitative differences occurred between the growth of campylobacters in a gas replacement jar and that in a Modular Atmosphere Controlled System cabinet (MACS), despite the fact that oxygen levels were comparable. It was therefore decided to investigate this further comparing the growth of a low passage

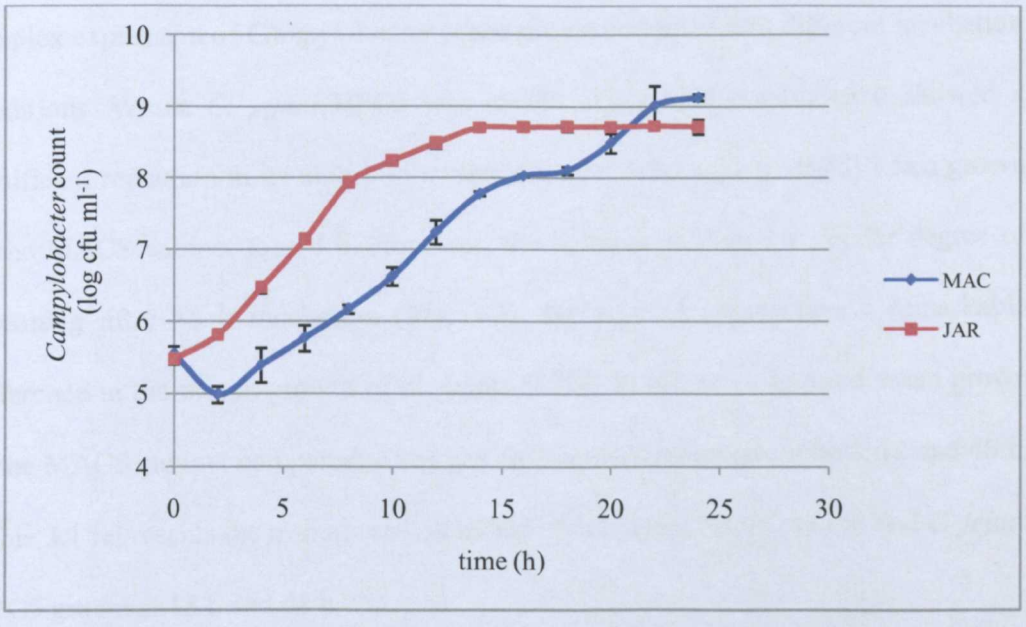
chicken *C. jejuni* isolate using the two methods, and to examine any accompanying physiological or transcriptional differences. The materials and methods utilised in this chapter are described in Chapter 2.

3.2. RESULTS

3.2.1. Growth studies of *C. jejuni* HPC5 to directly compare the two methods used to obtain a microaerobic atmosphere

Growth curve studies were performed under gas replacement conditions and compared to incubation in a MACS cabinet and are represented in Fig. 3.1. The growth rate constants during exponential growth were determined to be 0.84 cfu h^{-1} when *Campylobacter* was grown under gas replacement conditions in a jar and 0.54 cfu h^{-1} for *Campylobacter* grown in the MACS cabinet.

Figure 3.1. Comparison of the growth curves of *C. jejuni* HPC5 incubated under gas replacement conditions in a jar and MACS cabinet



The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the time in h. Red line represents the logarithmic growth of *C. jejuni* HPC5 in gas replacement conditions in a jar and Blue line represents the logarithmic growth of *C. jejuni* HPC5 in MACS.

A decline in cell numbers was observed during the first two h when *C. jejuni* HPC5 was incubated in the MACS cabinet.The viable count was reduced from $\log_{10}$ 5.5 cfu ml^{-1} (SD ± 0.11) to $\log_{10}$ 5.0 cfu ml^{-1} (SD ± 0.11), which implies that conditions were unfavourable for immediate growth when introduced into the MACS cabinet, although growth was observed after 6 h. Growth occurred immediately when the culture was introduced into the replacement gas jar. This difference in growth rate was attributed to the slower growth for cultures incubated in the MACS cabinet when compared to cultures incubated in the gas replacement jar.

3.2.2. Motility tests on cultures incubated under gas replacement conditions in a jar and incubated in a MACS cabinet

Motility tests were performed to detect possible changes in the flagella gene complex expression of *Campylobacter* when grown under the two different incubation conditions. Whilst *C. jejuni* HPC5 was motile under both conditions it showed a significant reduction in its ability to swarm on semi solid agar ($p<0.05$) when grown in the MACS cabinet for 18 h. However, the cultures exhibited a similar degree of swarming after 48 h incubation (Fig. 3.2). By way of comparison a remarkable difference in the motile growth of *C. jejuni* NCTC 11168 was observed when grown in the MACS cabinet compared to the gas replacement technique at both 18 and 48 h. Table 3.1 represents the average radius in 'cm' of *C. jejuni* NCTC 11168 and *C. jejuni* HPC5 grown at 18 h and 48 h.

Figure 3.2. Motility phenotypes of NCTC 11168 and *C. jejuni* HPC5

Fig. 3.2 (A)

Fig. 3.2 (B)

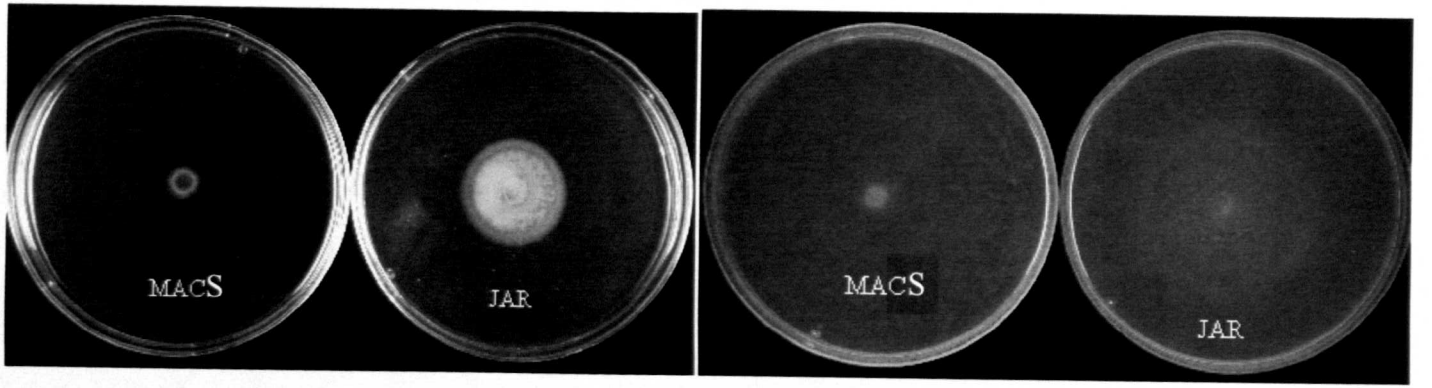
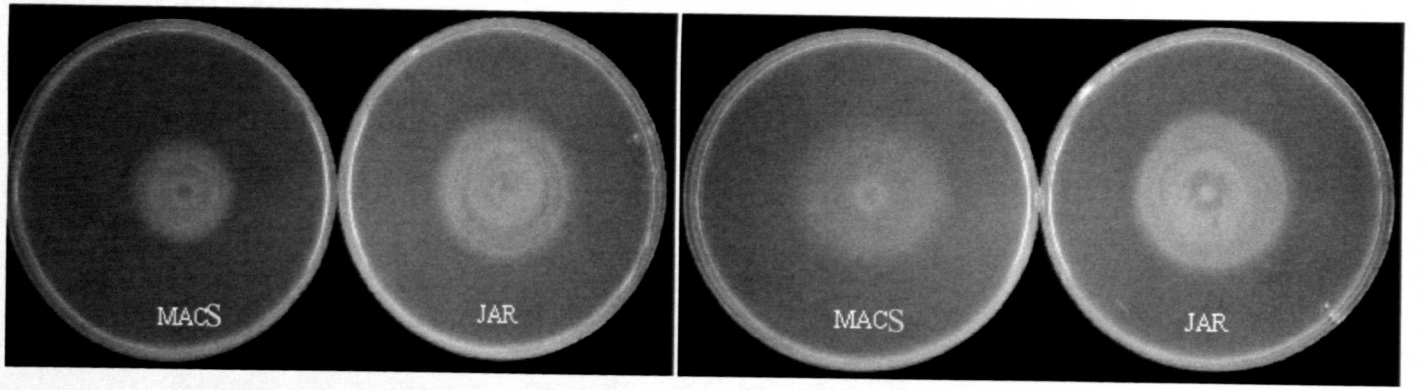


Fig. 3.2 (C)

Fig. 3.2 (D)



(A) *C. jejuni* NCTC 11168 after 18 h; (B) *C. jejuni* NCTC 11168 after 48 h; (C) *C. jejuni* HPC5 after 18 h; and (D) *C. jejuni* HPC5 when grown in MACS (left side) and in gas replacement jar (right side). Images obtained from BioRad Gel Doc EQ.

Table 3.1. Motility of *C. jejuni* NCTC 11168 and *C. jejuni* HPC5 at 18 h and 48 h

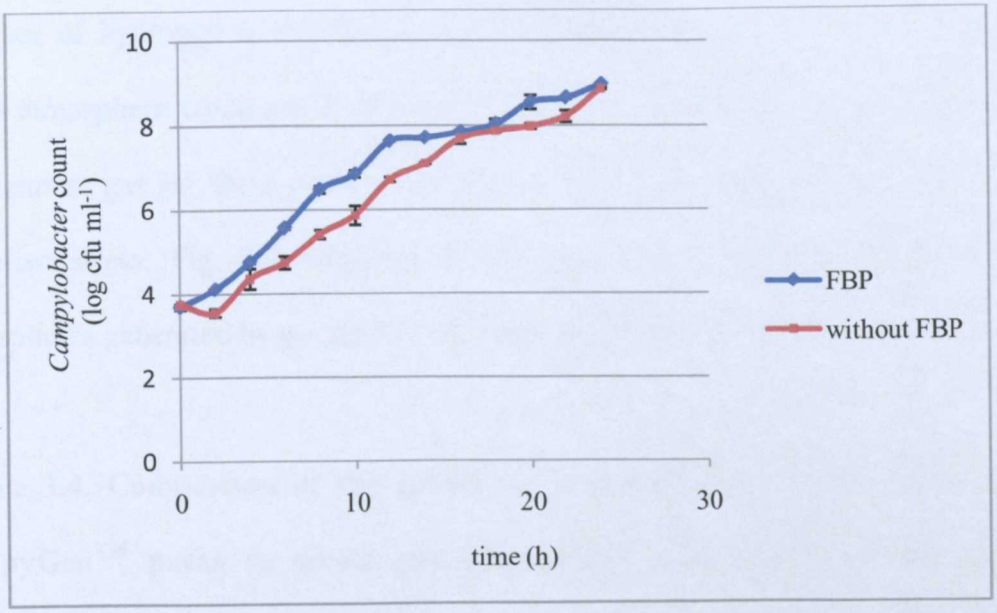
	18h		48h	
Strains	**MACS**	**JAR**	**MACS**	**JAR**
HPC5	1.7 (0.1)	2.43(0.05)	2.4 (0.1)	2.53 (0.05)
NCTC 11168	0.5 (0.05)	1.7 (0.05)	0.7 (0.05)	2.63 (0.1)

Average spread in 'cm' with standard deviation (SD) given in brackets.

3.2.3. Comparison of growth of *C. jejuni* HPC5 in the presence and absence of FBP reagent incubated in a MACS cabinet

An experiment was carried out using the growth supplement FBP to determine if the decline in growth observed in the previous section could be attributed to oxidative stress. *Campylobacter* grown in a MACS cabinet in the absence of FBP showed a reduction in the cell count during the first two hours although some cells remained viable. The numbers of cells was reduced from an initial inoculum of $\log_{10}$ 3.7 cfu ml^{-1} to $\log_{10}$ 3.5 cfu ml^{-1} (±0.01). In equivalent cultures to which FBP was added, the initial count of $\log_{10}$ 3.7 (±0.07) cfu ml^{-1} increased to $\log_{10}$ 4.1 (±0.01) cfu ml^{-1} after 2 h. The growth rate was determined to be 0.87 h^{-1} when *C. jejuni* HPC5 was grown in the presence of the FBP reagent compared to 0.65 h^{-1} without FBP.These results indicate that the cultures incubated in the MACS cabinet were likely to be under oxidative stress, and that this could be overcome by addition of FBP. Fig. 3.3 shows the growth of *C. jejuni* HPC5 in the presence and absence of FBP reagent.

Figure 3.3. Comparison of the growth of *C. jejuni* HPC5 in MACS cabinet in the presence and absence of FBP reagent



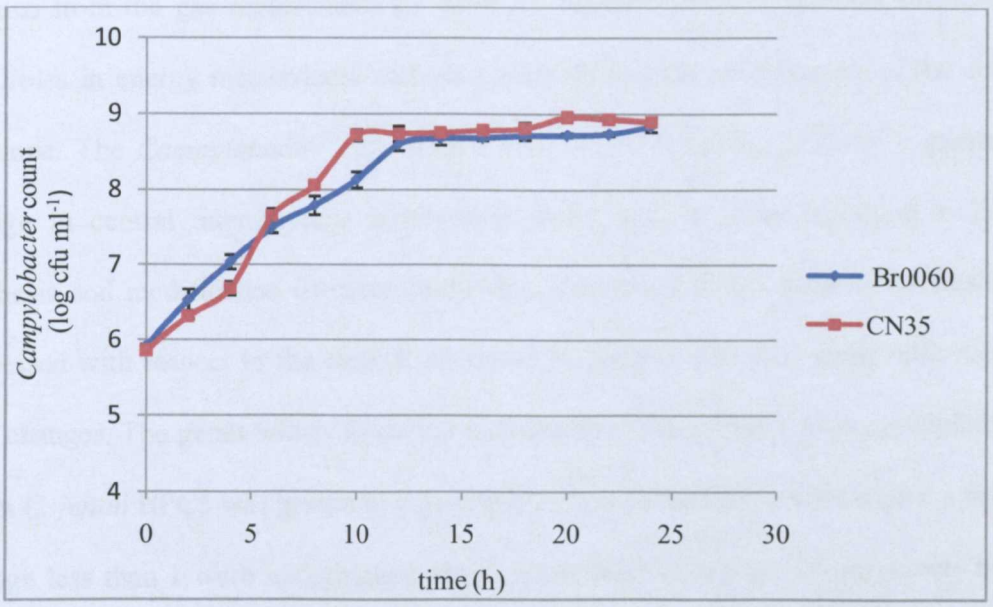
The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the growth time in h. FBP reagent includes filter sterilised Ferrous sulphate – 10% v/v, Sodium pyruvate – 10% w/v and Sodium metabisulphite – 10% w/v. Red line represents the logarithmic growth of *C. jejuni* HPC5 in MACS without FBP in the media and blue line represents the logarithmic growth of *C. jejuni* HPC5 in MACS in the presence of FBP in the media.

3.2.4. Comparative growth of *C. jejuni* HPC5 using hydrogen and non-hydrogen releasing CampyGen™ packs to produce the MACS atmospheres

Since the previous experiment indicated that oxidative stress occurred in the MACS cabinet but not in the hydrogen containing gas replacement jar atmosphere, an experiment was devised to determine the effect of hydrogen. This was achieved by using two types of gas pack, one which produced hydrogen and one which did not. The exponential growth rate was 0.64 h^{-1} for *C. jejuni* HPC5 grown in the atmosphere without hydrogen generated by CampyGen™ CN35, which could be compared with a

growth rate of 0.52 h^{-1} in the presence of hydrogen using CampyGen™ BR0060. However, it is notable that *C. jejuni* HPC5 actually shows a faster growth rate in the presence of hydrogen in the first 4 h of the experiment. Whilst growth in the gas packs atmosphere could not be directly compared to the growth in the cabinet and replacement gas jar, these results indicate that hydrogen could help to reduce initial oxidative stress. Fig. 3.4 compares the growth of *C. jejuni* HPC5 between the atmospheres generated by gas packs CN35 and BR0060.

Figure 3.4. Comparison of the growth of *C. jejuni* HPC5 using two types of CampyGen™ packs to create gas atmospheres with (BR0060) and without (CN35) hydrogen

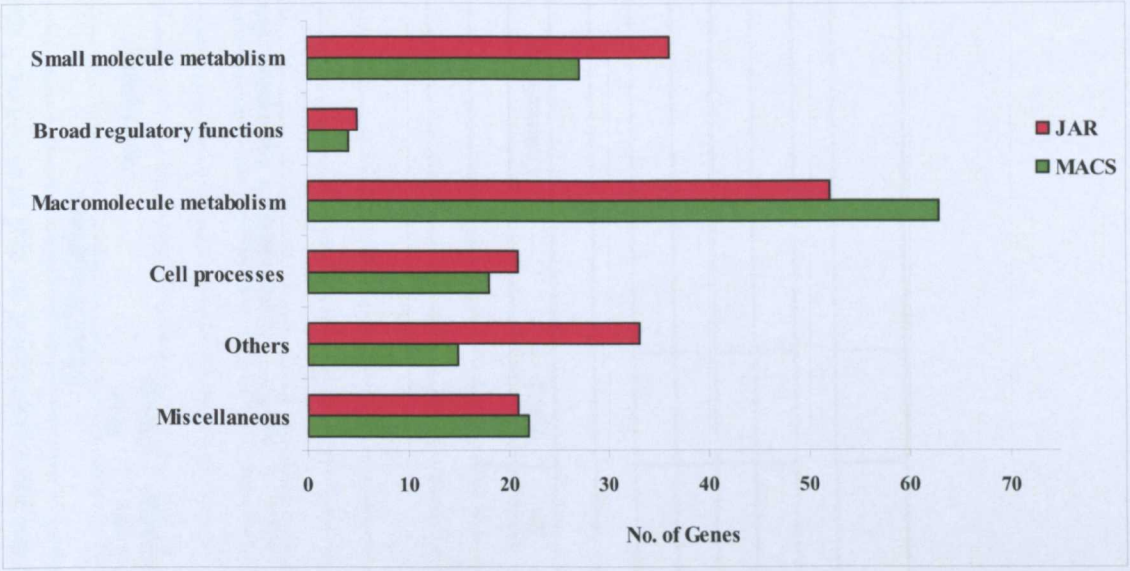


The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the growth time in h. Red line represents the logarithmic growth of *C. jejuni* HPC5 using CN35 and blue line represents the logarithmic growth of *C. jejuni* HPC5 using BR0060.

3.2.5. Transcriptomics study of *C. jejuni* HPC5 in gas replacement jars and in MACS

The gene transcription of the *C. jejuni* HPC5 growing in a MACS cabinet was compared with that of parallel cultures growing in gas replacement jars (GRJ). The total transcripts were measured using microarrays under conditions where equal quantities of cDNA were labelled with the same dye and competitively hybridised against a common control. The data obtained showed a large number of transcriptional changes in genes representing all functional categories. Around 147 genes registered a two fold or more increase in the GRJ compared to the MACS cabinet and 147 showing the opposite trend. The most striking changes observed in cultures from the gas replacement jar were the relatively high expression of genes with roles in energy metabolism, cellular processes and the modification of the cell envelope. The *Campylobacter* cells grown in the MACS cabinet showed a greater change in central intermediary metabolism genes and in genes involved in the synthesis and modification of macromolecules. The genes which were differentially expressed with respect to the control are listed in Table 3.2 to 3.11 along with their fold changes. The genes which showed a fold change greater than 1 were upregulated when *C. jejuni* HPC5 was grown in a gas replacement jar and those which gave a fold change less than 1 were upregulated when grown in MACS. Fig. 3.5 represents the functional classification of genes whch were differentially regulated when *C. jejuni* HPC5 was grown in a gas replacement jar and in MACS cabinet.

Figure 3.5. Functional classification of the genes regulated when *C. jejuni* HPC5 was grown in a gas replacement jar and in MACS cabinet



The X axis represents the number of differentially regulated genes and y axis represents the different functional classification of genes. This classification is based on the functional classification of *Campylobacter* genes given by Sanger Institute (http://www.sanger.ac.uk/Projects/C_jejuni/Cj_gene_list_hierarchical.html).

The red colour represents the genes which were differentially expressed in gas replacement jar and green colour represents the genes which were differentially expressed in MACS.

Table 3.2. Genes involved in energy metabolism which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
1.A. Energy metabolism							
1.A.1. Glycolysis							
				Cj1403c	*gap*A	0.479	Gyceraldehyde 3-phosphate dehydrogenase
1.A.2. Tricarboxylic acid cycle							
Cj0408	*frd*C	5.54	Fumarate reductase cytochrome b-556 subunit				
Cj0835c	*acn*B	2.96	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase				
1.A.3. Pentose phosphate pathway							
Cj0624	*hyp*C	3.108	Hydrogenase isoenzymes formation protein	Cj1645	*tkt*	0.472	Transketolase
1.A.4. Respiration							
1. A.4.i. Aerobic							
Cj1196c	*gps*A	2.84	Glycerol-3-phosphate dehydrogenase [NAD(P)+]				
Cj1579c	*nuo*A	2.556	NADH dehydrogenase subunit A				
Cj1578c	*nuo*B	2.231	NADH dehydrogenase subunit B				
Cj1577c	*nuo*C	2.013	NADH dehydrogenase subunit C				
1. A.4.ii. Anaerobic							
Cj1511c	*fdh*A	6.264	Putative formate dehydrogenase large subunit (Selenocysteine containing)				

Table 3.2. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
1. A.4.iii. Electron transport							
Cj0037c		2.448	Putative cytochrome c	Cj0874c		0.33	Putative cytochrome C
Cj0075c		7.642	Putative oxidoreductase iron-sulfur subunit	Cj1106		0.243	Putative periplasmic thioredoxin
Cj0535	*oor*D	2.945	2-oxoglutarate-acceptor oxidoreductase subunit OorD	Cj0465c	*ctb*	0.237	Group III truncated haemoglobin
Cj0536	*oor*A	2.47	2-oxoglutarate-acceptor oxidoreductase subunit OorA				
Cj0780	*nap*A	4.343	Nitrate reductase catalytic subunit				
Cj0781	*nap*G	3.224	Quinol dehydrogenase periplasmic component				
Cj0785	*nap*D	5.548	NapD protein homolog				
Cj1020c		2.173	Putative cytochrome C				
Cj1186c	*pet*A	14.34	Putative ubiquinol-cytochrome C reductase iron-sulfur subunit				
Cj1358c	*nrf*H	10.3	Putative periplasmic cytochrome C				
Cj1476c		3.714	Pyruvate-flavodoxin oxidoreductase				
1. A.4.iv. ATP proton motive force							
Cj0103	*atp*F	8.385	F0F1 ATP synthase subunit B	Cj0936	*atp*E	0.443	F0F1 ATP synthase subunit C
Cj0104	*atp*H	4.156	F0F1 ATP synthase subunit delta				
Cj0102	*atp*F'	6.144	F0F1 ATP synthase subunit B'				
Cj1204c	*atp*B	2.926	F0F1 ATP synthase subunit A				

Table 3.3. Genes involved in central intermediary metabolism and amino acid metabolism which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
1.B. Central intermediary metabolism							
1.B.1. General							
Cj0483	*uxa*A'	7.665	Putative altronate hydrolase C-terminus	Cj1407c		0.467	Putative phospho-sugar mutase
Cj0237	*cyn*T	3.39	Carbonic anyhydrase				
Cj0009	*glt*D	2.543	Glutamate synthase subunit beta				
1.B.2. Amino sugars							
				Cj1366c	*glm*S	0.33	Glucosamine-fructose-6-phosphate aminotransferase
1.C. Amino acid biosynthesis							
1.C.1. Aspartate family							
Cj1023c	*asd*	3.491	Aspartate-semialdehyde dehydrogenase				
1.C.2. Serine family							
				Cj0326	*ser*C	0.456	Phosphoserine aminotransferase
1.C.3. Aromatic amino acid family							
Cj0895c	*aro*A	2.042	3-phosphoshikimate 1-carboxyvinyltransferase	Cj0346	*trp*D	0.216	Anthranilate synthase component II
				Cj0349	*trp*A	0.178	Tryptophan synthase subunit alpha
				Cj0348	*trp*B	0.258	Tryptophan synthase subunit beta

Table 3.3. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
				1.C.4 Histidine			
				Cj1601	*his*A	0.365	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerise
				Cj1604	*his*I	0.329	Bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase protein
				1.C.5. Branched chain family			
				Cj1719c	*leu*A	0.407	2-isopropylmalate synthase
				1.D. Polyamine biosynthesis			
				Cj1208		0.47	Putative 5-formyltetrahydrofolate cyclo-ligase family protein
				Cj0514	*pur*Q	0.427	Phosphoribosylformylglycinamidine synthase I
				Cj0953c	*pur*H	0.351	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase

Table 3.4. Genes involved in the biosynthesis of purines, pyrimidines, nucleosides, nucleotides, cofactors, prosthetic groups and carriers which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene symbol**	**Fold change**	**Description**
1.E. Purines, pyrimidines, nucleosides and nucleotides							
1. E.1. Purine ribonucleotide biosynthesis							
CCOA0020		116.1	882aa long hypothetical purine, putative				
Cj0027	*pyr*G	2.122	CTP synthase				
1. E.2. 2'-deoxyribonucleotide biosynthesis							
				Cj1451	*dut*	0.332	dUTPase
1.F. Biosynthesis of cofactors, prosthetic groups and carriers							
1.F.1. Folic Acid							
Cj0861c	*pab*A	2.963	para-Aminobenzoate synthase glutamine amidotransferase component II				
Cj0065c	*fol*K	2.872	Putative 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase				
1.F.2. Molybdopterin							
Cj0161c	*moa*A	2.366	Mo cofactor biosynthesis protein A	Cj0725c	*mog*A	0.479	Mo cofactor biosynthesis protein
1.F.3. Thioredoxin							
				Cj0146c	*trx*B	0.361	Thioredoxin reductase
1.F.4. Heme							
				Cj0995c	*hem*B	0.444	delta-Aminolevulinic acid dehydratase
				Cj1243	*hem*E	0.297	Uroporphyrinogen decarboxylase

Table 3.4. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene symbol**	**Fold change**	**Description**
1.F.5. Thiamine							
				Cj1081c	*thi*E	0.471	Thiamin-phosphate pyrophosphorylase
				Cj1043c		0.462	Putative thiamine-phosphate pyrophosphorylase

Table 3.5. Genes involved in broad regulatory functions which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
2.A. Broad regulatory functions							
Cj0480c		3.499	Putative transcriptional regulator	Cj1533c		0.266	Putative helix-turn-helix containsing protein
Cj0368c	*cme*R	2.508	Transcriptional regulator CmeR	Cj0440c		0.241	Putative transcriptional regulator
2. B. Signal transduction							
Cj1110c		3.272	Putative MCP-type signal transduction protein	Cj1491c		0.46	Putative two-component regulator
Cj1608		2.37	Putative two-component regulator	Cj0951c		0.355	Putative MCP-domain signal transduction protein
Cj0889c		2.184	Putative sensory trasduction histidine kinase	Cj0890c		0.398	Putative sensory transduction transcriptional regulator

Table 3.6. Genes involved in synthesis and modification of macromolecules which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
3.A. Synthesis and modification of macromolecules							
3.A.1. Ribosomal protein synthesis and modification							
Cj1182c	*rps*B	3.378	30S ribosomal protein S2	Cj1696c	*rpl*X	0.46	50S ribosomal protein L24
				Cj0476	*rpl*J	0.489	50S ribosomal protein L10
				Cj1703c	*rps*S	0.468	30S ribosomal protein S19
				Cj1697c	*rpl*N	0.491	50S ribosomal protein L14
				Cj1695c	*rpl*E	0.455	50S ribosomal protein L5
				Cj1280c		0.447	putative ribosomal pseudouridine synthase
				Cj1479c	*rps*I	0.447	30S ribosomal protein S9
				Cj0245	*rpl*T	0.436	50S ribosomal protein L20
				Cj1182c	*rps*B	0.373	30S ribosomal protein S2
				Cj1596	*rpl*Q	0.458	50S ribosomal protein L17
				Cj1696c	*rpl*X	0.46	50S ribosomal protein L24
				Cj0475	*rpl*A	0.332	50S ribosomal protein L1
				Cj1691c	*rpl*R	0.31	50S ribosomal protein L18
				Cj1690c	*rps*E	0.226	30S ribosomal protein S5
				Cj1689c	*rpl*O	0.219	50S ribosomal protein L15

Table 3.6. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
3.A.2. Ribosome maturation and modification							
Cj0640c	*Asps*	9.336	aspartyl-tRNA synthetase	Cj0664c	*rplI*	0.487	50S ribosomal protein L9
Cj0370	*rps*U	8.181	30S ribosomal protein S21	Cj0206	*thr*S	0.471	threonyl-tRNA synthetase
Cj1288c	*glt*X	3.961	glutamyl-tRNA synthetase	Cj1596	*rplQ*	0.458	50S ribosomal protein L17
Cj0712	*Rim*	2.716	putative 16S rRNA processing protein				
Cj1061c	*ile*S	2.005	isoleucyl-tRNA synthetase				
3.A.3. DNA replication, restriction/modification, recombination and repair							
Cj0863c	*xer*D	6.128	DNA recombinase	Cj0003	*gyr*B	0.404	DNA gyrase subunit B
Cj0338c	*pol*A	2.955	DNA polymerase I	Cj0722c		0.365	putative DNA methylase
				Cj0690c		0.359	possible restriction /modification enzyme
				Cj0255c	*exo*A	0.298	Exodeoxyribonuclease
3.A.4. Protein translation and modification							
Cj0207	*inf*C	5.361	translation initiation factor IF-3	Cj1171c	*ppi*	0.355	peptidyl-prolyl cis-trans isomerase
3.A.5. RNA synthesis, RNA modification and DNA transcription							
Cj1006c		4.672	putative MiaB-like tRNA modifying enzyme	Cj0904c		0.424	putative RNA methylase
Cj0153c		4.232	putative rRNA methylase	Cj1253	*pnp*	0.418	polynucleotide phosphorylase/polyadenylase
Cj0287c	*gre*A	3.53	transcription elongation factor	Cj0479	*rpo*C	0.219	DNA-directed RNA polymerase beta' chain
Cj1156	*Rho*	3.154	transcription termination factor				

Table 3.7. Genes involved in the degradation of macromolecules and in the formation of cell envelope which were differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
3.B. Degradation of macromolecules							
3.B.1. DNA							
Cj1215		5.333	putative peptidase M23 family protein				
3. B. 2. Proteins, peptides and glycopeptides							
				Cj0929	*pep*A	0.453	leucyl aminopeptidase
				Cj0980		0.419	putative peptidase
				Cj0068	*psp*A	0.253	Protease
3.C. Cell Envelope							
3.C.1. Membranes, lipoproteins and porins							
Cj0818		51.84	putative lipoprotein	Cj0378c		0.2	putative ferric reductase-like transmembrane protein
Cj0201c		37.04	putative integral membrane protein	Cj0721c		0.488	putative integral membrane protein
Cj0928		34.17	putative integral membrane protein (dedA)	Cj0557c		0.455	putative integral membrane protein
Cj0457c		4.212	putative lipoprotein	Cj1649		0.441	putative lipoprotein
Cj1022c		3.409	putative integral membrane protein	Cj0362		0.403	putative integral membrane protein
Cj0937		3.273	putative integral membrane protein	Cj0952c		0.365	putative HAMP containing membrane protein
Cj0553		3.052	putative integral membrane protein	Cj1276c		0.343	putative integral membrane protein
				Cj0986c		0.326	putative integral membrane protein
				Cj1452		0.481	putative integral membrane protein
				Cj0091		0.408	putative lipoprotein
				Cj0950c		0.248	putative lipoprotein

Table 3.7. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
3.C.2. Surface polysaccharides, lipopolysaccharides and antigens							
Cj1134	*htr*B	2.954	lipid A biosynthesis lauroyl acyltransferase	Cj0685c	*cip*A	0.463	Invasion protein CipA
Cj1151c	*hld*D	2.554	ADP-glyceromanno-heptose 6-epimerase	Cj1121c	*pgl*E	0.459	UDP-4-keto-6-deoxy-GlcNAc C4 aminotransferase
Cj1311	*pse*F	2.304	putative acylneuraminate cytidylyltransferase	Cj1128c	*pgl*I	0.386	glucosyl transferase
Cj1316c	*pse*A	2.102	pseudaminic acid biosynthesis PseA protein	Cj1329		0.403	putative sugar-phosphate nucleotide transferase
Cj1320		2.073	putative aminotransferase (degT family)	Cj1327	*neu*B2	0.401	N-acetylneuraminic acid synthetase
				Cj1131c	*gne*	0.305	UDP-GlcNAc/Glc 4-epimerase
				Cj1424c	*gmh*A2	0.238	phosphoheptose isomerise
				Cj1423c	*hdd*C	0.213	putative D-glycero-D-manno-heptose 1-phosphate guanosyltransferase
3.C.3. Murein sacculus and peptidoglycan							
				Cj0432c	*mur*D	0.307	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase

Table 3.7. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
3.C.4. Surface structures							
Cj1339c	*fla*A	2.982	Flagellin	Cj1565c	*pfl*A	0.448	paralysed flagellum protein
Cj0547	*flaG*	6.905	possible flagellar protein	Cj1675	*fli*Q	0.399	flagellar biosynthesis protein FliQ
CCO1444		8.529	flagellin (flaA)	Cj0060c	*fli*M	0.413	flagellar motor switch protein FliM
Cj1312	*pse*G	2.518	nucleotidase specific for PseC product	Cj0882c	*flh*A	0.356	flagellar biosynthesis protein FlhA
Cj1294	*pse*C	2.787	C4 aminotransferase specific for PseB product				
Cj0548	*fli*D	2.699	flagellar capping protein				
Cj1338c	*fla*B	2.622	Flagellin				
Cj0320	*fli*H	2.614	flagellar assembly protein H				
Cj0351	*fli*N	2.306	flagellar motor switch protein				
3.C.5. Miscellaneous periplasmic proteins							
Cj1680c		221	putative periplasmic protein	Cj1670c	*cgp*A	0.487	putative periplasmic protein
Cj1041c		86.51	putative periplasmic ATP/GTP-binding protein	Cj0092		0.478	putative periplasmic protein
Cj1513c		13.31	possible periplasmic protein	Cj0093		0.352	putative periplasmic protein
Cj1632c		9.609	putative periplasmic protein	Cj0596	*peb*4cbf2	0.349	major antigenic peptide PEB-cell binding factor
Cj1240c		4.069	putative periplasmic protein	Cj1632c		0.277	putative periplasmic protein
Cj0114		3.301	putative periplasmic protein	Cj0034c		0.401	putative periplasmic protein
Cj1021c		2.87	putative periplasmic protein	Cj0515		0.401	putative periplasmic protein
Cj1193c		2.668	putative periplasmic protein	Cj0876c		0.386	putative periplasmic protein
Cj0776c		2.372	putative periplasmic protein	Cj0964		0.383	putative periplasmic protein
Cj1200		2.067	putative NLPA family lipoprotein				

Table 3.8. Genes involved in the cell processes which are differentially regulated in the gas replacement jar and in the MACS cabinet

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
4. Transport/ binding proteins							
4.A.1. Amino acids and amines							
				Cj1017c	*liv*H	0.348	branched-chain amino-acid ABC transport system permease protein
4.A. Carbohydrates, organic acids and alcohols							
Cj0613	*pst*S	9.423	putative periplasmic phosphate binding protein				
4.A.2. Cations							
				Cj0174c	*cfbp*B	0.375	putative iron-uptake ABC transport system permease protein
				Cj1617	*chu*D	0.304	putative haemin uptake system periplasmic haemin-binding protein
4.A.3. Anions							
				Cj1194		0.37	possible phosphate permease
4.A.4. Other							
Cj0484		5.002	putative MFS (Major Facilitator Superfamily) transport protein	Cj0025c		0.42	putative sodium:dicarboxylate family transmembrane symporter
Cj0238		4.53	putative mechanosensitive ion channel family protein	Cj1684c		0.361	putative transmembrane transport protein
Cj0203		3.39	putative citrate transporter	Cj0182		0.285	putative transporter
Cj0935c		2.166	putative sodium:amino-acid symporter family protein	Cj0426		0.285	putative ABC transporter ATP-binding protein
Cj1049c		2.126	putative LysE family transporter protein	Cj1663		0.261	putative ABC transport system ATP-binding protein
Cj1291c	*acc*B	2.112	putative biotin carboxyl carrier protein of acetyl-CoA carboxylase	Cj0182		0.285	putative transporter
Cj0263		2.094	zinc transporter ZupT	Cj0954c		0.469	putative dnaJ-like protein
				Cj1108	*clp*A	0.438	ATP-dependent Clp protease ATP-binding subunit

Table 3.8. (continued)

Gas replacement jar				MACS cabinet			
Locus Tag	**Gene Symbol**	**Fold change**	**Description**	**Locus Tag**	**Gene Symbol**	**Fold change**	**Description**
4.B. Chaperones, chaperonins, heat shock							
Cj0758	*grp*E	30.94	heat shock protein grpE				
Cj0759	*dna*K	8.282	molecular chaperone DnaK				
Cj0509c	*clp*B	6.127	ATP-dependent Clp protease ATP-binding subunit				
Cj1230	*hsp*R	3.158	heat shock transcriptional regulator				
Cj0193c	*Tig*	3.041	trigger factor				
Cj0518	*htp*G	2.794	heat shock protein 90				
Cj1221	*gro*EL	2.106	chaperonin GroEL				
4.C. Cell division							
Cj0101		3.766	parB family protein	Cj0276	mreB	0.311	rod shape-determining protein MreB
4.D. Chemotaxis and mobility							
Cj0285c	*che*V	2.404	chemotaxis protein				
4.E. Protein and peptide secretion							
Cj1093c	*sec*D	0.497	preprotein translocase subunit SecD	Cj1092c	*sec*F	0.427	preprotein translocase subunit SecF
				Cj1688c	*sec*Y	0.423	preprotein translocase subunit SecY
4.F. Pathogenicity							
Cj1349c		3.077	putative fibronectin/fibrinogen-binding protein	Cj1198		0.255	S-ribosylhomocysteinase
4.G. Detoxification							
				Cj0779	*tpx*	0.494	probable thiol peroxidase
				Cj0169	*sod*B	0.377	superoxide dismutase (Fe)

Table 3.9. Genes involved in the biosynthesis of fatty acids which are differentially regulated in the MACS cabinet

Locus Tag	Fold change	Description
Antibiotic resistance		
Cj1715	0.457	putative acetyltransferase
Cj1033	0.416	putative integral membrane component of efflux

Table 3.10. Genes differentially expressed in jar and MACS which coded hypothetical proteins

JAR			MACS	
CCO0807	Cj0539	Cj1460	Cj1501	Cj1164c
CCOA0056	Cj0550	Cj1465	Cj0118	CJE1727
CCOA0087	Cj0584	Cj1475c	Cj1330	CJE0599
CCOA0120	Cj0620	Cj1514c	Cj0703	CJE0256
Cj0044c	Cj0700	Cj1547	Cj0916c	CJE0600
Cj0070c	Cj0152c	Cj1606c	Cj0761	
Cj0073c	Cj0728	Cj1631c	Cj1249	
Cj0163c	Cj0787	CJE0264	Cj0427	
Cj0253	Cj0829c	CJE1102	Cj0849c	
Cj0286c	Cj1348c	Cj1060c	Cj1254	
Cj0418c	Cj1450	Cj0124c	Cj1429c	

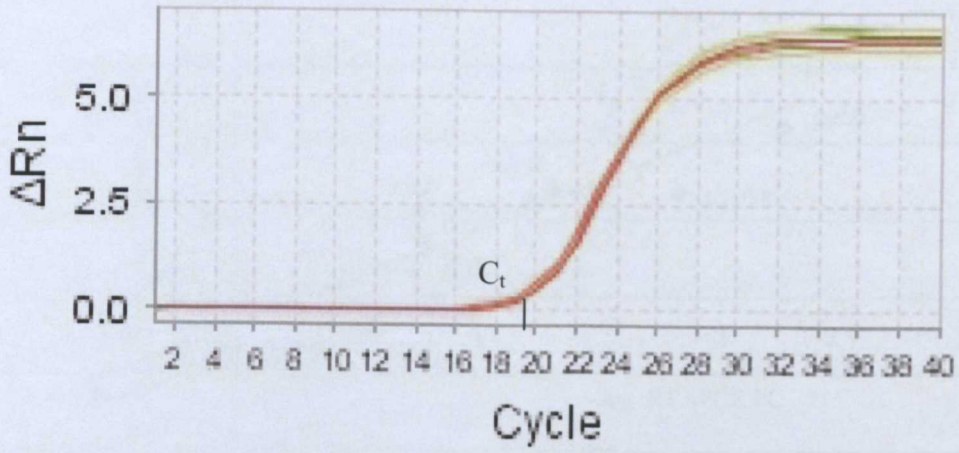
Table 3.11. Genes differentially expressed in jar and MACS which coded proteins with unknown functions

JAR	MACS	
Cj0796c	Cj0559	Cj1618c
Cj0377	Cj0462	Cj0807
Cj1100	Cj1115c	Cj0985c
Cj1152c	Cj1302	Cj1418c
Cj1585c	Cj0372	Cj0546
Cj1406c	Cj1337	Cj0791c
Cj1607	Cj0230c	Cj0119
CJE1720	CJE0670	Cj1477c
Cj0481	Cj0256	
Cj0419	Cj1417c	
Cj0487	Cj0356c	

3.2.6. Validation of Microarray results using qRT PCR

To support the reliability of the microarray data, the expression of 8 genes was confirmed by qRT PCR. Transcription of the gene for subunit S16 of the 30S ribosomal protein complex was selected as the control for the qRT PCR studies since it showed similar ΔC_t values ($p<0.05$) and zero fold change when *Campylobacter* was grown in the MACS cabinet and in the gas replacement jar. ΔC_t values for *rpo*S when grown in MACS and in jar using qRT PCR is represented in Fig. 3.6. The ΔC_t value for *rpo*S gene was 18.28. Table 3.12 confirms the trends observed in the transcriptomic study by comparing the microarray fold change and qRT PCR fold change. Fig. 3.7 showed the concordance between the microarray results and the qRT PCR data showing a correlation coefficient of 0.931.

Figure 3.6. ΔC_t value for *rpo*S when grown in MACS and in jar using qRT PCR



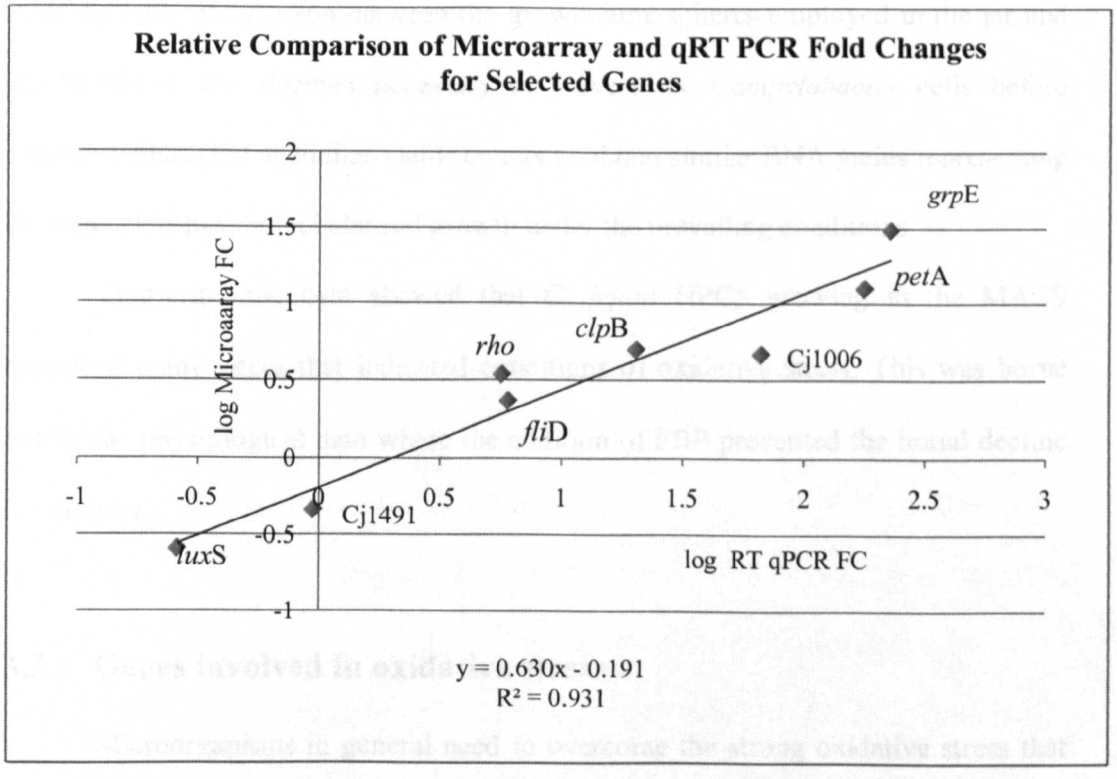
Ct is the intersection value where the amplification curve meets the threshold line which is the relative measure of the target. Y axis represent 'R_n' which represents the fluorescence of the reporter dye divided by the fluorescence of the passive dye reference and x axis denotes the number of cycles. C_t value for *rpo*S when grown in MACS and in jar gave the same value showing that there is no differential expression when grown in jar and in MACS.

Table 3.12. Relative comparison of Microarray and qRT PCR fold changes

Gene	ΔCt of gene	ΔCt of gene- ΔCt of control	qRT PCR fold change (2^ΔΔCt)	Log 10 qRT PCR	Microarray fold change	Log 10 Microarray FC
grpE	26.11	7.83	227.54	2.36	44.80	1.65
*clp*B	22.64	4.36	21.11	1.32	6.13	0.79
*fli*D	20.876	2.596	6.05	0.78	2.70	0.43
*lux*S	16.34	-1.94	0.26	-0.58	0.26	-0.59
Cj1491	18.21	-0.07	0.95	-0.02	0.46	-0.34
rho	20.79	2.51	5.70	0.76	3.15	0.50
*pet*A	25.77	7.49	179.77	2.25	14.34	1.16
Cj1006	24.34	6.06	66.72	1.82	4.67	0.67

The table represents the calculation of fold change in qRT PCR.

Figure 3.7. Comparison of the gene expression levels measured by microarray and qRT PCR of selected genes



The x axis represents the log ratio values of the microarray fold changes while the y axix represent the log qRT PCR fold change. R^2 represent coefficient of determination.

3.3. DISCUSSION

C. jejuni HPC5 growing under microaerobic conditions in a gas jar, exhibits a preferentially high rate in the expression of metabolic genes indicating a situation where conditions are favourable for high rates of growth. The growth curve and the enhanced motility also indicate favourable conditions commensurate with exponential growth in cell numbers. In contrast when *C. jejuni* HPC5 was introduced into the MACS cabinet, it showed reduced growth during the initial hours but showed similar growth to that in the gas replacement jar after 18 h. During the growth cycle of *Campylobacter*, it has been reported that a great number of changes in the expression levels of genes occur at stationary phase with 133 genes increased in expression and 151 genes decreased, from which it is concluded that this period is of high bacterial activity though there is overall less bacterial growth (Wright *et al.*, 2009). To enable transcriptomic comparison between the growth atmospheres employed in the jar and the MACS it was deemed necessary to harvest the *Campylobacter* cells before stationary phase but at similar viable counts to obtain similar RNA yields representing the transcripts present in balanced growth under the prevailing conditions.

Transcriptomic data showed that *C. jejuni* HPC5 growing in the MACS expressed many genes that indicated conditions of oxidative stress. This was borne out by the physiological data where the addition of FBP prevented the initial decline in numbers.

3.3.1. Genes involved in oxidative stress

Microorganisms in general need to overcome the strong oxidative stress that arises due to the oxidising potential of excess reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). ROS include superoxide anion (O^{2-}), hydrogen

peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and halogenated oxygen molecules while RNI include nitricoxide (NO), Peroxynitrite ($OONO^-$) S- nitrosothiols (RSNO), nitrogen dioxide ($NO_2^{\cdot}$), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4) and dinitrosyl- iron complexes (DNIC) (Hughes, 1999; Day Jr. *et al.*, 2000; Burney *et al.*, 1999; Fang, 1997). RNI can react with superoxide to yield the highly toxic peroxynitrite ion ($ONOO^-$) (Burney *et al.*, 1999).

$$NO^{\cdot} + O_2^{-} \longrightarrow ONOO^-$$

The interaction of hydrogen peroxide with reduced iron may also lead to the formation of more toxic intermediates, such as hypochlorous anion, hydroxyl radicals, hydroxide anions and nitrogen dioxide (Pacelli *et al.*, 1995). Superoxide anion radicals (O_2^-) are generated due to leakage from the electron transport chain and Krebs cycle with 0.2% of oxygen consumed being converted to superoxide (Addabbo *et al.*, 2009). ROS are also produced by metabolic oxidases that include NADPH oxidase and xanthine oxidase (Sauer, 2001; Nordberg and Arner, 2001). Oxide anion radicals will reduce iron and copper ions, and the reduced form of these ions will react with hydrogen peroxide to produce hydroxyl radicals *via* the Fenton reaction (Ohsawa, 2007). Hydroxyl radicals ($OH^{\cdot}$) represent the strongest oxidant species that can react indiscriminately with nucleic acids, lipids and proteins at the rate of diffusion (Cheng *et al.*, 2002).

The presence of hydrogen in the growth atmosphere can act as an antioxidant by combing with $OH^{\cdot}$ to produce water. The comparison of the two types of gas pack, with and without hydrogen, would seem to indicate that this could occur in the initial phases of growth and may prevent the initial decline observed when *C. jejuni* HPC5 was incubated in the MACS cabinet. In acidic solutions Fe^{2+} reacts with H_2O_2 to become oxidised to Fe^{3+} and H_2O.

$$2\ Fe^{2+}\ (aq) + H_2O_2 + 2\ H^+\ (aq) \rightarrow 2\ Fe^{3+}\ (aq) + 2H_2O\ (l)$$

C. jejuni HPC5 shows a relative increase in the expression of genes involved in detoxification of the cell. For example, the genes encoding the enzymes thiol peroxidase (Cj0779; *tpx*) and superoxide dismutase (CCO1706) show a two fold or more increase in the MACS cabinet to emphasise the importance of removing H_2O_2 under these conditions. Superoxide disproportionates to H_2O_2 and O_2 at a substantial rate, especially in the presence of Fe^{2+} ions. Thiol peroxidase in *Campylobacter* appears to be a dedicated H_2O_2 reductase since no *in vitro* activity was observed when organic peroxides or lipid peroxides were provided as substrates (Atack and Kelly, 2009). A differential increase in the expression of Cj1106 was observed in the MACS cabinet, which encodes periplasmic thioredoxin. It has been postulated that thioredoxin (*trx*B) like its orthologue in *H. pylori*, has a role in the defence against superoxide and peroxide mediated stresses by acting to recycle the alkyl hydroperoxidase (*aph*C) that reduces alkyl perioxides to their corresponding alcohols (Atack *et al.*, 2008).

Growth in the MACS cabinet was also associated with an increase in the expression of *lux*S which is thought to be responsible for the formation of autoinducer -2 (AI-2). A *lux*S mutant in the strain *C. jejuni* 81-176 has been shown to have high sensitivity to peroxide stress consistent with the low expression of the peroxidases *tpx* and *ahp*C (He *et al.*, 2008). However, the studies of Elvers and Park, (2002) indicated that *lux*S mutants of *C. jejuni* NCTC 11168 were able to resist oxidative stress, exhibiting growth patterns similar to wild type on paraquat (1-40 mM) and hydrogen peroxide (0.3-0.15% v/v).

The addition of FBP reagent to cultures grown in the MACS cabinet alleviated the initial fall in viable count likely associated with oxidative stress. Atack and Kelly

(2009) have suggested that *C. jejuni* grown in a medium with pyruvate, a component of FBP, relieves the need for inducing high levels of catalase during oxidative stress to allow growth under full aerobic conditions. Pyruvate reduced H_2O_2 to water thereby reducing H_2O_2 stress and in the process becomes degraded to acetate and carbon dioxide. The presence of carbon dioxide creates an acidic environment due to the production of the H^+ ions through the action of carbonic anhydrase (Cj0237), which also shows greater expression when *Campylobacter* was grown in the atmosphere of the jar. Carbonic anhydrases are ubiquitous zinc containing enzymes that catalyse the reversible hydration of carbon dioxide by nucleophilic attack of a zinc bound hydroxide ion on carbon dioxide, which is followed by the regeneration of the active site by ionization of the zinc bound water molecule and proton release (Lindskog, 1997).

The gene encoding *cme*R (Cj0368c) was differentially expressed in the jar. The product of the *cme*R gene is a *tet*R protein family member that acts as a transcriptional repressor of the downstream *cme*ABC operon. *Cme*ABC is a multidrug efflux system that can confer resistance against antimicrobials and toxic compounds (Guo *et al.*, 2008). A 2.5 fold greater level of expression of the repressor observed in the jar would suggest that any detoxifying function associated with the efflux system is not required during balanced growth in the jar.

3.3.2. Genes involved in motility associated functions

C. jejuni is motile by its polar flagellum, the function of which is associated with more than 40 structural and regulatory genes, including an O-linked glycosylation system that post translationally modifies the flagellin subunits (Guerry *et al.*, 2006). Flagella are notably required for the invasion of human epithelial cells,

adhesion and virulence (Wright *et al.*, 2009). Flagella genes that show increased differential expression for *C. jejuni* HPC5 growing in the jar include *fla*A, *fla*B, *fla*G, *fli*H, *fli*G, *fli*H and *fli*N that together code for flagellin proteins, flagella assembly proteins, hook associated proteins as well as the motor and switch components. Post translational modification of the flagellin proteins occurs due to an O- linked glycosylation system that includes modification by pseudaminic acid. The pseudaminic acid biosynthetic genes *pse*ACFG were also observed to have increased transcript levels for *C. jejuni* HPC5 which was grown in the gas replacement jar relative to the MACS cabinet. The general increase in expression of the flagella associated genes is consistent with the observations made on the motility of *C. jejuni* HPC5 growing in the gas replacement jar compared to motility in the MACS cabinet 18 h after inoculum.

A two-component system (TCS) signal transduction pathway represented by Cj1608 is differentially expressed in the gas replacement jar that is composed of a sensor receptor that receives external signal(s) and a response regulator that controls gene expression or physiological activities such as chemotaxis (Hoch, 2000). Most of the sensors of bacterial TCS are histidine kinases, where a membrane associated histidine kinase phosphorylates, a conserved histidine residue in response to the signal(s) in the environment. The putative sensory transduction histidine kinase (Cj0889c) also shows a 2.2 fold increase in expression during growth in the gas replacement jar. Hendrixson (2008a) in his studies on *Campylobacter* colonising chicks, has proposed that an uncharacterised signal emanating from the flagella export apparatus with *flh*F, is sensed by the *flg*S histidine kinase that results in autophosphorylation and activation of *flg*R in order to activate σ54 dependent transcription of the flagellin genes.

A 2.6 fold increase in the expression of Cj1110c encoding a methyl accepting chemotaxis type signal transduction protein (MCP) was observed in the gas replacement jar culture over the MACS cabinet grown culture. MCPs are components of the chemosensory apparatus (Kort *et al.*, 1975; Hazelbauer *et al.*, 1993) that link the cells chemotactic response to flagella and transmits signals to a non sensor (class II) histidine kinase, *che*A, which in turn transmits the signal to its cognate response regulator *che*Y (Zhulin *et al.*, 2003). These receptors are widely present in prokaryotes where they become methylated or demethylated depending upon the concentration of chemo attractants or repellents present in the surrounding medium (Morgan *et al.*, 1985). The chemotaxis gene *che*V (Cj0285c) which encodes a cheA-cheW hybrid protein with response regulator and scaffold protein functions that are essential for the formation of the ternary complex for chemotactic activity (Marchant *et al.*, 2002). These signalling pathways assist the organism in its intestinal life style and are an essential prerequisite to pathogenesis in human disease.

In contrast *C. jejuni* HPC5 grown in the MACS cabinet gave greater expression of *pfl*A (Cj1565c), *fli*M, *flh*A and a putative MCP domain signal transduction protein (Cj0951c). *Pfl*A is component of flagellar motor and switch mechanism, mutants of which result in paralysed flagella and thereby no motility (Yao *et al.*, 1994). The product of *fli*M is the motor switch C-ring protein and *flh*A codes for the pore of the flagellar export apparatus.

3.3.3. Genes involved in respiratory functions

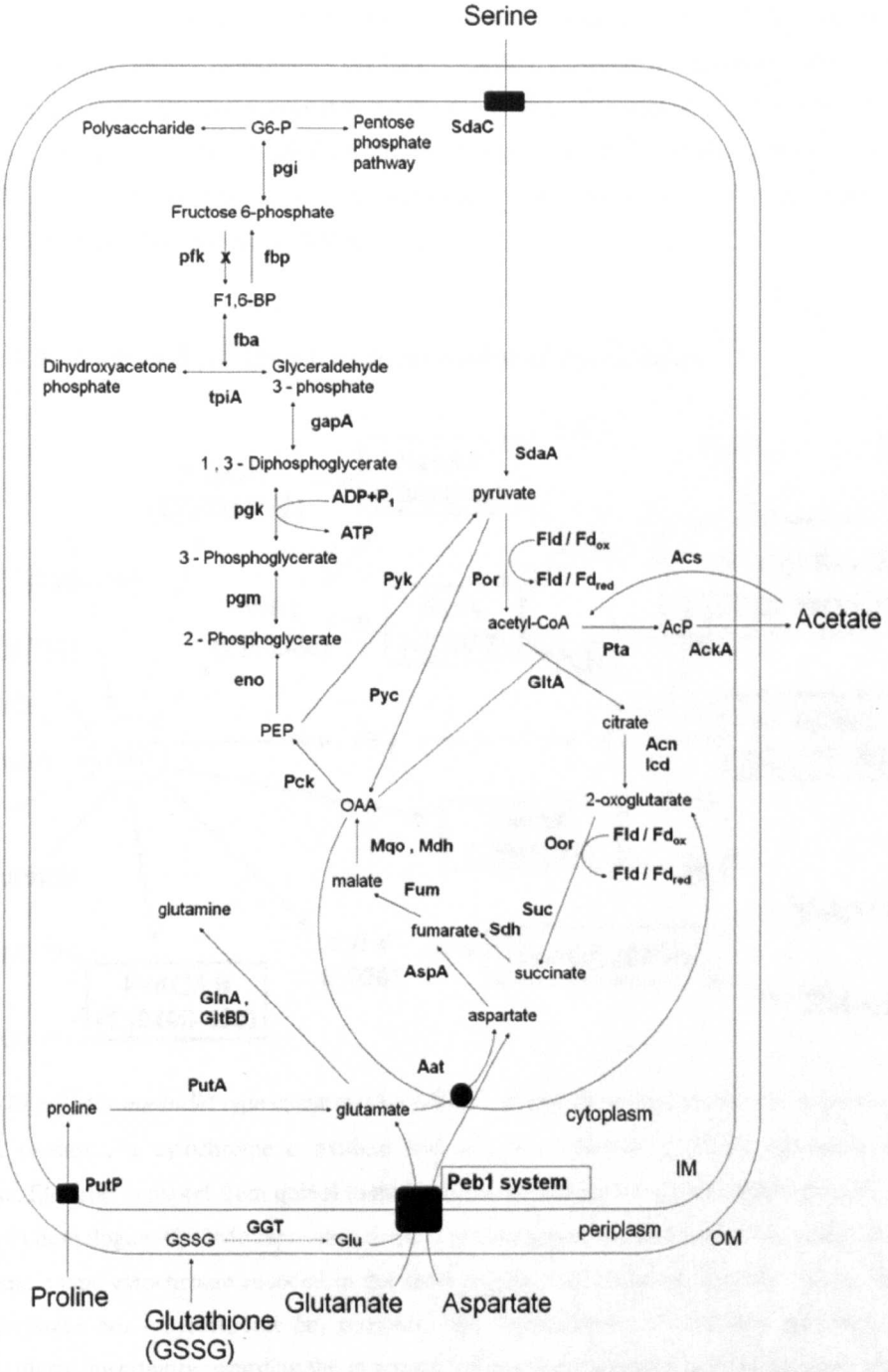
Studies have shown that campylobacters have a respiratory and chemo organotrophic type of metabolism, where cellular energy needs are met through the metabolism of amino acids and tricarboxylic acid cycle components (Debruyne *et al.*,

2008). The bacterium has a complex branched electron transport chain which allows oxygen limited respiration with a number of terminal reductases that allow the use of alternate electron acceptors (Kelly, 2008). Figure 3.8 shows the central carbon metabolism and amino acid utilisation pathways present in *C. jejuni*. It is widely accepted that *C. jejuni* neither oxidises nor ferments glucose due to the absence of 6-phospho fructokinase to convert fructose-6-phosphate to fructose 1,6-biphosphate. However, the genes encoding the Embden- Meyerhof and pentose phosphate pathways are present in its genome together with a complete citric acid cycle (CAC) (Parkhill *et al.*, 2000). The presence of fructose 1, 6- biphosphatase supports the role of Embden- Meyerhof pathway in gluconeogenesis (Velayudhan and Kelly, 2002). Figure 3.9 shows the major electron transfer pathways in *C. jejuni*, where notably organic acids, such as lactate, formate and succinate can act as electron donors.

Consistent with the view that *C. jejuni* has a complete citric acid cycle (CAC) the genome of *C. jejuni* NCTC 11168 encodes succinate dehydrogenase (*sdh* operon) that oxidizes succinate to fumarate. However, the organism also encodes a fumarate reductase (*frd* operon) to enable fumarate to act as an electron acceptor under anaerobic conditions. Each of these complexes consist of a membrane extrinsic component composed of a FAD binding flavoprotein, an iron-sulphur protein; and a hydrophobic component composed of a membrane anchor protein and/or a cytochrome B (Smith *et al.*, 1999b). *C.jejuni* HPC5 growing in the gas replacement jar showed 5.5 fold greater expression of Cj0480 (*frd*C) encoding the cytochrome B subunit of fumarate reductase over the MACS cabinet culture. The cytochrome b-556 subunit of fumarate reductase mediates electron transport from menaquinone (Smith *et al.*, 1999b). However in the same experiment the transcripts of Cj1476c encoding pyruvate flavodoxin oxidoreductase, Cj0835c (*acn*B) encoding aconitate hydratase,

Cj0535 and Cj0536 encoding 2-oxoglutarate: acceptor oxidoreductase (OOR) subunits A and D are all increased, and form functional components of the CAC that sink at succinate. It is possible that these components have alternative functions depending prevailing growth conditions. *C. jejuni* HPC5 grown in the gas replacement jar also showed greater expression of the beta subunit of glutamate synthase (NADPH) (Cj0009).

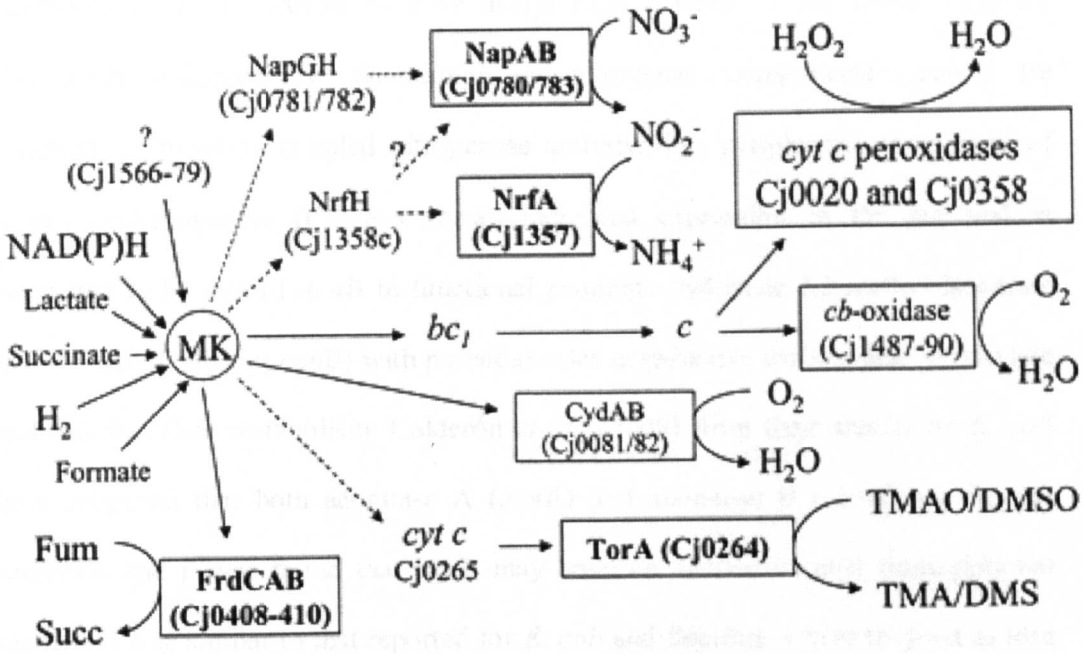
Figure 3.8. Central carbon metabolism and amino acid utilisation in *C. jejuni*



The cell is represented with outer membrane (OM) and inner membrane (IM) enclosing the periplasm. Key amino acid transport systems are represented as black rectangles which includes proline, serine, aspartate and glutamate. Black circles represent the transamination reaction that converts glutamate to aspartate. The dotted lines represent the conversion of glutathione to glutamate which occurs in the periplasm of some strains. Abbreviations include: SdaC- Serine transporter, PyK- Pyruvate kinase, PyC- Pyruvate carboxylase, Pck- PEP carboxy kinase, Por- Pyruvate: acceptor oxidoreductase, Acs- Acetyl CoA synthetase, Pta- Phosphotransacetylase, AckA- acetate kinase, GltA- citrate synthase, Acn-

Aconitase, Icd- Isocitrate dehydrogenase, OoR- 2 oxoglutarate acceptor oxidoreductase, Suc- Succinyl CoA synthetase, Sdh- Succinate dehydrogenase, Fum- Fumarase, Mqo- Malate:quinine oxidoreductase, Mdh- Malate dehydrogenase (NAD linked), AspA- Aspartase, Aat- Aspartate glutamate aminotransferase, GlnA- Glutamine synthase, GltBD- Glutamate synthase, PutA- Proline dehydrogenase, PutP- Proline transporter, GGT- γ glutamyl transpeptidase, Fld- flavodoxin, Fd- Ferrodoxin, OAA- Oxaloacetate, PEP- Phosphoenol pyruvate, AcP- Acetyl phosphate. The Peb1 system is an ABC transporter containing periplasmic aspartate/glutamate binding protein Peb1A (Kelly, 2008; Velayudhan and Kelly, 2002).

Figure 3.9. Predicted electron transport pathways in *C. jejuni*



Cj1566-79 encodes *nuo*/*ndh*I type enzyme. Oxygen linked respiration occurs *via* two membrane bound terminal oxidases, a cytochrome *c* oxidase and a quinol oxidase. FrdCAB represents fumarate reductase. Electron transport from quinol to nitrate is more likely to be *via* the napGH proteins than the *nap*C/*nrf*H homologue. Cj0264*c* represents *tor*A. It probably receives electrons from quinol *via* a small monoheme *c*-type cytochrome encoded in the same operon. Abbreviations include: - Fum- Fumarate; succ- Succinate; bc_1 - Cytochrome bc_1 complex, MK- Menaquinone, ?- unknown pathways. Dashed arrows indicate uncertainty regarding the exact electron transport pathway or the likely participation of additional redox proteins. Figure adapted from Sellars *et al.*, (2002) in growth of *C. jejuni* supported by respiration of fumarate, nitrate, nitrite, trimethylamine-*N*-oxide, or dimethyl sulfoxide requires oxygen.

The beta subunit is a flavin adenine dinucleotide-NADPH dependent oxidoreductase that provides electrons to the alpha subunit, which binds L-glutamine and 2-oxoglutarate to form L-glutamate. Glutamate enters the CAC by converting it to aspartate and results in the formation of pyruvate.

Pyruvate ferredoxin/flavodoxin oxidoreductases (PORs) are highly oxygen sensitive iron-sulfur proteins involved in the coenzyme A (CoA) dependent oxidative decarboxylation of pyruvate to form acetyl CoA (Pieulle *et al.*, 1999). Pyruvate flavodoxin oxidoreductase, flavodoxin, and nitrogenase components I and II are required for pyruvate coupled nitrogenase activity. The periplasmic component of quinol dehydrogenase (Cj0781) shows increased expression in the jar, and is suggested to be part of *Acn*B bi-functional aconitate hydratase 2,2-methyl isocitrate dehydratase (Cj0835c; *acn*B) with potential roles in reductive carboxylase, glyoxylate and dicarboxylate metabolism. Calderón *et al.*, (2009) from their studies on *E. coli* have proposed that both aconitase A (*acn*A) and aconitase B (*acn*B) are oxygen sensitive, and it was found that *acn*B may have an alternative post transcriptional regulatory role similar to that reported for *E. coli* and *Bacillus*, where they act as iron and oxidative stress responsive regulators, bind mRNA and alter transcript stability (Tang *et al.*, 2002). Oor is also oxygen sensitive enzyme that is responsible for the conversion of 2-oxoglutarate to succinyl- CoA whilst reducing ferrodoxin or flavodoxin.

In contrast an increase in the levels of transketolase (Cj1645; *tkt*) encoding transcript for *C. jejuni* HPC5 grown in MACS might suggest the operation of the pentose phosphate pathway and the production of sugars since the enzyme catalyses the formation of ribose 5-phosphate and xylulose 5-phosphate from sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate. Notably glyceraldehyde 3-phosphate

dehydrogenase (*gap*A) that converts glyceraldehyde-3-phosphate to 1, 3 disphophoglycerate, shows a relative increase in transcript level of 2.5 fold in the MACS over the gas replacement jar. This reaction is NAD dependent and is an important rate determining step in glycolysis.

Increased expression of genes coding for hydrogenases and dehydrogenases occurred when *C. jejuni* HPC5 was grown in jar compared to the MACS. Cj0624 (*hyp*C) encoding hydrogenase isoenzymes formation protein was increased 2.8 fold. The presence of hydrogen had a beneficial effect on the comparative growth of *C. jejuni* HPC5 in the gas jar with the culture reaching stationary phase two hours earlier than a parallel culture in its absence. NADH dehydrogenase subunits A, B and C (*nuo*A; Cj1579c, *nuo*B; Cj1578c and *nuo*C; Cj1577c) show increases of 2.5, 2.2 and 2 fold respectively. *Nuo*B acts as a point where the majority of electrons traverse the respiratory chain that eventually result in the reduction of oxygen. Subunits *nuo*A and *nuo*C help in the transfer of electrons from NADH to ubiquinone. Electron input to menaquinone is directed *via nuo*ABC, which is involved in the aerobic respiratory chain (Sellars *et al.*, 2002). Svensson *et al.*, (2008) has reported down regulation of the *nuo* operon in *spo*T mutants. *Spo*T codes for guanosine-3', 5'-bis (diphosphate) 3'-pyrophosphohydrolase that is important for the growth of *Campylobacter* at low CO_2 concentrations and for aerobic survival (Svensson *et al.*, 2009).

The large subunit of formate dehydrogenase (Cj1511c; *fdh*A) (selenocysteine containing unit) is a major component of nitrate respiration in bacteria and exhibits a 6 fold increase when *C. jejuni* was grown in the gas replacement jar over the MACS cabinet. *Fdh*N is a membrane protein that is a complex of three different subunits that acts as the major electron donor to the nitrate respiratory chain. The *fdh*A, B and C gene products are possibly involved in selenium incorporation, which affects the

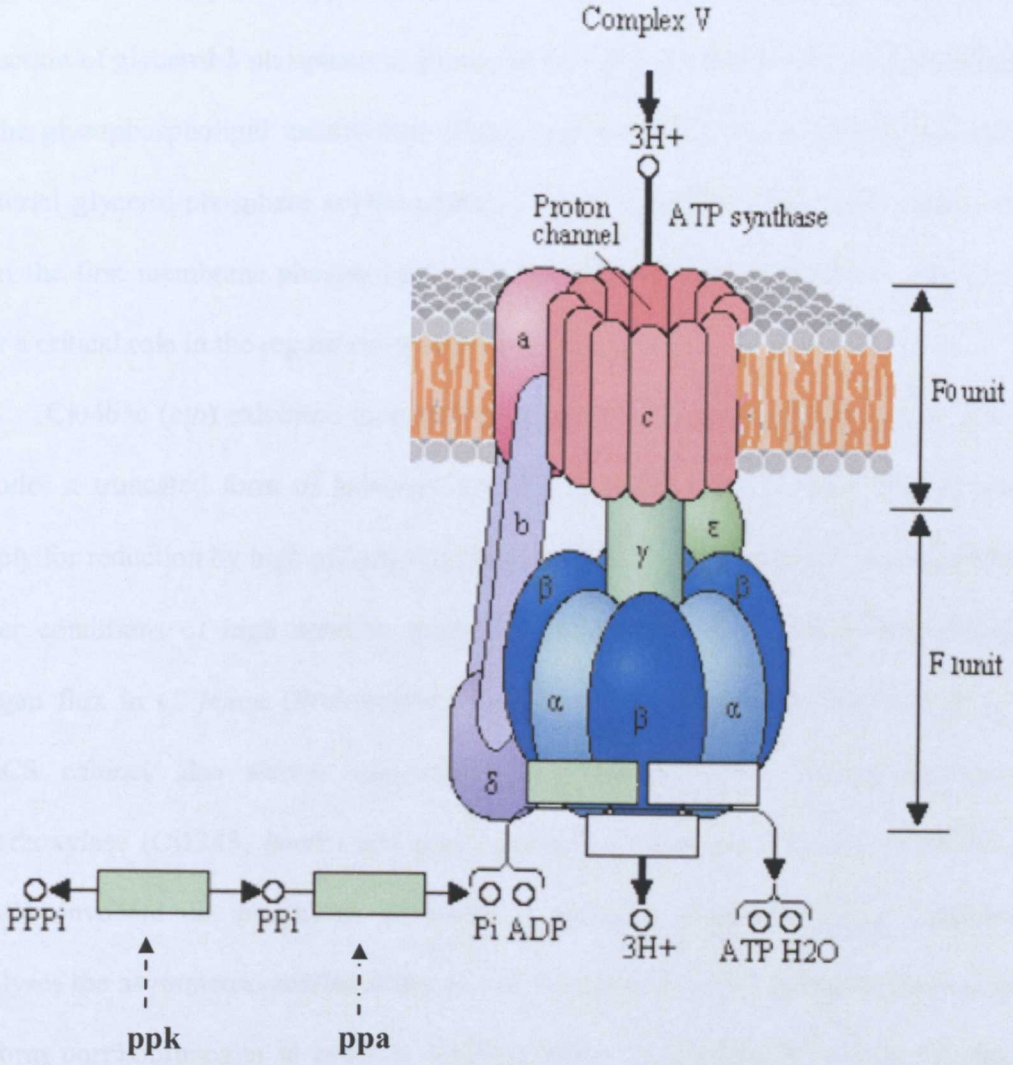
formate dehydrogenase pathway along with the involvement of *hyd*B (Wu and Berthelot, 1987). It is also involved in glyoxylate and dicarboxylate metabolism and methane metabolism. Schmitz and Diekert (2004) found high similarity between the *fdh* operons of *Sulphospirillum multivorans*, *C. jejuni* and *Wolinella succinogenes.* They have proposed that *fdh*A contains one Fe(4)/S(4) cluster and a selenocysteine residue, which is thought to be involved in the binding of a molybdopterin guanidine dinucleotide cofactor. The purified enzyme catalyses the oxidation of formate with oxidized methyl viologen as an electron acceptor.

Growth in the gas replacement jar also exhibited an increase in the expression in the *nap*ADG genes over the MACS. These genes encode components of the nitrate reductase system and iron sulphur proteins that help in the transmission of electrons from menaquinone upon nitrate reduction (Sellars *et al.*, 2002). The operon in *C. jejuni* does not contain a *nap*C gene as in other bacteria which encodes a membrane bound tetra heme cytochrome *C, a*lthough the *nap*C/*nir*T/*nrf*H homologue *Cj1358c* located directly upstream of *Cj1357c* encoding a pentaheme cytochrome C homologous to the periplasmic nitrite reductase (*nrf*A) of *E. coli* and other bacteria. Thus, *C. jejuni* appears to be equipped with both nitrate and nitrite reductases located in the periplasm, each predicted to receive electrons from menaquinol *via* distinct redox proteins. Cj0037c and Cj1020c that encode putative cytochrome c proteins with roles in electron transport also show increased transcription in the jar. It has been noted that *Campylobacter* spp. carry a great variety of c-type cytochromes (Elkurdi *et al.*, 1982, Lawson *et al.*, 1981), and in this context *pet*A also shows enhanced expression, the product of which is thought to assist in electron transport between cytochrome b to cytochrome c_1.

C. jejuni grown in the gas replacement jar showed an increase in the expression of *moa*A (Cj0161c) that code for molybdenum cofactor biosynthesis protein A. It is a member of the wider S-adenosyl methionine (SAM) dependent enzyme family and it catalyses the formation of protein and/or substrate radicals by reductive cleavage of SAM *via* a [4Fe-4S] cluster (Hu *et al.*, 2006). Molybdopterin when complexed with molybdenum forms molybdenum cofactor. Most of the enzymes that are involved in CAC in *C. jejuni* like the formate dehydrogenase (FDH), sulphite oxidase (sulphite: cytochrome c oxidoreductase), nitrate reductase and Trimethyl amine N- oxide (TMAO)/ Dimethyl sulphoxide (DMSO) reductases are all thought to be periplasmic molybdoenzymes (Smart *et al.*, 2009). Complex cofactor containing enzymes like the molybdoenzymes are assembled within the cell and exported as mature proteins. The export system is the twin arginine protein translocation (TAT) system that is widely present in the cytoplasmic membranes of bacteria and archaea, where direct use of proton motive force is used to transport folded protein substrates across the membranes. The TAT pathway precursor proteins have hydrophobic signal peptides similar to *Sec* pathway precursor proteins but are distinctive in possessing an essential twin arginine motif in their N domains. TAT pathway signal sequence domain containing protein (Cj1513c) is differentially increased 13 fold when *C. jejuni* HPC5 was grown in the gas replacement jar compared to the MACS cabinet. In contrast the molybdopterin biosynthesis protein (Cj0725c; *mogA*) is a novel superfamily of β-strand rich domains and was expressed in *C. jejuni* HPC5 grown in the MACS cabinet. This protein is involved in the final step of metal oxoanion ligation onto the dithiolene moiety of the pterin cofactor (Smart *et al.*, 2009).

Concomitant with the increase in the transcription of electron transport functions of *C. jejuni* HPC5 growing the jar atmosphere compared to the MACS, the transcript levels of the F_0 ATP synthase subunits *b'* (Cj1203c), *b* (Cj1204c) c (CJE1014) and ATP synthase F_1 sector delta subunit (Cj1204c) are also increased. ATP synthases represent one of natures' most unique enzyme classes that is responsible for energy transduction (Boyer 1997). The enzyme biochemically separates in to two units, a water soluble component F_1, containing five subunit types in the ratio $\alpha_3\ \beta_3\ \gamma\ \delta$ and ε, where as the other is a detergent soluble component, F_0, containing three subunit types (a, b, c) in bacteria (Ko *et al.*, 2000). The base unit is thought to translocate protons through membrane protein subunits conserved in the inner membrane in mitochondria, thylakoid membrane of plants and cytoplasmic membrane in bacteria. The *b* subunits are thought to interact with the stalk of the F_1 subunits. Fig. 3.10 represents ATP synthase and the components involved.

Figure 3.10. Representation of the F_0 and F_1 subunits of ATP synthase



ATP synthase included 2 components: F_0 which is detergent soluble (containing 3 subunits a, b, c) and F_1 which is water soluble (containing 5 subunits type in the ratio $\alpha_3\ \beta_3\ \gamma\ \delta$ and ε). Proton intake occurs through the proton channel and phosphorous intake occurs through the mediation of ppk: polyphosphate kinase and ppa: inorganic pyrophosphatase.

Transcription of membrane associated glycerol-3-phosphate dehydrogenase (Cj1196c; *gps*A) was almost 3 fold greater in the jar over the MACS. *Gps*A constitutes about 10% of the inner cytoplasmic membrane protein and is one of the key flavin linked primary dehydrogenases of the respiratory electron transport chain (Austin and Larson, 1991). *Gps*A catalyses the oxidation of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate (DHAP) with concurrent reduction of

flavin-adenine-dinucleotide (FAD) to $FADH_2$ and passes electrons on to ubiquinone (UQ) and ultimately to oxygen or nitrate. It helps in the NAD(P)H dependent reduction of glycerol 3 phosphate to glycerone phosphate, which is an important step in the glycophospholipid metabolism. Zhang and Rock, (2008) have proposed that bacterial glycerol-phosphate acyltransferases utilise completed fatty acid chains to form the first membrane phospholipid using a type II fatty acid synthase, and thus play a critical role in the regulation of membrane biogenesis.

Cj0465c (*ctb*) exhibited increased expression in the MACS cabinet. The gene encodes a truncated form of haemoglobin that is thought to moderate the oxygen supply for reduction by high affinity terminal oxidases. *Ctb* mutants have poor growth under conditions of high aeration implying the protein has a role in moderating oxygen flux in *C. jejuni* (Wainwright *et al.*, 2005). *C. jejuni* HPC5 grown in the MACS cabinet also shows relatively greater expression of uroporphyrinogen decarboxylase (Cj1243; *hem*E) and porphobilinogen synthases (PBGSs, CCO1057; *hem*B) involved in porphyrin biosynthetic process. Porphobilinogen synthase catalyses the asymmetric condensation of two molecules of delta-aminolevulinic acid to form porphobilinogen in order to produce heme. The active site of the enzyme consists of two zinc ions and a magnesium ion in most bacteria. Aminolevulinic acid and porphobilinogen are the biosynthetic precursors to all the tetrapyrrole pigments (e.g., porphyrin, chlorin, and corrin), which are essential to most life forms (Jaffe, 2004). These help in the acquisition of iron thereby reducing the free iron concentration in bacterial cells and controls the Haber-Weiss reaction:

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

These differences are accompanied by significant increases in the transcript levels of a putative iron uptake ABC transport system permease protein (Cj0174c) and haemin binding protein (*chu*D; Cj1617). Cj0174c and *chu*D are periplasmic binding proteins which assist in the acquisition of iron through modification and translocation of the cell membrane (Köster, 2001).

3.3.4. Genes involved in protein synthesis and stability

Several molecular chaperones including hsp90 family heat shock protein, *grp*E, *dna*K, ATP dependent *clp*B, *gro*EL and trigger factor (peptidyl prolyl cis/trans isomerase) showed increased expression when *C. jejuni* HPC5 was grown in a jar. These functions are often co regulated, for example, Anderson *et al.*, (2005) had shown that *Hsp*R is a negative regulator of several heat shock proteins including *dna*K, *clp*B and *groESL*. As a consequence mutants defective in *hsp*R show aerobic sensitivity, defects in adhesion and invasion, down regulation of flagella genes and motility defects as well (Svensson *et al.*, 2008). *C. jejuni* HPC5 grown in the MACS cabinet shows an increase in the relative expression of ATP- dependent Clp protease ATP- binding subunit (Cj1108; *clp*A) by 2.3 fold. Studies conducted on *E.* coli by Gottesman *et al.*, (1990) showed that *clp*A has an intrinsic ATP*ase* activity and that it associates with ClpP protease to degrade misfolded proteins generated at times of stress. Similarly the genes encoding aminopeptidase (*pep*A) and protease (*psp*A) show differential increases in expression in the MACS. PEB cell binding factor (*peb*A; Cj0596) is a major antigenic peptide that functions as a peptidyl prolyl cis-trans isomerase, and is located in the periplasm in association with the inner membrane is upregulated in MACS (Rathbun *et al.*, 2009). Mutant studies have shown that Cj0596 is involved in the folding of the proteins and changes in the

surface exposed proteins, although it negatively affects motility since Cj0596 mutants show increased motility (Rathbun *et al.*, 2009). Protein stability and increased turnover may be issue in the *C. jejuni* HPC5 cultures growing in the MACS as several genes involved in ribosomal protein synthesis and modification also showed significant increases in expression levels compared to the jar. These include the transcripts of *rplT*, *rplA*, *rplJ*, *rplQ*, *rplO*, *rplE* and *rplX* which function to modify the 50S ribosomal protein while *rpsB*, *rpsI*, *rpsE* and *rpsS* modify the 30S ribosomal protein.

3.3.5. Genes involved in amino acid biosynthesis

Growing in the MACS cabinet *C. jejuni* HPC5 showed an increased expression of genes required for the biosynthesis of aminoacids and polyamine biosynthesis over that found in growth in the gas replacement jar. Specifically the genes required for the biosynthesis of serine, histidine, tryptophan and polyamines show significant differential expression. L- Serine is the most favoured amino acid by *C. jejuni* for catabolism, where it uses an active serine dehydratase to convert serine to pyruvate and ammonia. Phosphoserine aminotransferase (Cj0326; *serC*) catalyses the formation of 3-phosphono oxypyruvate and glutamate from O-phospho-L-serine and 2-oxoglutarate. This enzyme is required for the phosphorylated pathways of serine biosynthesis and in the biosynthesis of pyridoxine (Basurko *et al.*, 1999). The pyruvate obtained will enter CAC and the Embden Meyerhof pathway for gluconeogenesis.

Studies have shown that *Campylobacter* synthesise tryptophan. *C. jejuni* HPC5 grown in MACS showed an upregulation of Cj0349 (*trpA*), Cj0348 (*trpB*) and Cj0346 (*trpD*) 5.6, 3.8 and 4.6 fold respectively. Tryptophan synthase is a bi-

functional tetrameric enzyme (2 alpha and 2 beta subunits) that catalyses the last two steps of L-tryptophan biosynthesis. The alpha and beta subunits catalyse two distinct reactions that are both strongly stimulated by the formation of the complex. The alpha subunit catalyses the cleavage of indole 3-glycerol phosphate (IGP) to indole and glyceraldehyde 3-phosphate (G3P). Indole is then channelled to the active site of the beta subunit that catalyses a replacement reaction to convert L-serine into L-tryptophan. Increased expression of anthranilate synthase component II (*trp*D; Cj0346) occurred 4.6 fold that catalyses the transfer of the phosphoribosyl group of 5-phosphoryl ribose-l-pyrophosphate to anthranilate, forming N-phospho ribosyl anthranilate in *E. coli* (Balderas-Hernández *et al.*, 2009). Anthranilate synthase phosphoribosyl transferase (*trp*E-*trp*D) is a multifunctional and heterotetrameric complex composed of two *trp*E and two *trp*D polypeptides (component I and II, respectively).

C. jejuni HPC5 grown in the jar showed a 2 fold increase in the differential expression of *aro*A (Cj0895c) over the MACS. The *aro*A encodes 3-phosphoshikimate 1-carboxyvinyltransferase that catalyses the formation of 5-O-(1-carboxyvinyl)-3-phosphoshikimate from phosphoenolpyruvate and 3-phosphoshikimate in tryptophan biosynthesis. Turner *et al.*, (2001) in his studies on *E. coli* proposed that the *aro* genes are required in the biosynthesis of the metabolic intermediate chorismate. Chorismate is required to synthesise folate which is a major methyldonor in many biosynthetic reactions and is also required for the synthesis of enterobactin, a protein involved in the *in vivo* acquisition of iron. Malcova *et al.*, (2009) had shown that *aro* mutants showed increased sensitivity to blood serum, albumen, EDTA, and ovotransferrin due to the defects in cell wall and outer membrane formation in *Salmonella* enteritidis, and similarly decreased resistance to

the components of the innate immune response even in the presence of aromatic amino acids.

A 3 fold increase in the transcripts for *his*A (Cj1601) and *his*I (Cj1604) occurred when *C. jejuni* HPC5 was grown in MACS. Genes *his*A and *his*I are part of the compact operon (*his*GDC[NB]HAF[IE]), in which three of them (*his*NB, *his*D and *his*IE) code for bi-functional enzymes. Histidine biosynthesis plays an important role in cellular metabolism being interconnected to both the *de novo* synthesis of purines and to nitrogen metabolism (Fani *et al.*, 2007).

A 2.4 fold increase in the transcript for 2-isopropylmalate syntase (Cj1719c; *leu*A) occurred when *C. jejuni* HPC5 was grown in the MACS cabinet. The product of this gene catalyses the reaction: acetyl-CoA + 3-methyl-2-oxobutanoate + H_2O to (2S)-2-isopropylmalate and CoA. This is required in the biosynthesis of leucine. The change was accompanied by an almost 3 fold increase in the transcript for the branched chain amino acid ABC transport system permease protein (*ilv*H; Cj1017c).

A significant increase in the transcript of *asd* (Cj1023c) encoding aspartate semialdehyde dehydrogenase occurred in *C. jejuni* HPC5 grown in the gas replacement jar. Similarly increase in *asp*S (Cj0640c) encoding aspartyl tRNA synthetase and *glt*X2 (Cj1288c) encoding glutamyl-tRNA synthetase were recorded by *C. jejuni* HPC5 grown in gas replacement jar.

3.3.6. Genes involved in fatty acid biosynthesis

The formation of para-aminobenzoate synthase glutamine amidotransferase component II (Cj0861c; *pab*A) was increased when *Campylobacter* was grown in jar. It catalyses the conversion of chorismate and glutamine to an unidentified intermediate, which is converted to para-aminobenzoate (PABA) (Nichols *et al.*,

1989). The formation of PABA is regulated by *pab*A and *pab*B and *fol* (Cj0065c) genes. *The fol* (Cj0065c) gene also showed increased expression when *C. jejuni* HPC5 was grown in a jar. PABA is essential for the biosynthesis of dihydrofolate, which in various forms participates in the synthesis of purines, pyrimidines, formyl methionyl-tRNA, some amino acids and vitamins. Dihydrofolate synthesis is essential in *E. coli* since it cannot transport or use exogeneous folates required for cell growth and division (Bognar *et al.*, 1985). Acetyl-CoA carboxylase, biotin carboxyl carrier (Cj1291c, *acc*B) subunit of acetyl coenzyme A (acetyl-CoA) carboxylase (ACC) which is the first enzyme of fatty acid biosynthesis (Cronan *et al.*, 2002) is increased 2 fold when *C. jejuni* HPC5 was grown in the gas replacement jar. The enzyme required for production of malonyl-CoA, the key precursor of fatty acid synthesis (Cronan *et al.*, 2002). However, the malonyl CoA-acyl carrier protein transacylase gene (*fab*D; Cj0116) also increases 2 fold for *C. jejuni* HPC5 in MACS cabinet. It is a key enzyme in the fatty acid biosynthesis pathway of bacteria. Oefner *et al.*, (2006) found that this enzyme functions in the transfer of a malonyl moiety from malonyl-CoA to holo acyl carrier protein (ACP), generating malonyl-ACP and free CoASH in *E. coli*. Malonyl-ACP, which is the product of this reaction, is the key building block for *de novo* fatty acid biosynthesis.

3.3.7. Genes involved in nucleotide biosynthesis

Genes *pur*Q (Cj0514) and *pur*H (Cj0953) are part of the *pur* operon that is required for purine metabolism, and shows increased expression in *C. jejuni* grown in the MACS cabinet. *The pur*Q product catalyses the ATP and glutamine dependent formation of formyl glycinamidine ribonucleotide, ADP, P (i) and glutamate in the fourth step of *de novo* purine biosynthesis (Hoskins *et al.*, 2004). *The pur*H product

(bi-functional phosphoribosylamino-imidazole carboxamide formyltransferase/IMP cyclohydrolase) operates in the synthesis of inosine monophosphate.

Changes occurred in the pyramidine biosynthesis of *C. jejuni* HPC5 when grown in the two different conditions. CTP syntase (Cj0027; *pur*G) which catalyses the ATP dependent amination of UTP showes a 2 fold increase in the gas replacement jar, whereas *C. jejuni* HPC5 grown in the MACS cabinet increases orotate phosphoribosyltransferase (Cj0233c; *pyrE*) that is functionally classified under 2`-deoxynucleotide biosynthesis. The latter will be expressed due to the coupled transcription and translation of the *pyr*BI and *pyr*E operons. Bacterial *pyr* gene regulation involves recognition of a regulatory sequence in the operon which causes premature transcription termination or translation inhibition in response to nucleotide signals (Turnbough *et al.*, 2008).

Deoxyuridine triphosphatase domain protein (Cj1451; *dut*) is another gene with increased expression in the MACS that is involved in nucleotide metabolism. The *dut* product is responsible for the production of dUMP, the immediate precursor of thymidine nucleotides. It decreases the intracellular concentration of dUTP so that uracil cannot be incorporated into DNA and thereby maintains the chromosomal integrity (Musso-Buendía *et al.*, 2009).

$$dUTP + H_2O = dUMP + diphosphate$$

3.3.8. Genes involved in nucleic acid synthesis and modification

C. jejuni HPC5 grown in the gas replacement jar showed 3 fold expression of DNA polymerase I, (*pol*A; Cj0338c) over that in the MACS cabinet, reflecting the strong commitment to cell divison and growth as a consequence metabolic competence. Growth in the jar also shows a differential increase in the transcript for

the chromosome partitioning protein (*par*B; Cj0101), which has a role in cell division. In contrast growth in the MACS exhibited an increase in *mre*B (Cj0276) transcript that encodes a homologue of *E. coli* rod shape determining protein. For *E. coli mre*B plays a crucial role by inserting subunits of muropeptides into the cell wall in a spatially correct way. This insertion occurs in the cells perimeter in order to maintain cell shape with a constant diameter (Varma and Young, 2009).

C. jejuni HPC5 grown in the MACS cabinet gave high expression of genes that resulted in the modification of nucleic acid. The expression of Cj0690c restriction modification enzyme was noted that contains a N-6 adenine specific DNA methylase protein signature. Bacterial DNAs are protected from restriction by methylating their DNA and *C. jejuni* HPC5 grown in MACS showed a 3 fold increase in the expression of DNA adenine methylase (CJE0220; *dam*) encoded in prophage Mu that is integrated in to the *C. jejuni* HPC5 genome. A putative DNA methylase is also produced by Cj0722c which is involved in the restriction modification system (Dale and Park, 2004). *Dam* has also been reported to positively or negatively regulate the expression of numerous genes, including those involved in virulence phenotypes, such as motility, adherence to and invasion of host cells, M-cell cytotoxicity and host colonization (Kim *et al.*, 2008). *Dam* affects gene expression by methylating GATC sites in promoter and operator regions, thus modifying interactions with the transcription apparatus and regulatory proteins (Kim *et al.*, 2008). Increased differential expression of exodeoxyribonuclease (Cj0255c; *xth*) occurred 3.3 fold that functions in base excision repair. It is the predominant DNA repair pathway which occurs when the cells undergo oxidative and alkylation damages. An increase in the transcript for extracellular deoxyribonuclease (CJE0256; *dns*) also occurred when *C. jejuni* HPC5 was grown in the MACS cabinet. Studies conducted by Lorenz *et al.*,

1991 have shown that the production of *dns* is down regulated when the cells are ready for transformation. These activities are consistent with the observation that growth in the MACS has to overcome oxidative stress, and is also notable that these conditions provoke an increase in the transcription of Mu prophage genes. *C. jejuni* HPC5 grown in the MACS cabinet showed increased expression of DNA gyrase subunit B (Cj0003) that negatively supercoils closed circular double stranded DNA. It is responsible for controlling the topological properties of DNA like the amount of supercoiling or catenation. An increase in transcription of the HIT family protein (Cj0419) occurred when it was grown in the gas replacement jar by 2.68 fold. These are a superfamily of nucleotide hydrolases and transferases, which act on the alpha phosphate of ribonucleotides. An increase in the expression of altronate hydrolase (C-terminus *uxa*; Cj0483 and N-terminus CJE0503) also occurred in the gas replacement jar by 7.4 fold which enables the hexuronate degradative pathway.

Growth of *C. jejuni* HPC5 in the gas replacement jar showed a 6 fold increase in the DNA recombinase *rec*A (CCO0982). RecA is a highly conserved bacterial protein that is responsible for homologous recombination (Roca and Cox, 1990). The *rec*A protein also has a regulatory role in the induction of the SOS response to extensive DNA damage and participates directly in SOS mutagenesis (Cox, 2003).

An increase in the differential expression of putative RNA methylases occurrs for *C. jejuni* HPC5 grown in MACS over the jar. The methylation of soluble RNA when the latter is in the polynucleotide form results in the alteration of the secondary and tertiary structure of the polynucleotides, and protects RNA from the attack of nucleases (Fleissner and Borek, 1962). Increases in the transcript levels of DNA directed RNA polymerase beta' chain and polyribonucleotide nucleotidyltransferase were observed for *C. jejuni* HPC5 grown in MACS.

3.3.9. Genes involved in Carbohydrate biosynthesis

The *hld*D gene (*hld*D/*waa*D; Cj1151c) encoding ADP-l -glycero-d - mannoheptose 6-epimerase that catalyses the interconversion of ADP-d -glycero-d - mannoheptose and ADP-l -glycero-d-mannoheptose showed increased expression in the jar which is the last step in the biosynthesis of the precursor of l-glycero-d - mannoheptose (Deacon *et al.*, 2000). Heptose is a highly conserved component of the LPS core among several genera of enteric and non enteric bacteria which forms one of inner portion of the cell membrane (Adams *et al.*, 1967). The *htr*B gene (Cj1134) involved in LOS biosynthesis and the ability to respond to stressful conditions also shows increased transcription in the jar (Phongsisay *et al.*, 2007).

There was an increase in several carbohydrate modifying enzymes when *C. jejuni* HPC5 was grown in the MACS cabinet. Modifications were produced by the genes which include *pgl*E, *gne*, Cj0685c, Cj1329, *gmh*A2, and *hdd*C. Among these *gmh*A2 (Cj1424) which codes for putative phosphoheptose isomerase which helps in the conversion of sedoheptulose 7 phosphate to d-*glycero*-d-*manno*-heptose 7-phosphate (Valvano *et al.*, 2002). Gram negative bacteria with a heptose LPS are highly sensitive to hydrophobic substances and they also display reduced virulence in animal infection models.

C. jejuni HPC5 grown in the MACS cabinet showed an increase in expression of a putative phospho sugar mutase (Cj1407c) and glucosamine fructose-6-phosphate aminotransferase (isomerising) (Cj1366), which are involved in fructose and mannose metabolism and amino sugar and nucleotide sugar metabolism respectively. Cj1407c is a bi-functional enzyme that functions in the reversible conversion of 1-phospho to 6-phospho sugars. Cj1366 is involved in hexosamine metabolism converting fructose 6 phosphate into glucosamine 6 phosphate using glutamine as a nitrogen source and is

the first and rate limiting enzyme of the hexosamine biosynthetic pathway. It is the only member of the amidotransferase subfamily of enzymes that does not display any ammonia dependent activity (Durand *et al.*, 2008). The final product of the hexosamine pathway, UDP-N-acetyl glucosamine is an active precursor of numerous macromolecules containing amino sugars (Milewski, 2002).

3.4. CONCLUSIONS

C. jejuni HPC5 cultures harvested at late logarithmic stages of growth from either an atmosphere created in a gas replacement jar (5.6% v/v oxygen, 3.6% v/v hydrogen, 7.3% v/v carbon dioxide and 83% v/v nitrogen) or that supplied in the MACS (5% v/v oxygen, 10% v/v carbon dioxide and 85% v/v nitrogen) show profound changes in their transcript levels. Overall the expression of genes that function in energy conservation were increased in the jar comparative to the MACS, and conversely genes that function to overcome oxidative stress exhibited greater expression levels in the MACS.

Studies have shown that in enteric bacteria energy conserving electron transfer pathways are usually depicted as a series of substrate specific dehydrogenases feeding electrons into a common quinone pool, from which they are transferred *via* specific quinol dehydrogenases to cytochrome oxidases during aerobic growth or terminal reductases during anaerobic growth (Brondijk, 2002). *C. jejuni* HPC5 grown in the gas replacement jar exhibited greater differential expression of genes encoding components of the citric acid cycle, electron transport chain and oxidative phophorylation. By comparison growth in the MACS cabinet occurs against a background of oxidative stress with a relative increase in the transcripts for superoxide dismutase and thiol peroxidase. The greater differential expression of extracellular deoxyribonuclease (*dns*) in *C. jejuni* HPC5 in MACS also resulted in reduced transformation effect in those strains. The observation that the initial fall in the viable count of *C. jejuni* HPC5 in the MACS can be overcome by the addition of antioxidants would suggest the organism is placed under oxidative stress and requires time to adapt to this condition, after which the viable count increases but at the expense of maintaining the proteins necessary for its defence.

This study is of paramount importance to scientists studying transcription in campylobacters because the method of atmospheric generation is shown here to significantly influence which genes are expressed and when.

CHAPTER FOUR

TRANSCRIPTOME STUDIES OF *C. JEJUNI* BACTERIOPHAGE RESISTANT MUTANTS

4.1. INTRODUCTION

4.1.1. Genomic rearrangement of *Campylobacter* in response to bacteriophage predation

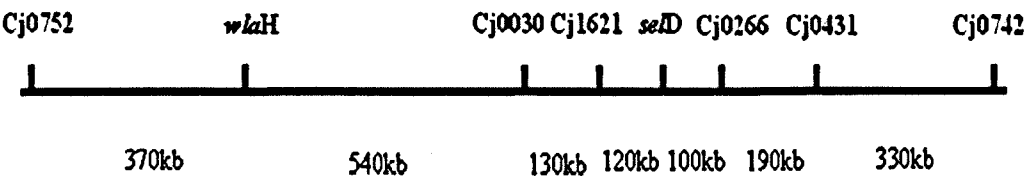
Bacteriophage treatment is a promising way of significantly reducing *Campylobacter* populations colonising chickens and thereby limiting their entry into the human food chain (Wagenaar *et al.*, 2005, Loc Carrillo *et al.*, 2005). Over 170 *Campylobacter* bacteriophages have been reported with the majority belonging to the *Myoviridae* family (Sails *et al.*, 1998). Resistance acquired by *Campylobacter* to phages following exposure is a potential problem for this treatment but the recovery rate of bacteriophage resistant *C. jejuni* post therapeutic treatment of chickens has been found to be relatively low at 4% (Loc Carrillo *et al.*, 2005). DNA recombination occurring *in vivo* is a potential way of overcoming the sub optimum biotic conditions by the organism and this leads to the development of a heterogeneous population. Bacteriophage predation can also influence the evolution and the *Campylobacter* genome (Connerton *et al.*, 2008) by influencing which subtypes survive and provoke recombination (Scott *et al.*, 2007).

4.1.2. Genomic Recombination in *C. jejuni* HPC5

Chickens colonised with *C. jejuni* HPC5 were treated with bacteriophage. The surviving campylobacters were tested for phage resistance to the therapeutic phage CP34. The molecular characteristics of the 4% of campylobacters determined to be phage resistant post phage therapy were investigated using *Sma*I digestion of the genomic DNAs that were then separated by pulsed field gel electrophoresis (PFGE) (Scott *et al.*, 2007). Fig. 4.1 represents the *Sma*I sites present in *C. jejuni* HPC5. The physical map of *C. jejuni* HPC5 showed similarity with that of the *C. jejuni* NCTC

11168 genome, however *C. jejuni* HPC5 exhibits a polarity change which involves inversion of Cj0030 and *selD*. *C. jejuni* HPC5 shares 8 *Sma*I sites with *C. jejuni* NCTC 11168, the exception being the ones present within the *tsf* gene and between Cj0056 and Cj0057 (Scott, 2006).

Figure 4.1. Linearised *Sma*I restriction map of *C. jejuni* HPC5



Representation of the *Sma*I restriction map of the whole genome of *C. jejuni* HPC5. The sites are located between Cj0742 and Cj0752, *wla*H, Cj0029 and Cj0030, Cj1621, *sel*D, Cj0266 and Cj0431. The fragment sizes are given below. Diagrams are not drawn to scale.

Phage resistant *C. jejuni* isolates R14 and R20 recovered from phage treated chickens shared five of seven *Sma*I fragments with *C. jejuni* HPC5 but also contained two new fragments of approximately 420 kb and 240 kb in R14 and 170 kb and 125 kb in R20 that were not present in *C. jejuni* HPC5. Physical maps of the genomes of these strains were constructed, which indicated that an intra genomic recombination of 590 kb had occurred from the parental strain. The combined size of the novel fragments were approximately equal to the size of the missing fragments in *C. jejuni* HPC5 indicating that there was no gain or loss of genetic material and recombination was the cause of the change in restriction pattern. Fig 4.2. A and B represents the rearrangements that occurred in *C. jejuni* HPC5 on exposure to bacteriophage CP34 and the novel fragment formed in *C. jejuni* HPC5 R14 and R20.

Figure 4.2. PFGE and Southern hybridisations of HPC5, R14 and R20

Fig. 4.2.(A)

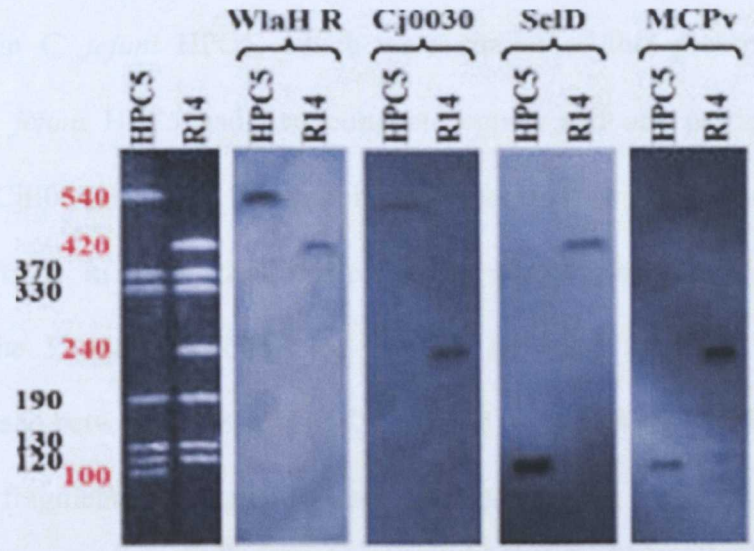
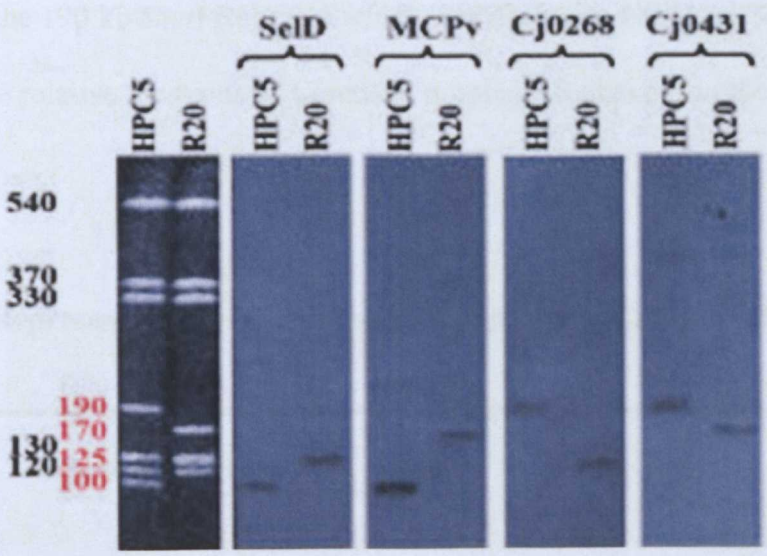


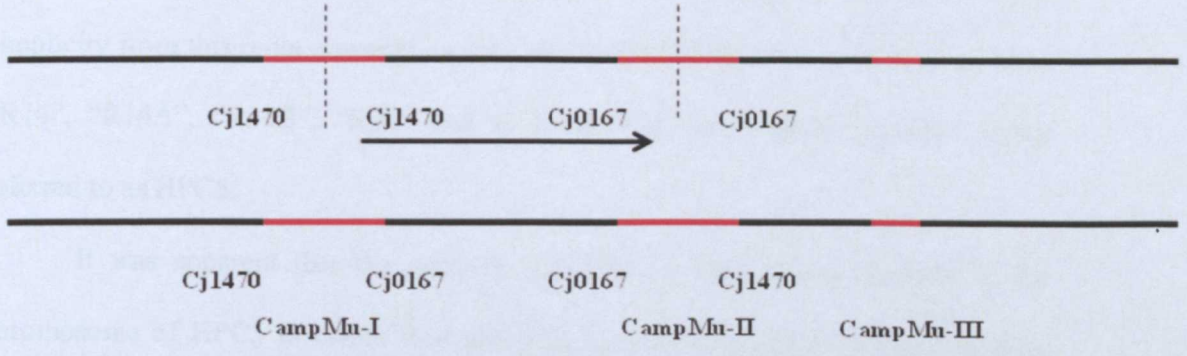
Fig. 4.2.(B)



(A) CP34 sensitive *C. jejuni* HPC5 and CP34 resistant *C. jejuni* HPC5 R14 and (B) CP34 sensitive *C. jejuni* HPC5 R20. The left panel indicates the *Sma*I fragments produced by *C. jejuni,* R14 and R20 while the right panels indicate the southern blots hybridised to the genes indicated above. The genes that retain their relevant positions are marked in black, and those involved in the genome rearrangements are marked in red. The figure is adapted from Scott *et al*., 2007, Genome Dynamics of *Campylobacter jejuni* in Response to Bacteriophage Predation.

Intra chromosomal recombination within *C. jejuni* HPC5 was confirmed by determining genomic sequences over the *Sma*I sites. Mu like prophage sequences were found in *C. jejuni* HPC5, which were similar to that present in RM1221. However, *C. jejuni* HPC5 had two complete copies and one partial copy (ORFs CjE0227 to CjE0241) of Mu like prophage sequences that were located at distinct genomic locations. In HPC5, the complete copies were between the 3′ end of Cj1470 (*ctsF*) and the 5′ end of Cj0167c in the 540 kb *Sma*I fragment (designated as CampMu-I), and between the 3′ end of Cj0167 and the 3′ end of Cj1470c (*ctsF*) in the 100 kb *Sma*I fragment (designated as CampMu-II), resulting in the disruption of both *ctsF* and Cj0167c. The partial copy was between an unknown gene and a paralog of CJE0225 in the 190 kb *Sma*I fragment which was designated as CampMu-III. Fig. 4.3 represents the relative locations of CampMu prophage sequences in the genome of *C. jejuni* HPC5.

Figure 4.3. Representation of the CampMu prophage in *C. jejuni* HPC5



The Mu sequence were inserted within Cj1470 and Cj0167. Recombination must have occurred within these regions resulting in the production of CampMu I which is flanked by 3′ end of Cj1470 (*cts*F) and the 5′ end of Cj0167c and CampMu II flanked by 3′ end of Cj0167 and the 3′ end of Cj1470c (*cts*F). CampMu III is a partial copy and it is present between unknown genes. The region shown in red represents the Mu sequence. The arrow represents the direction of recombination. The figure is not drawn to scale. Data adapted from Scott *et al.*, 2007, Genome Dynamics of *Campylobacter jejuni* in Response to Bacteriophage Predation.

The CampMu containing regions were found to be the substrates for recombination events resulting in the inversion of regions of *C. jejuni* HPC5 genome to create HPC5 R14 and HPC5 R20 (Scott *et al.*, 2007). The *C. jejuni* HPC5 R14 and R20 phage resistant phenotypes were found to be stable in the laboratory following multiple subcultures and to produce infectious CampMu phage (Scott *et al.*, 2007).

These isolates were used to colonise chickens to establish the colonisation potential of the recombinant strains compared to the original. It was found that the strains isolated from these birds were now sensitive to CP34 with colonisation potentials similar to that of the parent strain (Scott *et al.*, 2007). To establish whether this reversion was associated with further genetic changes a selection of the strains were examined by *Sma*I digestion of genomic DNA and PFGE. Comparison of the PFGE macro restriction pattern (MRPs) showed further recombination events had occurred resulting in five new unique MRPs termed as, HPC5 R14A, HPC5 R14B, HPC5 R20A, HPC5 R20B and HPC5 R20C. Of the R14 derived isolates examined 75% had the R14B MRP and for R20 derived isolates 80% had the R20A MRP. For simplicity from this point onwards each *C. jejuni* HPC5 MRP would be referred to as "R14", "R14A", "R14B", "R20" and so forth, with the original genotype being referred to as HPC5.

It was apparent that the genomic arrangement that caused changes in the chromosome of HPC5 to create R14 and R20 had also resulted in the reversion to bacteriophage sensitivity with further changes from the HPC5 parental strain. It was evident that the R14 and R20 strains were unstable *in vivo* and were rapidly out grown when in competition with their sensitive derivatives in the absence of bacteriophage CP34 (Scott *et al.*, 2007). In contrast 90% of the bacterial survivors after *in vitro* treatment of HPC5 with CP34, were resistant to CP34 and non motile, where as the

bacterial survivors after *in vivo* treatment arose at 4% and were motile in nature. This indicated that the bacteria could mutate to be non motile to confer phage resistance but these non motile phenotypes were unable to compete in the environment of the chicken intestine or subsequently colonise efficiently without reversion (Connerton *et al.*, 2008).

Recombination is a major source of genetic diversity in pathogens thus influencing their epidemiology. Consequently understanding recombination processes is necessary to understand the roots of biological diversity in pathogen populations, and from these strategies for their control and prevention may be devised. Access to the rearranged HPC5 strains gave us a unique opportunity to compare the transcription dynamics of these strains and establish the evolutionary consequences of such rearrangement on the fitness of these strains.

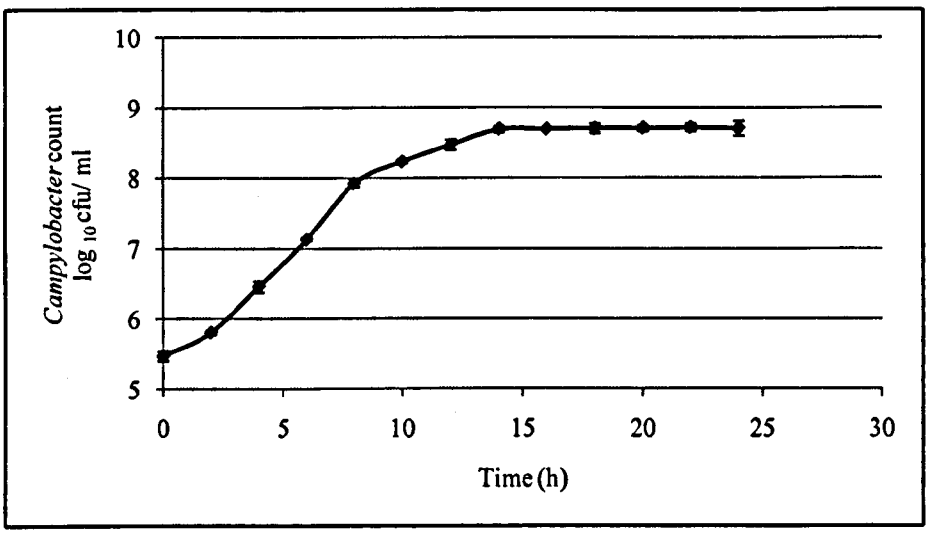
The objective of this study was to find the genes that showed changes in the regulation pattern due to the effect of recombination in HPC5 and their pseudo revertant strains and to investigate the mechanisms responsible for the generation of phage resistance. The materials and methods utilised in this chapter are described in Chapter 2.

4.2. RESULTS

4.2.1. Growth characteristics of HPC5 and its variants

The growth characteristics of HPC5 and the recombinant variants were determined by plotting growth curves; by calculation of growth rates during the exponential growth phase and by determination of the time taken to reach the maximum exponential growth phase. Figures 4.4, 4.5 and 4.6 represented the growth curves of HPC5, R14 series and R20 series and Table 4.1 gives the growth rate and doubling time of these strains.

Figure 4.4. Growth characteristics of HPC5



The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the growth time in h. The black line represents the growth of HPC5 in gas replacement jar.

The doubling time of HPC5 and its daughter strains were calculated using the formula

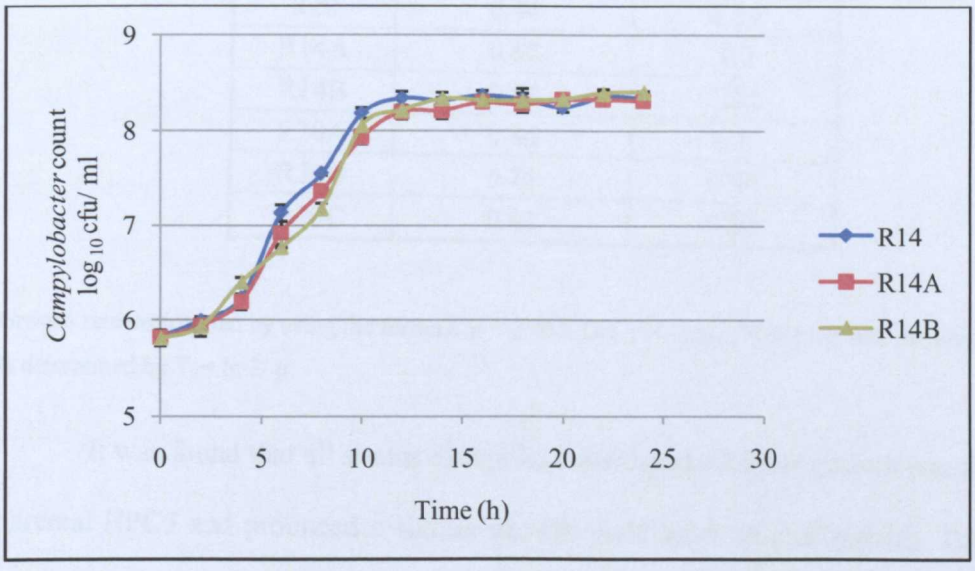
$$Td = \ln 2 / \mu$$

where μ is the growth rate constant which was determined using the formula,

$$\mu = 2.303((\log_{10} N - \log_{10} N_0)/ t - t_0$$

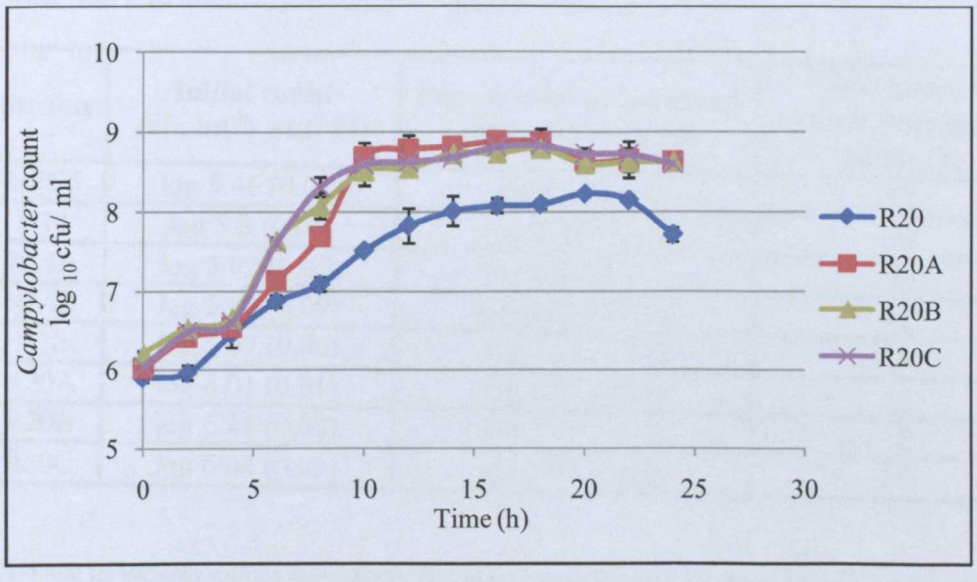
where N and N_0 were the growth at time intervals t and t_0.

Figure 4.5. Growth characteristics of R14 series



The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the growth time in h. Blue line represents the growth curve of R14, red represents the growth curve of R14A and green represents the growth curve of R14B.

Figure 4.6. Growth characteristics of R20 series



The y axis represents the *Campylobacter* count in log cfu ml^{-1} and x axis represents the growth time in h. Blue line represents the growth curve of R20, red represents the growth curve of R20A, green represents the growth curve of R20B and purple represent the growth curve of R20C.

Table 4.1. Growth rate and doubling time of HPC5 and its variants

Strains	Growth rate constant μ (h^{-1})	Doubling time Td (h)
HPC5	0.58	1.18
R14	0.6	1.14
R20	0.44	1.56
R14A	0.57	1.2
R14B	0.52	1.32
R20A	0.86	0.8
R20B	0.74	0.94
R20C	0.82	0.84

Growth rate determined by using the formula $\mu = 2.303((\log_{10} N - \log_{10} N_0)/ t - t_0$ and doubling time is determined by $T_d = \ln 2/ \mu$.

It was found that all strains except R20 displayed a higher growth rate than the parental HPC5 and produced a similar growth yield after 10 h of growth. Table 4.2 shows the initial and final counts and the time take for it to reach the stationary phase. HPC5 and all its variants reached 10^8 cfu ml^{-1} at 10 h, with the exception of R20 which took 14 h.

Table 4.2. Growth analysis of HPC5 and its variants

Strains	Initial count (cfu ml^{-1}) with SD	Exponential phase count (cfu ml^{-1}) with SD	Time taken to reach stationary phase (h)
HPC5	log 5.46 (0.01)	log 8.4 (0.07)	12
R14	log 5.8 (0.06)	log 8.33 (0.07)	12
R14A	log 5.02 (0.05)	log 8.19 (0.07)	12
R14B	log 5.80 (0.09)	log 8.21 (0.008)	12
R20	log 5.07 (0.06)	log 7.55 (0.21)	14
R20A	log 6.01 (0.01)	log 8.67 (0.17)	12
R20B	log 6.21 (0.02)	log 7.26 (0.02)	12
R20C	log 6.04 (0.03)	log 7.70 (0.05)	12

HPC5 and its variants with its initial count and exponential phase count along with the time required to reach stationary phase.

4.2.2. Motility of HPC5 and its variants

Motility tests (Fig. 4.7. A and B) were performed to determine if the genomic rearrangements of HPC5 resulted in changes in the motility that could result in decreased competitive fitness. All the derivatives of HPC5 were found to be motile. The R14 derivative was diminished in its motility compared to the other derivatives in the R14 series, whilst R20 showed diminished growth in the R20 series. Among the third generation variants R20B showed a reduction in the spread in the motility agar. Table 4.3 represented the average spread in cm^{-1} of HPC5 and its variants.

Table 4.3. Mean spread of HPC5 and its variants

Strains	Average growth in cm	SD
HPC5	2.77	0.05
R14	2.1	0.1
R14A	2.7	0.1
R14B	2.57	0.1
HPC5	2.77	0.05
R20	1.97	0.05
R20A	2.57	0.1
R20B	2.03	0.05
R20C	2.7	0.1

Mean spread in 'cm' after 24 h growth on motility agar with SD (number of replication =3)

On performing a 1 tailed, 2 sample unequal variance t test with $p< 0.05$ on the average spread of each strains it was found that HPC5 showed significant difference in its motility to generation 2 strains, R14 and R20 as well generation 3 strains R14B, R20A and R20B. No significant difference was observed in the motility of HPC5 R14A and R20C with the parental strain HPC5.

Figure 4.7. Motility characteristics of HPC5 variants

Fig. 4.7(A)

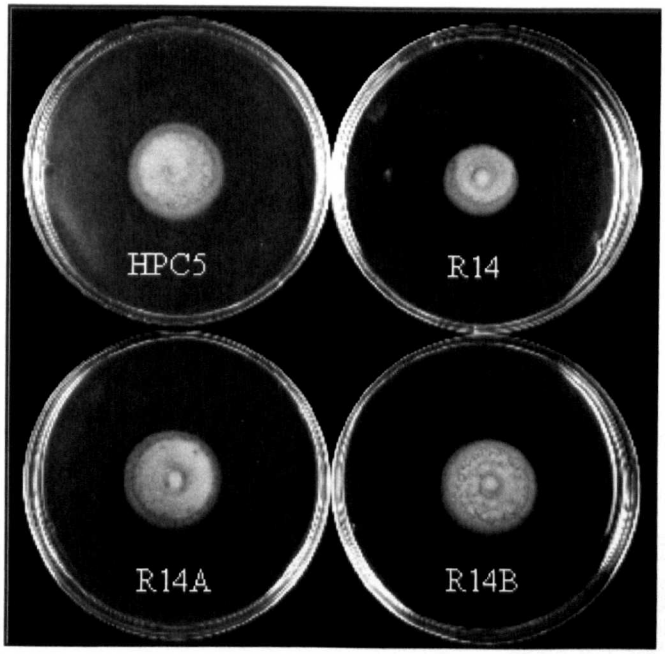
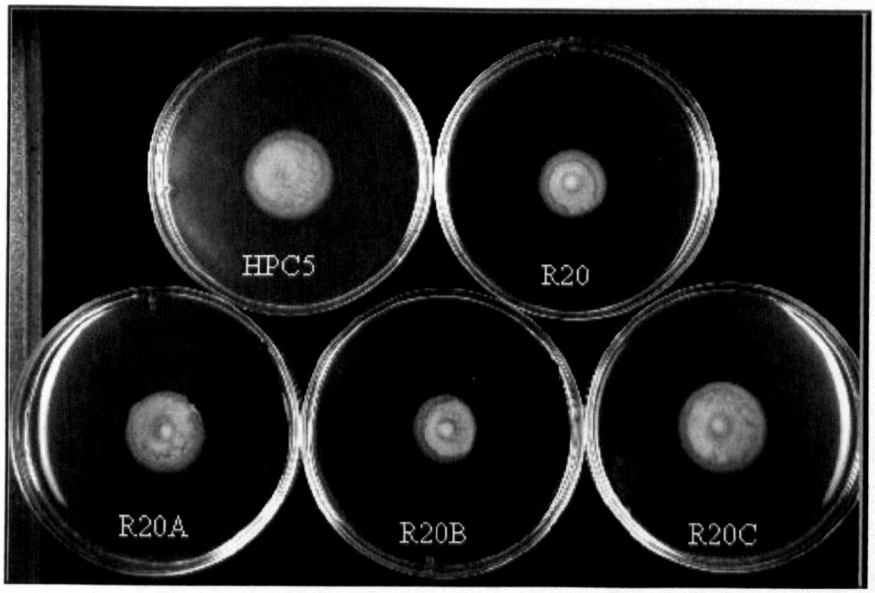


Fig. 4.7(B)



(A) R14 series and (B) R20 series. Images obtained from BioRad Gel Doc EQ. R14 and R20 has similar motility to that of HPC5 and it is coherent with the t test.

4.3.3. RNA extraction for Transcriptomics

The growth curves were used to determine the optimum point at which the cells could be harvested to extract RNA for transcriptomics experiments. RNA extraction was performed with HPC5 and its variants following growth on NB No 2 (2.1.8) for 8 h. However, R20 was grown for 12 h in order to for it to reach the same mid exponential growth phase. The quantity and quality of the extracted RNAs were estimated using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Fig. 2.1) and then by using an Agilent bioanalyser (Fig. 2.2).

4.2.4. cDNA extraction and labelling of HPC5 and its variants

The RNAs extracted from HPC5 and its variants were used to make cDNA. Control and the sample cDNAs were labelled with AF555 and AF645 (Fisher, UK) respectively. Fig. 2.3 represented the absorption norm of AF555 labelled HPC5 using a Nanodrop® ND- 1000 UV- Vis Spectrophotometer.

As signal level control for each experiment, hybridisations were performed with AF555 labelled HPC5 and AF645 labelled HPC5 to normalise the data. The resultant array should contain only yellow coloured spots representative of equal signals from each labelled probe.

4.2.5. Transcriptome analysis of HPC5 and its variants

Competitive hybridisations were performed using HPC5 and its variants. The relative abundance of each transcript was analysed by the levels of pixels developed in the microarray chip and their relative expression was recorded as fold change (FC) from the levels found in the normalised HPC5 sample. Hybridisation levels which

showed a two fold change with a $p<0.05$ were selected for further studies. The number of genes that were up regulated and down regulated in R14 and R20 using this criteria are given in table 4.4 whilst a schematic diagram showing the differences in genome structure caused by rearrangements is given in Fig. 4.8.A and B.

Table 4.4. Number of genes up regulated and down regulated due to the genomic rearrangements in R14 series and R20 series

Strains	Up regulated genes	Down regulated genes
R14	16	17
R14A	50	14
R14B	14	11
R20	7	4
R20A	19	10
R20B	21	24
R20C	4	3

Figure 4.8. Genomic rearrangement in HPC5 variants (Scott *et al.*, 2007)

Fig. 4.8(A)

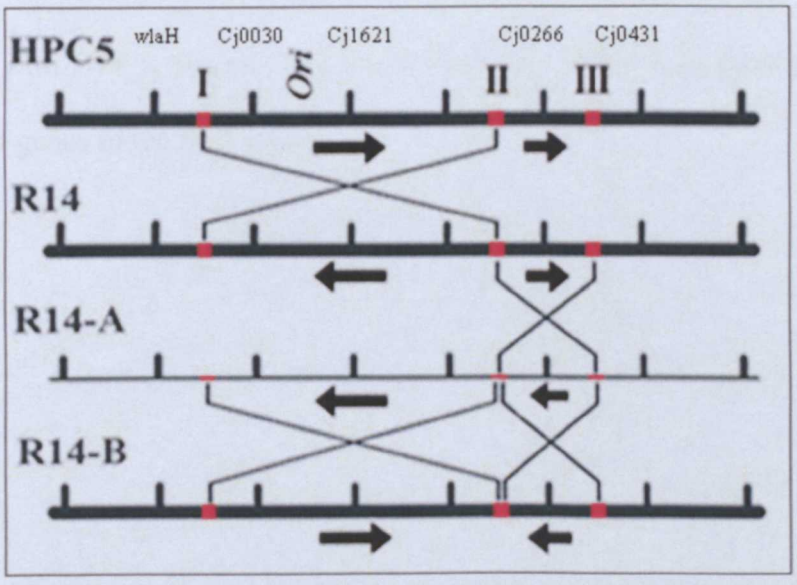
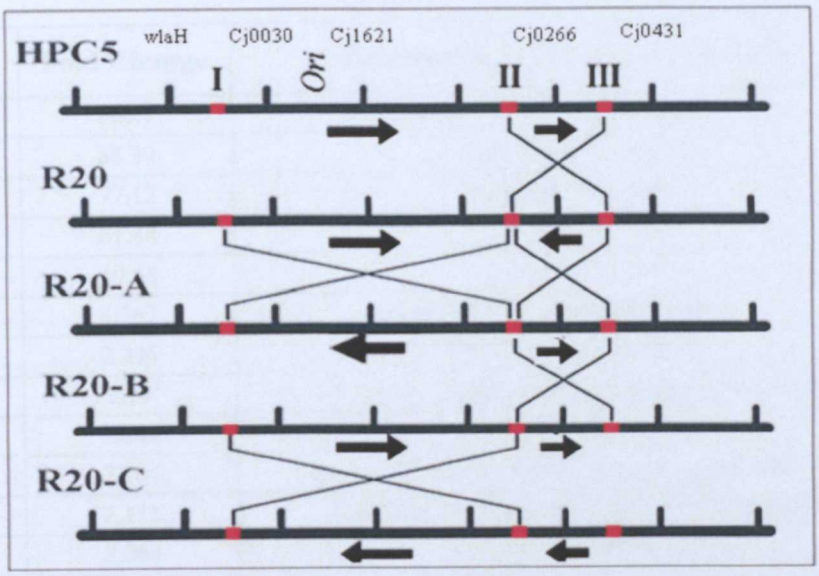


Fig. 4.8(B)



(A) R14 and it variants, (B) R20 and its variants. The HPC5 genome is represented by the black line and the three copies of Mu genes are represented by the red line. '*Ori*' denotes the origin of replication. The region where the intra genomic recombination had occurred is represented by the cross lines and the direction of recombination is shown by the arrows. The *Sma*I restriction sites are represented by an upward line.

4.2.5.1. Transcriptome analysis of R14 and its variants

4.3.5.1.1. Genes differentially regulated in R14 and its variants

Tables 4.5 to 4.7 listed the description of genes that were differentially regulated with their corresponding fold changes for R14 and its variants by comparison with HPC5. Figure. 4.9 gave a summary of the functional categories of the regulated genes in the R14 series

Table 4.5. Genes in R14 that are up regulated and down regulated compared to HPC5

Locus tag	Fold Change	Description of the encoded protein
Cj0705	140.7	conserved hypothetical protein
Cj0591c	88.39	lipoprotein, putative
Cj0788	77.12	hypothetical protein
*rlp*A	61.88	putative lipoprotein
CJE1141	40.48	hypothetical protein
*pdx*J	4.762	pyridoxine 5'-phosphate synthase
*rps*E	2.295	30S ribosomal protein S5
*pet*B	2.27	ubiquinol- cytochrome c reductase, cytochrome b
CJE1357	2.242	sensor histidine kinase
*pet*C	2.164	ubiquinol- cytochrome c reductase, cytochrome c1
CJE0201	2.115	D12 class N6 adenine-specific DNA methylase
*rpl*O	2.064	50S ribosomal protein L15
*rps*C	2.031	30S ribosomal protein S3
*rpl*R	2.018	50S ribosomal protein L18
*rpl*W	2.007	50S ribosomal protein L23
Cj0805	0.00348	putative zinc protease
CJE1461	0.0828	hypothetical protein
Cj0636	0.204	NOL1/NOP2/sun family protein
Cj1251	0.207	conserved hypothetical protein
Cj0948c	0.208	cation efflux family protein
Cj1450	0.215	putative ATP/GTP-binding protein
Cj1464	0.245	flgM hypothetical protein
Cj1168c	0.253	putative integral membrane protein (dedA homolog)
*fla*G	0.318	flagellar protein FlaG
Cj0550	0.32	hypothetical protein
Cj0613	0.376	putative periplasmic phosphate binding protein
Cj1315c	0.385	imidazole glycerol phosphate synthase subunit HisH
*clp*B	0.4	ATP-dependent Clp protease ATP-binding subunit
Cj1079	0.422	putative periplasmic protein
Cj0236	0.494	putative integral membrane protein

Table 4.6. Genes in R14A that are up regulated and down regulated compared to HPC5

Locus Tag	Fold Change	Description of the encoded proteins
*hrc*A	11.93	heat inducible transcription repressor
Cj1631c	8.278	conserved hypothetical protein
*ptm*B	7.08	acylneuraminate cytidylyltransferase
*grp*E	6.4	heat shock protein GrpE
Cj1466	6.19	flagellar hook associated protein FlgK
Cj1314c	5.619	imidazole glycerol phosphate synthase subunit HisF
Cj1315c	5.473	imidazole glycerol phosphate synthase subunit HisH
Cj1451	5.386	dUTPase
CJE0050	5.023	hypothetical protein
Cj1034c	4.946	adenylosuccinate lyase
Cj1465	4.931	hypothetical protein
Cj1316c	4.532	pseudaminic acid biosynthesis PseA protein
*uxa*A	4.49	putative altronate hydrolase N-terminus
Cj1337	4.295	PseE protein
*clp*B	4.289	ATP dependent Clp protease ATP binding subunit
*fli*D	4.25	flagellar capping protein
*ptm*A	4.236	flagellin modification protein A
Cj0550	4.072	hypothetical protein
Cj1229	4.031	putative curved-DNA binding protein
*dna*K	4.016	molecular chaperone DnaK
Cj1230	3.969	heat shock transcriptional regulator
Cj0887c	3.86	flagellar hook associated protein FlgL (FlaD)
*fli*S	3.84	flagellar protein FliS
CJE0555	3.8	hypothetical protein
Cj0045c	3.755	putative iron binding protein
Cj0480c	3.587	putative transcriptional regulator
Cj0454c	3.476	hypothetical protein
Cj0760	3.429	hypothetical protein
Cj0455c	3.241	hypothetical protein
Cj0457c	2.874	putative lipoprotein
Cj0977	2.81	hypothetical protein
Cj1026c	2.795	putative lipoprotein
*trx*B	2.76	thioredoxin reductase
Cj0874c	2.646	putative cytochrome C
*rpo*D	2.582	RNA polymerase sigma factor RpoD
*cds*A	2.489	phosphatidate cytidylyltransferase

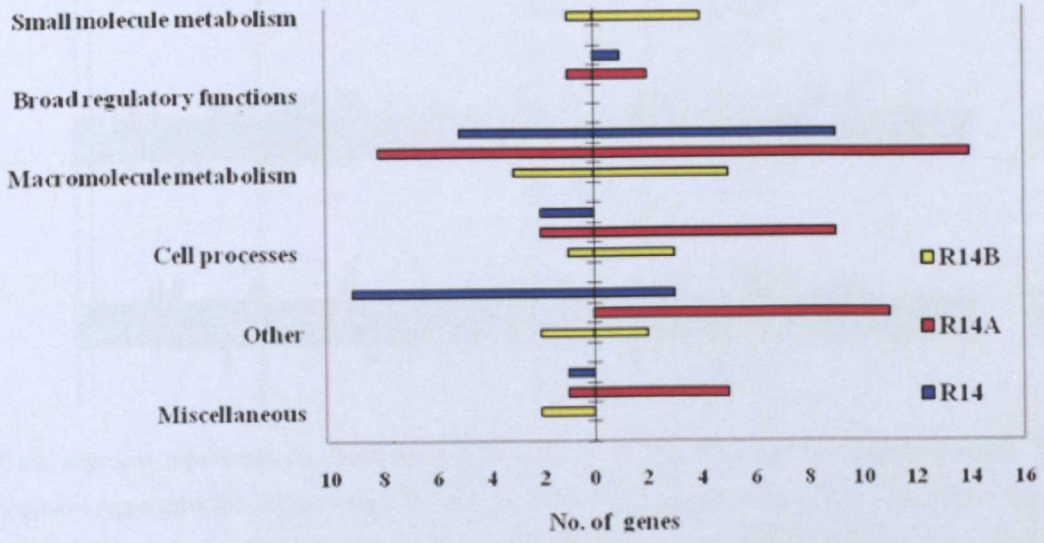
Table 4.6. (continued)

Locus Tag	Fold Change	Description of the encoded proteins
Cj1648	2.268	putative ABC transport system periplasmic substrate binding protein
Cj0418c	2.243	hypothetical protein
*flm*A	2.118	polysaccharide biosynthesis protein
*leu*B	2.093	3-isopropylmalate dehydrogenase
CJE0246	2.048	hypothetical protein
*hld*E	2.012	beta-D-heptose 1-phosphate adenylyltransferase
Cj1348c	2.012	putative coiled coil protein
*cas*1	2.011	CRISPR associated protein Cas1
Cj1183c	2.001	cyclopropane-fatty-acyl-phospholipid synthase
*rps*O	0.291	30S ribosomal protein S15
Cj0289c	0.351	major antigenic peptide PEB3
*his*A	0.387	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase
*put*P	0.428	putative sodium/proline symporter
*nap*G	0.438	quinol dehydrogenase periplasmic component
Tpx	0.441	thiol peroxidase
Cj0362	0.447	putative integral membrane protein
Cj0998c	0.449	putative periplasmic protein
Gne	0.451	DP-GlcNAc/Glc 4-epimerase
*fli*R	0.456	flagellar biosynthesis protein FliR
Era	0.47	GTP-binding protein Era
Cj0466	0.471	transcriptional regulator
Cj0872	0.486	putative protein disulphide isomerase
*pgl*B	0.498	oligosaccharide transferase to N-glycosylate proteins

Table 4.7. Genes in R14B that are up regulated and down regulated compared to HPC5

Locus tag	Fold Change	Description of the encoded proteins
*cts*W	151.8	transformation system protein
Cj0886c	133.6	putative cell division protein
Cj0974	114.8	hypothetical protein
Cj0337c	95.87	flagellar motor protein MotA
Cj1729c	86.57	flagellar hook protein FlgE
*fli*S	83.34	flagellar protein FliS
Cj0945c	73.59	putative helicase
*bio*F	60.35	8-amino-7-oxononanoate synthase
Cj1313	4.346	N-acetyltransferase specific for PseC product
Cj0465c	3.91	group III truncated haemoglobin
Cj0839c	3.682	hypothetical protein
*neu*B	2.546	N-acetylneuraminic acid synthetase
*leu*B	2.296	3-isopropylmalate dehydrogenase
Cj1532	0.209	putative periplasmic protein
Cj0190c	0.276	conserved hypothetical protein
Cj1457c	0.324	tRNA pseudouridine synthase D
CJE0265	0.358	host-nuclease inhibitor protein Gam, putative
Cj0248	0.395	hypothetical protein
pth	0.423	peptidyl-tRNA hydrolase

Figure 4.9. Summary of the functional categories of the regulated genes in the R14 series

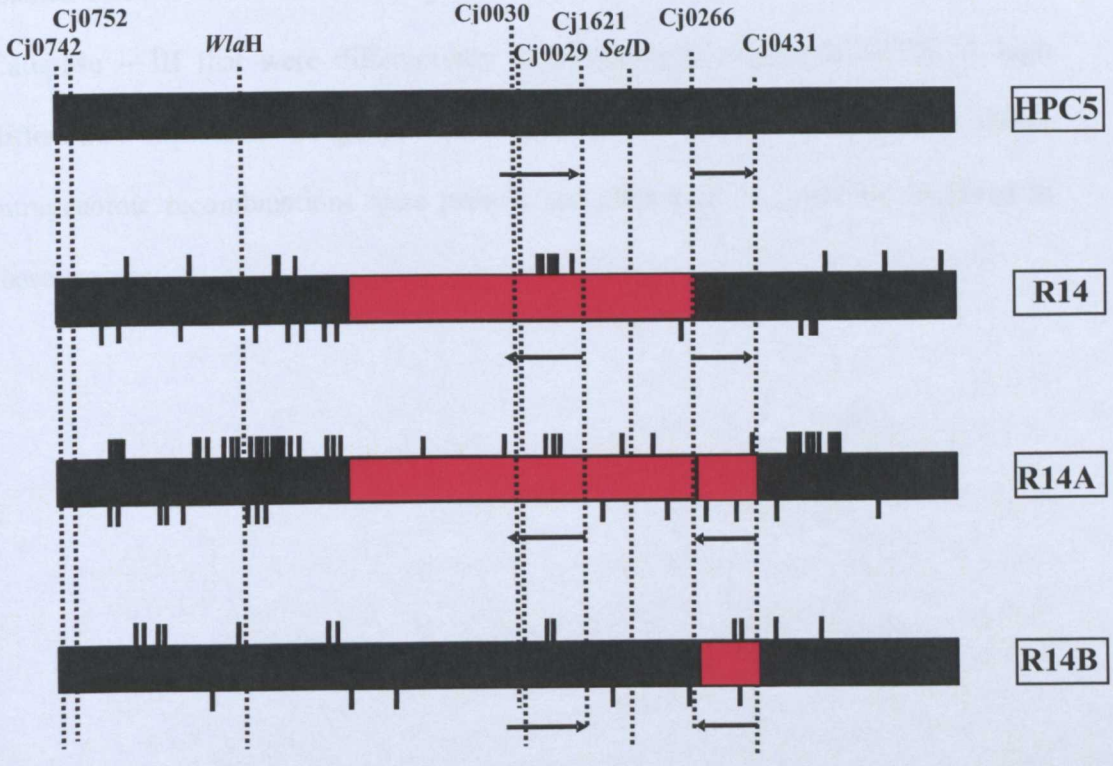


The axis towards left represent the number of down regulated genes while to the right represent the number of upregulated genes and the y axis represents the different functional classification of genes. The classification is based on the functional classification of *Campylobacter* genes given by Sanger Institute. Blue represents R14, Red represent R14A and Yellow represents R14B.

(http://www.sanger.ac.uk/Projects/C_jejuni/Cj_gene_list_hierarchical.html)

The genes which showed changes in their expression were not uniquely localised to the region where the intra genomic recombination had occurred. Fig. 4.10 shows the relative positions of the differentially regulated genes in the R14 series. The differentially expressed genes were located throughout the genome, however a few of the differentially expressed genes could be observed to be located in the regions containing Mu prophage genes that mark the boundaries of the recombination events.

Figure 4.10. Genomic representation of the positions of the differentially regulated genes in the R14 series



Each segment represents the *Sma*I restriction map of HPC5, R14 and its variants genome. The red segment represents the region where the rearrangement had occurred. The arrows represent the relative orientation of the invertible genome segments with reference to HPC5. The positions of the *Sma*I restriction sites were represented using dotted lines. Upregulated genes are represented by upward line segment and down regulated genes are represented by the downward line segment. Diagrams are not drawn to scale.

The up regulated and down regulated genes that were located in the regions implicated in the intragenomic recombinations to form R14, R14A and R14B are presented in Table 4.8. Table 4.9 shows the genes located between Cj1124 (*wla*H) and Cj1470 which include the genomic DNA sequence adjacent to the CjE0213 540 kb *Sma*I fragment of HPC5 which was identified as the 3′ end of Cj1470c.

Table 4.10 presents the genes located between Cj0259 (*pyr*C) and Cj0030 which include the genomic DNA sequence on the 3′ end of the CampMu-I sequence. Table 4.11 presented those genes located between Cj0260 which was supposed to be located close to the 3′ end of CampMu-II and Cj0431 which lies near the 3′ end of CampMu – III that were differentially expressed with respect to HPC5. A high differential expression of genes occurred at these regions on the strains where intragenomic recombinations were present and clustering of genes are localised in these regions.

Table 4.8. Genes located in the rearranged regions of the R14 series exhibiting differential regulation with respect to HPC5

R14	R14A	R14B
Genes up regulated		
CJE0201 (putative DNA modification methylase) Cj1678 (Lipoprotein) Cj1689c (50S ribosomal protein L15) Cj1690c (30S ribosomal protein S5) Cj1691c (50S ribosomal protein L18) Cj1701c (30S ribosomal protein S3) Cj1705c (50S ribosomal protein L23)		Cj0166 (tRNA delta(2)-isopentenylpyrophosphate transferase)
Genes down regulated		
Cj0236c (putative integral membrane protein)	Cj0289c (major antigenic peptide PEB3)	CJE0265 (host nuclease inhibitor protein Gam, putative) Cj0248 (hypothetical protein) Cj1532 (putative periplasmic protein) Cj0190c (conserved hypothetical protein) Cj0312 (peptidyl-tRNA hydrolase) Cj1457c (tRNA pseudouridine synthase D)

Table 4.9. Genes located between Cj1124 and Cj1470 which were differentially regulated with respect to HPC5

R14	R14A	R14B
Genes up regulated		
Cj1184c (putative ubiquinol-cytochrome C reductase cytochrome C subunit)	Cj1150c (D-beta-D-heptose 7-phosphate kinase)	Cj1313 (pseH)
Cj1185c (putative ubiquinol-cytochrome C reductase cytochrome B subunit)	Cj1183c (cyclopropane-fatty-acyl-phospholipid synthase)	Cj1317 (pseI)
Cj1222c (two-component sensor (histidine kinase)	Cj1229 (putative curved-DNA binding protein)	Cj1457 (tRNA pseudouridine synthase D)
Cj1238 (pdxJ pyridoxine 5'-phosphate synthase)	Cj1230 (heat shock transcriptional regulator)	
	Cj1293 (UDP-GlcNAc-specific C4,6 dehydratase)	
	Cj1314c (imidazole glycerol phosphate synthase subunit HisF)	
	Cj1315c (imidazole glycerol phosphate synthase subunit HisH)	
	Cj1316c (pseudaminic acid biosynthesis)	
	Cj1318 (motility accessory factor)	
	Cj1331 (acylneuraminate cytidylyltransferase)	
	Cj1332 (flagellin modification protein A)	
	Cj1337 (PseE protein)	
	Cj1347c (phosphatidate cytidylyltransferase)	
	Cj1348c (putative coiled coil protein)	
Genes down regulated		
Cj1168c (putative integral membrane protein)	Cj1126c (oligosaccharide transferase to N-glycosylate proteins)	
Cj1251 (putative integral membrane protein)	Cj1131c (Glc 4-epimerase)	
Cj1283 (putative K+ uptake protein)	Cj1179c (flagellar biosynthesis protein FliR)	
Cj1315c (imidazole glycerol phosphate synthase subunit)		
Cj1450 (putative ATP/GTP-binding protein)		
Cj1464 (hypothetical protein)		

Table 4.10. Genes located between Cj0259 and Cj0030 which were differentially regulated with respect to HPC5

R14	R14A	R14B
Genes up regulated		
Cj0208 (putative DNA modification methylase)	Cj0045c (putative iron binding protein) CJE0050 (hypothetical protein) Cj0146c (thioredoxin reductase) CJE0246 (hypothethical protein)	
Genes down regulated		
Cj0236c (putative integral membrane protein)		CJE0265 (host nuclease inhibitor protein Gam, putative) Cj0248 (hypothetical protein) Cj0190c (hypothetical protein)

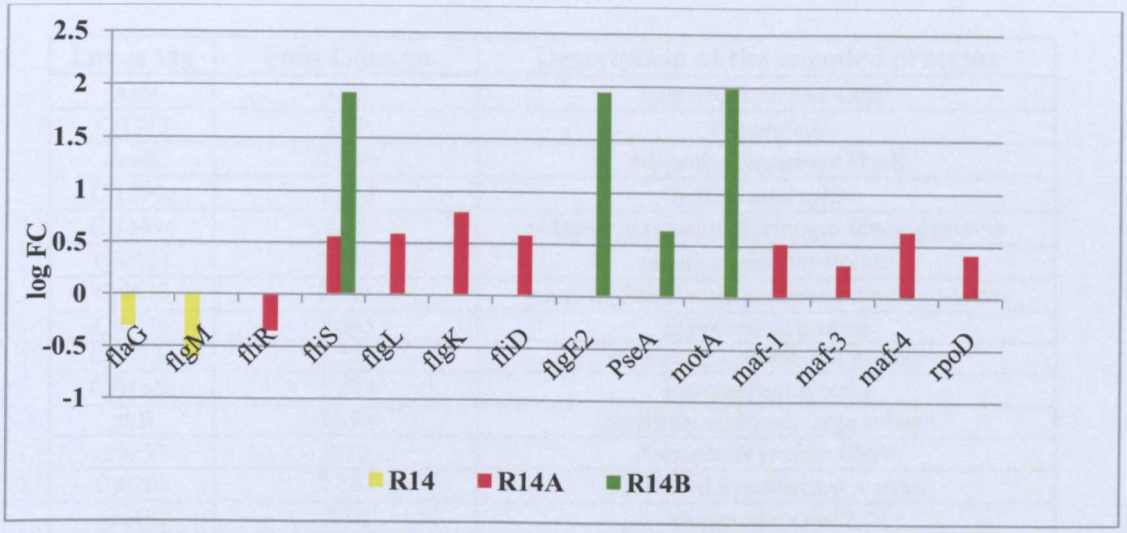
Table 4.11. Genes located between Cj0260 and Cj0431 which were differentially regulated with respect to HPC5

R14	R14A	R14B
Genes up regulated		
	Cj0418c (hypothetical protein)	Cj0306c (8-amino-7-oxononanoate synthase) Cj0337c (flagellar motor protein MotA)
Genes down regulated		
	Cj0289c (major antigenic peptide PEB3) Cj0362 (putative integral membrane protein)	Cj0312 (peptidyl-tRNA hydrolase)

4.2.5.1.2. Differential regulation of the flagellar genes in the R14 series

The interactions of the flagellar genes are shown in the Fig. 4.11 where the changes in the expression were represented as log FC. There was a down regulation of *fla*G in R14 and *fli*R in R14A, while *fli*S, *flg*K, *fli*D wereup regulated in R14A and *fli*S, *flg*E2, Cj1313 and *mot*A in R14B.

Figure 4.11. Differential expression of flagella associated genes of the R14 series



The y axis represents log fold change (log FC) and x axis represents the flagellar genes which were differentially expressed in R14 series. Yellow represents R14, red represents R14A and green represents R14B. Upregulated genes gave a log FC value above 0 and down regulated genes gave a value below 0.

4.2.5.2. Transcriptome analysis of the R20 series

4.3.5.2.1. Genes differentially regulated in R20 and its variants

Table 4.12 to Table 4.15 lists the description of genes that were differentially regulated along with their fold changes for R20, R20A, R20B and R20C in comparison with HPC5.

Table 4.12. Genes of R20 differentially regulated with respect to HPC5

Locus tag	Fold Change	Description of the encoded proteins
Cj0620	5.682	conserved hypothetical protein
Cj0501	5.601	Pseudogene
CJE0253	2.146	tail protein X, putative
CJE0593	2.047	hypothetical protein
Cj1530	2.044	dephospho-CoA kinase
Cj0135	2.006	conserved hypothetical protein
Cj1100	0.136	hypothetical protein Cj1100
CJE1461	0.13	hypothetical protein
Cjt2	0.362	tRNA-Asp

Table 4.13. Genes of R20A differentially regulated with respect to HPC5

Locus tag	Fold Change	Description of the encoded proteins
*grp*E	5.03	heat shock protein GrpE
Cj1337	3.79	Pse protein
*dna*K	2.768	molecular chaperone DnaK
Cj1290c	2.638	biotin carboxylase
Cj1349c	2.52	putative fibronectin/fibrinogen binding protein
Cj0487	2.426	putative amidohydrolase
Cj0613	2.391	putative periplasmic phosphate binding protein
Cj0036	2.295	hypothetical protein
Cj0073c	2.238	conserved hypothetical protein
Cj0456c	2.204	hypothetical protein
*glt*B	2.193	glutamate synthase, large subunit
*che*V	2.19	chemotaxis protein CheV
Cj0705	2.185	conserved hypothetical protein
*gro*EL	2.16	chaperonin GroEL
*glt*X	2.154	glutamyl-tRNA synthetase
Cj1060c	2.142	hypothetical protein
Cj0075c	2.009	putative oxidoreductase iron-sulfur subunit
*pol*A	2.006	DNA polymerase I
Cj1514c	2.002	hypothetical protein
Cj0184c	0.0195	putative serine/threonine protein phosphatise
*rpl*E	0.304	50S ribosomal protein L5
*cco*Q	0.382	cb-type cytochrome C oxidase subunit IV
*his*A	0.419	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerise
Cj0236c	0.451	putative integral membrane protein
gne	0.454	UDP-glucose 4-epimerase
*lep*B	0.459	signal peptidase I
*his*I	0.459	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase protein
Cj0998c	0.475	putative periplasmic protein

Table 4.14. Genes in R20B differentially regulated with respect to HPC5

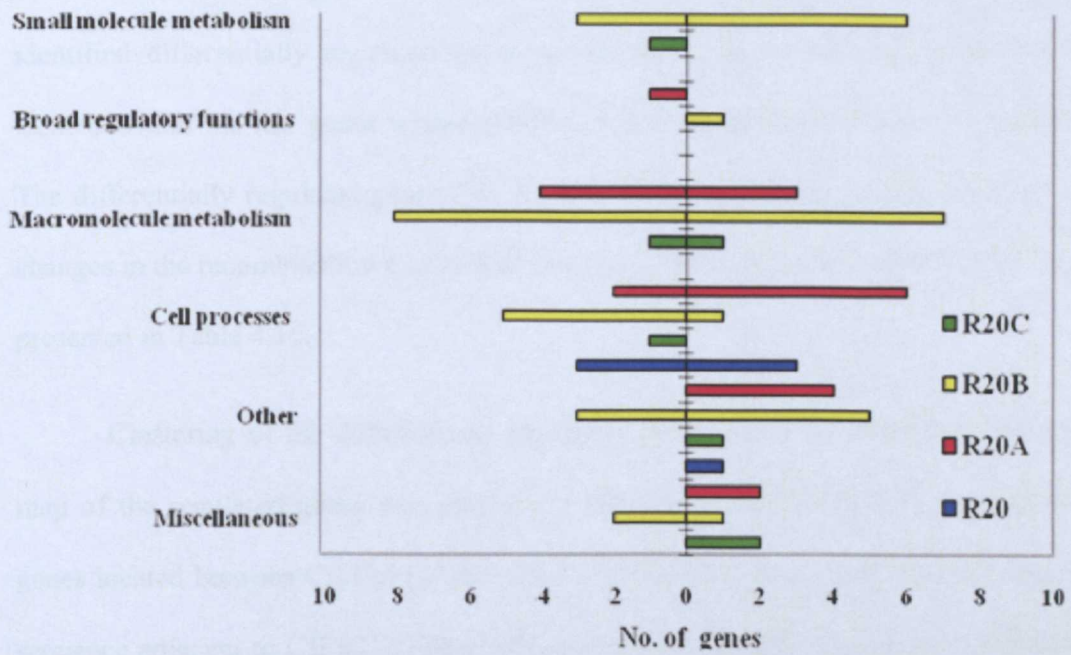
Locus Tag	Fold Change	Description of the encoded proteins
*nus*B	180.1	transcription antitermination protein NusB
Cj0777	142.8	putative ATP dependent DNA helicase
*fol*E	131.9	GTP cyclohydrolase I
Cj0886c	130.8	putative cell division protein
*nrf*A	130.5	putative periplasmic cytochrome C
*gly*A	127.5	serine hydroxymethyltransferase
Cj1496c	111.8	putative periplasmic protein
Cj0926	108.4	hypothetical protein
CJE1145	99.18	hypothetical protein
*hyp*F	95.52	Carbamoyltransferase
Cj0945c	91.03	putative helicase
Cj1361c	69.39	hypothetical protein
*bio*F	66.67	8-amino-7-oxononanoate synthase
*neu*B	3.148	N-acetylneuraminic acid synthetase
Cj1242	2.516	hypothetical protein
*leu*B	2.391	3-isopropylmalate dehydrogenase
Cj1189c/CJE1323	2.092	bipartite energy taxis response protein cetB
Cj0111	0.108	putative periplasmic protein
CJE0265	0.269	host nuclease inhibitor protein Gam, putative
*mre*B	0.486	rod shape determining protein MreB
Cj0292	0.216	Pseudogene (putative glycerol-3-phosphate transporter)
*acc*A	0.296	acetyl-CoA carboxylase carboxyltransferase subunit alpha
*cys*E	0.446	serine acetyltransferase
Cj0789	0.383	putative multifunctional Cca protein
*jlp*A	0.488	surface exposed lipoprotein
ssb	0.372	single stranded DNA binding protein
Cj1275c	0.231	peptidase, M23/M37 family
CJE1551	0.421	hypothetical protein
Cj1457c	0.376	tRNA pseudouridine synthase D
Cj1587c	0.172	multidrug transporter membrane component/ATP binding component
Cj1622	0.212	riboflavin specific deaminase/reductase
Cj0444	0.3	Pseudogene
Cj0190c	0.29	conserved hypothetical protein
Cj0561c	0.34	putative periplasmic protein
Cj1241	0.43	putative MFS (Major Facilitator Superfamily) transporter protein
Cj1381	0.46	lipoprotein, putative

Table 4.15. Genes in R20C differentially regulated with respect to HPC5

Locus Tag	Fold Change	Description of the encoded proteins
Cj0529c	2.607	putative aminodeoxychorismate lyase family protein
*mia*A	2.431	tRNA delta(2)-isopentenylpyrophosphate transferase
*thi*D	0.01	phosphomethylpyrimidine kinase
Cj1659	0.453	periplasmic protein p19
Cj1584c	0.489	putative peptide ABC transport system periplasmic peptide binding protein

A summary of the differentially regulated genes based upon their functional classification are presented in Fig. 4.12.

Figure 4.12. Functional categories of the regulated genes in the R20 series



The axis towards left represent the number of down regulated genes while to the right represent the number of upregulated genesand y axis represent the functional classification of genes. The classification is based on the functional classification of *Campylobacter* genes given by Sanger Institute

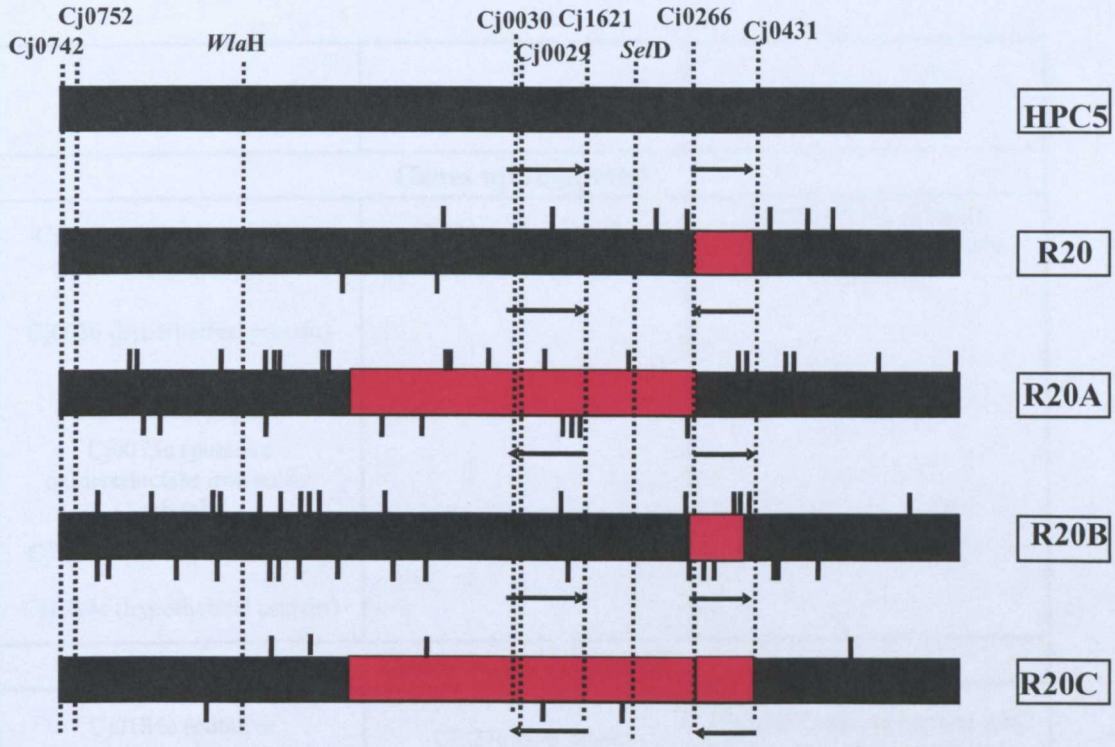
(http://www.sanger.ac.uk/Projects/C_jejuni/Cj_gene_list_hierarchical.html)

The functional classification of the R20 differentially regulated genes placed them largely in cell processes affecting the cell envelope and DNA metabolism. In the generation 3 variants R20A showed changes in amino acid biosynthesis, modifications of cellular envelope, cellular processes, fatty acid metabolism, protein synthesis and several conserved genes of unknown function. The differentially expressed R20B genes were associated with the biosynthesis of amino acids, biosynthesis of cofactors, modification of the cell envelope, cellular process, fatty acid metabolism, DNA metabolism, protein synthesis and some of unknown function in comparison with HPC5. In comparison R20C exhibited relatively few genes with expression levels significantly different to HPC5.

The relative genomic locations of the up and down regulated genes for the R20 series are given in Fig. 4.13. In common with the R14 series, the R20 series also identified differentially regulated genes across the genome, although a few were located within the Mu genes whose orientation had changed with respect to HPC5. The differentially regulated genes that are located in the regions subject orientation changes in the recombination events that gave rise to R20, R20A, R20B and R20C are presented in Table 4.16.

Clustering of the differentially expressed genes was seen when the physical map of the regulated genes was plotted for the R20 series. Table 4.17 showed the genes located between Cj1124 (*wla*H) and Cj1470 which included the genomic DNA sequence adjacent to CJE0213 540 kb *Sma*I fragment of HPC5 which was identified as the 3′ end of Cj1470c.

Figure 4.13. Genomic representation of the differentially regulated genes in R20 series



Each segment represents the *Sma*I restriction map of HPC5, R20 and its variants genome. The red segment represents the region where the rearrangement had occurred. The arrows represent the relative orientation of the invertible genome segments with reference to HPC5. The positions of the *Sma*I restriction sites were represented using dotted lines. Upregulated genes are represented by upward line segment and down regulated genes are represented by the downward line segment. Diagrams are not drawn to scale.

Table 4.18 presented the genes located between Cj0259 (*pyr*C) and Cj0030 which include the genomic DNA sequence on the 3′ end of the CampMu-I sequence. Table 4.19 gave genes located between Cj0260 which were supposed to be located close to the 3′ end of CampMu-II and Cj0431, which lies near the 3′ end of CampMu-III that were differentially expressed with respect to HPC5. A clustering and localisation of genes which were differently expressed were seen in R20 series as similar to R14 series.

Table 4.16. Genes located in the rearranged region in the R20 series which were differentially regulated with respect to HPC5

R20A	R20B	R20C
Genes up regulated		
Cj0007 (glutamate synthase (NADPH) large subunit) Cj0036 (hypothetical protein) Cj0073c (conserved hypothetical protein) Cj0075c (putative oxidoreductase iron sulfur subunit) Cj0338c (DNA polymerase I) Cj1514c (hypothetical protein)	Cj0306c (8-amino-7-oxononanoate synthase)	Cj0166 (tRNA delta(2)-isopentenylpyrophosphate transferase)
Genes down regulated		
Cj0184c (putative serine/threonine protein phosphatase) Cj0236c (putative integral membrane protein) Cj1488c (b-type cytochrome C oxidase subunit IV) Cj1601 (*hisA*) Cj1604 (*hisI*) Cj1695c (50S ribosomal protein L5)	Cj0276 (rod shape determining protein MreB) Cj0292c (putative glycerol 3-phosphate transporter)	Cj1584c (putative peptide ABC transport system periplasmic peptide binding protein) Cj1659 (periplasmic protein p19)

Table 4.17. Genes located between Cj1124 and Cj1470 in the R20 series which were differentially regulated with respect to HPC5

R20	R20A	R20B	R20C
Genes up regulated			
CJE1461 (hypothethical protein)	Cj1221 (chaperonin GroEL)	Cj1189c (bipartate energy taxis response protein)	Cj1255 (putative isomerase)
	Cj1288c (glutamyl-tRNA synthetase)	Cj1242 (hypothetical protein)	Cj1309c (hypothetical protein)
	Cj1290c (biotin carboxylase)	Cj1317 (Pse synthetase)	
	Cj1337 (PseE protein)	Cj1357c (putative periplasmic cytochrome C)	
	Cj1349c(putative fibronectin)	Cj1361c (hypothetical protein)	
Genes down regulated			
	Cj1131c (UDP-GlcNAc/Glc 4-epimerase)	Cj1457c (tRNA pseudouridine synthase D)	
		Cj1241 (putative MFS transporter protein)	
		Cj1275c (putative peptidase M23 family protein)	
		CJE1551 (hypothetical protein)	
		Cj1381 (putative lipoprotein)	

Table 4.18. Genes located between Cj0259 and Cj0030 in the R20 series which were differentially regulated with respect to HPC5

R20	R20A	R20B	R20C
Genes up regulated			
Cj0135 (hypothetical protein) CJE0253 (tail protein X, putative)	Cj0036 (hypothetical protein) Cj0073c (hypothetical protein) Cj0075c (putative oxidoreductase iron sulfur subunit)	Cj0194 (GTP cyclohydrolase I)	Cj0166 (tRNA delta(2)-isopentenylpyrophosphate transferase)
Genes down regulated			
	Cj0184c (putative serine/threonine protein phosphatase) Cj0236c (putative integral membrane protein)	Cj0111 (putative periplasmic protein) Cj0190c (conserved hypothetical protein) CJE0265 (host nuclease inhibitor protein Gam, putative)	

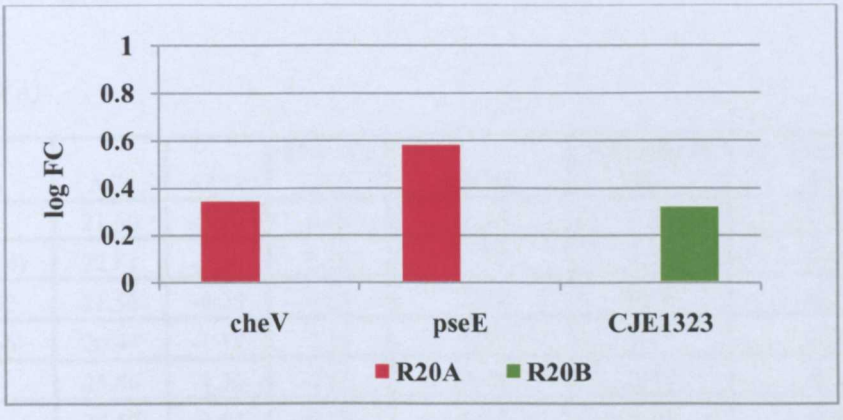
Table 4.19. Genes located between Cj0260 and Cj0431 in the R20 series which were differentially regulated with respect to HPC5

R20	R20A	R20B	R20C
Genes up regulated			
	Cj0285c (chemotaxis protein) Cj0338c (DNA polymerase I)	Cj0382c (transcription antitermination protein NusB) Cj0402 (serine hydroxymethyltransferase) Cj0306c (8-amino-7-oxononanoate synthase)	
Genes down regulated			
		Cj0276 (rod shape determining protein MreB) Cj0292c (putative glycerol-3-phosphate transporter) Cj0429c (conserved hypothetical protein)	

4.2.5.2.2. Differential regulation of the flagella associated genes in R20 series

The interactions of the flagellar genes in R20 series where the changes in the expression were represented as log FC. There was an up regulation of *che*V and *pse*E in R20A and CJE1323 in R20B. Fig. 4.14 represented the expression of flagellar genes in R20 series.

Figure 4.14. Differential expression of flagella associated genes in HPC5 R20 series



The y axis represents log fold change and x axis represents the flagellar genes which were differentially expressed in R20 series. Red represents R20A and green represents R20B. No flagellar genes were differentially expressed in R20 and R20C.

4.2.5.3. Validation of microarray results using qRT PCR for the R14 and R20 series

To support the reliability of the microarray data, the expression of 10 genes from R14 series and 7 genes from the R20 series was confirmed by qRT PCR. A high level of concordance was observed between the microarray results and the qRT PCR data (with a correlation coefficientof 0.78 and 0.87 respectively from the experiment).

*Gap*A which encodes glyceraldehyde 3 phophate dehydrogenase was used as the control for the qRT PCR studies since it showed zero fold change in the transcriptomic study conducted for R14, R20 and daughter strains. Table 4.20 A and B confirms the transcriptomic study by comparing the microarray fold change and qRT PCR fold change. Figures 4.15. A and B compares the microarray and qRT PCR fold change values.

Table 4.20. ΔCt values and calculation of fold changes for selected genes in (A) R14 series (B) R20 series

Table 4.20(A)

Genes	ΔCt	ΔΔCt	2^Δ ΔCt (FC)	log FC	Microarray FC	log Microarray FC
*fla*G	21.60	-3.40	0.09	-1.02	0.32	-0.50
*clp*B (R14)	22.61	-2.39	0.19	-0.72	0.41	-0.38
CJE1302	21.53	-4.25	0.05	-1.28	0.25	-0.60
CJE1466	20.44	-1.13	2.18	0.34	6.19	0.79
*peb*3	25.86	-4.30	0.05	-1.29	0.35	-0.45
*his*A	24.57	-3.01	0.12	-0.91	0.39	-0.41
CJE0265	18.70	-0.40	0.76	-0.12	0.36	-0.45
*clp*B (R14A)	15.34	6.22	74.54	1.87	4.29	0.63
*grp*E	14.57	6.99	60.86	1.78	6.40	0.81
CJE1703	26.77	-7.67	0.00	-2.31	0.21	-0.68

Table 4.20(B)

Genes	ΔCt	ΔΔCt	2^Δ ΔCt (FC)	log qRT PCR FC	Microarray FC	log Microarray FC
CJE0253	28.75	1.28	2.43	0.39	2.15	0.33
*grp*E	16.39	0.30	1.23	0.09	5.03	0.70
*mre*B	18.28	-1.11	0.46	-0.34	0.49	-0.31
CJE0183	25.15	-7.98	0.00396	-2.40	0.22	-0.66
CJE01755	20.17	-1.07	0.48	-0.32	0.49	-0.31
*bio*F	33.37	16.20	75281.10	4.88	66.67	1.82
*his*A	28.02	-11.33	0.00033	-3.41	0.42	-0.38

Figure 4.15. Comparison of the gene expression levels measured by microarray and qRT PCR for (A) R14 series and (B) R20 series

Fig. 4.15(A)

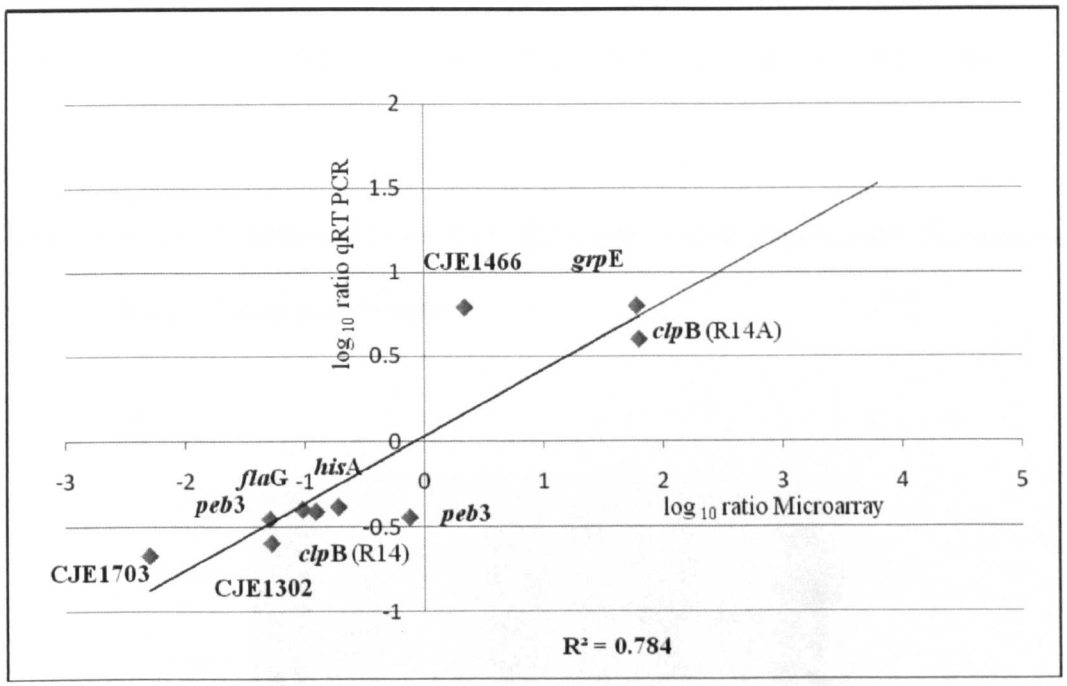
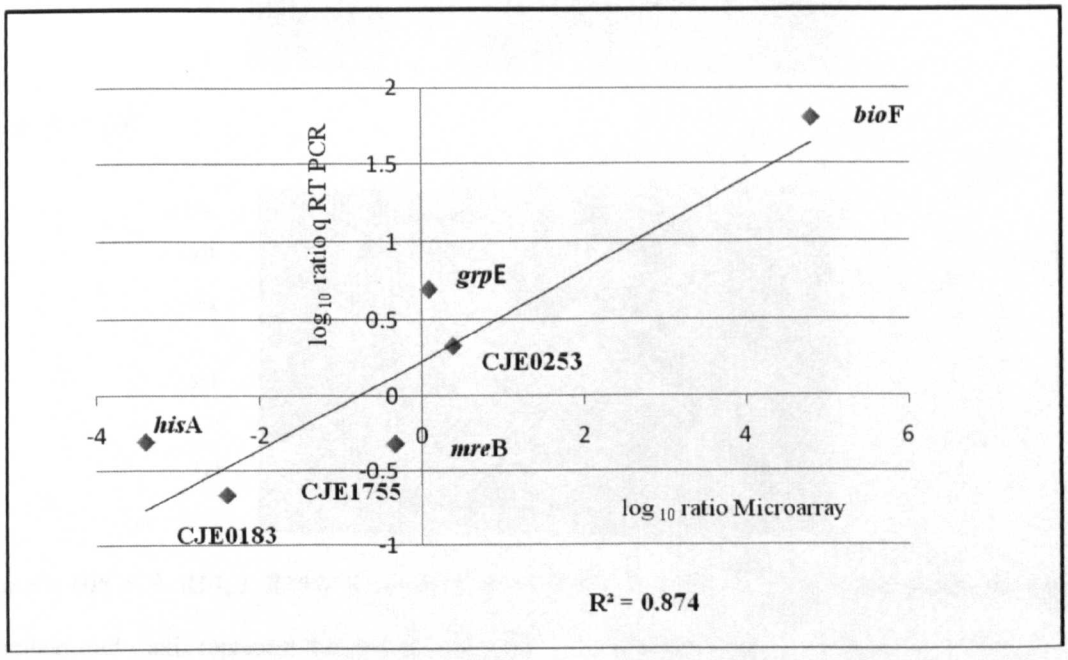


Fig. 4.15 (B)



The x axis represent the log ratio values of the microarray fold changes while the y axix represent the log qRT PCR fold change. R^2 represent coefficient of determination.

4.2.6. Secreted proteins of HPC5 and its variants

Assays were performed on HPC5 and its variants to determine whether protein secretion was affected by the rearrangements and transcriptional changes. The protein profiles for the whole cell lysates of HPC5 and variants were presented in Fig. 4.16.A and B. No marked difference in the production of secretary proteins was observed.

Figure 4.16. (A) Whole cell proteins in HPC5 and its variants, and (B) Secretary proteins in HPC5 and its variants

Fig. 4.16 (A)

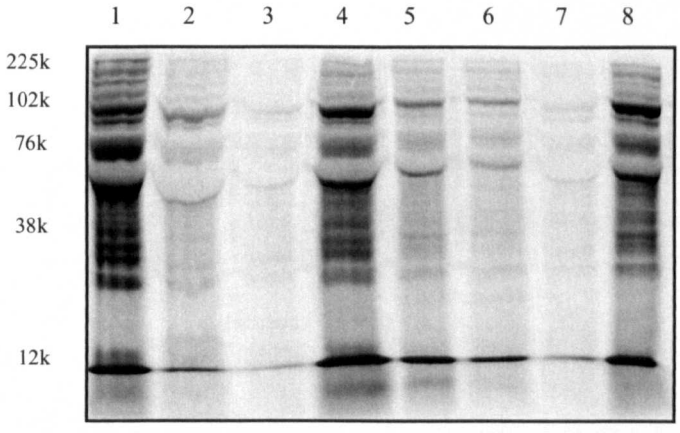
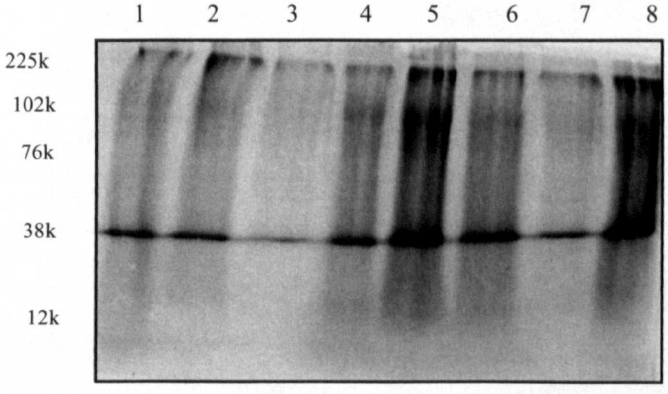


Fig. 4.16(B)



Lane 1: HPC5, 2: R14, 3: R20 4: R14A 5: R14B, 6: R20A, 7: R20B, 8: R20C. x axis denote the lane numbers and y axis represent the molecular weight of the protein in kDa.

4.2.7. Temperature influence on HPC5 and its variants

4.2.7.1. *Hsp*R mutant genes differentially regulated in HPC5 and its variants

Transcriptomic analysis indicated changes in the expression of temperature regulated genes of the HPC5 variants. Table 4.21 lists the *hsp*R mutant genes which were differentially regulated (A) R14 (B) R14A (C) R14B (D) R20A

Table 4.21. *Hsp*R mutant genes that were differentially expressed in (A) R14 (B) R14A (C) R14B and (D) R20A

Table 4.21 (A)

Gene No	Gene name	Fold Change	Proposed gene function
Cj0509c	*clp*B	0.4	ATP dependent chaperone protein *Clp*B

Table 4.21 (B)

Gene No	Gene name	Fold Change	Proposed gene function
Cj0509c	*clp*B	4.28	ATP dependent chaperone protein ClpB
Cj0757	*hrc*A	11.93	heat inducible transcription repressor
Cj0758	*grp*E	6.4	co-chaperone protein GrpE
Cj0759	*dna*K	4.016	molecular chaperone DnaK
Cj0760	-	3.429	hypothetical protein
Cj1230	-	3.969	transcriptional regulator, *Mer*R family

Table 4.21 (C)

Gene No	Gene name	Fold Change	Proposed gene function
Cj0515	-	3.91	hypothetical protein

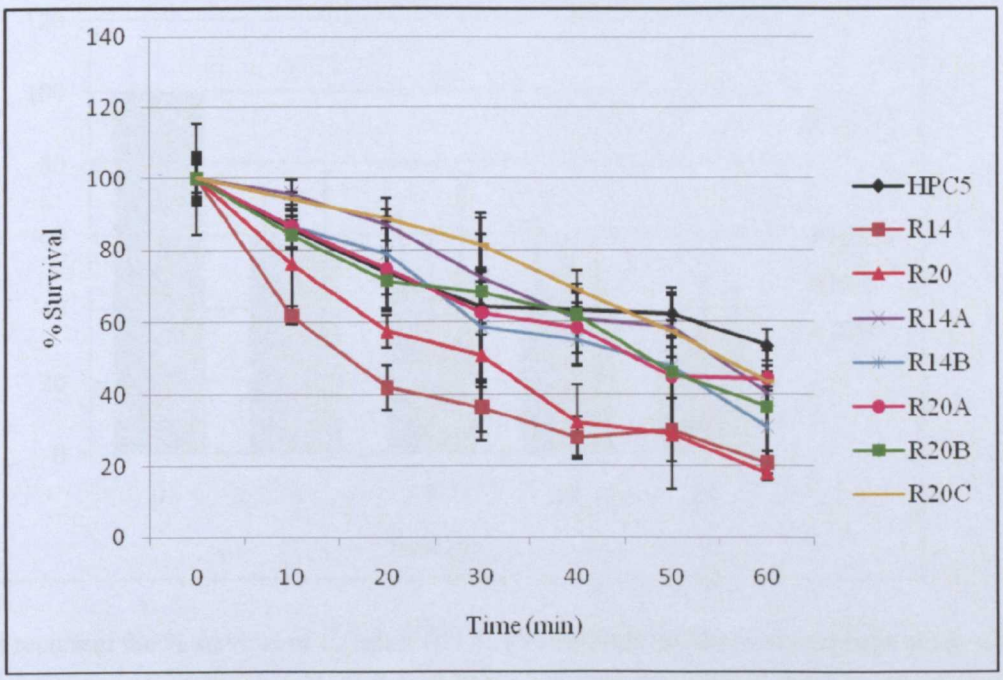
Table 4.21 (D)

Gene No	Gene name	Fold Change	Proposed gene function
Cj0758	*grp*E	5.03	co-chaperone protein GrpE
Cj0759	*dna*K	2.76	molecular chaperone DnaK

4.2.7.2. Thermotolerance of HPC5 and its variants at 50 °C

Thermotolerance at 50 °C showed that 2nd generation variants R14 and R20 showed a marked reduction in thermotolerance compared with HPC5. However, the 3rd generation variants R14A, R14B, R20A, R20B and R20C were as good if not better than HPC5. An initial inoculm of approximately 7 $\log_{10}$ cfu ml^{-1} of each strains were introduced into NB No 2 (2.1.8) which is considered as the 0 time point where all the strains showed 100% survival. Fig. 4.17 presents the survival curves of HPC5 and its variants at 50 °C.

Figure 4.17. Thermotolerence of HPC5 and its variants at 50 °C

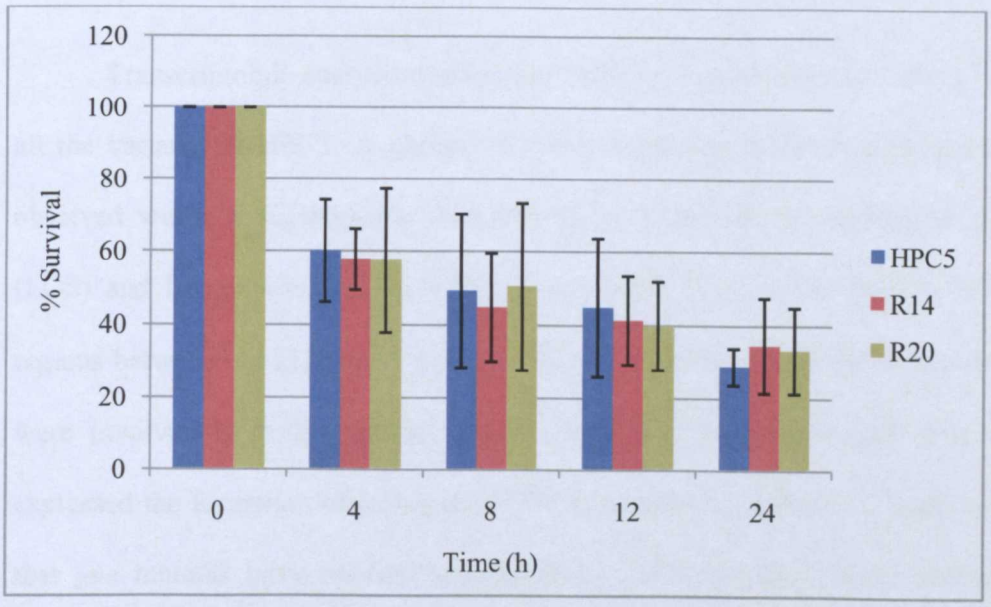


The y axis represent the percent survival rate while the x axis represent growth time in 'min'. The error bars represent the positive and negative error value for each time point. The colour codes are represented in the figure.

4.2.8. Intracellular survival of HPC5, R14 and R20 in HD11 macrophages

The intacellular survival of HPC5, R14, R20 in chicken HD11 macrophage cells were investigated (Fig. 4.18). The intracellular survival of HPC5, R14 and R20 were determined by plate count assay after treating strains with HD11 macrophages. The survival of the isolates varied between 10^7 cfu ml^{-1} (100% of input bacteria) to 10^2 cfu ml^{-1} (approximately 0.025% of the input bacteria) after 24 h. Significant differences were not observed between HPC5, R14 and R20 with respect to their ability to survive intracellularly in HD11 macrophages. The survival rate of HPC5 was comparably higher during the first 12 h but declined after that.

Figure 4.18. Survival of HPC5, R14 and R20 in HD11 macrophages



Y axis represent the % survival of *C. jejuni* HPC5, R14 and R20 on HD11 macrophage along with the error bars and x axis represent the experimental time in 'h'. Blue represents HPC5, red represents R14 and green represents R20.

4.3. DISCUSSION

The selective pressure imposed by bacteriophage CP34 caused bacteriophage induced genome evolution of the HPC5 genome *via* an intrachromosomal rearrangement to escape infection. These rearrangement events caused changes in motility, bacteriophage sensitivity and colonisation potential. The availability of the variants of HPC5 provided a unique opportunity to study the changes in transcription and relate them to these physiological changes. The observed transcriptional differences observed led to further investigations of altered physiology such as temperature sensitivity, macrophage survival and protein secretion.

4.3.1. Effect on the location of the differentially regulated genes due to recombination

Transcriptomic analysis demonstrated differential regulation of many genes in all the variants of HPC5. A cluster of genes exhibiting differential regulation was observed within a region associated with genes that code for lipooligosaccharides (LOS) and lipopolysaccharides (LPS) biosynthesis. In *C. jejuni* NCTC 11168 the regions between Cj1131c (*gne*; *gal*E) and Cj1152 (*rfa*D) encoded gene products that were involved in both inner and outer core LOS biosynthesis and *gne* mutants expressed the formation of a truncated LOS (Parkhill *et al.*, 2000). It is also reported that *gne* mutants have reduced transformation efficiency due to the altered outer membrane composition which interferes with the assembly or the stability of the transformation complex (Wiesner and DiRita, 2008). In R14A Cj1150 was up regulated 2.8 fold and Cj1131c (*gne*) down regulated 0.45 fold. R20A also showed the

relative down regulation of Cj1131c by 0.454 fold. Cj1150 (*hld*E) encodes D-beta-D-heptose 7-phosphate kinase that was involved in the biosynthesis of the lipopolysaccharide (LPS) core precursor ADP-L-glycero-D-manno-heptose. LPS plays an important role in maintaining the structural integrity of the bacterial outer membrane of gram negative bacteria (Gilbert *et al.*, 2008). *Gne*, which codes for UDP-galactose 4-epimerase in campylobacters has been implicated in triggering the onset of GBS. Studies by Fry *et al.* (2000) have shown that a *gne* mutant was reduced in its ability to adhere to and invade INT407 cells. However, it was still able to colonise chickens to the same level as an isogenic wild type strain. Serum resistance and haemolytic activity of this mutant were also not impaired when compared to the parent strain. The down regulation of *gne* in R14A and R20A must have resulted in its reduced transformation ability (Wiesner and Di Rita, 2008).

4.3.2. Operons differentially regulated in the R14 and R20 series

R14 exhibited the up regulation of Cj1184c and Cj1185c that encode cytochrome C and B subunits of a putative ubiquinol cytochrome C reductase that played a potential role in the electron transport system. Bacterial ubiquinol: cytochrome c oxidoreductase or bc1 complex helps in the transfer of two electrons from hydroquinone or hydroubiquinone to cytochrome b, creating an electrochemical gradient across the cytoplasmic membrane (Smith *et al.*, 2000).

R14A exhibited a 4 fold increase in the expression of Cj1229 (*cbp*A) that codes for putative curved DNA binding protein and the adjacent gene Cj1230 encoding the heat shock transcriptional regulator *hsp*R. *Hsp*R is organized in an operon with the *cbpA* gene, which belongs to the heat shock protein *dna*J family.

*Hsp*R regulates the expression of *dna*K, *gro*ESL, and *cbp*A genes in *C. jejuni*. *C. jejuni* use more than one strategy to simultaneously regulate distinct sets of heat shock genes and this complex regulatory network may allow the bacterium to fine tune heat shock gene expression in response to a temperature up shift or other stimuli such as oxidative shock, acid shock or osmotic shock. *Hsp*R mutants show a down regulation of the flagellar genes resulting in less motile bacteria and thereby reduced bacterial colonisation potential but the disruption of heat-inducible transcription repressor (*hcr*A) does not alter colonisation ability in the rabbit ileal loop model (Stintzi *et al.*, 2005).

R14A also showed an upregulation of Cj1314c, Cj1315c, Cj1316c and Cj1318 when compared with HPC5. Cj1314c (*his*F) and Cj1315c (*his*H) encoding subunits of imidazole glycerol phosphate synthase were upregulated 5.5 fold, which clearly reverses the down regulation observed in R14 (0.385 fold). The products of the *his*F and *his*H genes are integral to cellular metabolism being connected to both histidine biosynthesis and nitrogen metabolism by acting as the final source of nitrogen (Fani *et al.*, 2007). The adjacent gene Cj1316c is also up regulated 4.5 fold in R14A. Cj1316c (*pse*A) codes for a component of the pseudaminic acid biosynthesis pathway that leads to O-linked glycosylation of the flagella in *C. jejuni* 81-176 (Thibault *et al.*, 2001). Studies have shown that mutants of *pse*A failed to autoagglutinate which is a recognised marker of virulence when compared to the parent strain. It is also associated with flagellar expression and glycosylation in *C. jejuni* and *H. pylori* (Golden and Acheson, 2002). Cj1318 (*maf*1) can functionally substitute for *pse*E that is involved in the assembly and transfer of O- linked glycosylation groups to flagellin subunits and the mutants of *pse*E are non motile (Karlyshev *et al.*, 2002). R14A also shows upreglation of Cj1331 (*ptm*B) and Cj1332

(*ptm*A) by 7 and 4.2 fold respectively. Studies had shown that mutants of *ptm*A and *ptm*B affect legionaminic acid biosynthesis and loss of immunoreactivity indicative of a change in glycosylation profile (Guerry *et al.*, 1996).

Upregulation of Cj1347c (*cds*A) and Cj1348c occurred in R14A by 2.5 and 2 fold respectively compared to HPC5. *Cds*A (phosphatidate cytidyl transferase) is involved in glycerophospholipid metabolism and thereby fatty acid biosynthesis. It converts 1,2- Diacyl- sn- glycerol 3- phosphate to CDP diacylglycerol. Cj1348c codes for a putative coiled coil protein of unknown function.

4.3.3. Motilty associated genes differentially regulated in R14 and R20 series

C. jejuni is a motile species driven by polar flagella. It involves over 40 structural and regulatory genes and it includes a glycosylation system that post translationally modifies the flagellin subunits (Guerry *et al.*, 2006; Hendrixson, 2008b). The *C. jejuni* NCTC 11168 genome has 3 predicted sigma factors; σ^{70} (encoded by *rpo*D) which regulate class I genes involved in the assembly of the basal body and the export apparatus, σ^{54} (encoded by *rpo*N) regulate class II genes that encode proteins forming part of the hook and basal body and σ^{28} (encoded by *fli*A) regulate class III genes which are involved in filament biosynthesis, capping and flagellin post translational modification (Carrillo *et al.*, 2004). The transcriptome analysis of R14 series and R20 series showed an up regulation and down regulation of various sets of genes that affect motility.

4.3.3.1. Motility associated genes differentially regulated in R14

The cell surface structure of R14 was modified due to the down regulation of Cj0547 (*flaG*) which codes for the flagellar filament along with *fla*A, *fla*B and *fli*A, mutants in which result in long flagellar filament formation and the absence of pellicle formation even after long days of incubation. This is consistent with the observation that R14 is significantly impaired in its motility compared to HPC5. Studies reported by Kalmokoff *et al.*, (2006) found that *fla*G mutants retained its motility though it stopped producing pellicle due to the presence of extra thin filaments. The reduction in *fla*G expression was accompanied by down regulation of Cj1464 (*flg*M) by 0.245 fold. Studies in *E. coli* indicated that *flg*M inhibits σ^{28} until the expression of σ^{54} dependent flagellar genes and the formation of the flagellar export apparatus, basal body and hook are completed. Secretion of *flg*M out of the cytoplasm through the filament structure will relieve the inhibition of σ^{28} and allow the expression of *fla*A (Wösten *et al.*, 2010; Hendrixson, 2008a). However, recent evidence suggests that the *C. jejuni* homologue functions to prevent unlimited elongation of the flagellum, which otherwise leads to reduced bacterial motility and does not silence σ^{28} dependent genes until the hook basal body is completed (Wösten *et al.*, 2010). Of particular interest to the temperature dependence observed here is that the *flg*M product has a temperature dependent association with σ^{28} (*fli*A) (Wösten *et al.*, 2010).

4.3.3.2. Motility associated genes differentially regulated in R14A

In R14A modification in the cell surface structure had been shown by the up regulation of genes such as *fli*D, *fli*S, *fla*D, *flm*A, *pse*A, *pse*E, *ptm*A, *ptm*B, CJE1525 and CJE1640. The motility assays of the R14 series showed a significant increase in

the motility of R14A when compared to HPC5 which clearly reverses the impaired motility of R14.

Cj0548 (*fli*D) is involved in filament cap formation, and with homology to other bacterial flagellins could serve as a minor flagellin. *Fli*D mutants give a unipolar tuft of truncated sheath flagella as of *fla*A mutants since the expression of both genes are controlled by σ^{28} dependent promoter (Josenhans *et al.*, 1995). These showed the requirement of *fli*D in the morphogenesis of flagella and more specifically in the elongation of the flagellar filament. Studies have shown that *fli*D is not required for flagellar motility *in vitro* even though it constitutes part of the flagellum, but could play a role in motility when expressed in other environments (Golden and Acheson, 2002). It also forms a part of the *fli*D operon along with *fla*G and *fliS*. Cj0549 (*fli*S) mutants are reported to be aflagellate and non motile in nature, as it constitutes the main chaperone for flagellin (Hendrixson, 2008a). The gene encoding the flagellin like sequence *fla*D is upregulated in R14A by a factor of 3.86.

CJE1485 (*flm*A), which is involved in motility, flagellar formation and adherence, was up regulated in R14A by a factor of 2.1. It helps in the biosynthesis of pseudaminic acid in the conversion of UDP-N-acetylglucosamine to UDP-4-keto-6-deoxy-N-acetylglucosamine. As noted above R14A also up regulated for the pseudaminic acid biosynthetic components *pse*A and *pse*E in addition to up regulation of *ptm*A and *ptm*B. The increase in motility observed for R14A was clearly associated with an increase in the components of the flagellin post translational modification machinery. The genes involved in *Campylobacter* flagellin glycosylation were found (Guerry *et al.*, 1996; Linton *et al.*, 2000a) to be a part of a larger group of genes that were involved in sugar biosynthesis and transport, which included seven closely related genes of unknown function (Parkhill *et al.*, 2000). These genes were termed as

the motility accessory factor (*maf*) family of flagellin associated proteins.Two *maf* genes (*maf1* and *maf4*) appear to be identical, by both containing homopolymeric G tracts and are involved in the post translational modification of the flagellin.

Cj1466 (*flg*K) which encodes a flagellar hook associated protein was also up regulated in R14A by a factor of 4.3. Ueki *et al.*, (1988) isolated a flagellar protein of 92,000 daltons (Da) associated with the hook region. The hook protein was more associated to the distal rod protein, *flg*G and proximal hook associated protein which are attached to the proximal and distal regions of the hook.

In R14A Cj0887c (*flg*L) was up regulated which affects flagellar hook production. Studies have shown that in *flg*L mutants the overproduction of *flg*E also resulted in longer than normal hooks (Muramoto *et al.*, 1999).

However, R14A also showed down regulation of Cj1179c (*fli*R) by a factor of 0.456. *Fli*R is thought to be involved in flagellar biosynthesis, and in particular the inner membrane localised flagellar export apparatus. Studies have shown that the deletion of *fli*R reduced transcription of the σ^{54} dependent genes *flg*DE2 that are involved in flagellar hook formation and minor flagellin *fla*B (Hendrixson and Di Rita, 2003). However, R14A also showed a 2.5 fold upregulation of Cj1001 encoding primary sigma factor σ^{70} (*rpo*D), that regulates the genes involved in flagellar hook formation.

4.3.3.3. Motility associated genes differentially regulated in R14B

The impaired motility observed in R14 was also overcome in R14B, which may also feature modification of the flagella in response to transcriptional changes in

the genes *fli*S, *pse*H, *flg*E2 and *mot*A. Studies had shown that aflagellate *fli*S mutants lack the ability to attach to cell surfaces and also form biofilms (Joshua *et al.*, 2006). CJE0382 (*mot*A) is a putative flagellar motor component that is thought to be involved in cell motility. *Flg*E2 encodes a flagellar hook associated protein that is regulated by σ^{54} class II genes. Phenotypically *flg*E2 mutants resemble the *mot*$^-$ phenotype and they failed to produce *fla*A which was determined by immunoblot analysis and hence they failed to produce Cia proteins. However these mutants synthesize *fla*B which resulted in the formation of truncated filaments (Konkel *et al.*, 2004).

4.3.3.4. Motility associated genes differentially regulated in R20

R20 showed a marked reduction in its motility though there was no notable down regulation of any flagellar genes.

4.3.3.5. Motility associated genes differentially regulated in R20A

R20A showed upregulation of *che*V that is a chemotaxis signal processing protein consisting of an N terminal *che*W domain fused to C terminal response regulator (RR domain). *Che*V in *C. jejuni*, *H. pylori* and *B. subtilis* show highly conserved sequences. In *B. subtilis*, *che*V can be phosphorylated by *che*A and can partly substitute for *che*W. Strains lacking the RR domain of *che*V are defective in MCP methylation and thus *che*V might have a role in *B. subtilis* adaptation (Marchant *et al.*, 2002). Receptors at the bacterial cell surface receive information from the surrounding regions and these signals are then passed from the receptors to

cytoplasmic chemotaxis components: *che*A, *che*W, *che*Z, *che*R, and *che*B. These proteins function to regulate the level of phosphorylation of a response regulator designated *che*Y that interacts with the flagellar motor switch complex to control swimming behaviours (Lukat and Stock, 1993). The conservation of these residues suggests that the *C. jejuni che*Y protein also interacts with the flagellar motor (when phosphorylated by *che*A) to influence the direction of flagellar rotation.

R20A also showed an upregulation of Cj1337 (*pse*E) which codes for putative pseudaminic acid transferase that helps in flagellin modification. Studies have shown that *pse*E is functionally substituted by motility accessory factor-1 (*maf*1) which is involved in O- linked glycosylation of flagellin subunits. Interruption of *pse*E resulted in a non motile and aflagellated phenotype (Karlshev *et al.*, 2002).

4.3.3.6. Motility associated genes differentially regulated in R20B

In R20B CJE1323 was observed to be up regulated, which encodes a methyl accepting chemotaxis protein with receptor and signal transducer activity. R20B exhibited a motility phenotype when compared to HPC5 but the transcriptional analysis had not shown any decrease in the flagellar proteins or proteins that help in the motility.

4.3.3.7. Motility associated genes differentially regulated in R20C

R20C had not shown any differential expression of flagellar associated genes but clearly had overcome the impaired motility phenotype of R20.

4.3.4. Mu genes differentially expressed in the R14 and R20 series

R14B and R20B showed down regulation of the putative host nuclease inhibitor protein *gam* (CJE0265). The *gam* gene of bacteriophage Mu encodes a protein which protects linear double stranded DNA of Mu genomes from exonuclease degradation *in vitro* and *in vivo* (http://www.ncbi.nlm.nih.gov/gene?term=CJE0265). Studies in *E. coli* had shown that transfection was highly reduced in cells which produce *rec*BC due to degradation of the double stranded DNA (Hoekstra *et al.*, 1980). Akroyd *et al.*, (1986) had purified the protein and found that it is a dimer which consists of identical subunits of 18.9 kD each. It aggregates DNA into large, rapidly sedimenting complexes and acts as a potent exonuclease inhibitor when bound to DNA.

In R20, tail X putative Mu Phage gene, (CJE0253) was up regulated by 2.1 fold when compared to HPC5. This domain is a member of the family of phage tail proteins that would be required for the production of infectious bacteriophage Mu.

4.3.5. CRISPR genes differentially regulated in R14 and R20 series

Clustered, regularly interspaced short palindromic repeats (CRISPR) consist of near perfect direct repeats interspaced with similarly sized non repetitive spacer sequences (Price *et al.*, 2007). Studies have shown that these elements confer sequence directed immunity against bacteriophage (Marraffini and Sontheimer, 2008). The resistance is assisted by a set of cas proteins which are encoded by *cas* genes in addition to the spacer sequences matched with fragments of extra chromosomal elements (mainly from plasmids and virus genomes). This led to the hypothesis that

the CRISPR–Cas system might be a novel defence system that is able to protect a host cell against invading alien nucleic acid (van der Oost *et al.*, 2009). A set of *cas* genes had been identified which produce enzymes that modify extra chromosomal nucleic acid, of which the main ones are *cas*1, *cas*2 and *cas*3. *Cas*1 is a metal dependent DNA endonuclease which has been predicted to be an α helical nuclease/integrase. *Cas*2 has a ferredoxin like fold and it has metal dependent endo ribonuclease activity. *Cas*3 encodes a polypeptide that consists of phosphohydrolase/ nuclease domain and a helicase domain though experimental details are still to be determined on this domain (van der Oost *et al.*, 2009). R14A exhibits 2.0 fold upregulation of *cas*1 and could contribute to the resistance against bacteriophage CP34.

4.3.6. Temperature influence in the growth of HPC5 and its variants

The exposure of bacteria to stressfull conditions leads to the expression of a set of heat shock proteins which fall into two categories, the chaperones and the ATP dependent proteases (Anderson *et al.*, 2005). The genome sequence of *C. jejuni* NCTC 11168 revealed the presence of several heat shock protein homologues that include the major chaperones *gro*EL, *gro*ES, *grp*E, *dna*K and *dna*J and several ATP dependent proteases (Parkhill *et al.*, 2000). *C. jejuni* possesses homologues of *hrc*A and *hsp*R regulators, which negatively control the heat stress response in other bacteria (Narberhaus, 1999). Anderson *et al.*, (2005) undertook a microarray based comparison of the relative transcript levels in *hsp*R mutants and their corresponding wild type strains. This study noted the high expression (4.8 to 28.2 fold) of putative heat shock operon genes *hrc*A, *grp*E and *dna*K as well as two down stream genes encoding hypothetical proteins (Cj0760 and Cjl761). The gene

encoding the ATP dependent chaperone *clp*B, which interacts with *dna*K to reactivate proteins that have become aggregated after heat shock was also transcribed at higher levels in *hsp*R mutants.

In R14A and R20A *grp*E transcripts were increased (5.0 to 6.4 fold) when compared to HPC5. The *grp*E product modulates the activity of Hsp70/Hsc70 class, by binding to the ATP*ase* domain of Hsp70 catalysing the dissociation of ADP which stimulates the hydrolysis and exchange of adenyl nucleotides by other proteins. An up regulation of *cbp*A occurred by 4 fold in R14A which plays an important role in protein translation, folding, unfolding, translocation and degradation (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=99751). *Clp*B heat shock protein is necessary for the survival of *Campylobacter* cells upon sudden increase in temperature and it has a remarkable ability to rescue proteins from an aggregated state. *Clp*B has a cooperative action with *dna*K, *dna*J and *grp*E in the reactivation of chemical and heat denatured substrate proteins (Konieczny and Liberek, 2001). In R14 the *clp*B transcript showed a 0.4 fold reduction in expression but in R14A there was an upregulation by 4.3 fold when compared to HPC5. The *mer*R family proteins were up regulated by 3.9 fold in R14A when compared to HPC5, which help in the elimination of Hg(II) from the cells since it has no beneficial roles in bacteria. Once it is removed the *mer*R operon is switched off quickly to reduce the production of protein which is not required (Hobman *et al.*, 2005).

Changes in the transcript levels of heat shock regulatory proteins prompted the thermotolerence experiments of R14 and R20 series. The phage resistant strains R14 and R20 showed a reduction in the survival rate when compared to their parental strain and phage sensitive derivatives. Transcriptomic analysis had shown that in R14 there was a down regulation of *clp*B and studies proved that *clp*B

mutants are defective for aerobic survival, host cell adherence and invasion (Svennsson *et al.*, 2009). The low intracellular survival of R14 and R20 compared to HPC5 after 12 h of incubation could be related to the reduced expression of *clpB*.

4.3.7. Genes involved in small molecule metabolism which were differentially expressed in R14 and R20 series

4.3.7.1. Genes involved in energy metabolism which were differentially expressed in R14 and R20 series

Cj1184c and Cj1185c which were involved in electron transport were up regulated in R14 and their roles were discussed in section 4.4.2. Cj0874c encodes a putative cytochrome C with weak similarity to many of the cytochrome C proteins involved in energy metabolism. This gene was up regulated in R14A by 2.6 fold (http://www.ncbi.nlm.nih.gov/gene/905168?ordinalpos=1&itool=EntrezSystem2.PEntrez.Gene.Gene_ResultsPanel.Gene_RVDocSum).

Cj0781 (*nap*G) encodes the periplasmic component of quinol dehydrogenase that was down regulated in R14A. Electron transport from quinol to nitrate is more likely to be *via* the *nap*GH proteins than the *nap*C/*nrf*H homologue (Pittman *et al.*, 2007).

R14B exhibited up regulation of Cj0465c (*ctb*) which codes for group III truncated haemoglobin. *Ctb* is involved in the electron transport system, reflecting a role in respiration at physiologically relevant external oxygen concentrations and in R14B the expression of *ctb* was 3.9 fold more than HPC5.

In R20A an up regulation of Cj0075c was observed by a factor of 2.0, which codes for a putative oxidoreductase ferredoxin type electron transport protein. Ferredoxin NADP(H) reductases (FNRs) are flavoenzymes that catalyze electron transfer between NADP(H) and the iron sulfur protein ferredoxin (Fd) or Flavin mononucleotide containing flavodoxin (Ceccarelli *et al.*, 2004). These enzymes are normally present as monomeric proteins in plastids, bacteria and mitochondria where they catalyze the reaction described in the equation using noncovalently bound FAD as a prosthetic group.

$$2\ Fd(Fe^{+2}) + NADP^{+} + H^{+} \leftrightarrows 2\ Fd(Fe^{+3}) + NADPH.$$

Down regulation of Cj1488c (*cco*Q) that codes for cb-type cytochrome C oxidase subunit IV occurred in R20A by a factor of 0.382. Members of this protein family are restricted to the epsilon branch of the Proteobacteria. All members are found in operons containing the other three structural subunits of the cbb3 type of cytochrome c oxidase. These small proteins show remote sequence similarity to the *cco*Q subunit in other cytochrome c oxidase systems, so this family is assumed to represent the epsilon proteobacterial variant of *cco*Q (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=140575).

R20 B exhibited up regulation of Cj0622, which codes for carbamoyltransferase. It acts as a precursor for the synthesis of both the CO and CN groups, which are the non protein ligands present in the large subunits of hydrogenases and acts as the reaction centre (Rangarajan *et al.*, 2008).

Up regulation of Cj1357c (*nrf*A) was observed in R20B, which is predicted to encode a periplasmic pentahaem cytochrome c nitrite reductase that is the terminal

enzyme in the six electron dissimilatory reduction of nitrite to ammonia (Pittman and Kelly, 2005). According to the work in *E. coli* by Poock *et al.*, 2002, this reaction may be physiologically relevant under microaerobic conditions, and it has been proposed that *nrf*A plays a significant role in nitric oxide detoxification in addition to flavohaemoglobin (Hmp) and flavorubredoxin (NorV), which are part of the *E. coli* NO detoxification systems.

R14B showed a down regulation of Cj0443 (*acc*A) which codes for alpha acetyl-CoA carboxylase carboxyltransferase subunit. It is involved in propanoate metabolism and catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (http://www.ncbi.nlm.nih.gov/gene/904768).

4.3.7.2 Genes involved in central intermediary metabolism which were differentially expressed in R14 and R20 series

Cj0483 (*uxa*) which enables the hexuronate degradative pathway was up regulated in R14A and is involved in Pentose and glucuronate interconversions.

Up regulation of Cj0007c (*glt*B) glutamate synthase (NADPH) large subunit was observed in R20A by a factor of 2.2. The C-terminus of the large subunit of glutamate synthase (*glt*S) contains a complex iron-sulfur flavoprotein that catalyzes the synthesis of L-glutamate from L-glutamine and 2-oxoglutarate

(http://www.ncbi.nlm.nih.gov/sites/Structure/cdd/cddsrv.cgi?uid=29611).

4.3.7.3 Genes involved in the biosynthesis of co-factors, prosthetic groups and carriers which were differentially expressed in R14 and R20 series

Cj1238 (*pdx*J) encoding pyridoxine 5'- phosphate (PNP) synthase was up regulated by 4.7 fold in R14. *Pdx*J codes for the PNP synthase domain which is the active form of vitamin B6 that is an essential, ubiquitous coenzyme in amino acid metabolism. It catalyses the condensation of 1 amino acetone 3 phophate and 1-deoxy-D-xylulose 5-phophate which leads to the formation of Pyridoxine 5'-phosphate (vitamin B6) (Wetzel *et al.*, 2004).

The expression of Cj0146c (*trx*B) was increased by 2.7 fold in R14A which is a probable thioredoxin reductase that catalyzes the transfer of electrons between pyridine nucleotides and a specific disulphide containing substrate (Wang *et al.*, 1996). It also helps in reducing the oxidative stress by acting to recycle the alkyl hydroperoxidase (*aph*C) that reduces alkyl perioxdes to their corresponding alcohols (Atack *et al.*, 2008).

Cj1530 (*coa*E) encoding dephospho-CoA kinase was up regulated by a factor of 2.0 in R20. This enzyme catalyses the phosphorylation of the 3'-hydroxyl group of dephosphocoenzyme A to form coenzyme A which is the final step in CoA biosynthesis (ATP + dephospho-CoA = ADP + CoA) (Zhyvoloup *et al.*, 2003).

R14B and R20B show up regulation of Cj0306c (*bio*F) which codes for 8-amino-7-oxononanoate synthase. It helps in biotin metabolism by catalyzing the conversion of pimeloyl CoA to 8-amino7-oxononanoate (http://www.genome.jp/dbget-bin/show_pathway?cje00780+Cj0306c). R20B also showed an upregulation of Cj0194 (*fol*E) which codes for *fol*E which codes for GTP cyclohydrolase I that catalyzes the conversion of GTP into dihydroneopterin

triphosphate. The enzyme product is the precursor of folate analogs in methanogenic bacteria. Cj1622 (*rib*D) which codes for riboflavin specific deaminase/reductase in R14B by a factor of 0.212. It catalyzes the deamination of 2, 5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5'-phosphate, which is an intermediate step in the biosynthesis of riboflavin (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=29827).

4.3.7.4. Genes involved in amino acid biosynthesis which were differentially expressed in R14 and R20 series

R14A, R14B and R20B showed up regulation of Cj1718c (*leu*B) which codes 3-isopropylmalate dehydrogenase. It catalyzes the oxidation of 3-isopropylmalate to 3-carboxy-4-methyl-2-oxopentanoate in leucine biosynthesis by synthesising 3-isopropylmalate dehydrogenase (http://www.ncbi.nlm.nih.gov/gene?term=Cj1718c). The down regulation of *his*A (Cj1601) and *his*I (Cj1604) was observed in R20A by a factor of 0.4 with respect to HPC5. *His*A and *his*I are involved in histidine metabolism and thereby connected to purine and nitrogen metabolism (Fani *et al.*, 2007).

4.3.8. Genes involved in the broad regulatory functions which were differentially expressed in R14 and R20 series

R14 shows up regulation of Cj1222 (*dcc*S) that encodes a two component sensor histidine kinase. Studies of MacKichan *et al.*, (2004) had shown that the two-

component signal transduction system (TCSTS) designated *dcc*R-*dcc*S (Cj1223c-Cj1222c), had been found important for *in vivo* colonization but was dispensable for *in vitro* growth. *Dcc*R and *dcc*S mutants were found to be defective in chicken colonisation. On receiving the appropriate signal from the environment, histidine kinase anchored in the membrane undergoes an autophosphorylation reaction. The response regulator is activated by auto phosphorylation to a conserved aspartate residue and in this state the activated response regulator is able to bind the promoter region of its target genes to effect changes in their expression (Stock *et al.*, 2000). Though there is an up regulation of *dcc*S in R14 the effect could be counteracted due to the reduction in the expression of *flg*M where the expression of σ^{54} genes were down regulated.

An up regulation of Cj1001 (*rpo*D) occurred in R14A which is the major RNA polymerase sigma factor. This protein belongs to the σ^{70} family which comprises the primary or housekeeping sigma factors (Petersen *et al.*, 2003).

R14A had shown an up regulation of Cj0480c which codes for putative transcriptional regulator which includes the glycerol operon regulatory protein and acetate operon repressor both of which are members of the *icl*R family (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=31604). Members of the *icl*R family control genes whose products are involved in the glyoxylate shunt in *Enterobacteriaceae*, multidrug resistance, degradation of aromatics, inactivation of quorum sensing signals, determinants of plant pathogenicity and sporulation (Molina-Henares *et al.*, 2006).

R14A showed a down regulation of Cj0466 (*nss*R) which codes for a transcriptional regulator, which induces the production of *Campylobacter* globin

(Cgb) which helps in nitric oxide (NO) detoxification in *Campylobacter* (Elvers *et al.*, 2005). Studies have also shown that the nitrosative stress responsive regulon include Cj0465c (*ctb*) that encodes a group III truncated haemoglobin, which was also up regulated in R14B.

R20A showed down regulation of Cj0184c which codes for serine threonine protein phosphatase which participates in complex interacting signalling pathways in eukaryotes, prokaryotes, and Archae. In *Bacillus subtilis* they regulate the activity of sigma factors in response to environmental stress (Shi *et al.*, 1998).

Cj1189c was up regulated in R20B, which codes for bipartate energy taxis response protein. Mutants lacking *cetA* or *cetB* are deficient in energy taxis. *Cet*B sense environments having high electron transport and ATP generation possibly using FAD as a redox sensor. On recognising an environment with poor electron transport it interacts with *cet*A to transduce signals and alter the flagellar rotation and direct their motility towards new environments with higher energy producing capabilities (Taylor and Zhulin, 1999).

4.3.9. Genes involved in macromolecule metabolism which were differentially expressed in R14 and R20 series

4.3.9.1. Genes involved in synthesis and modification of macromolecules which were differentially expressed in R14 and R20 series

R14 showed an upregulation of Cj1689c (*rplO*), Cj1690c (*rpsE*), Cj1691c (*rplR*), Cj1701c (*rpsC*) and Cj1705c (*rplW*) which help in the maturation and modification of ribosomes. *RplO* codes for the 50S ribosomal protein L15 subunit,

*rps*E codes for the 30S ribosomal protein S5 subunit and *rpl*R codes for 50S ribosomal protein L18. L15 and L18 are late assembly proteins that help in the formation of active particles during late assembly of ribosomes. In *E.coli,* S5 is important in the assembly and function of the 30S ribosomal subunit and mutations in S5 have been shown to increase translational error frequencies. *Rps*C codes for the 30S ribosomal protein S3 and *rpl*W which 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 on either the 23S or 26S rRNA. In *E. coli,* S3 is involved in the binding of initiator Met- tRNA. It is one of the territory binding proteins of the S7 binding protein group (Grondek and Culver, 2004). The need to increase the availability of functional ribosomes in R14 may reflect a defect in translation or perhaps misfolding of the protein products.

There was an up regulation of the D12 class N6 adenine specific DNA methyl transferase gene (CJE0201) in R14, which is involved in the covalent transfer of methyl group to either N-6 of adenine or C-5 or N-4 of cytosine and it affects the functions of housekeeping functions, such as DNA replication and mismatch repair. Kim *et al.*, 2008 had reported that the DNA methylases are often involved in restriction modification.

R14A showed down regulation of Cj0872 (*dsb*A) that codes for a putative protein disulphide isomerase and Cj0884 (*rps*O) that codes for 30S ribosomal protein S15, which act on ribosomal protein synthesis and modification. *Dsb*A is involved in the oxidative protein folding pathway in prokaryotes, and is the strongest thiol oxidant known, due to the unusual stability of the thiolate anion form. The highly unstable oxidized form of *dsb*A directly donates disulfide bonds to reduced proteins secreted into the bacterial periplasm. This rapid and unidirectional process helps to catalyze

the folding of newly synthesized polypeptides. To regain catalytic activity, reduced *dsb*A is then reoxidized by the membrane protein *dsb*B, which generates its disulfides from oxidized quinones, which in turn are reoxidized by the electron transport chain (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=48568).

Cj0312 (*pth*) encoding peptidyl tRNA synthase was down regulated 0.40 fold in R14B. Peptidyl tRNA hydrolase, (*pth*), an esterase, releases tRNA from peptidyl-tRNAs by cleaving the ester bond between the C-terminal end of the peptide and the 2'- or 3'-hydroxyl of the ribose at the end of the tRNA (Cuzin *et al.*, 1967; Kössel and RajBhandary, 1968). It also hydrolyses an amide bond between the peptide and the 3'-amino group of the modified ribose at the end ofthe tRNA in synthetic substrates (Jost and Bock, 1969). Down regulation of Cj1457c (*tru*D) encoding tRNA pseudouridine synthase was also observed in R14B and R20B. *Tru*D belongs to pseudouridine synthases (*Psi*S) which isomerises uridine to form pseudouridine and is one of the most abundant modified nucleosides in RNAs (Chan and Huang, 2009).

Cj0945c encoding a putative helicase was up regulated in R14B. The helicase is an ATP-dependent exoDNase which involve in DNA and RNA metabolism, such as replication, recombination, repair and transcription (Bochman *et al.*, 2010).

R20A exhibits up regulation of Cj1288c (*glt*X) which codes for glutamyl-tRNA synthetase (GluRS) that helps in the translation of proteins. It causes aminoacylation of both $tRNA^{Glu}$ and $tRNA^{Gln}$ with glutamate (Siatecka *et al.*, 1998).

Up regulation of Cj0338c (*pol*A) in R20A codes for DNA polymerase 1 and causes modification of the DNA. It has 3'-5' exonuclease, 5'-3' exonuclease and 5'-3' polymerase activities, primarily functions to fill gaps during DNA replication and repair (http://www.ncbi.nlm.nih.gov/gene/904662). The 3'-5' exonuclease is involved

in methyl – directed mismatch repair (MMR) system thereby reducing polymerase errors and is involved in prrof reading activity (Miller, 2008). There was also a down regulation of Cj1695 (*rplE*) in R20A which codes for the maturation of the 50S ribosomal protein L5.

Another down regulated transcript in R20B was *ssb*, which encodes a single stranded DNA (ssDNA) binding protein that resolves secondary structure in ssDNA. R20B also showed up regulation of Cj0777 and Cj0945c that code for a putative helicase which helps in the modification of DNA. An up regulation of Cj0382c (*nus*B) which codes for transcription anti termination protein was up regulated in R20B. Studies have shown that they are required for the efficient expression of *rrn* operon which helps in the modification of RNA polymerase. It is also implicated in rRNA transcription antitermination, but their precise role has not been determined (Quan *et al.*, 2005).

Cj0166 (m*ia*A) which codes for tRNA delta(2)-isopentenylpyrophosphate transferase is up regulated in R20C which cause GC to CA transversions. Studies have shown that in *S.* Typhimurium, the *miaA*, *miaB*, and *miaAB* double mutants specify enzymes for tRNA modification. The *miaA* mutation reduced the level of tetracycline resistance mediated by both Tet(O) and Tet(M) (Taylor *et al.*, 1998).

4.3.9.2. Genes involved in modification of cell envelope which were differentially expressed in R14 and R20 series

R14 differentially expressed many genes that modify the cell envelope of the bacteria. An up regulation of Cj0591c and Cj0646 were observed which codes for putative lipoprotein and a down regulation of Cj0236 and Cj1168 (*ded*A family) which codes for putative integral membrane protein.

R14A also showed modification of the membrane lipoproteins in the cell membrane by the up regulation of Cj0454c, Cj0455c, Cj0457c and Cj1026. Down regulation of Cj0362 occurred which coded for an integral membrane protein. Modification in LOS also occurred by the up regulation of Cj1150c (*hld*E) and Cj1316c (*pse*A). Cj1183c was upregulated by a factor of 2 which codes for the cyclopropane-fatty-acyl- phopholipid syntase which occur in the phopholipid of bacteria. Cyclopropane fatty acids (CFA) are formed by the addition of a methylene group, derived from the methyl group of S- adenosyl methionine, across the carbon-carbon double bond of unsaturated fatty acids (UFAs). It can be considered as the post synthetic modification of bacterial membrane lipid bilayers (Grogan and Cronan, 1997).

4.3.10. Genes involved in cellular processes which were differentially expressed in R14 and R20 series

In R14 a down regulation of Cj0613 (*pst*S) was seen which encoded a putative periplasmic phosphate binding protein. It is a part of the ABC transporters two component system which help in the transmission of phosphate anion (http://www.genome.jp/dbget-bin/show_pathway?cje02010+Cj0613). *Pst*S was also up regulated in R20A.

R14 also showed the down regulation of Cj0948c, which encodes a cation efflux protein. Cj0599c (*clp*B) was also down regulated which modified the cellular process and their function was explained in section 4.4.6.

R14A showed an up regulation of a cation efflux protein Cj0045c which is a putative iron binding protein that helps in transporting inorganic ions (http://www.ncbi.nlm.nih.gov/gene/904381?ordinalpos=1&itool=EntrezSystem2.PEntrez.Gene.Gene_ResultsPanel.Gene_RVDocSum).

It also showed an up regulation of Cj1648 which is a putative ABC transport system periplasmic substrate binding protein involved in resistance to organic solvents. This family of proteins contains the MCE (mammalian cell entry) proteins from *Mycobacterium tuberculosis*. The archetype (Rv0169) which was isolated was found necessary for colonisation and survival within the macrophage. This family contains proteins of unknown functions in other bacteria. (http://www.ncbi.nlm.nih.gov/gene/905921?ordinalpos=1&itool=EntrezSystem2.PEntrez.Gene.Gene_ResultsPanel.Gene_RVDocSum). An up regulation of *clp*B, *hrc*A, *grp*E, *dna*K, *cbp*A and *hsp*R also occurred in R14A and their functions were discussed in section 4.4.6. R20A also showed an up regulation of *gro*EL, *dna*K and *grp*E.

A down regulation of Cj0779 (*tpx*) which coded thiol peroxidase was seen in *C jejuni* R14A which reduce the hydrogen peroxide concentration in the cell and thereby reducing oxidative stress (Atack *et al.*, 2008).

R14B and R20B have showed an upregulation of Cj0886 (*fts*K) which is a putative cell division protein. The N-terminal domain ($FtsK_N$) serves to localize the protein to the division septum and is required for cell division while the C-terminal domain ($FtsK_C$) forms the translocation motor involved in chromosome segregation (Begg *et al.*, 1995; Draper *et al.*, 1998).

R20A showed a down regulation of Cj0856 (*lep*P) which codes for signal peptidase 1. Signal peptidases are unique serine proteases that cleave the mature protein from the membrane and allows them to locate to their final destination in the periplasm, outer membrane or extra-cellular milieu (Geukens *et al.*, 2001).

In R20B the down regulation of Cj1241 had occurred which belongs to Major Facilitator Superfamily (MFS) transporter protein. Using the electrochemical potential of the transported substrates, these proteins facilitate the transport across cytoplasmic or internal membranes of a variety of substrates including ions, sugar phosphates, drugs, neurotransmitters, nucleosides, amino acids, and peptides

(http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=119392). Though R20B had shown an up regulation of *fts*K which promotes cell division, *mre*B which is a homolog of *E.coli* rod shape determining protein which makes up the bacterial cytoskeleton was down regulated. Genes coding for *mre*B/*mbl* are only found in elongated bacteria and not in coccoid forms

(http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=115385).

R20C showed the down regulation of Cj1587c (*opp*A) which encoded a putative peptide ABC-transport system periplasmic peptide-binding protein which helps in the transferring of nickel and peptides (http://www.genome.jp/dbget-bin/show_pathway?cje02010+Cj1584c).

4.4. CONCLUSION

HPC5 challenged with CP34 gives rise to a minor population of genotypic variants that are resistant to bacteriophage. Each variant had a unique transcription profile that was altered from the parental strain. The positions of the genes exhibiting transcriptional changes were not localised to the region where the intra genomic recombination had occurred, suggesting that the physical rearrangement itself did not appear to be a factor in which genes were altered in their transcription. The phage resistant variants showed increased transcription of Mu related genes consistent with their production of Mu bacteriophage and their phage sensitive variants showed down regulation of Mu host nuclease inhibitor protein. The up regulation of CRISPR genes and genes that assist in DNA repair were more prevalent in bacteriophage sensitive strains. The reduction in the expression of heat shock proteins in R14 and R20 compared to HPC5, R14A, R14B, R20A, R20B and R20C is a likely explanation of the lack of thermal tolerance in the phage resistant strains. These strains also showed high modification in the membrane structure by differentially regulating many integral membrane proteins and putative lipoproteins. Though the secretary protein analysis had not shown any remarkable difference between HPC5 and its variants, intra genomic recombination had caused significant changes in the expression of flagellar proteins and the altered transcription patterns in R14 and R20 series which are consistent with differences in flagellar function that will affect colonisation ability. The ability to survive harsh environments such as high temperature was also compromised in some of the variants particularly the phage resistant types but the ability to survive engulfment by macrophages did not appear to be greatly affected. This study reveals the extreme plasticity of the transcriptome of this *C. jejuni* HPC5 strain with the rearrangement events resulting in significant changes that no doubt

contribute to a much greater chance of the bacteria ultimately being able to survive any eventuality.

CHAPTER FIVE

COMPARATIVE GENOMIC ANALYSIS OF *C. JEJUNI* AND *C. COLI* ISOLATED FROM PIGS

5.1. INTRODUCTION

Pigs are a natural reservoir of *Campylobacter* spp. with prevalence rates of between 50 and 100% and excretion levels ranging from 10^2 to 10^7 cfu g^{-1} of faeces (Jensen *et al.*, 2006). *C. coli* is the dominant species found in pigs but *C. jejuni* has also frequently been isolated (Jensen *et al.*, 2005). Besides *C. jejuni* and *C. coli* several other species of *Campylobacter* have also been isolated from pigs which include *C. hyointestinalis, C. mucosalis, C. sputorum, C. lanienae, C. lari* and *C. hyoilei* (On, 1996; Varela *et al.*, 2007). More than one type of the same species may be isolated from the same pig (Jensen *et al.*, 2006). Where attempts have been made to determine the prevalence of different species of *Campylobacter* in pigs, it is likely that where co- existing minority populations are present within the same samples; only the more prevalent dominant species would be reliably detected using standard methods.

The co-existence of different species and different types of the same species within the same animal raises several important questions:

1. What is the relative proportion of each species? This has not been addressed in a systematic way probably due the practical difficulties of detecting low numbers of such closely related species within a much larger *C. coli* population. However, advances in molecular technology now make it possible to start to answer this question. The difficulty in screening for different species of *Campylobacter* from the same plate may be overcome by a direct plating approach combined with differential colony blot hybridisation or PCR primers (Jensen *et al.*, 2006). More genetic comparisons of the strains can be identified

through comparative genomic hybridisation by which the presence or absence of a particular gene can be identified (Taboada *et al.*, 2007).

2. The second question relates to the ecological niche inhabited. Do different species and different genotypes colonise different parts of the intestine? In other words do the minority population of *Campylobacter* survive because they are not competing equally with the majority population? This has never been addressed before.

3. Does exposure to an outdoor environment increase the likelihood of colonisation by more than one species? Jensen *et al.*, (2006) reported that most of the *C. jejuni* and *C. coli* isolated from outdoor reared pigs, their paddock environment and indigenous rodents were of similar serotypes. Exposure of pigs to other infected animals, rodents and their surroundings was also reported to lead to a rapid rate of spread and a high prevalence rate (Young *et al.*, 2000).

4. Do the *C. coli* and *C. jejuni* populations in individual pigs comprise of single or multiple genotypes, and are pigs from the same environment colonised with the same genotypes and or species? Other studies have indicated that in outdoor reared pigs, some subtypes seem to dominate, and that the genotype pool stabilizes with fewer genotypes as the age of the pigs increases (Jensen *et al.*, 2006; Weijtens *et al.*, 1997 and Moore *et al.*, 2002). A particular serotype that dominates the faecal material of a pig at one time may not necessarily be isolated from the same pig at subsequent sampling times (Jensen *et al.*, 2006).

5. Another important question arises as a consequence of intestinal of co-colonization as to how much, if any, genetic exchange occurs between strains

and/or species through natural transformation, conjugation or bacteriophage mediated transduction. Wang and Taylor, (1990) and Wassenaar *et al.*, (1993b) have demonstrated the competency of *C. jejuni* for natural transformation where *C. jejuni* and *C. coli* can co-exist together (Wilson *et al.*, 2003). Lateral gene transfer of DNA between different species of enteric pathogens is well documented and it is important in the diversification of colonization capabilities and virulence traits.

In order to collect data towards answering these questions this chapter describes a study conducted on both outdoor and indoor reared pigs to determine the excretion levels of *Campylobacter* sp, the distribution of *Campylobacter* spp. in different regions of the pig's intestine and differences in the genotypes present. In addition the study examines the likelihood of genetic exchange between species due to the co-existence of *C. jejuni* and *C. coli* in pigs.

Species level detection and differentiation of *Campylobacter* were based on, hippuricase gene sequence which identified most of the *C. jejuni* strains and putative aspartokinase which identified *C. coli* strains and 16S rRNA gene sequences which identified both *C. coli* and *C. jejuni* (Linton *et al.*, 1997). It was suggested that 3% variation between two rRNAs was the threshold at which two strains may be considered to represent distinct species (Clayton *et al.*, 1995; Fox *et al.*, 1992; Kolbert *et al.*, 1999). Gorkiewicz *et al.*, (2003) concluded that 16S rRNA based differentiation of Campylobacters displaying sequence variation below 3% has practical application and several *C. jejuni* and *C. coli* strains shared identical 16S rRNA sequences and

hence species wise differentiation using 16S rRNA is not accurate. The differentiation is also difficult due to the acquisition of DNA due to horizontal gene transfer.

Comparative genomic hybridization (CGH) is a technology used to compare whole genome sequences which enables us to know the presence and absence of genes in a given genome as compared to the reference genome on the microarray. Whole genome comparisons identify the genes which are shared by all strains and also the accessory genes present that often result from gene acquisition (Champion *et al.*, 2008).

Based on the MLST of 7 housekeeping genes of 4507 *Campylobacter* spp. isolates, Sheppard *et al.*, 2008 reported that ~18.6% of the unique alleles found in *C. coli* isolates may have been imported through horizontal gene transfer from *C. jejuni.* Caro Quintero *et al.*, 2009 had re evaluated the available *Campylobacter* MLST data set and found that more than 98% of the available data set does not contain imported alleles and the interspecies genetic exchange is biased and heavily limited to few genes and they proposed to have a clear species boundary between *C. jejuni* and *C. coli.*

C. jejuni and *C. coli* were isolated from different regions of the gastrointestinal tract of the same pig. To analyse the genetic transfer between these strains, CGH was performed. The DNA isolated from each strain was labelled with Cy3 and it was hybridised with Cy5 labelled control DNA of *C. jejuni* RM1221. The microarray contained differential alleles designed from the genomes of *C. jejuni* NCTC 11168, RM1221 and *C. coli* RM2228. The results were analysed in terms of the patterns of presence and absence of genes based on hybridisation with *C. jejuni* NCTC 11168, RM1221 and *C. coli* RM2228 genes located on the microarray. An analysis of

plasmids present in selected isolates was also undertaken as these are potentially important vectors for the spread of genetic information, determinants that include those for pathogenicity. The materials and methods utilised in this chapter are described in Chapter 2.

5.2. RESULTS

5.2.1. *Campylobacter* spp. excretion from pigs

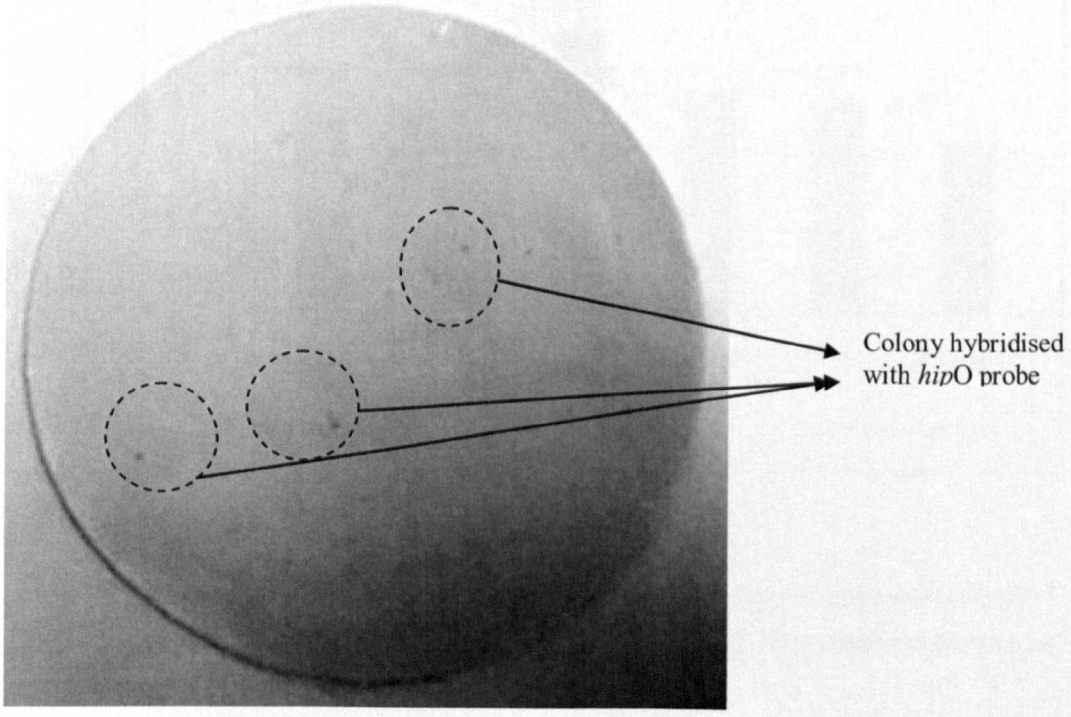
All pigs sampled (n=67), excreted thermophilic campylobacters. All isolates were confirmed as Gram negative, spiral, oxidase positive and catalase positive typical of *Campylobacter* spp. The number of *Campylobacter* present ranged from 10^3 to 10^7 cfu g^{-1} of intestinal contents. Campylobacters could not be isolated from all parts of the intestines of all pigs. Campylobacters were not isolated from the upper and lower intestines of pigs from herds 3, 5 and 6 or from the upper intestine of herds 4 and 7. The absence of campylobacters in the upper intestine correlated with the pigs being reared in an indoor environment except for pigs from herd 8. The *Campylobacter* counts from outdoor reared pigs varied between 10^5 and 10^7 cfu g^{-1} of the intestinal contents whilst the counts recovered from indoor pigs were lower, ranging between 10^3 and 10^5 cfu g^{-1} of the intestinal contents.

5.2.2. Detection of *C. jejuni* in intestinal contents

The intestinal contents from the pigs were screened for the presence of *C. jejuni* and *C. coli* and by colony blot hybridisation method using a *hipO* probe to detect *C. jejuni.* The distribution between the two species was calculated as the ratio between the *C. jejuni* target probe hybrids and the total number of *Campylobacter* isolated from that dilution. The colonies which showed positive results in the colony blot assay were selected, sub cultured and confirmed as being *C. jejuni* by the hippurate test. *C. jejuni* was isolated from 20 of the 67 pigs tested where the majority

of the pigs from which *C. jejuni* were isolated were reared outside. In pigs where *C. jejuni* was present, the species represented a minority of the total *Campylobacter* population ranging from 0.2- 9.9%. Fig. 5.1 presents a colony blot membrane with target probe hybrids of the *hip*O gene. *C. jejuni* was generally confined to the caecal region of the intestine from the indoor reared pigs except for those sampled from herd 8. In outdoor reared pigs, *C. jejuni* were isolated from all the intestinal regions except for the caecal region of pigs from herd 1. The percentage of *C. jejuni* isolated from outdoor reared pigs was between 0.2% and 9.9% of the total *Campylobacter* population. For indoor reared pigs the percentage of *C. jejuni* isolated ranged from 0 to 6.95% of the total *Campylobacter* population. Fig. 5.2 and 5.3 presents the distribution of total campylobacters and the proportion of *C. jejuni* isolated from the caecum, upper and lower intestine of outdoor and indoor reared pigs respectively.

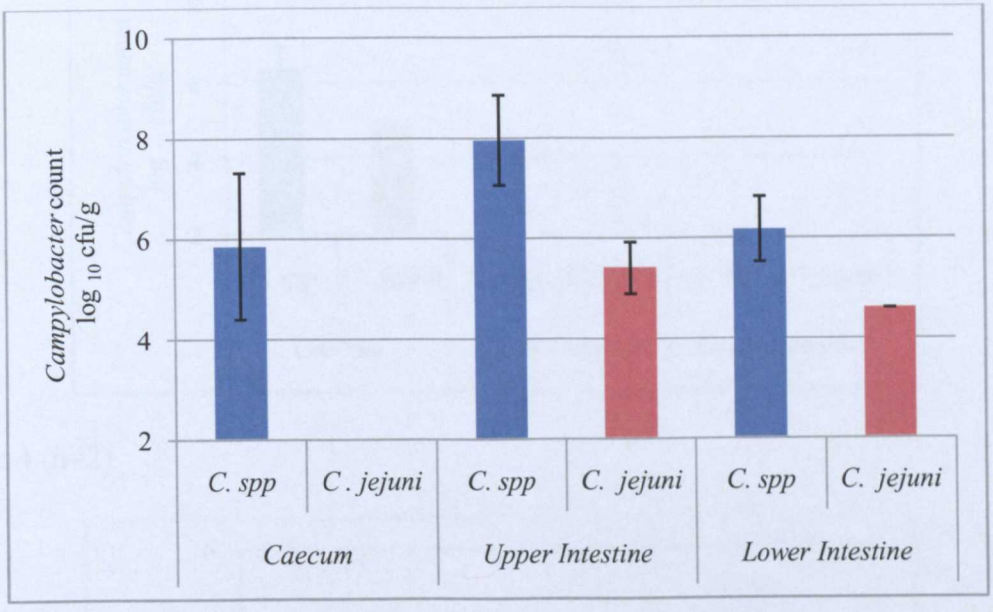
Figure 5.1. Colony blot membrane with target hybridised using *hip*O probe



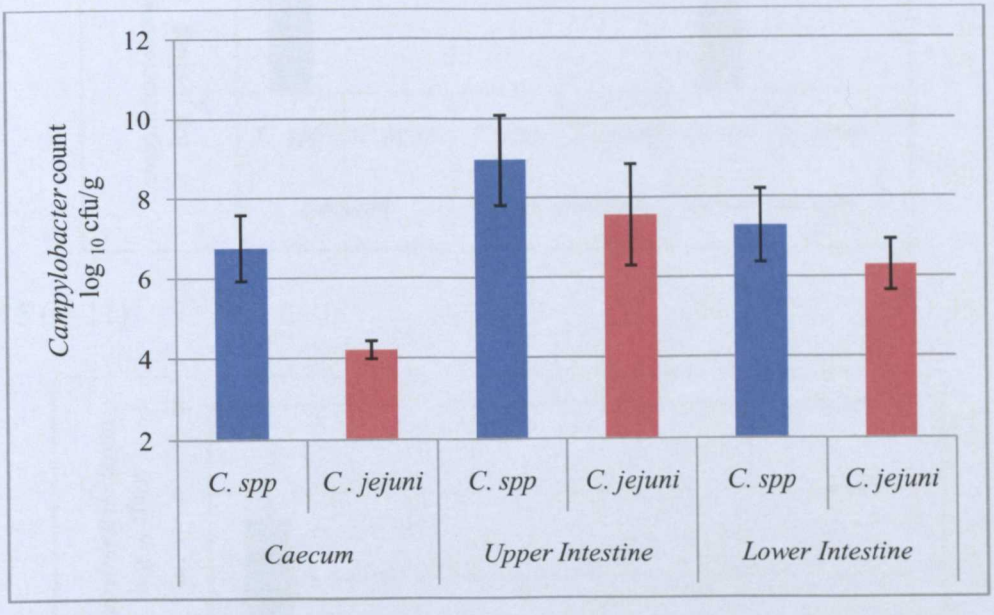
The purple blots in the colony blot membrane shows the presence of *hip*O probe in the colony which represent *C. jejuni*.

Figure 5.2. *Campylobacter* count from different regions of the pig gut in outdoor reared pigs (herds 1 and 2)

Herd: 1 (n=12)



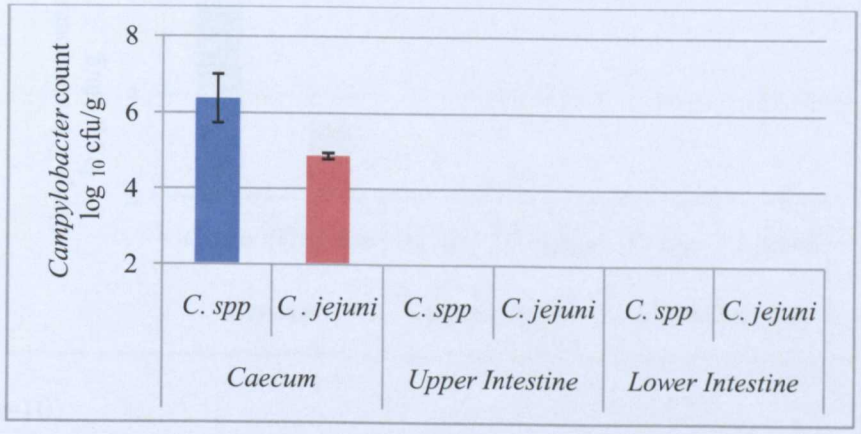
Herd: 2 (n=10).



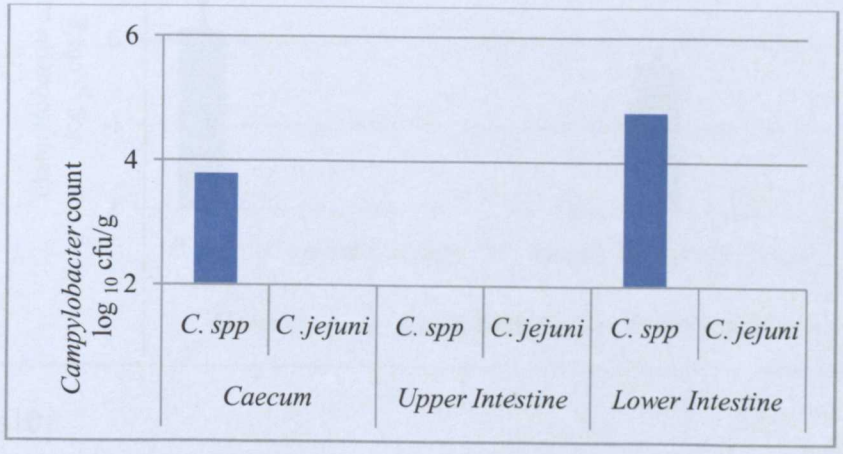
Y axis represent the *Campylobacter* count in log cfu g^{-1} and x axis shows *Camplobacter* sp and *C. jejuni* distributed in caecum, upper intestine and lower intestine. Error bars represent positive and negative error value.

Figure 5.3. *Campylobacter* count from different regions of the pig gut in indoor pigs (herds 3 to 8)

Herd: 3 (n=10)



Herd: 4 (n=2)



Herd: 5 (n=11)

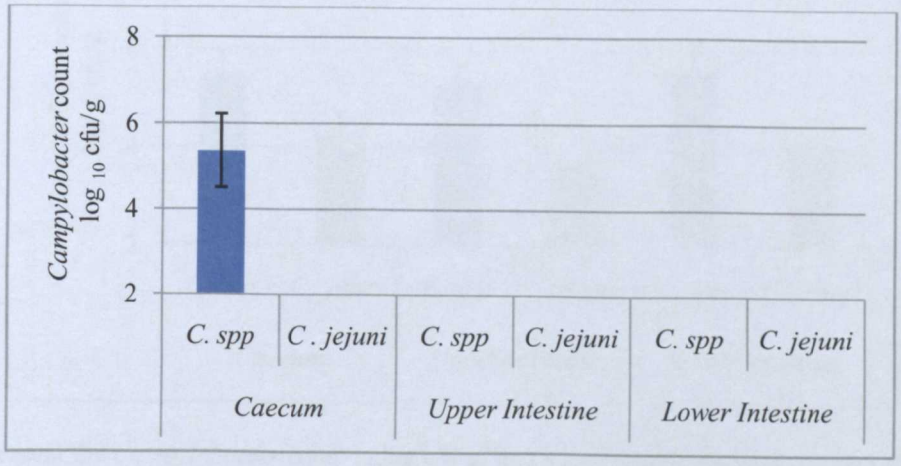
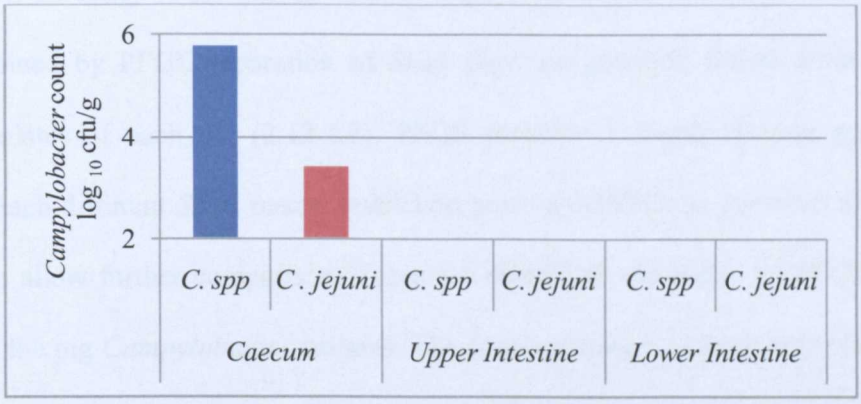
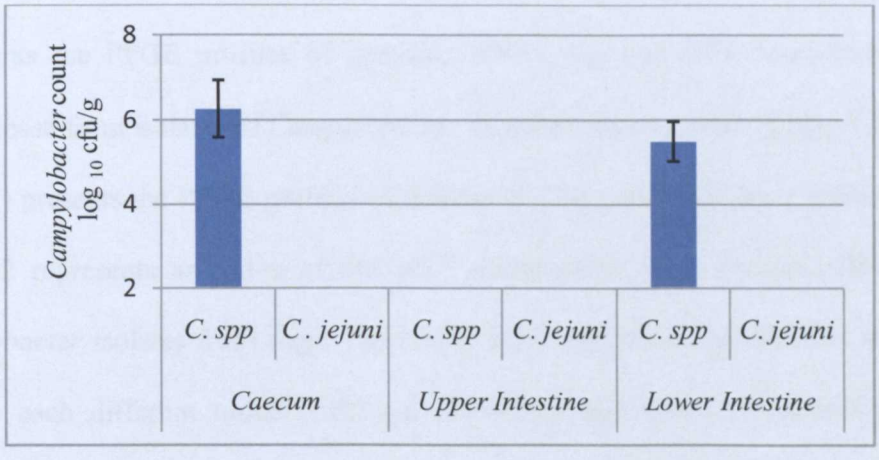


Figure 5.3. (continued)

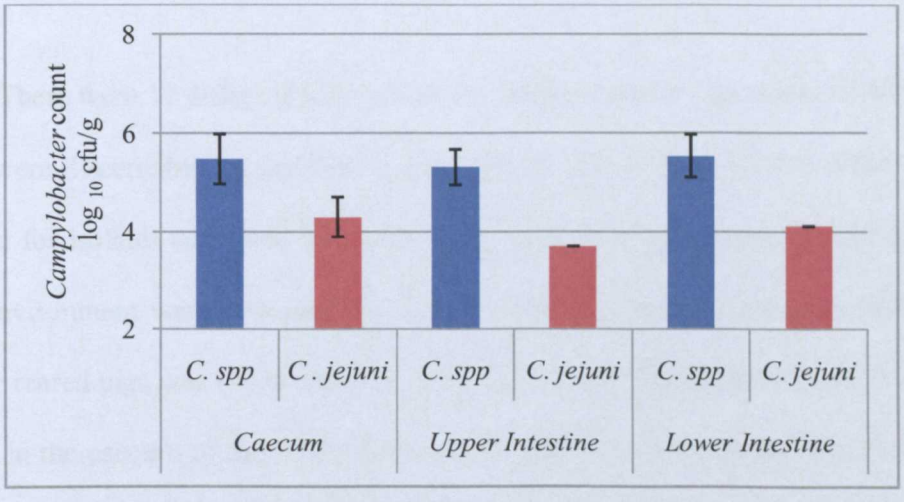
Herd: 6 (n=2).



Herd: 7 (n=10).



Herd: 8 (n=10)



Y axis represent the *Campylobacter* count in log cfu g^{-1} and x axis shows *Camplobacter* sp and *C. jejuni* distributed in caecum, upper intestine and lower intestine. Error bars represent the positive and negative error values.

5.2.3. Pulsed Field Gel Electrophoresis (PFGE) typing

The genetic diversity of the strains isolated from the indoor and outdoor reared pigs was examined by PFGE separation of *Sma*I digested genomic DNAs from single colony isolates of each pig (2.12.2.2). PFGE revealed a highly diverse group of isolates. Each different *Sma*I macro restriction pattern (MRP) was given an arbitrary number to allow further comparison. Table 5.1 represents the index for PFGE lanes related to the pig *Campylobacter* isolates. The *Campylobacter* isolates are referenced with respect to herd number, the intestinal region from which they were isolated (C- caecum, U- Upper intestine, L- Large intestine) and the pig number. Fig. 5.4 (A and B) presents the PFGE profiles of genomic DNAs digested with *Sma*I restriction endonuclease from isolates of *Campylobacter* from the outdoor reared pigs while Fig. 5.5 (A-E) presents the PFGE profiles of isolates of *Campylobacter* from indoor pigs. Table 5.2 represents an index of the MRP designations from genomic DNAs of *Campylobacter* isolates from pigs. The PFGE MRP represents the arbitrary number given to each different pulsed field gel electrophoresis macro restriction pattern identified when the *Campylobacter* genomic DNA was digested with *Sma*I restriction enzyme.

There were 17 different MRPs from the outdoor reared pigs while 21 different MRPs were discernable for isolates recovered from indoor pigs. Similar MRPs were obtained for isolates recovered from different pigs indicating that pigs inhabiting the same environment were colonised by related isolates. The most common MRP for outdoor reared pigs was P5, which was shared by two different pigs, of which it was present in the caecum of the 1st pig and caecum, upper and lower intestine of the 2nd pig. Generally particular genotypes were found in all parts of the intestine where campylobacters were isolated rather than being confined to one area. The most

common MRPs for indoor pigs were P24, P25, P26 and P35 which was shared by *Campylobacter* strains isolated from all three anatomical regions.

Table 5.1. Index for PFGE lanes and to *Campylobacter* isolates from pigs.

Lane No	Colony No	Lane No	Colony No	Lane No	Colony No	Lane No	Colony No	Lane No	Colony No
1	50 kb Marker	21	T2U4	41	T3C8	61	T5C7	81	50 kb Marker
2	T1C1	22	T2C5	42	T3C9	62	T5C8	82	T8U101*
3	T1L1	23	T2L5	43	T2C10	63	T5C9	83	T8C101
4	T1C2*	24	T2C6	44	50 kb Marker	64	T5C101	84	T8L101
5	T1C3	25	T2U6	45	T4C11	65	T5C102	85	50 kb Marker
6	T1C4*	26	T2C7	46	T4C12	66	T5C111		
7	T1L4	27	T2C8	47	T4C13	67	T5C112		
8	T1C5*	28	T2C9	48	T4C14	68	50 kb Marker		
9	T1C6	29	T2C10	49	T4C21	69	T6C1		
10	T1U6	30	T3C1	50	T4C22	70	T6C2		
11	T1L6	31	T3C2	51	T4C33	71	T7C1		
12	T1C7	32	T3C3*	52	T4C44	72	T7C2		
13	T1C8	33	T3C32*	53	50 kb Marker	73	T7C3		
14	T1C9	34	T3C4*	54	T5C1	74	T7C4		
15	50 kb Marker	35	T3C5*	55	T5C2	75	T7C5		
16	T2C1	36	T3C61	56	T5C3	76	T7C6		
17	T2C2	37	T3C62	57	T5C41	77	T7C7		
18	T2U2	38	T3C71	58	T5C42	78	T7C8		
19	T2C3	39	T3C72	59	T5C5	79	T7C9		
20	T2C4	40	T3U81	60	T5C6	80	T7C10		

Lane number represents the lanes in fig 5.4 and fig. 5.5. The *Campylobacter* colony number are referenced with respect to herd number, the intestinal region from which they were isolated (C- caecum, U- Upper intestine, L- Large intestine) and the pig number. *C. jejuni* were represented by *.

Figure 5.4. PFGE profiles of genomic DNAs digested with *Sma*I restriction endonuclease from isolates of *Campylobacter* from the outdoor reared pigs

Fig. 5.4 (A)

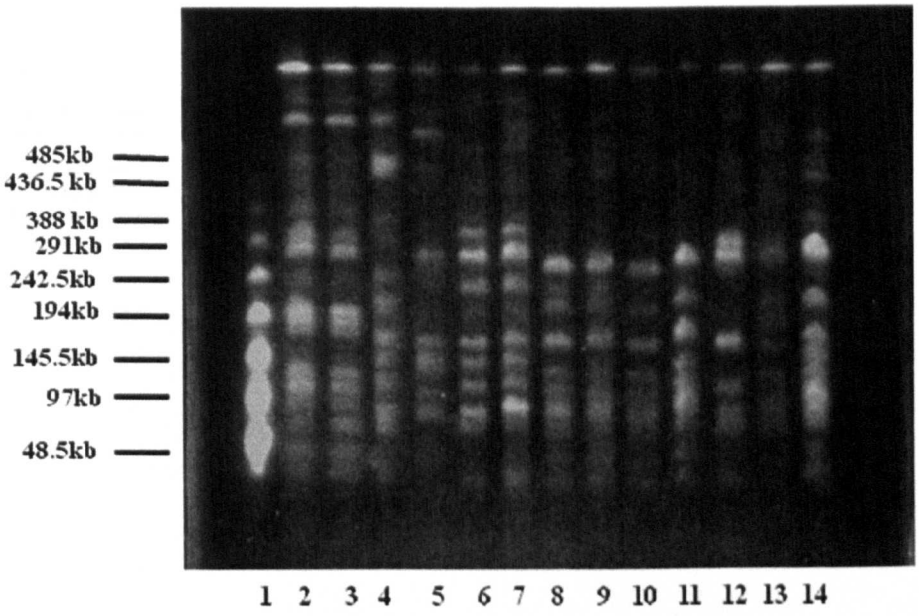
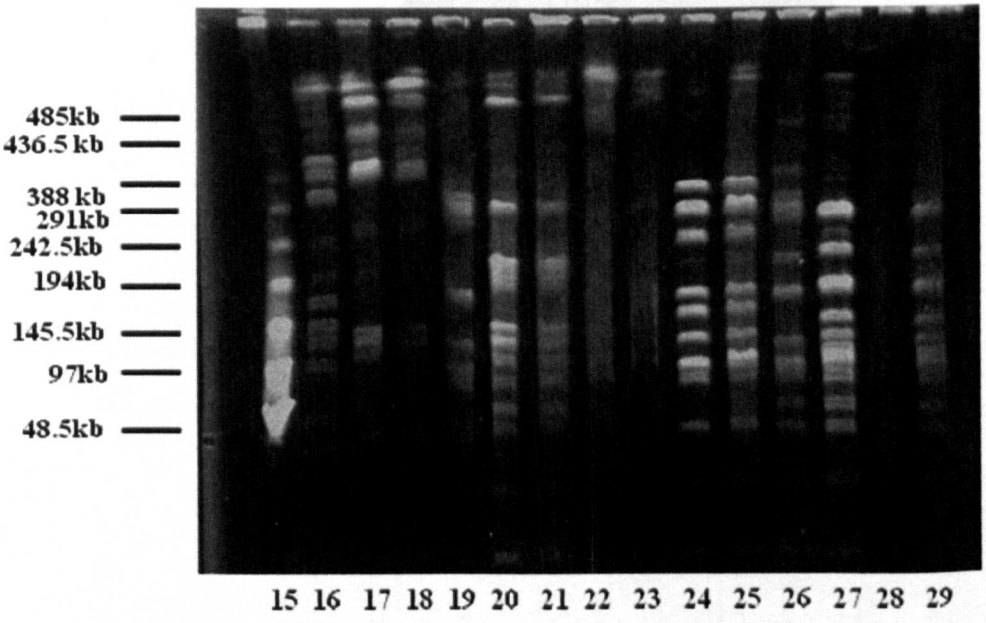


Fig. 5.4 (B)



(A) Lanes 1 to 14, and (B) Lanes 15 to 29. Y axis denotes the molecular size of the 50-1000 kb PFGE marker. Image obtained from BioRad GelDoc EQ.

Figure 5.5. PFGE profiles of genomic DNAs digested with *Sma*I restriction endonuclease from isolates of *Campylobacter* from indoor reared pigs

Fig. 5.5 (A)

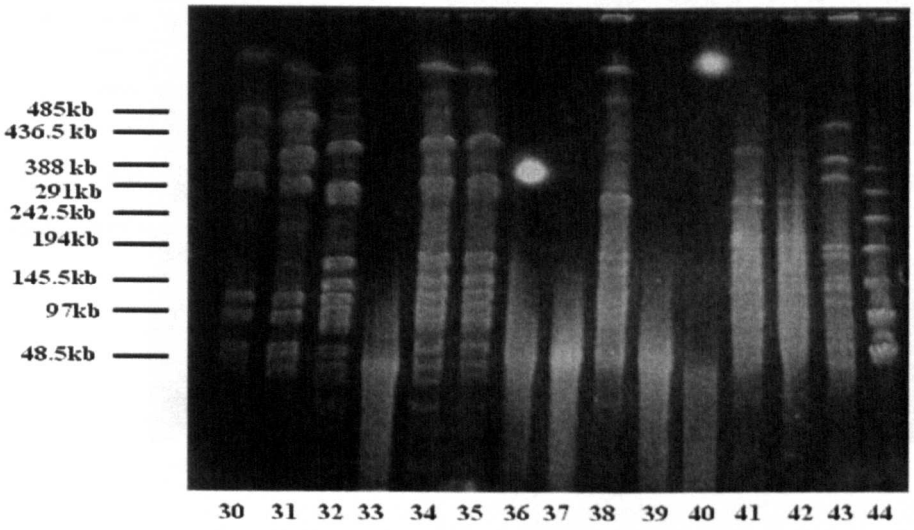


Fig. 5.5 (B)

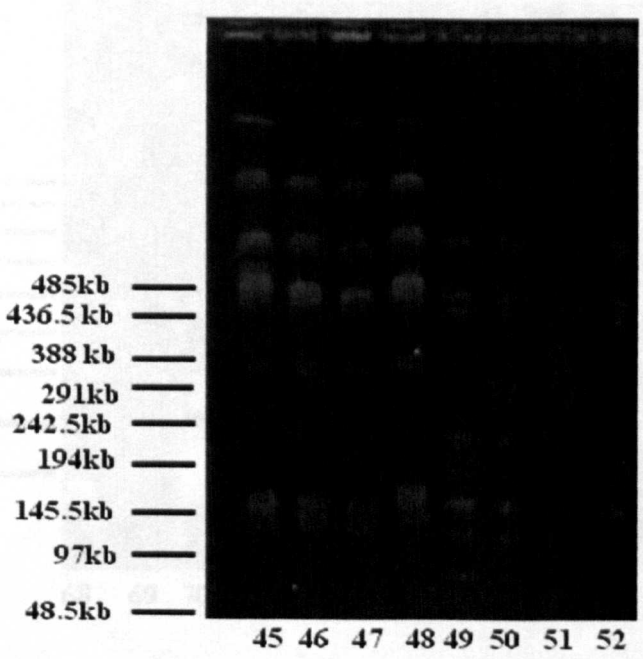


Fig. 5.5 (C)

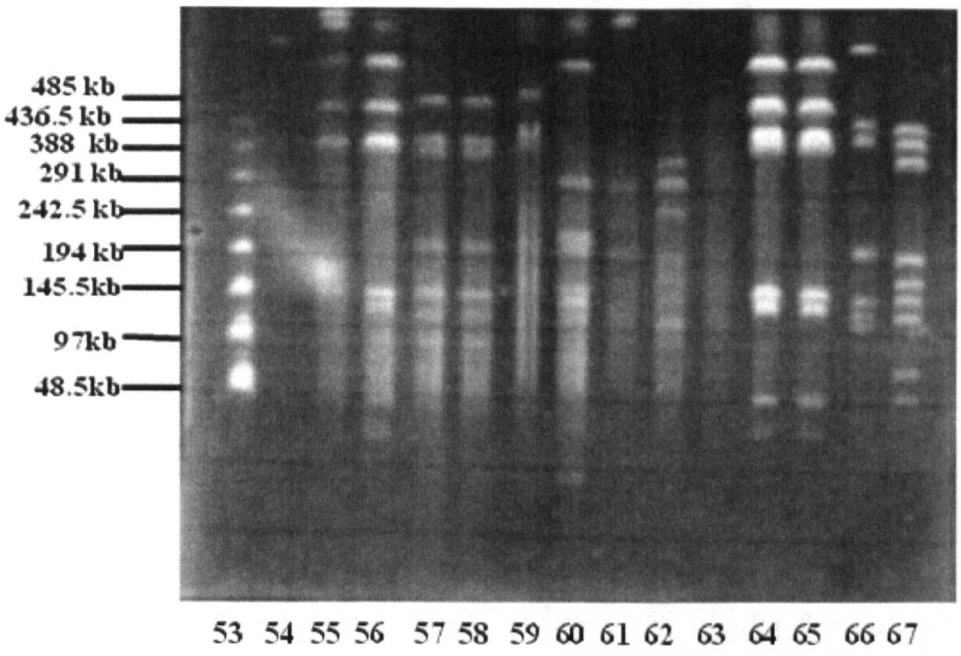


Fig. 5.5 (D)

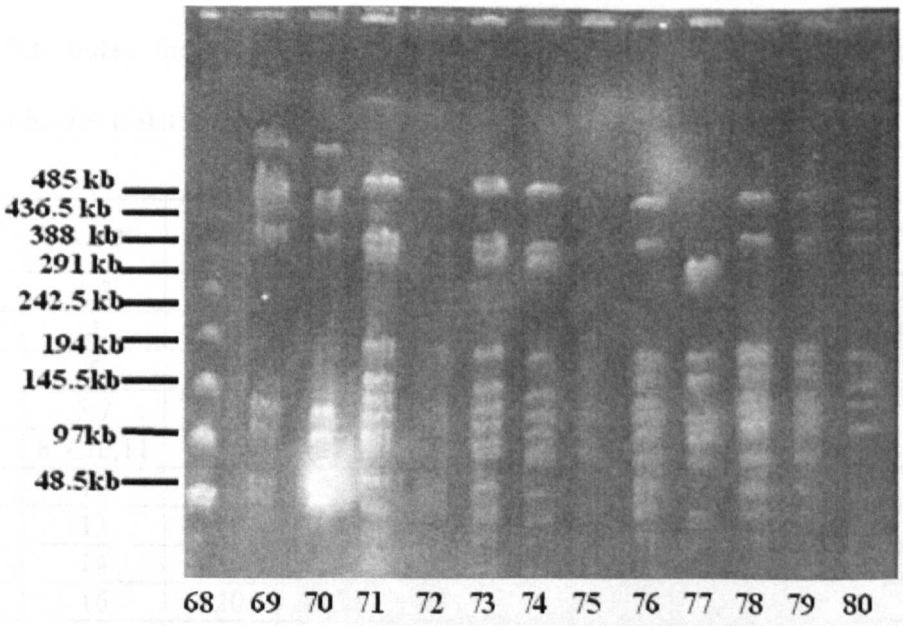
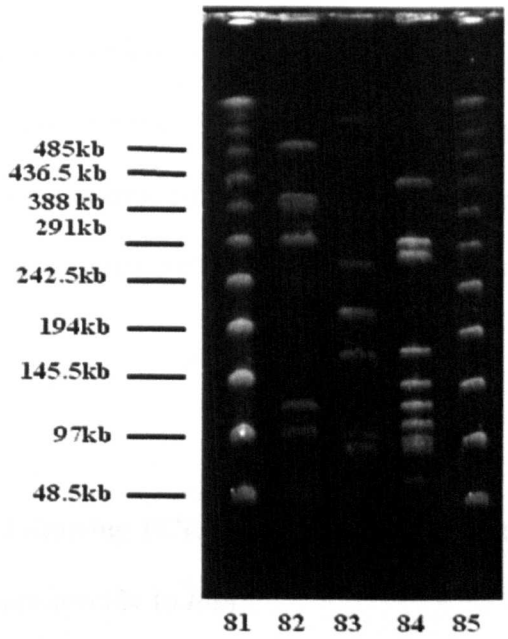


Fig. 5.5 (E)



(A) Lanes 30-44, (B) Lanes 45-52, (C) Lanes 53-67, (D) Lanes 68-80, (E) Lanes 81-85 . Y axis denotes the molecular size of the 50-1000 kb PFGE marker. Image obtained from BioRad GelDoc EQ.

Table 5.2. Index for PFGE and the MRP designations from genomic DNAs of *Campylobacter* isolates from pigs.

MRP	Lane No	MRP	Lane No	MRP	Lane No	MRP	Lane No
P1	2,3	P12	20,21	P23	43	P34	69,70
P2	4	P13	23	P24	45,46,47,48	P35	71,72,73,74
P3	5	P14	24,25	P25	49,50,51,52	P36	76,78,79
P4	6,7	P15	26	P26	55,56,64,65	P37	77
P5	8,9,10,11	P16	27	P27	57,58	P38	80
P6	12	P17	29	P28	59	P39	81
P7	13	P18	30,31	P29	60	P40	82
P8	14	P19	32	P30	61	P41	83
P9	16	P20	34,35	P31	62		
P10	17,18	P21	38	P32	66		
P11	19	P22	41,42	P33	67		

The PFGE Macro Restriction Profile (MRP) is an arbitrary number given to each different pulsed field gel electrophoresis macro restriction pattern identified when genomic DNA was digested with *SmaI* restriction enzyme.

5.2.4. PCR identification using the *hip*O gene

In order to confirm the colony hybridisation results, a PCR assay for the *hip*O gene was performed (2.5.3). Genomic DNAs of *C. jejuni* isolates gave amplification products from the *hip*O gene as anticipated. Figure 5.6 (A and B) shows *hipO* positive and negative isolates, the positive isolates produce a DNA fragment of 750 bp as indicated.

Figure 5.6. Agarose gel showing PCR products obtained from genomic DNAs of pig isolates using primers specific to *hip*O

Fig. 5.6(A)

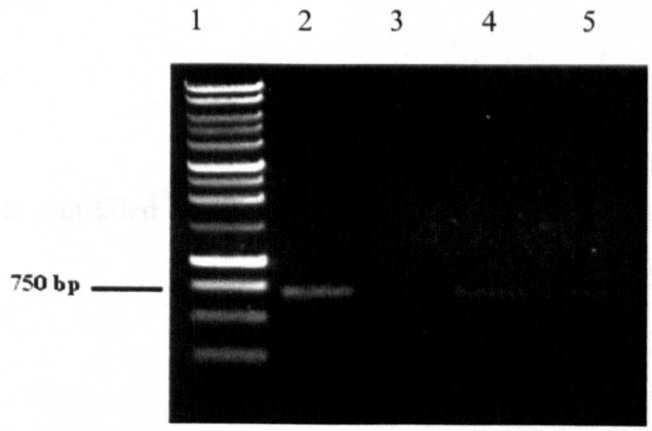
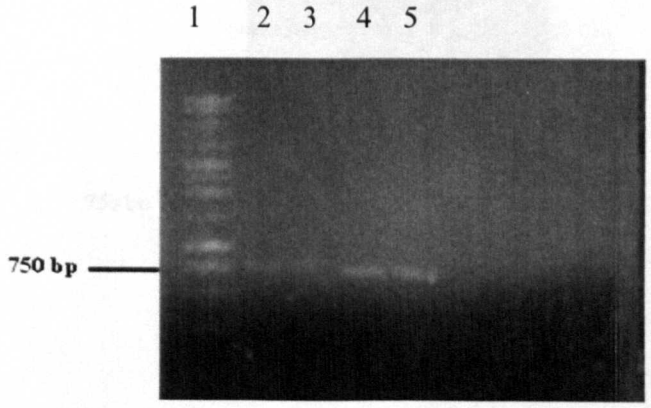


Fig. 5.6(B)



X axis represent lanes (A) Lane 1. 1kb marker, 2: T1C2, 3: T1C7, 4: T1L4, 5: T1C5, and (B) Lane 1: 1 kb marker, 2: T3C3, 3: T3C32, 4: T3C4, 5: T3C5 and y axis shows the Amplicon size of *hip*O primers which is 750 bp.

5.2.5. Genomic analysis of C101, U101 and L101

5.2.5.1. PCR analysis for Hippurate and Aspartokinase genes

To analyse the genetic diversity of the different isolates of *Campylobacter* from the same pig, three *Campylobacter* strains from a pig in herd 8 were selected (C101 isolated from caecum, U101 isolated from the upper intestine and L101 isolated from the large intestine) and comparative genomic DNA hybridisation was carried using a DNA microarray. To confirm the species designation of each isolate PCR analyses were performed for hippurate and aspartokinase genes. C101 and L101 were *C. coli* while U101 was *C. jejuni* (Fig. 5.7 A and B). The MRP of each strain is given in Fig. 5.5 (D).

Figure 5.7. Bands amplified using Hippurate and Aspartokinase genes in C101, U101 and L101

Fig. 5.7 (A)

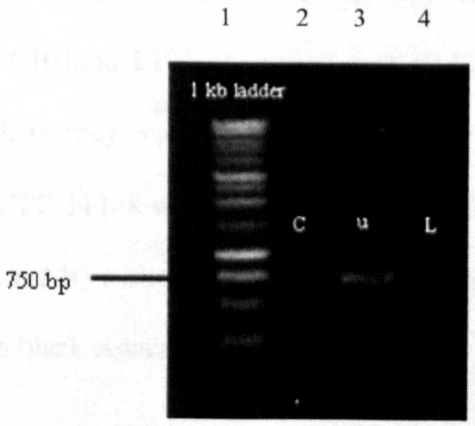
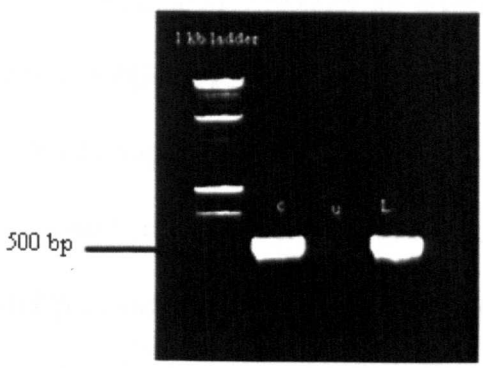


Fig. 5.7 (B)



(A) *hip*O primers for *C. jejuni* isolates encoding 750 bp, and (B) ASK CC18F and ASK CC519 primers encoding 500 bp for *C. coli* isolates. Lane representations are: Lane 1: 1 kb ladder, Lane2: *Campylobacter* strain from caecal sample (C101), Lane3: *Campylobacter* strain from upper intestine (U101) and Lane: 4: *Campylobacter* strain from lower intestine (L101).

5.2.5.2. Sequencing of the 16s rRNA gene locus and phylogenetic analysis

The sequences of the 16s rRNA genes (2.11.1.2) were determined for C. *coli* C101, L101 and *C. jejuni* U101 to confirm the species designation of these strains. The sequence results were analysed using ClustalW along with the control sequences of NCTC 11168 and RM2228 (2.14). Aligning the 16S rRNA gene sequences of NCTC 11168 and RM2228 shows 3 nucleotide base pairs difference between them (see appendix). *C. coli* C101and L101 align to *C. coli* RM2228 except for a single nucleotide base pair where they match to *C. jejuni* NCTC 11168. *C. jejuni* U101 aligns with *C. jejuni* NCTC 11168 without any nucleotide mismatch. The results are presented in Fig. 5.8 (A and B) with the mismatches to the type strain 16S rRNA gene sequences highlighted in black colour.

When the 16S ribosomal RNA gene sequences of *C. jejuni* NCTC 11168 and *C. coli* RM2228 were aligned to each other there were three differences in nucleotide bases. However when *C. jejuni* RM1221 and *C. coli* RM2228 16S ribosomal RNA

gene sequences were aligned, only a single difference in the nucleotide bases was evident (data in appendix). For *C. coli* RM2228 an adenine base is replaced by a guanine in the sequence 'CGAAGATACGC' found in *C. jejuni* RM1221.Both the *C. coli* strains analysed here (C101 and L101) also had an adenine at this position, which is more typical of the *C. jejuni* strains. *C. jejuni* U101 did not deviate from the *C. jejuni* type sequence, and was therefore a typical *C. jejuni* with regard to its 16S ribosomal gene sequence.

Figure 5.8. Sequence alignment of 16S rRNA coded by *C. jejuni* NCTC 11168 and *C. coli* RM2228 with (A) *C. coli* C101, and (B) *C. coli* L101

Fig. 5.8 (A)

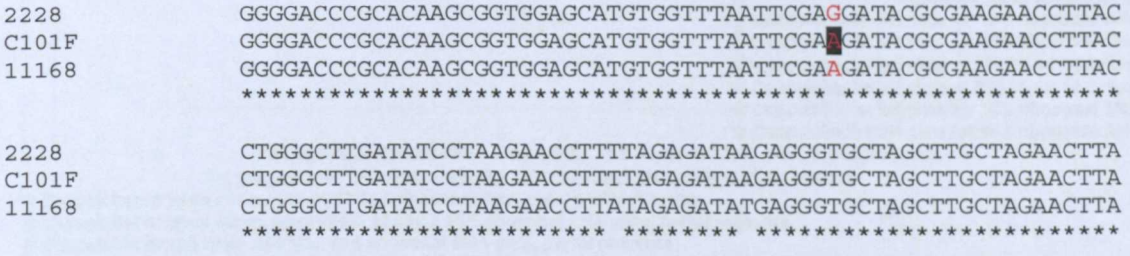


Fig. 5.8 (B)

The base pair mismatch is highlighted black in (A) and (B) and the corresponding base pair in *C. jejuni* NCTC 11168 and *C. coli* RM2228 are shown in red.

5.2.5.3. Phylogenetic analysis

Phylogenetic analyses of the three selected *Campylobacter* isolates are presented as a tree structure based of the 16S rRNA gene sequences in Fig. 5.9 (A-C). The *C. coli* C101 isolate was positioned between two *C. jejuni* isolates (*C. jejuni* TGH9011 and *C. jejuni* subsp. *jejuni* strain 958204). The *C. coli* isolate L101 was positioned between *C. coli* strain 869304 and *C. jejuni* subsp. *jejuni* strain 1167-3195. The position of *C. jejuni* U101 lies between *C. jejuni* subsp. *jejuni* strain 1160-2195 and *C. jejuni* subsp. *jejuni* strain B991224.

Figure 5.9. Phylogenetic analysis of *Campylobacter* strains

Fig. 5.9 (A)

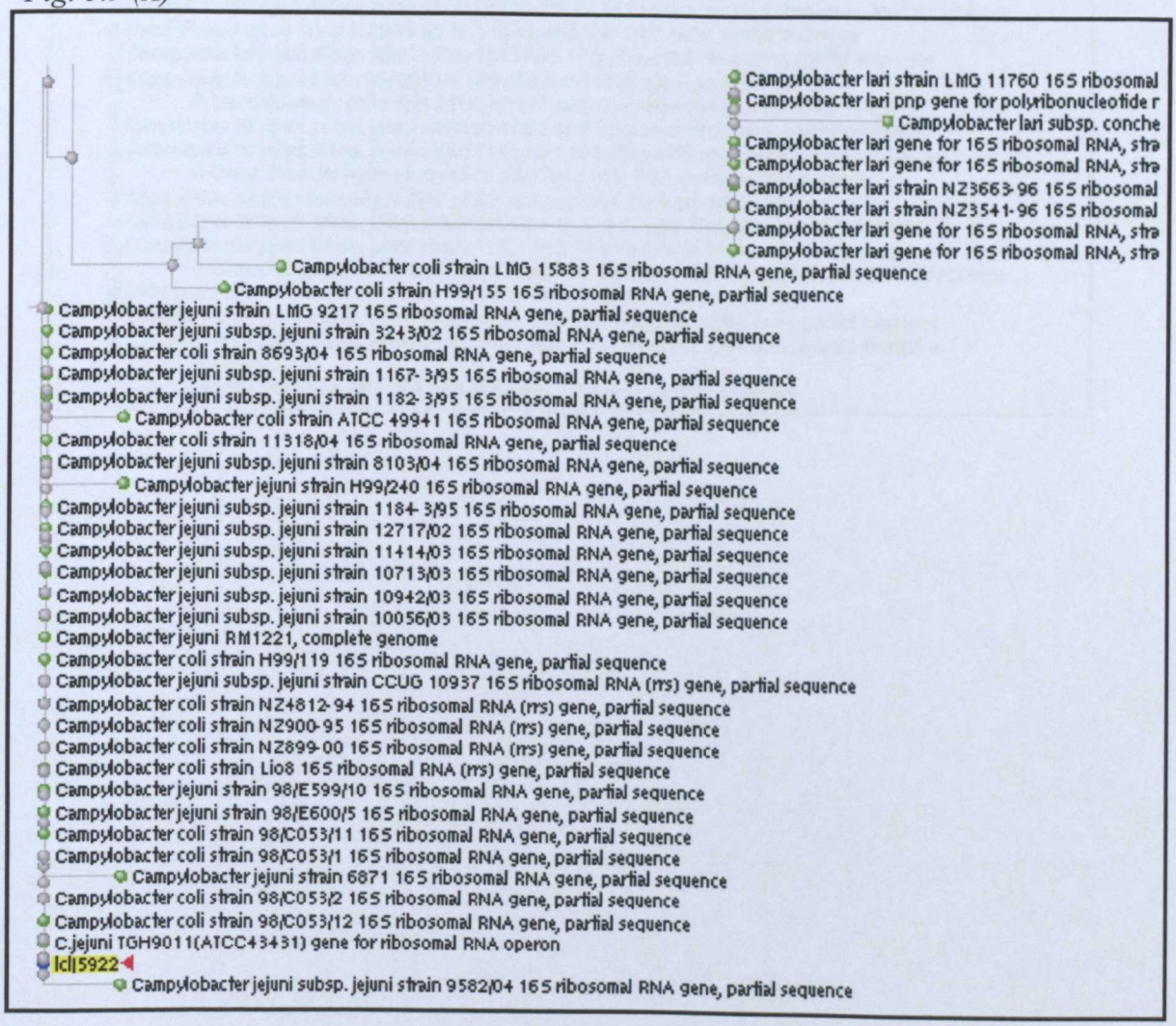


Fig. 5.9 (B)

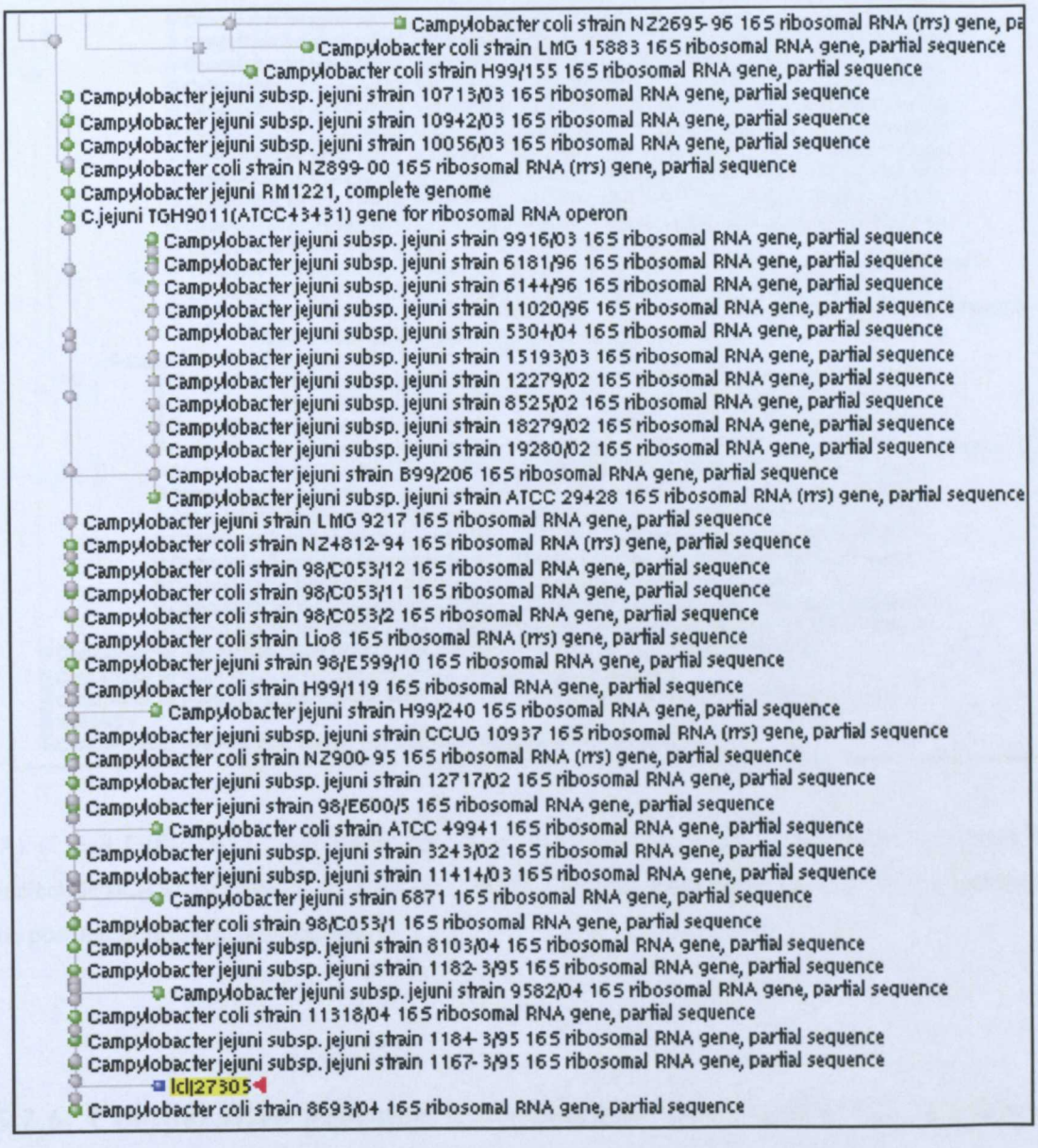
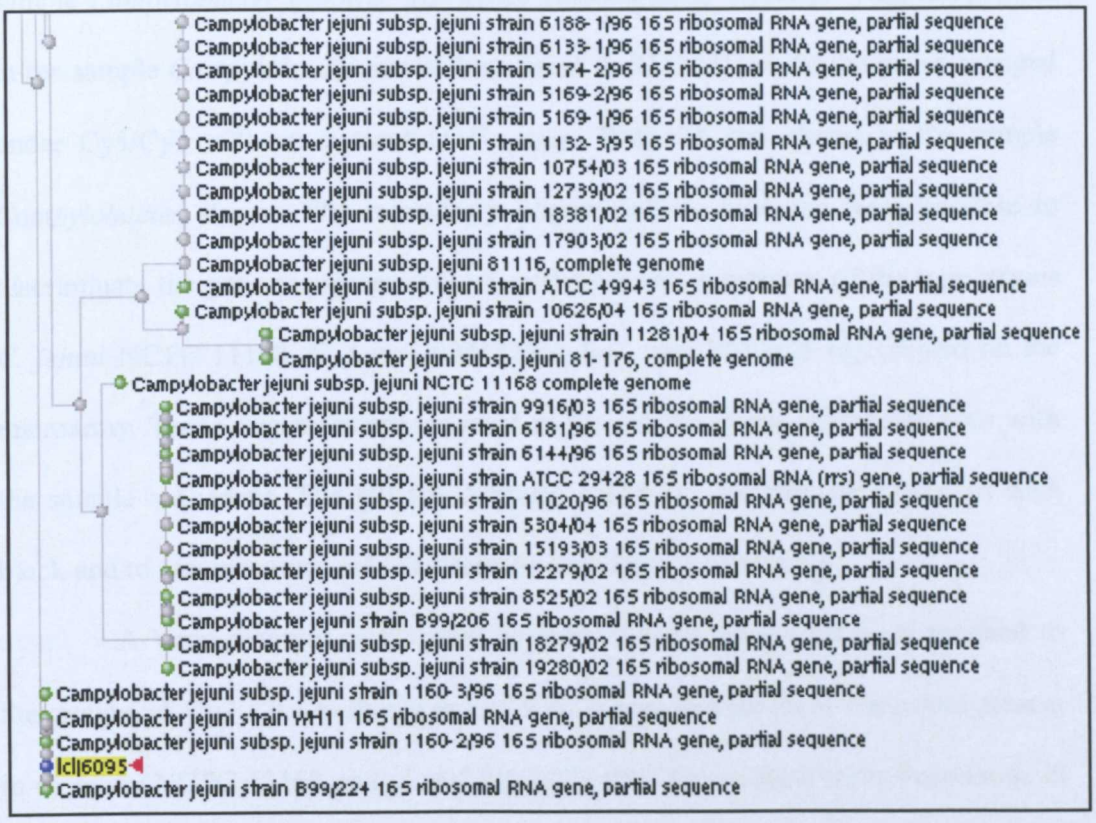

Campylobacter coli strain NZ2695-96 16S ribosomal RNA (rrs) gene, pa
Campylobacter coli strain LMG 15883 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain H99/155 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 10713/03 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 10942/03 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 10056/03 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain NZ899-00 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter jejuni RM1221, complete genome
C.jejuni TGH9011(ATCC43431) gene for ribosomal RNA operon
Campylobacter jejuni subsp. jejuni strain 9916/03 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 6181/96 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 6144/96 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 11020/96 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 5304/04 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 15193/03 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 12279/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 8525/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 18279/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 19280/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni strain B99/206 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain ATCC 29428 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter jejuni strain LMG 9217 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain NZ4812-94 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter coli strain 98/C053/12 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain 98/C053/11 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain 98/C053/2 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain Lio8 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter jejuni strain 98/E599/10 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain H99/119 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni strain H99/240 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain CCUG 10937 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter coli strain NZ900-95 16S ribosomal RNA (rrs) gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 12717/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni strain 98/E600/5 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain ATCC 49941 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 3243/02 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 11414/03 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni strain 6871 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain 98/C053/1 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 8103/04 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 1182-3/95 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 9582/04 16S ribosomal RNA gene, partial sequence
Campylobacter coli strain 11318/04 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 1184-3/95 16S ribosomal RNA gene, partial sequence
Campylobacter jejuni subsp. jejuni strain 1167-3/95 16S ribosomal RNA gene, partial sequence
lcl|27305
Campylobacter coli strain 8693/04 16S ribosomal RNA gene, partial sequence

Fig. 5.9 (C)



(A) *C. coli* C101, (B) *C. coli* L101, and (C) *C. jejuni* U101, based on 16S rRNA generated by nucleotide BLAST pairwise alignments from NCBI. The highlighted region in these figures represents the position of the respective strains.

5.2.6. Comparative genomic hybridisation of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 with respect to *C. jejuni* RM 1221

The diversity of the whole genomes of *C. jejuni* U101, *C. coli* C101 and *C. coli* L101 isolates were compared by comparative genomic hybridisation (CGH) with respect to *C. jejuni* RM1221. The analysis was performed based on the available sequence data from the genome sequenced strains *C. jejuni* NCTC 11168, RM1221 and *C. coli* RM 2228. The presence or absence of each gene from these three strains was determined on the basis of the Cy5 to Cy3 ratio, of which Cy5 represents hybridisation with the genome of *C. jejuni* RM1221 and Cy3 hybridisation with the

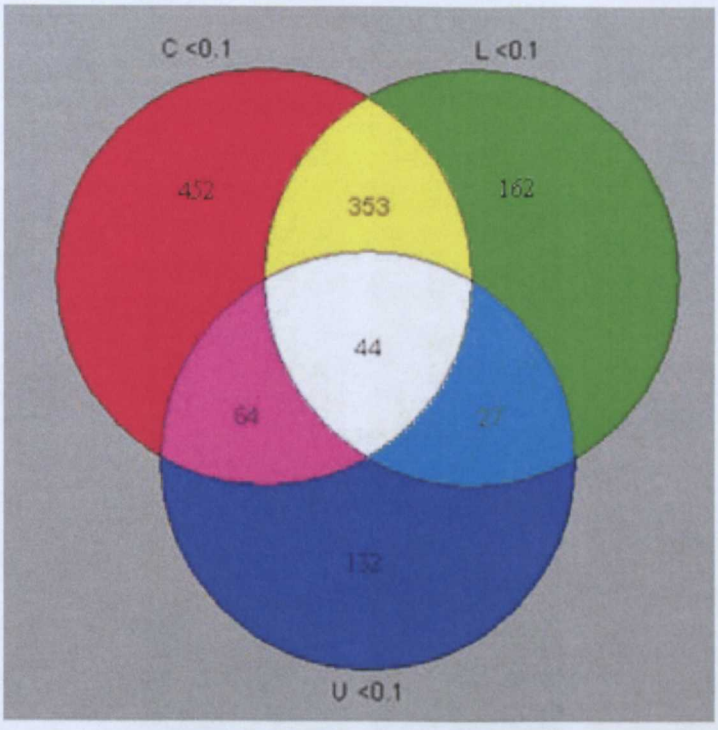
sample *Campylobacter* genome. The genes grouped under Cy5/Cy3 < 0.1 are present in the sample *Campylobacter* strains but absent in RM1221 whilst the genes grouped under Cy5/Cy3 >20 are present in *C. jejuni* RM1221 and absent in the sample *Campylobacter* strains. The microarray oligonucletides designed were possible to discriminate the gene sequences present in the genome sequences of the type strains *C. jejuni* NCTC 11168, *C. jejuni* RM1221 and *C. coli* RM2228 represented on the microarray. These sequences are recorded in the gal file of the microarray slide with the sample sequences. The gal file contains entries to describe the layout of each block and to assign names and identifiers to each feature of an array.

A Venn diagram of the total number of hybridising sequences ascribed to the group Cy5/Cy3 < 0.1 is shown in Fig. 5.10. These include those sequences present in *C. jejuni* NCTC 11168 and *C. coli* RM2228 that discriminate them from those in RM1221. *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 share 44 genes with each other that are absent in RM1221. The *C. coli* strains C101 and *C. coli* L101 shared 353 of this class of genes whilst *C. coli* C101 and *C. jejuni* U101 shared 64 genes not present in *C. coli* L101. Conversely *C. coli* L101 and C. *jejuni* U101 shared 27 hybridising genes not present in *C. coli* C101 between them. *C. coli* C101 produced 452 hybridisation signals which it did not share with any of the other isolates, whereas *C. coli* L101 and *C. jejuni* U101 hybridised to 162 and 132 probes respectively that were specific to these isolates. Fig. 5.11 presents a dendrogram view of the clustering map which shows a genomic comparison of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 using GeneSpring. The map includes all the *C. jejuni* NCTC 11168, RM1221 and *C. coli* RM2228 gene sequences present in the *Campylobacter* strains on which CGH was performed. The blue line represents the presence of a gene in the sample strain whilst the red represents its absence in sample strains but presence in the

control *C. jejuni* RM1221. The yellow line represents the presence of genes in both control and sample strains. Fig. 5.12 shows the dendrogram view of the genome comparison of *C. coli* C101, *C. coli* L101 and *C. jejuni* U101 with respect to (A) *C. jejuni* NCTC 11168 probes, (B) RM1221, and (C) *C. coli* RM2228. The colour comparison was similar to that in Fig. 5.11. Unique genes were those genes which were represented by a single locus tag. Tables 5.3 to 5.8 lists the hybridising genes which were unique and showed sequence similarity to either NCTC 11168 and RM2228 from the selected *Campylobacter* strains with Cy5/Cy3< 0.1.

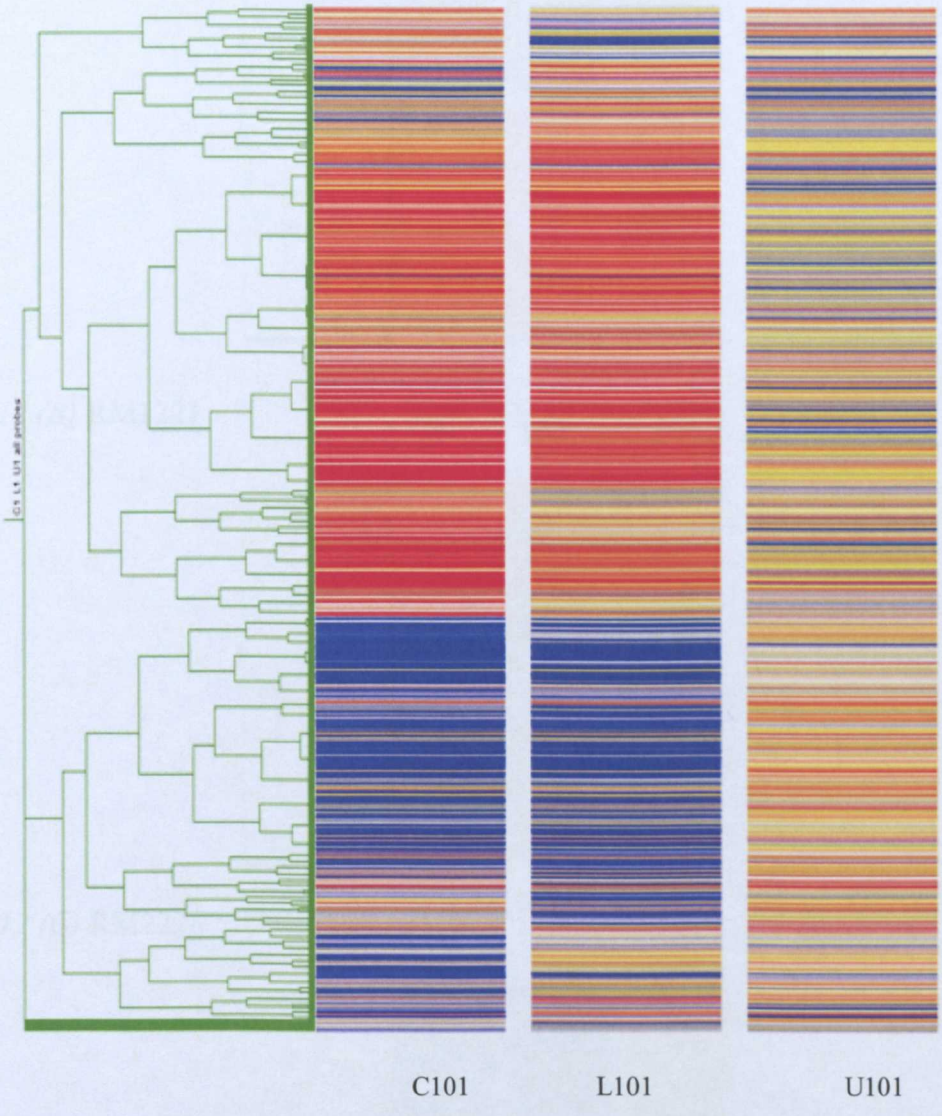
An overview of the functional classification of the hybridising genes identified in *C. coli* C101, L101 and *C. jejuni* U101 that showed sequence differences with the genes in *C. jejuni* RM1221 was presented in Fig. 5.13. Most of the genes were classified as conserved hypothetical proteins, hypothetical proteins and plasmid related functions and therefore were grouped as 'Others'. Figure 5.14 represents the venn diagram which shows the distribution of *C. jejuni* NCTC 11168, RM1221 and RM2228 genes which were absent in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101.The NCTC 11168 and *C. coli* RM2228 genes present in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 are listed in the appendix 2

Figure 5.10. Venn diagram showing the cumulative data from microarray based CGH surveys



Venn diagram obtained from Genespring GX 7.3. Hybridising gene sequences of *C. coli* C101 (C), *C. jejuni* U101 (U) and *C. coli* L101 (L) where Cy5/Cy3<0.1. These genes are present in the pig *Campylobacter* sample genomes but are absent in RM1221

Figure 5.11. Dendrogram view of the genome comparison of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 using DNA microarray



The gene sequences were compared using probes of NCTC 11168, RM1221 and RM2228. Red represents the presence of hybridising genes in the control strain *C. jejuni* RM1221, blue represents the presence of hybridising genes in the sample strain, yellow represents the presence of genes hybridising in both control and sample, while white represents hybridisation to neither and therefore absence. Image obtained from Genespring GX 7.3.

Figure 5.12. Dendrogram view of the genome comparison of *C. coli* C101 (lane 1), *C. coli* L101 (lane 2) and *C. jejuni* U101 (lane 3)

Fig. 5.12 (A) NCTC 11168

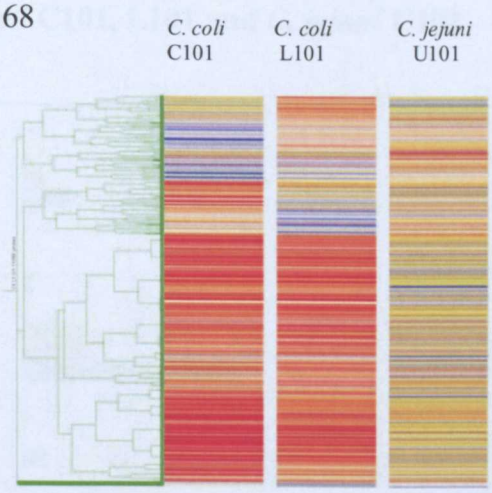


Fig. 5.12 (B) RM1221

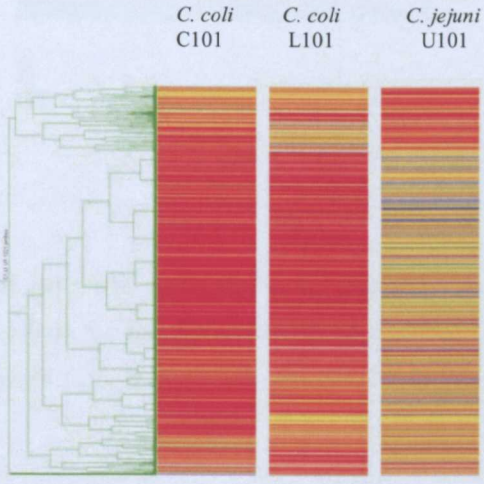
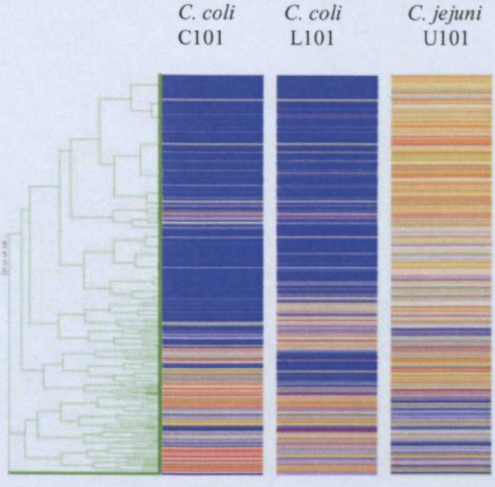
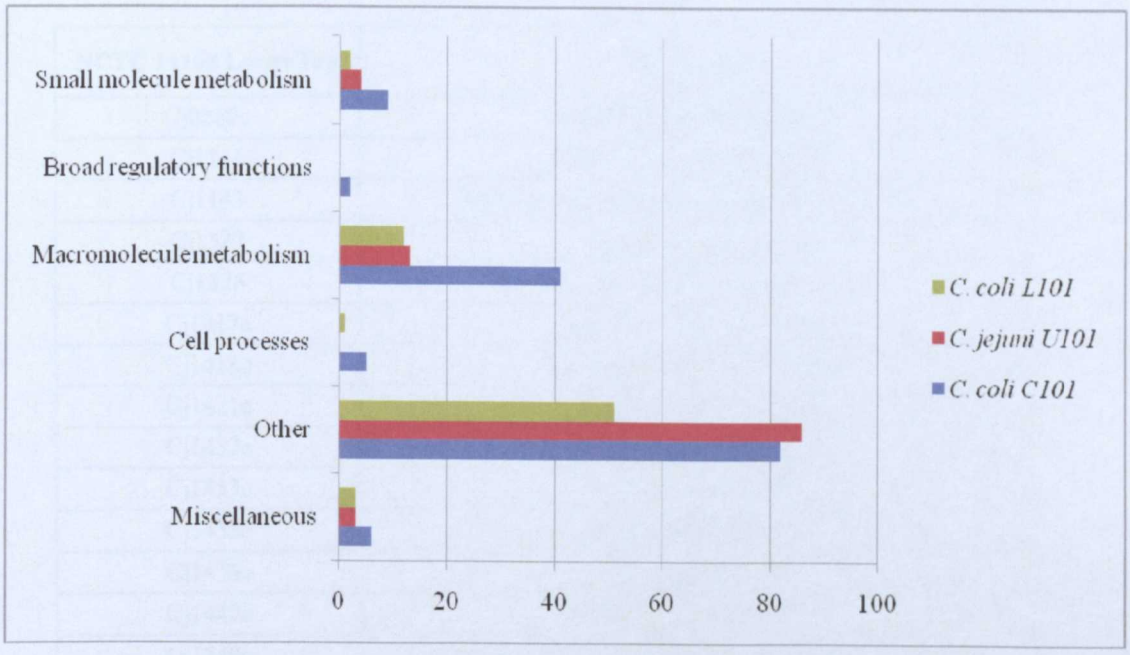


Fig. 5.12 (C) RM2228



Comparisons are with respect to (A) NCTC 11168 probes, (B) RM1221 probes, and (C) RM2228 probes. Red represents the presence of hybridising genes in the control strain *C. jejuni* RM1221, blue represents the presence of hybridising genes in the sample strain, yellow represents the presence of genes hybridising in both control and sample, while white represents hybridisation to neither and therefore absence. Images obtained from Genespring GX 7.3.

Figure 5.13. Overview of the functional classification of the unique hybridising genes identified in *C. coli* C101, L101 and *C. jejuni* U101



x axis represent the number of genes and y axis shows the functional classification of *Campylobacter* genes. The classification is based on the functional classification of the *Campylobacter* genes given by Sanger Institute. Green represents *C. coli* L101, red represents *C. jejuni* U101 and blue represents *C. coli* C101.

(http://www.sanger.ac.uk/Projects/C_jejuni/Cj_gene_list_hierarchical.html).

Table 5.3. *C. jejuni* NCTC 11168 genes shared with *C. coli* C101 that are not present in RM1221

NCTC 11168 Locus Tag	Description
Cj0260c	small hydrophobic protein
Cj1141	sialic acid synthase (*neu*B1)
Cj1143	acylneuraminate cytidylyltransferase (*neu*A1)
Cj1322	hypothetical protein
Cj1326	hypothetical protein
Cj1417c	putative amidotransferase
Cj1418c	hypothtical protein Cj1418c
Cj1421c	putative sugar transferase
Cj1432c	putative sugar transferase
Cj1433c	hypothetical protein
Cj1435c	putative phosphatase
Cj1436c	aminotransferase
Cj1442c	putative sugar transferase
Cj1549c	putative type I restriction enzyme R protein (*hsd*R)

Table 5.4. *C. coli* RM2228 genes shared with *C. coli* C101 that are not present in RM1221

RM 2228 locus tag	Description	RM 2228 locus tag	Description	RM 2228 locus tag	Description
CCO0026	YeeE/YedE family protein family	CCO0536	citE, putative	CCO1514	hypothetical protein
CCO0035	Tat (twin-arginine translocation) pathway signal	CCO0601	conserved hypothetical protein	CCO1532	D-3-phosphoglycerate dehydrogenase
CCO0039	sodium:solute symporter family protein	CCO0603	B. subtilis YxjH and YxjG proteins homolog	CCO1534	acylneuraminate cytidylyltransferase, putative
CCO0040	Protein of unknown function, DUF485 superfamily	CCO0654	hypothetical protein	CCO1536	hypothetical protein
CCO0098	hypothetical protein	CCO0658	conserved hypothetical protein	CCO1587	DnaJ-related protein
CCO0104	conserved hypothetical protein	CCO0815	hypothetical protein	CCO1651	Pseudogene
CCO0105	hypothetical protein	CCO0847	transporter, LysE family	CCO1658	major facilitator family transporter, putative
CCO0127	methyl-accepting chemotaxis protein, putative	CCO0917	ferric uptake regulation protein, putative	CCO1662	conserved hypothetical protein
CCO0128	conserved hypothetical protein	CCO0919	periplasmic solute binding protein for ABC	CCO1667	conserved hypothetical protein
CCO0138	conserved hypothetical protein	CCO0921	membrane protein, putative	CCO1669	NADP(H) oxidoreductase CC0205
CCO0182	hemagglutinin/hemolysin-related protein	CCO0926	hypothetical protein	CCO1688	hypothetical protein
CCO0183	Hemolysin, putative	CCO0955	inner membrane protein, putative	CCO1689	hypothetical protein
CCO0184	Hemolysin, putative	CCO0970	hypothetical protein	CCO1694	major facilitator family transporter, putative
CCO0185	hypothetical protein	CCO1005	sodium/alanine symporter VC2356	CCO1707	integral membrane protein, putative
CCO0187	filamentous hemagglutinin 1, putative	CCO1041	conserved hypothetical protein	CCO1708	integral membrane protein, putative
CCO0190	hemolysin activation protein HecB, putative	CCO1049	surface-exposed lipoprotein	CCO1738	Protein of unknown function DUF262 family
CCO0203	hypothetical protein	CCO1076	hypothetical protein	CCO1740	hypothetical protein
CCO0204	hypothetical protein	CCO1077	hypothetical protein	CCO1758	conserved hypothetical protein

Table 5.4.(continued)

RM 2228 locus tag	Description	RM 2228 locus tag	Description	RM 2228 locus tag	Description
CCO0212	conserved hypothetical protein	CCO1078	hypothetical protein	CCO1784	hypothetical protein
CCO0213	methyl-accepting chemotaxis protein, putative	CCO1142	conserved hypothetical protein	CCO1785	hypothetical protein
CCO0251	lipoprotein, putative	CCO1170	oxidoreductase, short-chain	CCO1786	hypothetical protein
CCO0252	probable integral membrane protein Cj0564	CCO1171	hypothetical protein	CCO1801	hypothetical protein
CCO0254	hypothetical protein	CCO1218	conserved hypothetical protein	CCOA_CctmRNA2	sRNA
CCO0285	CAAX amino terminal protease family protein	CCO1277	hypothetical protein	CCOA0049	hypothetical protein
CCO0292	conserved hypothetical protein	CCO1280	conserved hypothetical protein	CCOA0053	conserved hypothetical protein
CCO0293	penicillin-binding protein, putative	CCO1298	sodium/pantothenate symporter , putative	CCOA0062	conserved hypothetical protein
CCO0349	carboxyphosphonoenolpyruvate phosphonomutase	CCO1299	hypothetical protein	CCOA0064	hypothetical protein
CCO0350	2-methylcitrate synthase	CCO1303	Ribbon-helix-helix protein, copG family domain	CCOA0078	hypothetical protein
CCO0353	membrane protein , putative	CCO1306	cytochrome c family protein	CCOA0109	conserved hypothetical protein
CCO0354	hypothetical protein	CCO1307	conserved hypothetical protein	CCOA0122	hypothetical protein
CCO0355	probable periplasmic protein Cj0413 , putative	CCO1308	putative periplasmic protein	CCOA0130	conserved hypothetical protein
CCO0368	cytolethal distending toxin C	CCO1309	putative periplasmic protein	CCOA0131	conserved hypothetical protein
CCO0384	Helix-turn-helix domain protein	CCO1310	putative periplasmic protein	CCOA0147	Fic family protein, putative
CCO0413	tricarboxylate transport protein TctC, putative	CCO1311	hypothetical protein	CCOA0184	single-strand binding protein, putative
CCO0509	lipoprotein, putative	CCO1312	filamentous haemagglutinin domain protein	CCOA0203	ISCco1, transposase orfA
CCO0510	integral membrane protein	CCO1325	hypothetical protein	CCOA0206	tetracycline resistance protein
CCO0511	integral membrane protein	CCO1326	hypothetical protein	CCOA0207	conserved hypothetical protein
CCO0518	methyl-accepting chemotaxis protein, putative	CCO1340	methyltransferase Atu0936 , putative	CCO0535	L-carnitine dehydratase
CCO0534	conserved hypothetical protein	CCO1341	phospholipid N-methyltransferase, putative	CCO1342	hypothetical protein

Table 5.5. *C. jejuni* NCTC 11168 genes shared with *C. jejuni* U101 that are not present in RM1221

NCTC 11168 Locus Tag	Description
Cj0170	hypothetical protein
Cj0171	hypothetical protein
Cj0265c	putative cytochrome C-type haem-binding periplasmic protein
Cj1324	hypothetical protein
Cj1325	putative methyltransferase
Cj1415c	adenylylsulfate kinase
Cj1416c	putative sugar nucleotidyltransferase
Cj1418c	hypothetical protein
Cj1420c	putative methyltransferase
Cj1427c	putative sugar-nucleotide epimerase
Cj1436c	aminotransferase (cysC)

Table 5.6. *C. jejuni* NCTC 11168 genes shared with *C. coli* L101 that are not present in RM1221

NCTC 11168 Locus Tag	Description
Cj0260c	small hydrophobic protein
Cj0567	hypothetical protein
Cj1138	putative glycosyltransferase
Cj1322	hypothetical protein
Cj1324	hypothetical protein
Cj1325	hypothetical protein
Cj1417c	putative amidotransferase

Table 5.7. *C. coli* RM2228 genes shared with *C. jejuni* U101 that are not present in RM1221

RM 2228 locus tag	Description	RM 2228 locus tag	Description
CCO0041	probable integral membrane protein Cj0006	CCOA0072	TnpV
CCO0347	hypothetical protein	CCOA0074	conserved hypothetical protein
CCO0508	integral membrane protein	CCOA0076	hypothetical protein
CCO0509	lipoprotein, putative	CCOA0077	hypothetical protein
CCO0510	integral membrane protein	CCOA0078	hypothetical protein
CCO1215	bifunctional alpha-2,3/-2,8-sialyltransferase	CCOA0081	hypothetical protein
CCO1303	Ribbon-helix-helix protein, copG family domain	CCOA0083	hypothetical protein
CCO1369	conserved domain protein	CCOA0105	hypothetical protein
CCO1446	conserved hypothetical protein	CCOA0119	hypothetical protein
CCO1484	integral membrane protein, putative	CCOA0136	lipoprotein, putative
CCO1541	sulfate adenylyltransferase, subunit	CCOA0145	Pseudogene
CCO1610	hypothetical protein	CCOA0146	ISCco1, transposase orfB
CCO1656	modulator of drug activity (mdaB)	CCOA0171	conserved hypothetical protein
CCO1662	conserved hypothetical protein	CCOA0175	VapD-related protein
CCO1738	Protein of unknown function DUF262 family	CCOA0178	virulence-associated protein 2,
CCO1785	hypothetical protein	CCOA0188	lipoprotein, putative
CCOA0013	conserved hypothetical protein	CCOA0189	type IV secretion system protein VirB8,
CCOA0017	hypothetical protein	CCOA0198	hypothetical protein
CCOA0018	conserved hypothetical protein	CCOA0199	DNA topoisomerase III
CCOA0020	882aa long hypothetical purine NTPase, putative	CCOA0201	conserved hypothetical protein
CCOA0027	hypothetical protein	CCOA0205	conserved hypothetical protein
CCOA0055	hypothetical protein	CCOA0206	tetracycline resistance protein, (tetO)
CCOA0058	hypothetical protein	CCOA0208	hypothetical protein
CCOA0060	hypothetical protein		
CCOA0061	hypothetical protein		
CCOA0062	conserved hypothetical protein		

Table 5.8. *C. coli* RM2228 genes shared with *C. coli* L101 that are not present in RM1221

RM 2228 locus tag	Description	RM 2228 locus tag	Description
CCO0040	Protein of unknown function, DUF485 superfamily	CCO1280	conserved hypothetical protein
CCO0102	hypothetical protein	CCO1281	cobalamin synthesis protein/P47K family protein
CCO0109	hypothetical protein	CCO1310	putative periplasmic protein
CCO0137	transcriptional regulator, Crp family, putative	CCO1484	integral membrane protein, putative
CCO0183	Hemolysin, putative	CCO1707	integral membrane protein, putative
CCO0184	Hemolysin, putative	CCO1708	integral membrane protein, putative
CCO0185	hypothetical protein	CCO1758	conserved hypothetical protein
CCO0291	Hypothetical cytosolic protein, putative	CCO1784	hypothetical protein
CCO0347	hypothetical protein	CCO1785	hypothetical protein
CCO0351	Pseudogene	CCO1786	hypothetical protein
CCO0354	hypothetical protein	CCOA0023	hypothetical protein
CCO0355	probable periplasmic protein Cj0413 , putative	CCOA0075	transcriptional regulator, Cro/CI family
CCO0412	tricarboxylate transport protein TctB, putative	CCOA0077	hypothetical protein
CCO0535	L-carnitine dehydratase	CCOA0105	hypothetical protein
CCO0601	conserved hypothetical protein	CCOA0107	hypothetical protein
CCO0603	B. subtilis YxjH and YxjG proteins homolog	CCOA0115	hypothetical protein
CCO0658	conserved hypothetical protein	CCOA0117	hypothetical protein
CCO0764	hypothetical protein	CCOA0122	hypothetical protein
CCO0921	membrane protein, putative	CCOA0131	conserved hypothetical protein
CCO1040	conserved hypothetical protein	CCOA0134	conserved hypothetical protein
CCO1076	hypothetical protein	CCOA0137	conserved hypothetical protein
CCO1143	transporter, MFS superfamily	CCOA0170	hypothetical protein
CCO1587	DnaJ-related protein		
CCO1654	conserved hypothetical protein		
CCO1668	sarcosine oxidase, putative		

Figure 5.14. Venn diagrams which represents the number of (A) *C. jejuni* RM1221, (B) NCTC 11168 and (C) RM2228 genes absent in *C. coli* C101, L101 and *C. jejuni* U101

Fig. 5.14 (A)

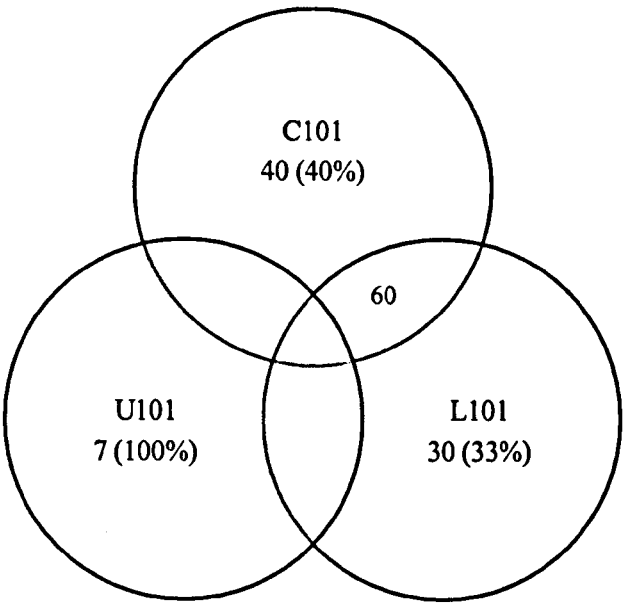


Fig. 5.14 (B)

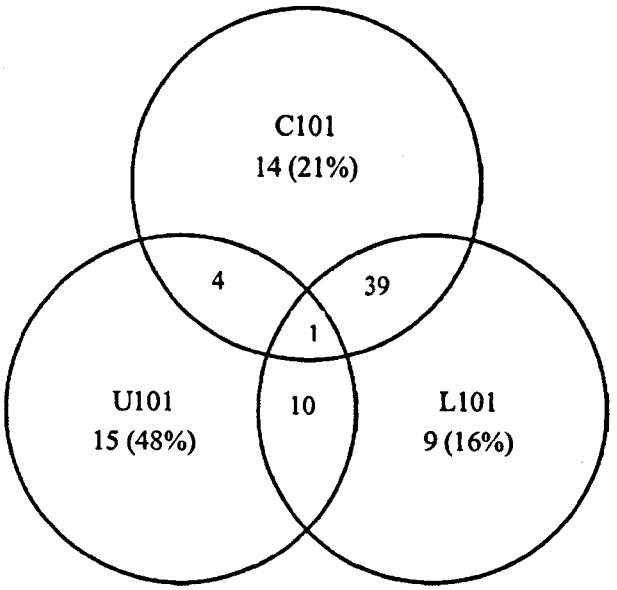
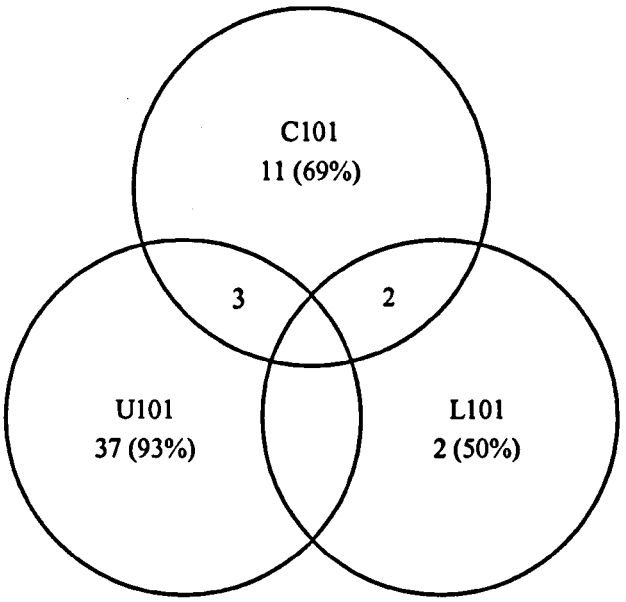


Fig. 5.14 (C)



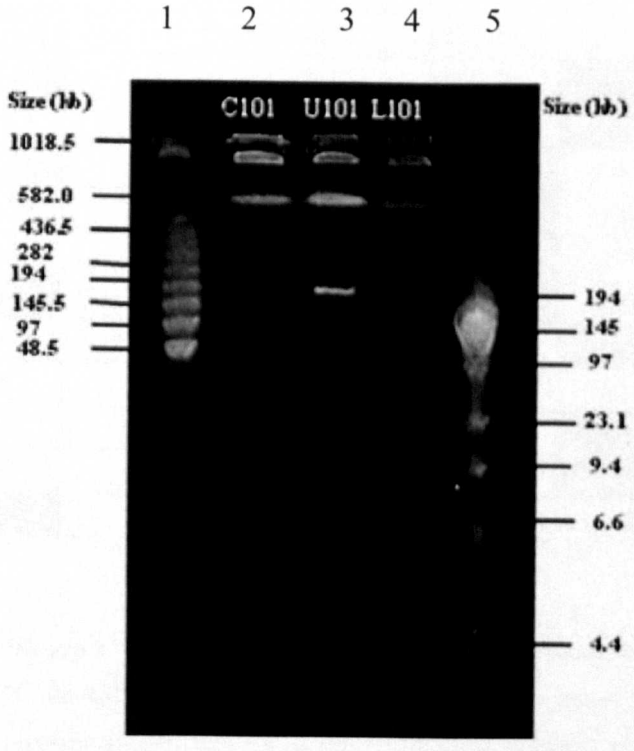
C. coli C101, L101 and *C. jejuni* U101 are samples from the caecum, upper intestine and lower intestine. This data shows the number of genes which were not present in the sample strains but are present in RM1221. The percentage of genes which were unique to each strain is listed in brackets.

5.2.7. Genomic plasmid analysis

Extra chromosomal *Campylobacter* plasmid sequences were found to hybridise to *C. coli* C101, *C. jejuni* U101 and *C. coli* L101. Some of the plasmid associated genes were found to be common between the three pig isolates with *C. coli* C101 notably sharing most of the plasmid genes present in *C. coli* L101. However, the majority of the type IV secretion proteins, mobilisation and replication genes of *cpp* and pTet were also shared between *C. jejuni* U101 and *C. coli* L101. Figure 5.15 presents the PFGE separation of the undigested genomic DNAs of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101. Plasmid genes of approximately 19.4 kb and 4.4 kb were present in *C. jejuni* U101 while *C. coli* L101 showed a band size of

approximately 6 kb. These data suggest that plasmid DNA exchanges may have occurred between different isolates of the same species and between different *Campylobacter* species present in the same pig intestinal environment. Table 5.9 presents the plasmid gene content of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101. Figure 5.16 represents the Venn diagram for the plasmid distribution in *C. coli* C101, L101 and *C. jejuni* U101.

Figure 5.15. PFGE of total DNAs of *C. coli* C101, L101 and *C. jejuni* U101 for 6h



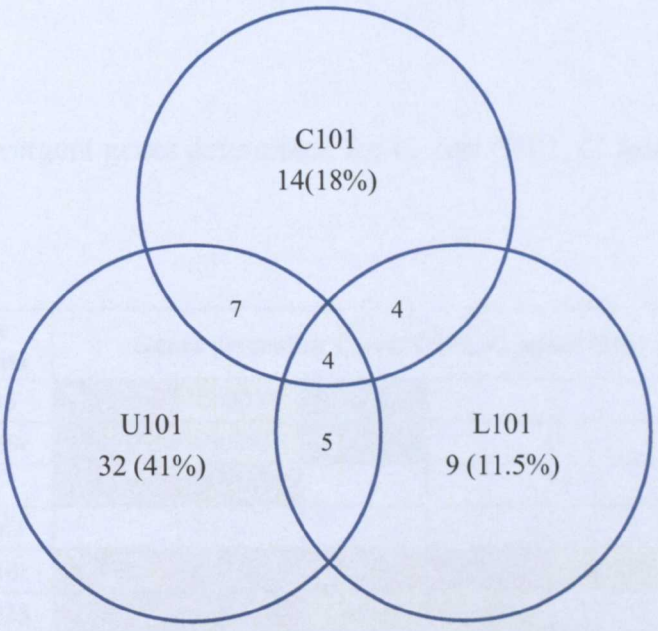
Lane 1: 50-1000 kb PFGE marker (Sigma Aldrich), Lane 2: *C. coli* C101, Lane 3: *C. jejuni* U101, Lane 4: *C. coli* L101, Lane 5: 200bp marker (Sigma Aldrich) Y axis denote the molecular size of PFGE markers. Image obtained from BioRad GelDoc EQ.

Table 5.9. Plasmid gene content of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

C101		U101		L101
Cjp22	pTet_35	Cjp04	pCC31p35	Cjp04
Cjp26	pTet_37	Cjp16	pCC31p36	Cjp47
Cjp30	pTet_39	Cjp17	pCC31p38	p3384_03
Cjp33	pTet_40	Cjp21	pCC31p41	p3386_02
Cjp44	pTet_41	Cjp30	pCC31p42	p3386_03
Cjp47	virB11	p3384_02	pCC31p43	pCC31p01
p3384_01	virB9	pCC31p02	pCC31p48	pCC31p02
p3386_01		pCC31p03	pTet_03	pCC31p03
p3386_02		pCC31p04	pTet_05	pCC31p10
pCC31p01		pCC31p05	pTet_11	pCC31p12
pCC31p02		pCC31p07	pTet_13	pCC31p13
pCC31p04		pCC31p09	pTet_15	pCC31p18
pCC31p06		pCC31p14	pTet_16	pCC31p22
pCC31p25		pCC31p15	pTet_19	pCC31p30
pCC31p26		pCC31p16	pTet_21	pCC31p36
pCC31p33		pCC31p17	pTet_31	pCC31p43
pCC31p38		pCC31p18	pTet_34	pCC31p49
pCC31p39		pCC31p23	pTet_37	pTet_27
pCC31p47		pCC31p24	pTet_39	pTet_30
pCC31p48		pCC31p25	pTet_41	pTet_33
pCC31p50		pCC31p26	pTet_44	pTet_34
pTet_06		pCC31p27	RepA	pTet_35
pTet_30		pCC31p29	virD4	pTet_37
pTet_33		pCC31p32		pTet_41
pTet_34		pCC31p34		virB11

Blue represent the plasmid genes which were present in all three strains. Yellow represent those which were common to *C. coli* C101 and L101. Red represents those which were common to *C.coli* L101 and *C. jejuni* U101. Green represents those plasmid genes which were common to *C. coli* C101 and *C. jejuni* U101 and white represent plasmid gene which are specific to each strain.

Figure 5.16. Venn diagram representing the plasmid distribution in *C. coli* C101, L101 and *C. jejuni* U101



C. coli C101, *C. jejuni* U101 and *C. coli* L101 are samples from caecum, upper intestine and lower intestine. The percentage of genes which were unique to each strain is listed in brackets.

5.2.8. Determination of divergent genes in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

Divergent genes are those where there is a percent difference in nucleotide sequence between two related DNA sequences or in amino acid sequences between two proteins (Lewin, 2008). The divergent genes in this study were selected based on the previous studies performed by Taboada *et al.*, (2004) and Pearson *et al.*, (2003). On comparing the genes which showed a Cy5/Cy3 ratio < 1, 39, 32 and 22 variable genes were present in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 respectively. Table 5.10 lists all the variable genes which were present in these three strains. Most of the variable genes were present in the lipooligosaccharide, capsular polysaccharide, and flagellar biosynthetic loci. UDP-glucose 6-dehydrogenase (Cj1441c; *kfi*D) and

putative sugar nucleotide epimerase (Cj1427c) which were the variable genes which was found common in all the three strains.

Table 5.10. Divergent genes determined for *C. coli* C101, *C. jejuni* U101 and *C. coli* L101.

Hypervariable regions endpoints	Genes present in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101						
Cj0032- Cj0036	Cj0036	Cj0032	Cj0033				
Cj0055c- Cj0059c	Cj0059c	Cj0055c	Cj0056c				
PR3	Cj0141c	Cj0142c					
Cj0177- Cj0182							
Cj0294- Cj0310c	Cj0295	Cj0302c	Cj0309c	Cj0310c	Cj0294	Cj0306c	
Cj0421c- Cj0425	Cj0417	Cj0422c	Cj0416	Cj0424			
Cj0480c- Cj0490							
Cj0561c- Cj0571	Cj0561c	Cj0564	Cj0570	Cj0570	Cj0571	Cj0567	
Cj0625- Cj0629	Cj0627	Cj0626					
Cj0727- Cj0755	Cj0729	Cj0731					
Cj0967- Cj0975	Cj0974	Cj0973	Cj0968	Cj0974			
PR4	Cj1125c						
Cj1135- Cj1151c	Cj1139c	Cj1141	Cj1143	Cj1136	Cj1143	Cj1138	Cj1139c
Cj1293- Cj1343	Cj1300	Cj1311	Cj1313	Cj1322	Cj1326		
	Cj1324	Cj1325	Cj1332	Cj1333	Cj1341c	Cj1343c	
	Cj1299	Cj1308	Cj1309c	Cj1310c	Cj1318	Cj1322	
	Cj1324	Cj1325					
Cj1414c- Cj1449c	Cj1417c	Cj1418c	Cj1421c	Cj1427c	Cj1432c	Cj1433c	Cj1435c
	Cj1436c	Cj1441c	Cj1442c	Cj1443c	Cj1415c	Cj1416c	Cj1418c
	Cj1420c	Cj1427c	Cj1429c	Cj1432c	Cj1435c	Cj1437c	Cj1440c
	Cj1441c	Cj1436c	Cj1417c	Cj1423c	Cj1427c	Cj1441c	
Cj1543c- Cj1563c	Cj1549c						
Cj1677- Cj1679							
Cj1717c- Cj1729	Cj1721c	Cj1722c	Cj1729c	Cj1718c	Cj1719c	Cj1729c	

The hypervariable region end points were determined by Taboada *et al.*, (2004) and Pearson *et al.*, (2003). Red represents *C. coli* C101, yellow represents *C. jejuni* U101 and blue represents *C. coli* L101.

5.3. DISCUSSION

5.3.1. *Campylobacter* excretion from pigs

Detailed studies of the populations of campylobacters present in pigs have not previously been reported. The work described in this chapter aimed to answer a number of questions raised by the co-existence of different species and strains of *Campylobacter* within pigs.

5.3.2. What is the relative proportion of each species?

In outdoor reared pigs the prevalence of *C. jejuni* varied between 0.2% and 9.9% and for indoor pigs the *C. jejuni* prevalence ranged between 0 to 7%. The remainder were likely to be *C. coli*. Clearly the pig intestinal environment favours *C. coli* over *C. jejuni* but the latter may still colonise and maintain a population despite numerical domination by the former.

5.3.3. Do different species and different genotypes colonise different parts of the intestine?

Campylobacters could not always be isolated from every part of the intestine indicating some differences in colonisation patterns. They were most frequently isolated from the caecum and the large intestine with the upper intestine being mainly negative for indoor pigs with one exception. In some cases the same PFGE pattern was observed from isolates from different parts of the intestine indicating single genotypes may colonise the entire length. Where *C. jejuni* were isolated there was no

particular correlation between the species with the location it was found. The *C. coli* strains were always dominant and *C. jejuni* the lesser population.

5.3.4. Does exposure to an outdoor environment increase the likelihood of colonisation by more than one species?

Campylobacters in the intestinal content of outdoor reared pigs varied between 10^5 to 10^7 cfu g^{-1} of the intestinal contents while those in indoor pigs it was between 10^3 to 10^5 cfu g^{-1} of the intestinal contents. The answer to this question is therefore that exposure to the environment was associated with higher carriage levels and generally but not necessarily, a more diverse *Campylobacter* population.

5.3.5. Do the *C. coli* and *C. jejuni* populations in individual pigs comprise single or multiple genotypes, and are pigs from the same environment colonised with the same genotypes and/or species?

A large number of different PFGE MRP types were observed for pigs reared in each environment indicating that pigs are colonised by very diverse populations of campylobacters. Multiple genotypes were isolated from some pigs while some genotypes were shared between different pigs particularly those reared in an indoor environment.

5.3.6. If the different species are closely associated with each other in the intestine, how much, if any, genetic exchange occurs through natural transformation, transduction by bacteriophages or conjugation?

The results presented in the following sections provide evidence for genetic exchange between subtype and species based on shared gene content.

5.3.7. Sequence analysis of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

Gorkiewicz (2003) reported that 16S rRNA sequence analysis was an effective and rapid procedure for the species specific identification of campylobacters. However, discrimination between *C. coli* and *C. jejuni* strains may be problematic as identical 16S rDNA sequences for *C. coli* and *C. jejuni* can be obtained. This was particularly evident here where both the *C. coli* strains analysed were more typical of *C. jejuni* with regard to their 16S ribosomal rRNA sequences.

5.3.8. Comparative genomic hybridisation (CGH) of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

The hybridisation profile for each of the three isolates analysed in this study was obtained by hybridising Cy3 labelled genomic DNA with Cy5 labelled *C. jejuni* RM1221 DNA to microarrays featuring oligonucleotides representing the genomes of *C. jejuni* NCTC 11168, *C. jejuni* RM1221 and *C. coli* RM2228. CGH with *C. coli*

C101, *C. jejuni* U101 and *C. coli* L101 genomic DNAs was employed to identify the presence and absence of genes using *C. jejuni* RM1221 as the control strain. Hybridisation also provided an inventory of the virulence and plasmid genes present in these strains. This approach provided information regarding the genes present in *C. jejuni* NCTC 11168, *C. jejuni* R1221 and *C. coli* RM2228 which showed marked sequence differences with the pig strains. Hybridisation to the microarray oligonucleotides also identified genes that were present in only one of the strains present in the same pig, suggesting the strains *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 maintained some diversity despite colonising the same individual. Differential hybridisation to the oligonucleotides present on the microarray reveal distinct differences in gene content between these strains. However, of equal significance the microarray also indicated a core group of genes which were common between them. Dendrogram clustering of the gene presence/absence data indicated that the two *C. coli* strains, *C. coli* C101 and *C. coli* L101, exhibited significant gene conservation across different gene regions which are presented in Figs. 5.11 and 5.12 and are therefore maintain a clear relationship to each other. Many genes from the 44 common genes comprising the core set are predicted to be involved in vital functions such as energy metabolism, regulatory functions, macromolecule metabolism, formation of surface structures and pathogenicity. Known virulence genes in this core set included the cytolethal distending toxin (Cj0079c; *cdt*C), putative haemolysin (Cj0588; *tly*A) and flagellar motor switch protein (Cj0351; *fli*N). *Tly*A encoded haemolysin of *Serpulina hyodysenteriae* is an important virulence factor in swine dysentery but their role in *C. jejuni* mediated diarrhoeal disease is still unknown (Ketley and Konkel, 2005). The DNA modifying gene exodeoxyribonuclease VII large subunit (Cj0325; *xse*A), which bidirectionally degrades single-stranded DNA

into large acid insoluble oligonucleotides was also present in all three strains. They also share 11 hypothetical proteins and 2 conserved hypothetical protein. All the three *Campylobacter* strains also shared β 1-3 galactosyl transferase (Cj1139c; *wla*N), which is a putative phase variable gene responsible for the addition of terminal galactose and thereby converting GM_2 ganglioside-mimicking lipo-oligosaccharide (LOS) structures to GM_1 like LOS structures (Linton *et al.*, 2000a). Whole genome analyses of 11 *C. jejuni* strains has shown that species specific or conserved genes include those involved in metabolic, biosynthetic, cellular and regulatory processes, putative virulence factors (CDT (Pickett *et al.*, 1996), flagellar structural proteins (Wassenaar *et al.*, 1991), phospholipase A (Grant *et al.*, 1997), PEB antigenic surface proteins (Pei *et al.*, 1991)) and proteins involved in host pathogen interactions such as ciaB (Konkel *et al.*, 1999), cadF (Konkel *et al.*, 1997) and cheY (Yao *et al.*, 1997).

Many of the strain specific variable genes which were identified in the pig strains were involved in modification of cell envelope. Pearson *et al.*, 2003 showed that many of the variable genes from 18 *Campylobacter* strains, were clustered in seven large distinct regions referred to as plasticity regions (PR) that contain up to 45 genes. Three major plasticity regions include those related to the structural and biosynthetic components for the assembly of capsular polysaccharide, lipo-oligosaccharide and flagellar glycosylation, Pearson *et al.*, 2003 recorded the newly identified plasticity regions as PR1, PR2, PR3, PR4 and the major plasticity regions as PR5, PR6 and PR7. More genes were included in this group by Taboada *et al.*, 2005.

PR1 contains genes which encode molybdenum transport apparatus (Cj0300c, Cj0301c and Cj0302c), pantothenate biosynthesis genes (Cj0296c, Cj0297c and

Cj0298c) and hypothetical proteins of putative or unknown function (Pearson *et al.*, 2003). Another highly divergent gene present is located just upstream of PR1 is the molybdoenzyme reductase gene (Cj0264c) which is responsible for respiration of trimethylamine-*N*-oxide and dimethyl sulfoxide under oxygen limiting conditions. CGH analysis had shown the presence of a putative molybdenum-pterin binding protein (Cj0302c) and molybdopterin containing oxidoreductase (Cj0264c) in *C. coli* C101. Nitrate reductase (Cj0780), a flavoprotein enzyme containing both molybdenum and cytochrome b is involved in the reduction of nitrate to nitrite. *Campylobacter* uses nitrate as a terminal electron acceptor in the place of oxygen under oxygen deficient conditions (Sellars *et al.*, 2002). Pearson *et al.*, 2003 had suggested that the acquisition of these genes provided some strains with a selective advantage in specific ecological niches. None of the *Campylobacter* strains showed the presence of putative altronate hydrolase (Cj0483), aldehyde dehydrogenase (Cj0490), putative oxidoreductase (Cj0580c) and sugar transporter (Cj0486) which is present in PR2.

Genes involved in glycosylation were identified in *C. coli* C101 including N-acetyl galactosamine transferase (Cj1125c; *pgl*A), beta-1,3 galactosyltransferase (Cj1139c, *wla*N), sialic acid synthase (Cj1141; *neu*B1) and acylneuraminate cytidylyltransferase (Cj1143; *neu*A1) which are involved in N-linked glysolylation of surface proteins and the modification of LOS. *C. jejuni* U101 also possesses Cj1139c while the *C. coli* L101 genome included both putative glycosyl transferases Cj1138c and Cj1139c. *Pgl*A, *pgl*H, *pgl*I and *pgl*J genes encode specific glycosyltransferases responsible for sequential addition of monosaccharides to form the heptasaccharide glycan. Inactivation of *pgl*A resulted in the formation of a fragmentation pattern of a glycan with a single residue of bacillosamine attached to a peptide (Linton *et al.*,

2005). The *pgl/wla* gene locus NCTC 11168 is distinct and located some distance from the flagellin biosynthesis locus. Thus, it appears that there are two distinct loci involved in *C. jejuni* and *C. coli* protein glycosylation: the flagellin modification locus involved in O-linked glycosylation of the flagellin subunit proteins, and the *pgl/wla* gene locus involved in a system of general protein (including flagellin) glycosylation (Linton *et al.*, 2002). *C. coli* C101 showed the presence of Cj1143 (*neu*A) and Cj1141 (*neu*B) which modifies the LOS. *Neu*A, encodes for cytidine monophosphate (CMP) - N- acetylneuramic acid (NANA) synthetase, responsible for the activation of NANA prior to incorporation into polysialic acid chains. *Neu*B1 catalyses the formation of NANA through the condensation of *N*-acetyl-d-mannosamine and phosphoenolpyruvate and the absence of NANA has resulted in increased motility. The property of the LOS structure mimicking ganglioside GM_1 is lost in *neu*B1 mutants (Linton *et al.*, 2000b).

The longest stretch of variable regions occurs in the LOS biosynthesis and flagellin modification region which stretches from Cj1293 to Cj1343. This region contains a large number of hypothetical proteins of unknown functions and also genes involved in fatty acid biosynthesis (Pearson *et al.*, 2003). Within this region, *C. coli* C101 showed the presence of putative S-adenosylmethionine dependent methyltransferases (Cj1300), putative acylneuraminate cytidylyltransferase (Cj1311; *pse*F), N-acetyltransferase (Cj1313; *pse*H), Cj1322, Cj1326 (hypothetical proteins) and motility accessory factor (Cj1341; *maf*6). *C. jejuni* U101 contained flagellin modification protein A (Cj1332; *ptm*A), Cj1333; *pse*D and motility accessory factor (Cj1341c; *maf*6). *C. coli* L101 also showed the presence of Cj1322, putative methyltransferase (Cj1325) and motility accessory factor Cj1341.

The capsular polysaccharide biosynthesis locus includes Cj1414c to Cj1449c that is flanked by genes with similarity to capsule transport genes found in *E. coli,* i.e. *kps*S, C (Cj1413c and Cj1414c) and F, D, E, T and M (Cj1443c – Cj1445c, Cj1447c-Cj1448c) (Pearson *et al.*, 2003). Genes present in *C. coli* C101 included putative sugar transferase (Cj1421c and Cj1432c), Cj1433c (hypothetical protein) and putative phosphatase (Cj1435c), while *C. coli* L101 included only the capsular polysaccharide heptosyltransferase (Cj1423c; *hdd*C) which supports deoxyheptose biosynthesis (Karlyshev *et al.*, 2005).

Genes responsible for restriction modification enzymes were absent from *C. coli* L101 and C. *jejuni* U101. *C. coli* C101 had putative type I restriction enzyme R protein (Cj1549c; *hsd*R) which is a part of the multisubunit complex containing products of the *hsd*R, Cj1551c (*hsd*S) and Cj1553 (*hsd*M) that supports endonuclease activity (Miller *et al.*, 2005). Cj1549 and Cj1553 had a G+C content of 34.3% and 37.8%, respectively compared to 26.6% and 27.8% in the capsule biosynthesis region and LOS biosynthesis region and the significantly variable G+C content of these regions indicates that they may have been acquired by horizontal genetic exchange (Dorrell *et al.*, 2001).

In *C. coli* C101 the divergent genes were often present in large clusters, suggesting that they were acquired or lost from the genome in groups during evolution. Unlike the pathogenicity islands found in the genomes of *E. coli* and *Salmonella, C. jejuni* PR do not have a markedly different G+C content to the bulk of the genome and they are not associated with mobile elements important in horizontal DNA transfer.

5.3.9. Plasmid Analysis in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

Plasmid genes play a key role in the ability of the bacteria to exploit new environments and in the transfer of DNA to promote bacterial genome plasticity (Batchelor *et al.*, 2004; Drysdale *et al.*, 2005). The analysis of the CGH of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 showed that these strains shared 4 plasmid genes, pCC31p02, pTet34, pTet37 and pTet41 between them. Excluding these four, *C. coli* C101 shared seven plasmid genes between *C. jejuni* U101 and *C. coli* L101. Five plasmid genes were shared by *C. jejuni* U101 and *C. coli* L101 including Cjp04, pCC31p03, pCC31p18, pCC31p36 and pCC31p43 (see Appendix 3). Above all *C. coli* C101 and *C. jejuni* U101 hybridised several *C. coli* RM2228 oligonucleotides which represent pCC31 plasmid genes. These plasmid genes may be integrated into the chromosome through recombination, subsequently acquire chromosomal genes through transposition or integration events, and thus play an important role in genetic diversity (Ochman *et al.*, 2000).

The plasmid genes present in these pig strains contained a large number of genes coding for type IV secretion proteins and plasmid like proteins, which are involved in conjugative plasmid transfer or the secretion of virulence factors (Fouts *et al.*, 2005). The *C. coli* plasmid gene pCC31p36 present in *C. jejuni* U101 and *C. coli* L101 contain the *vir*B6 protein. Abril *et al.*, (2007) found the gene for virB6 in *C. fetus* sub sp *veneralis* but was truncated by an IS*cfe*1 insertion element. Several contigs within *C. fetus* sub sp *veneralis* contain a number of sequences that exist as extrachromosomal DNA plasmids elsewhere. The pCC31p27 gene present in *C. jejuni*

U101 encodes the CRISPR associated protein cas2 that confers resistance to infection by bacteriophages (Schouls *et al.*, 2003). It also contains pTet03, which modifies DNAs by introducing a nick in the double stranded DNA and thereby promoting horizontal transfer of genetic information through plasmids (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=141263). The pTet11 plasmid in *C. jejuni* U101 encodes a protein similar to the repair protein *Rad*A belonging to the recombinase superfamily, which facilitate DNA strand exchange process between a single stranded DNAs (ssDNA) and homologous double stranded DNAs (dsDNA) (Li *et al.*, 2009). *C. jejuni* U101 also has the pTet15 plasmid gene, which encodes a protein similar to traC that acts as a cell surface receptor thereby helping in bacterial conjugation process (Alfieri *et al.*, 2008). pTet19 codes for single stranded binding protein and binds with high affinity to ssDNA intermediates during metabolic process. Although the DNA transport process has not been formally demonstrated in *Campylobacter*, it is known that in other bacteria dsDNA is degraded during the uptake process and only a single strand is transported to the cytosol (Jeon and Zhang, 2007).

C. jejuni U101 also has plasmids p3384_02 and pTet37, which encode proteins similar to *rep*A that are present on *C. coli* plasmids (Luo and Zhang, 2001). pCC31p48 shows significant homology to topoisomerase, which removes negative super coils in DNA (http://www.ncbi.nlm.nih.gov/gene?term=pCC31p48).

C. coli C101 and L101 contained only a few DNA modification genes of known function when compared to that of *C. jejuni* U101. In addition to pTet37 *C. coli* C101 also housed the *C. coli* plasmid gene pCC31p39 encoding a putative DNA

invertase (http://www.ncbi.nlm.nih.gov/gene?term=pCC31p26). pTet27 is only present in *C. coli* L101 amongst all the three isolates. pTet27 encodes a protein similar to traN which helps in mating pair stabilisation during bacterial conjugation. This function is also supported by traG. Klimke and Frost (1998) demonstrated that mutated *tra*N and *tra*G strains show drastic reductions in the transfer efficiency of plasmids. The main function of *tra*N is in recognition of a receptor and interactions with other plasmid specific components. *C. coli* L101 also carries putative *rep*A genes that are plasmid borne in p3386_02 and pTet37.

5.3.10. Type IV secretion systems in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

C. coli C101, *C. jejuni* U101 and *C. coli* L101 exhibited many genes that are required for the assembly of a type IV secretion system (T4SS). This class of macromolecular transfer system functions to translocate proteins and nucleoprotein complexes from donor to recipient bacterial cells in processes related to bacterial conjugation. In pathogenic bacteria, T4SSs transmit effector molecules with virulence functions to eukaryotic hosts (Bacon *et al.*, 2002). pVir is 37.5kb plasmid that contains components of a type IV secretion system (T4SS) known to be important for the virulence of a number of major bacterial pathogens (Bacon *et al.*, 2000). It is also suggested that the pVir plasmid is important for both *in vitro* adherence and invasion of intestinal epithelial cells in culture (Bacon *et al.*, 2000). The pTet plasmid co-exists in *C. jejuni* strain 81-176 with a smaller, previously characterized, non conjugative plasmid pVir that also encodes a type IV secretion system (T4SS) that may affect

virulence (Bacon *et al.*, 2000). Table 5.11 enlist the plasmids involved in type IV secretion system in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101.

Table 5.11. Plasmids involved in type IV secretion system in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

C. coli C101		*C. jejuni* U101		*C. coli* L101	
locus tag	**Gene description**	**locus tag**	**Gene description**	**locus tag**	**Gene description**
Cjp22	pVir_p22	CCOA0178	virulence associated protein 2,	pTet_35	similar to type IV secretion system VirD2 component
Cjp26	pVir_p26	CCOA0189	type IV secretion system protein VirB8,	CCOA0186	type IV secretion system protein, putative
Cjp30	pVir_p30	Cjp04	pVir_p04	Cjp04	pVir_p04
Cjp33	pVir_p33	Cjp17	pVir_p17	Cjp47	pVir_p47
Cjp44	pVir_p44	Cjp30	pVir_p30	pCC31p30	putative type IV secretion system component
Cjp47	pVir_p47	pCC31p29	cmgb2	pCC31p36	putative type IV secretion system component
pCC31p39	putative type IV secretion system component	pCC31p35	cmgb5 putative type IV secretion system component	VirB11	
pTet_35	similar to type IV secretion system VirD2 component	pCC31p36	cmgb6 putative type IV secretion system component		
VirB9		pCC31p38	cmgb8, putative type IV secretion system component		
VirB11		pCC31p41	cmgb11		
pCC31p38	cmgb8, putative type IV secretion system component	pCC31p42	cmgd4,putative type IV secretion system component		
		pTet_21	similar to type IV secretion system VirB5 component		
		virD4	Transfer coupling protein		

The genome of these strains harbour different genes that code for T4SS and bear homology to several plasmid borne genes including several present in pCC31 and pTet that are found in *C. coli* and *C. jejuni* respectively. The pCC31 and the pTet genes that code for T4SS are functionally distinct from the T4SS carried on pVir genes (Batchelor *et al.*, 2004). The hybridization signals indicate *C. coli* C101 houses 6 pVir genes, while *C. jejuni* U101 has 3 and *C. coli* L101 has 2 pVir genes. Studies had shown that although the pVir virulence plasmid does not correlate with all clinical symptoms of infected patients suffering from *C. jejuni* individuals infected with *C. jejuni* carrying pVir were more likely to produce bloody stools than those infected with a pVir negative strain (Tracz *et al.*, 2005). The frequency of pVir was significantly higher (53%) in patients with bloody diarrhoea when compared to patients with non bloody diarrhoea (21%) on the basis of DNA- DNA hybridisations on 104 human isolates (Tracz *et al.*, 2005). The presentation of bloody stools in *C. jejuni* gastroenteritis indicates the progression of the infection into the tissues of the colon and rectum (Skirrow and Blaser, 2000). Invasion of the intestinal epithelium leads to mucosal damage and inflammatory lesions, observed in *C. jejuni* infections, and is a major component of the pathogenesis (Tracz *et al.*, 2005). pVir was previously reported to be important for the *in vitro* invasion of intestinal epithelial cell lines (Bacon *et al.*, 2002; Bacon *et al.*, 2000). The precise role of the T4SS carried on pVir is unknown, although mutation of several pVir genes and T4SS homologues resulted in reductions of invasion into INT407 cells *in vitro* (Bacon *et al.*, 2002; Bacon *et al.*, 2000). It was also noted that mutation of a subset of pVir genes also affected natural transformation (Bacon *et al.*, 2000).

The Agrobacterial T4SS proteins mediating T-DNA transfer are the 11 VirB proteins (encoded by *vir*B1–*vir*B11), as well as the transfer coupling protein, *vir*D4, a

nucleoside triphosphatase. *Vir*B2 encodes the membrane pilus while *vir*B4 and *vir*B11 code for a cytoplasmic membrane ATPase and virB6-10 a trans-envelope pore complex (Batchelor *et al.*, 2004). However, recent reports from Nielsen *et al.*, (2010) had shown that *vir*B was not detected by PCR in any of the 143 *Campylobacter* isolates from Danish patients recovered from blood and stools.

5.3.11. Virulence genes identified in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

The CGH analysis of *C. coli* C101, *C. jejuni* U101 and *C. coli* L101 found genes coding for virulence factors such as cytolethal distending toxin (*cdt*), chemotaxis, flagellar biosynthesis, and 2 component systems present in these strains. Fouts *et al.*, (2005) had suggested that the pathogenic mechanisms responsible for intestinal infections by *Campylobacter* involve adhesion, cellular invasion and toxin production but not all clinical isolates of *C. jejuni* are able to invade cultured human cells or produce defined toxins. *C. coli* C101 contained the genes CCO0127, CCO0213 and CCO0518 while *C. coli* L101 contained CCO0280 that encode methyl accepting chemotaxis proteins, which have been reported to be required for caecal colonisation of chickens (Hendrixson and DiRita, 2004).

C. coli C101 possessed the gene CCO0368 encoding cdtC (cytolethal distending toxin C) that has been reported to irreversibly block eukaryotic cells in the G2 phase of the cell cycle (Whitehouse *et al.*, 1998). Toxin proteins were also produced in *C. coli* L101 which included putative toxin like outer membrane protein encoded by CCO1467 and putative vacuolating cytotoxin precursor coded by CCO1468. Although *Campylobacter* sub species have largely conserved genomes,

sub species display variable virulence phenotypes in animal models and this phenotypic virulence has been speculated to be due to hyper variable antigenic diversity and immune evasion (Moolhuizen *et al.*, 2009). In *C. jejuni* U101 virulence associated proteins were coded by virulence associated protein-2 (CCOA0178). CJE0731 and CJEO732 which coded type III restriction/modification enzymes were also present in *C. coli* L101.

5.3.12. Phage related genes present in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

C. jejuni U101 and *C. coli* L101 were found to contain genes for Mu like phage proteins. *C. jejuni* U101 housed many Mu genes that included the phage major tail tube protein (CJE0226), major tail sheath protein (CJE0227), putative tail fiber protein H (CJE0231), putative base plate assembly protein J (CJE0233), putative Mu like prophage I protein (CJE0244), putative prophage MuSo1, F protein (CJE0251), putative tail protein X (CJE0251) and putative bacteriophage DNA transposition protein B (CJE0269). The Mu related gene CJE0232 encoding a putative phage tail protein was also present in *C. coli* L101.

It has been long recognised that bacteriophages have critically important roles in genome diversification, the evolution of virulence and host adaptation in other enteric bacteria (Wagner and Walder, 2002). CampMu bacteriophages are from a temperate bacteriophage family that is widely distributed among *C. jejuni* populations (Clark and Ng, 2008) whilst rarely seen in *C. coli*.

The presence of CRISPR genes were also found in *C. jejuni* U101 and *C. coli* L101. CJE1695 in *C. coli* L101 coded *cas*1 family protein while CJE1694 in *C. jejuni*

U101 coded for *cas*2 family protein. *C. jejuni* U101 also housed CCOA0175 encodes a putative virulence associated protein similar to *cas*2. CRISPR form peculiar genetic loci that provide acquired immunity against viruses and plasmids by targeting nucleic acids in a sequence specific manner. These hypervariable loci take up genetic material from invasive elements and build up heritable DNA encoded immunity against phages (Horvath and Barrangou, 2010). CRISPR elements were found in *C. lari* RM2100 and *C. upsaliensis* RM3195 though it was not present in *C. coli* RM2228 (Fouts *et al.*, 2005). However, Price *et al.*, (2007) and Schouls *et al.*, (2003) had reported the presence of CRISPR in other strains of *C. coli.*

5.3.13. Convergence of *Campylobacter* genes

This study supports convergence as supported by Sheppard *et al.*, 2008 between the *C. jejuni* and *C. coli* strains as a result of their existence together in the gastro intestinal tract of pig. In addition to the 44 genes which were shared by the 3 strains, *C. jejuni* U101 shared 24% of its genes with *C. coli* C101 and 10% of its genes with *C. coli* L101. *C. coli* L101 showed the presence of Mu genes and *cas*1 genes which are unique to *C. jejuni* strains. The *C. coli* strains C101 and L101 shared 34% and 36% of its plasmid genes with *C. jejuni* U101. All pCC31 and pTet genes are involved in plasmid replication and conjugative transfer (Batchelor *et al.*, 2004). The *C. jejuni* U101 has 26 (54%) pCC31 plasmid genes which are unique to *C. coli* strains. The non coding region between pCC31p18 and pCC31p19 act as a putative origin of replication region and is present both in *C. jejuni* U101 and *C. coli* L101 (Batchelor *et al.*, 2004). Caro-Quintero *et al.*, (2009) proposed that interspecies gene transfer events were too infrequent to account for species convergence (2% of 4507 *Campylobacter* isolates) which might have resulted due to the selection of strains

from different ecological niches. The strains which showed genetic exchange gave ~10% of exchanged genes between *C. jejuni* and *C. coli.*

Genomic comparisons revealed that a total of ~ 117 genes had potentially exchanged between *C. jejuni* RM1221 and *C. coli* RM2228 and *vice versa* which constitute 10% of the genome (Caro-Quintero *et al.*, 2009). The pool of 117 genes was heavily enriched in hypothetical proteins, motility accessory factors and flagellar genes (16/117), and genes related to metallo-beta-lactamases, multi drug efflux pumps, two ABC transport systems, endonucleaseIII, lipopoligosaccharide synthesis, and membrane associated proteins. *C. coli* C101 showed the presence of many flagellar associated genes, which include flagellar motor switch protein (Cj0059c; *fli*Y), flagellar motor switch protein (Cj0060c; *fli*M), flagellar MS-ring protein (Cj0318; *fli*F), flagellar basal body rod protein (Cj0698; *flg*G), flagellar biosynthesis protein (Cj0820; *fli*P), flagellar basal body associated protein (Cj1408; *fli*L) and *C. coli* L101 showed the presence of flagellar motor protein (Cj0337; *mot*A) and putative flagellar motility protein (Cj0371). *C. coli* C101 showed the presence of CCO0919 which coded for periplasmic solute binding protein for ABC which was also reported by Caro Quintero *et al.*, 2009 as one of the imported alleles between *C. jejuni* and *C. coli.* Section 5.4.3 enlisted the membrane associated proteins and LOS modification genes which were present in these strains. These data supported the fact that genetic exchange did occur between the 3 *Campylobacter* strains and convergence had occurred.

5.3.14. Miscellaneous groups present in *C. coli* C101, *C. jejuni* U101 and *C. coli* L101

C. coli C101 and L101 housed genes encoding haemolysins. These included CCO0183 and CCO0184, which are present in both *C. coli* C101 and L101, whilst CCO0182, CCO0187 and CCO0190 were only observed in *C. coli* C101. The presence of these genes makes these *Campylobacter* strains candidates to cause tissue damage upon infection of humans and animals (On *et al.*, 2006).

5.4. CONCLUSION

The majority of the pigs were dominated by *C. coli* with *C. jejuni* either absent or present at low frequency. Multiple genotypes were isolated from some pigs while some genotypes were shared between different pigs particularly those reared in an indoor environment. The PFGE MRP of the campylobacters isolated from pigs showed that a single genotype was present in more than one pig. The analysis after CGH hybridisation grouped the genes present in these strains and it showed that these strains contained genes that encode several virulence proteins and type IV secretion proteins. The *C. coli* strains housed several pVir genes which indicated that they might be highly pathogenic to humans. The majority of the variable genes present in these strains were associated with capsule biosynthesis, flagellin modification and LOS biosynthesis supporting the occurrence of horizontal gene transfer between these strains. The presence of more variable genes increases the chance of genome diversity and they play an important role in the avoidance of innate and adaptive immune responses in the host. Approximately 0.2% of the genes present in the *C. coli* strains showed high nucleotide sequence similarity to the sequences present in *C. fetus* and *C. lari*. This study also highlighted the genetic factors that might be linked to phenotypic variation and adaptation to different ecological niches.

CHAPTER SIX

DISCUSSION

6.1. FINAL DISCUSSION

Farm animals harbour a large number of campylobacters in their intestine which serve as a major cause of bacterial gastroenteritis in humans. It is generally believed that human infection with different genotypes of *C. jejuni* and *C. coli* may have different disease outcomes, for example, determining the intensity or duration of infection. There is however little direct evidence to support this due to the lack of volunteer data and the enormous genetic diversity of the two species. Genotypes have been identified which were able to undergo spontaneous genomic rearrangements in response to phage predation increasing diversity still further (Scott *et al.*, 2007). The effects of such rearrangements on transcription and the regulation of any particular set of genes are completely unknown. Such rearrangements may affect the virulence and colonisation potential of the organism. In order to compare the physiological and transcriptional effects of such rearrangements it was first necessary to standardise procedures for RNA extraction. During this process quantitative and qualitative differences were observed in the growth of cells to be used for RNA extraction depending on the method of atmosphere generation used. To determine the reasons for these differences a study comparing transcription and physiological effects of different methods of atmosphere generation was carried out and this is described in Chapter 3. Chapter 4 describes the transcriptional and physiological differences of the genetically rearranged variants described by Scott *et al.*, (2007). Chapter 5 describes an investigation of the genotypes of *C. coli* and *C. jejuni* where the species co-colonise pigs. The phenomenon of the two species colonising the same pig intestine raised some important questions regarding the exchange of genetic material between species as well as basic questions regarding, the frequency of co-colonisations,

relative proportions of the two species, parts of intestine infected and whether the method of rearing was a factor.

One of the main findings of the work described in Chapter 3 describing an investigation of the growth characteristic of *C. jejuni* HPC5 in the two different microaerobic conditions, was the importance of hydrogen which was present in the gas replacement atmosphere but not in the MACS cabinet. The possibility that the presence of hydrogen in the microaerobic atmosphere was protecting the *Campylobacter* cells from oxidative damage was tested by addition of the anti-oxidation protective supplement FBP to the cells grown in hydrogen free atmosphere. Growth curves that had previously indicated a lag in the first hours of grown in a hydrogen free atmosphere, now showed comparable growth with the hydrogen containing atmosphere. Further experiments using sachet type atmosphere generation with and without hydrogen also appeared to confirm the hypothesis that hydrogen acted as an antioxidant in the gas replacement atmosphere. Motility was also affected depending on the atmosphere used. As the growth curves of the apparent oxidatively stressed cells eventually "caught up" with the non stressed cells upon reaching stationary phase, one might be forgiven for thinking that the outcomes from the condition were similar. However, the transcription experiments revealed that profound changes in the gene expression and regulation occurred depending on the atmosphere generation method used and this may have a significant effect on the results of further experiments to be performed with these cells. In reality exponential growth is supported by differential gene expression between the conditions employed. Cells for the studies described in Chapter 4 were therefore grown in hydrogen containing gas replacement atmospheres.

The main questions that were addressed in chapter 3 were:

- Does the transcriptome of *C. jejuni* HPC5 grown in MACS without the presence of hydrogen show comparable gene expression to that of cells grown in microaerobic jar in the presence of hydrogen?
- Are there any mechanisms adopted for reducing the oxidative stress by campylobacters grown in MACS?
- Is there any identifiable difference in the motility regulated genes which resulted in the reduced motility of *C. jejuni* HPC5 in MACS?
- Does *C. jejuni* HPC5 grown in MACS showed any characteristic difference in their genetic recombination ability?

Analysis of the transcriptomic data obtained from growing *C. jejuni* HPC5 in the two different atmospheres showed that a large number of expression changes in genes representing all functional categories occurred. Of the 294 genes exhibiting differential transcript levels between the GRJ and MACS, 147 genes registered a two fold or more increase in the GRJ compared to the MACS cabinet and 147 showing the opposite trend. Although the number of genes which showed changes in expression was similar for each category they differed in their functional classification, and therefore marked an unambiguous difference between the transcription profiles of *C. jejuni* HPC5 grown in the microaerobic conditions in the presence and absence of hydrogen.

A relative increase in the transcripts for superoxide dismutase and thiol peroxidase occurred when *C. jejuni* HPC5 was grown in the MACS. These genes help the organism to overcome the oxidative stress adding weight to the original hypothesis that hydrogen in the GRJ was providing protection so these genes were

only required to be up-regulated in the non hydrogen containing atmosphere of the MACS cabinet.

C.jejuni HPC5 grown in GRJ showed a relative up-regulation of 17 flagellar related genes which encode proteins involved in flagellar assembly, hook associated proteins as well as the motor and switch components. The flagellum of *Campylobacter* has a major role in invasion, adhesion, virulence, pathogenesis and colonisation. Thus the presence of hydrogen or other antioxidant during growth is likely to have a major impact on the ability of cells grown in this way and how they behave in experiments that mimic *in vivo* conditions, for example in colonisation assays. Analysis of the transcription data comparing growth in the two atmospheres revealed another important finding regarding the increased expression of genes which reportedly reduce the ability of campylobacters to undergo natural transformation in the MACS cabinet. The reason for this is not clear but may indicate a general stress response to unfavourable conditions where incorporation of foreign DNA would be inadvisable.

Many genes which were involved in energy conservation were up-regulated in the GRJ indicative of balanced growth comparative to the MACS while many ribosomal genes were up-regulated in *C. jejuni* HPC5 grown in MACS which indicated energy expenditure on growth and survival mechanisms.

Chapter 4 describes a study of *C. jejuni* HPC5 and its variants which were first described by Scott *et al.*, (2007). The resistance of some of the *C. jejuni* HPC5 variants (R14 and R20) to bacteriophage CP34 and their inefficiency in colonising chickens were of particular interest. Five further variants that were sensitive to

bacteriophage CP34 and efficient chicken colonisers were also studied. The main points which were addressed in this study included:

- Identification of genes that showed changes in the regulation pattern due to the effect of recombination in *C. jejuni* HPC5 revertant.
- Identification of possible mechanisms responsible for the generation of resistance in *C. jejuni* HPC5 R14 and R20.
- Investigation of the expression of flagellar genes in *C. jejuni* HPC5 and its variants.
- Investigate of the secretion of proteins by *C. jejuni* HPC5 and its variants.
- Identification of differences in temperature sensitivity of *C. jejuni* HPC5 variants

C.jejuni HPC5 colonised chickens administered with bacteriophage CP34 gave rise to a minor population of phage resistant genotypic variants, identified by *Sma*I digestion of genomic DNA separated by PFGE. In these strains genes associated with the prophage Mu were up-regulated in bacteriophage resistant strains compared to phage sensitive strains. It therefore appeared that prophage genes were involved both in the mechanism of rearrangement as proposed by Scott *et al.*, (2007) and in the subsequent resistance to the lytic phage CP34. Flagellar function appeared to be compromised in the phage resistant R14 and R20 series transcription patterns, which may influence their reduced colonisation ability. The up-regulation of the flagellar genes together with an observed increase in motility in bacteriophage sensitive strains that were proficient colonisers also is also consistent with contention that motility effects influence the colonisation proficiency of these strains. Expression of the CRISPR genes and genes that function in DNA repair were notably higher in

bacteriophage sensitive strains derived from passage in chickens of the phage resistant strains. The recovery of bacteriophage sensitivity was accompanied by an increased ability to respond to infection even in the absence of bacteriophage predation.

Other observations included the up-regulation of heat shock proteins in the bacteriophage sensitive variants whilst the bacteriophage resistant variants could be demonstrated to be significantly more temperature sensitive. The bacteriophage sensitive strains also showed a high level of modification in their membrane structure by differentially regulating many integral membrane proteins and putative lipoproteins. Secretary protein analysis did not show any remarkable difference between *C. jejuni* HPC5 and its variants.

Chapter 5 describes a study of campylobacters isolated from pigs and their genotypic characterisation. Pigs are a significant and stable reservoir of *C. coli.* Intestinal contents were collected from pigs slaughtered in the abattoir at different points in time and the *Campylobacter* content was analysed. Colony blot hybridisation using the *hip*O probe was used to identify minor populations of *C. jejuni* present amongst *C. coli.* By amplification of the hippurate and aspartokinase gene using specific PCR primers, the presence of *C. jejuni* and *C. coli* were confirmed respectively. Pigs mostly harboured *C. coli* and if *C. jejuni* was present it occurred as a minority population. Genotypic characterisation was performed using *Sma*I digestion of genomic DNA and separation by PFGE. It was found that multiple genotypes were isolated from pigs but certain genotypes were shared between individuals reared in a common indoor environment. The main objectives of this study were as follows:

- To identify the proportion of *C. jejuni* and *C. coli* present in pigs and to study the localisation of these species at different parts of the intestine.

- To determine whether the *C. coli* and *C. jejuni* populations in individual pigs comprised single or multiple genotypes.
- To investigate if genetic exchange between the *Campylobacter* species was occurring when they were present in the same intestine.

In this study *Campylobacter* was present in 100% of the pigs though it differed for the different parts of the intestine. Mostly it was isolated from the caecum and large intestine and rarely from the upper intestine. In outdoor reared pigs the prevalence of *C. jejuni* varied between 0.2% and 9.9% and for indoor pigs the *C. jejuni* prevalence ranged between 0 to 7%. Campylobacters were isolated from the caecum, upper intestine and lower intestine of outdoor reared pigs while it was present mostly in the caecum of indoor pigs.

Pigs were colonised by campylobacters with diverse genotypes. In some pigs multiple genotypes were present at different regions of the intestine. *C. coli* C101 and L101, identified as different genotypes, were isolated from the caecum and large intestine of the same pig whilst *C. jejuni* U101 was isolated from the upper intestine of this same pig. These three strains were selected to study the potential genetic exchange that may be occurring between them through natural transformation, bacterial conjugation and transduction. The *C. coli* stains had a nucleotide mismatch in their 16S ribosomal RNA that showed more similarity to typical *C. jejuni* strains. Phylogenetic analysis confirmed the resemblance of these *C. coli* strains to the *C. jejuni* strains. Competitive genomic hybridisation analysis showed that *C. coli* C101 and *C. jejuni* U101 shared many genes between them but *C. coli* L101 was less similar in gene content to *C. jejuni* U101. The *C. coli* strains housed several plasmids which encode virulence proteins and type IV secretion proteins.

In conclusion this thesis has shed valuable insights into some fundamental aspects of the physiology and transcriptome of *Campylobacter*. Understanding the importance of selecting suitable methods of atmosphere generation before even considering an experimental variable is clearly of paramount importance. A better understanding of the way *Campylobacter* is able to evade phage and the fitness cost associated with this evasion will contribute to the development of phage therapy treatments. The detailed investigation of the relationships between *C. coli* and *C. jejuni* populations from the same pig intestine will add to the debate as to whether the two species are converging or diverging. In addition the data on levels and degree of colonisation by the species provides valuable new information to researchers in the field as previously studies of pigs have been somewhat neglected in comparison to those of chickens.

6.2. FUTURE WORK

A study can be carried out to find the critical adaptation of campylocacters experiencing the fall in count when cultured under conditions such as those experienced in the MACS during their 2nd h of growth. Changes in transcription could be identified using transcript sequencing approaches with next generation sequencing methods. Rapid changes in transcription could also be monitored *in situ* using responsive reporter assays such as lux. Mutational studies (knock-out and knock-in strategies) can also be carried out to decipher the role of selected genes which were differentially expressed in this study and their role in the adaptation to each condition. This should provide valuable information to extend our knowledge concerning the mechanisms employed by campylobacters to survive the stress conditions occurring in MACS. Studies can also be carried out on the genes identified in this study as impotant in the transition of chicken colonization observed in *C. jejuni* HPC5 and its variants. A combination of transcriptional and mutational studies should identify the genes critical for colonization and discriminate those that are generally impaired in growth. A greater understanding of the transfer of genes between different species of campylobacters in pigs can be obtained by amplifying the target genes and analysing flanking regions within the genome, and in this way ascribe the genome regions transferred and any structural requirements necessary for the DNA transfer.

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Appendix 1

Sequence alignment of 16S rRNA gene of *C. coli* RM2228 and *C. jejuni* RM1221

```
RM2228          CCACGCCCTAAACGATGTACACTAGTTGTTGGGGTGCTAGTCATCTCAGTAATGCAGCTA 780
RM1221          CCACGCCCTAAACGATGTACACTAGTTGTTGGGGTGCTAGTCATCTCAGTAATGCAGCTA 780
                ************************************************************

RM2228          ACGCATTAAGTGTACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATAGAC 840
RM1221          ACGCATTAAGTGTACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATAGAC 840
                ************************************************************

RM2228          GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAGGATACGCGAAGAACCTTAC 900
RM1221          GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGATACGCGAAGAACCTTAC 900
                ************************************** ********************

RM2228          CTGGGCTTGATATCCTAAGAACCTTTTAGAGATAAGAGGGTGCTAGCTTGCTAGAACTTA 960
RM1221          CTGGGCTTGATATCCTAAGAACCTTTTAGAGATAAGAGGGTGCTAGCTTGCTAGAACTTA 960
                ************************************************************
```

Sequence alignment of 16S rRNA gene of *C. jejuni* NCTC 11168 and *C. coli* RM2228

```
RM2228          ACGCATTAAGTGTACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATAGAC 840
11168           ACGCATTAAGTGTACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATAGAC 830
                ************************************************************

RM2228          GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAGGATACGCGAAGAACCTTAC 900
11168           GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGATACGCGAAGAACCTTAC 890
                ************************************** ********************

RM2228          CTGGGCTTGATATCCTAAGAACCTTTTAGAGATAAGAGGGTGCTAGCTTGCTAGAACTTA 960
11168           CTGGGCTTGATATCCTAAGAACCTTATAGAGATATGAGGGTGCTAGCTTGCTAGAACTTA 950
                ************************** ******** **************************

RM2228          GAGACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC 1020
11168           GAGACAGGTGCTGCACGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC 1010
                ************************************************************
```

Appendix 2

C.jejuni NCTC 11168 oligonucleotides shared with *C. coli* C101									
Cj0001	Cj0198c	Cj0386	Cj0573	Cj0792	Cj0933c	Cj1152c	Cj1359	Cj1494c	Cj1721c
Cj0002	Cj0199c	Cj0390	Cj0574	Cj0795c	Cj0940c	Cj1161c	Cj1363	Cj1503c	Cj1722c
Cj0010c	Cj0226	Cj0392c	Cj0577c	Cj0800c	Cj0941c	Cj1163c	Cj1364c	Cj1504c	Cj1729c
Cj0014c	Cj0243c	Cj0401	Cj0590	Cj0802	Cj0945c	Cj1167	Cj1370	Cj1507c	
Cj0015c	Cj0245	Cj0402	Cj0591c	Cj0810	Cj0946	Cj1168c	Cj1372	Cj1533c	
Cj0022c	Cj0251c	Cj0404	Cj0598	Cj0811	Cj0948c	Cj1176c	Cj1377c	Cj1539c	
Cj0024	Cj0259	Cj0406c	Cj0604	Cj0812	Cj0949c	Cj1199	Cj1379	Cj1542	
Cj0036	Cj0260c	Cj0410	Cj0606	Cj0813	Cj0962	Cj1204c	Cj1381	Cj1549c	
Cj0038c	Cj0264c	Cj0418c	Cj0608	Cj0818	Cj0964	Cj1208	Cj1386	Cj1569c	
Cj0059c	Cj0282c	Cj0419	Cj0621	Cj0820c	Cj0980	Cj1209	Cj1387c	Cj1572c	
Cj0060c	Cj0288c	Cj0422c	Cj0627	Cj0823	Cj0982c	Cj1211	Cj1405	Cj1573c	
Cj0082	Cj0295	Cj0449c	Cj0631c	Cj0825	Cj0997	Cj1218c	Cj1407c	Cj1580c	
Cj0086c	Cj0302c	Cj0454c	Cj0634	Cj0830	Cj1000	Cj1219c	Cj1408	Cj1582c	
Cj0089	Cj0303c	Cj0455c	Cj0642	Cj0831c	Cj1014c	Cj1229	Cj1409	Cj1583c	
Cj0091	Cj0304c	Cj0456c	Cj0660c	Cj0833c	Cj1015c	Cj1230	Cj1410c	Cj1595	
Cj0097	Cj0309c	Cj0458c	Cj0682	Cj0834c	Cj1017c	Cj1232	Cj1411c	Cj1600	
Cj0108	Cj0310c	Cj0459c	Cj0689	Cj0836	Cj1027c	Cj1240c	Cj1412c	Cj1608	
Cj0109	Cj0313	Cj0464	Cj0692c	Cj0837c	Cj1029c	Cj1244	Cj1417c	Cj1609	
Cj0110	Cj0316	Cj0466	Cj0693c	Cj0838c	Cj1041c	Cj1249	Cj1418c	Cj1618c	
Cj0112	Cj0317	Cj0494	Cj0695	Cj0842	Cj1058c	Cj1253	Cj1421c	Cj1620c	
Cj0119	Cj0318	Cj0498	Cj0698	Cj0844c	Cj1062	Cj1257c	Cj1432c	Cj1625c	
Cj0120	Cj0320	Cj0504c	Cj0705	Cj0853c	Cj1064	Cj1260c	Cj1433c	Cj1627c	
Cj0128c	Cj0321	Cj0507	Cj0707	Cj0854c	Cj1071	Cj1269c	Cj1435c	Cj1629	
Cj0138	Cj0322	Cj0510c	Cj0710	Cj0855	Cj1076	Cj1275c	Cj1436c	Cj1632c	
Cj0141c	Cj0323	Cj0511	Cj0711	Cj0862c	Cj1077	Cj1277c	Cj1442c	Cj1634c	
Cj0142c	Cj0324	Cj0512	Cj0714	Cj0881c	Cj1078	Cj1279c	Cj1443c	Cj1636c	
Cj0143c	Cj0328c	Cj0515	Cj0715	Cj0892c	Cj1079	Cj1283	Cj1445c	Cj1637c	
Cj0148c	Cj0332c	Cj0522	Cj0717	Cj0893c	Cj1083c	Cj1284	Cj1448c	Cj1646	
Cj0149c	Cj0333c	Cj0523	Cj0718	Cj0894c	Cj1095	Cj1289	Cj1449c	Cj1650	
Cj0153c	Cj0338c	Cj0524	Cj0719c	Cj0895c	Cj1096c	Cj1300	Cj1459	Cj1663	
Cj0155c	Cj0350	Cj0529c	Cj0721c	Cj0899c	Cj1097	Cj1311	Cj1461	Cj1667c	
Cj0156c	Cj0352	Cj0538	Cj0722c	Cj0903c	Cj1099	Cj1313	Cj1468	Cj1673c	
Cj0160c	Cj0361	Cj0539	Cj0767c	Cj0908	Cj1101	Cj1322	Cj1473c	Cj1674	
Cj0164c	Cj0365c	Cj0541	Cj0774c	Cj0914c	Cj1106	Cj1326	Cj1476c	Cj1680c	
Cj0166	Cj0366c	Cj0551	Cj0775c	Cj0917c	Cj1107	Cj1346c	Cj1477c	Cj1682c	
Cj0183	Cj0374	Cj0560	Cj0777	Cj0919c	Cj1115c	Cj1349c	Cj1483c	Cj1684c	
Cj0185c	Cj0378c	Cj0561c	Cj0778	Cj0923c	Cj1125c	Cj1350	Cj1484c	Cj1689c	
Cj0186c	Cj0380c	Cj0564	Cj0783	Cj0925	Cj1141	Cj1351	Cj1486c	Cj1712	
Cj0187c	Cj0382c	Cj0570	Cj0786	Cj0927	Cj1143	Cj1358c	Cj1493c	Cj1714	

Appendix 2 (continued)

***C. jejuni* NCTC 11168 oligonucleotides shared with *C. jejuni* U101**		***C. jejuni* NCTC 11168 oligonucleotides shared with *C. coli* L101**			
Cj0006	Cj0803	Cj0012c	Cj0582	Cj0996	Cj1513c
Cj0018c	Cj0823	Cj0029	Cj0584	Cj1002c	Cj1515c
Cj0027	Cj0850c	Cj0033	Cj0599	Cj1003c	Cj1529c
Cj0032	Cj0947c	Cj0040	Cj0610c	Cj1022c	Cj1541
Cj0038c	Cj0949c	Cj0056c	Cj0613	Cj1028c	Cj1570c
Cj0055c	Cj0964	Cj0069	Cj0639c	Cj1056c	Cj1653c
Cj0113	Cj0971	Cj0070c	Cj0648	Cj1057c	Cj1718c
Cj0117	Cj1011	Cj0078c	Cj0650	Cj1062	Cj1719c
Cj0170	Cj1072	Cj0093	Cj0702	Cj1068	Cj1722c
Cj0171	Cj1077	Cj0103	Cj0706	Cj1069	
Cj0207	Cj1082c	Cj0108	Cj0716	Cj1075	
Cj0208	Cj1108	Cj0123c	Cj0759	Cj1076	
Cj0263	Cj1113	Cj0130	Cj0768c	Cj1090c	
Cj0265c	Cj1139c	Cj0147c	Cj0786	Cj1102	
Cj0294	Cj1166c	Cj0188c	Cj0788	Cj1109	
Cj0303c	Cj1220	Cj0252	Cj0794	Cj1138	
Cj0304c	Cj1223c	Cj0260c	Cj0799c	Cj1139c	
Cj0306c	Cj1238	Cj0337c	Cj0805	Cj1165c	
Cj0332c	Cj1262	Cj0369c	Cj0814	Cj1201	
Cj0385c	Cj1276c	Cj0371	Cj0815	Cj1210	
Cj0386	Cj1324	Cj0383c	Cj0816	Cj1235	
Cj0412	Cj1325	Cj0395c	Cj0822	Cj1245c	
Cj0414	Cj1333	Cj0397c	Cj0852c	Cj1247c	
Cj0418c	Cj1341c	Cj0418c	Cj0900c	Cj1248	
Cj0420	Cj1343c	Cj0442	Cj0902	Cj1260c	
Cj0424	Cj1357c	Cj0461c	Cj0918c	Cj1265c	
Cj0430	Cj1415c	Cj0472	Cj0922c	Cj1267c	
Cj0473	Cj1416c	Cj0474	Cj0932c	Cj1288c	
Cj0570	Cj1418c	Cj0501	Cj0933c	Cj1299	
Cj0571	Cj1420c	Cj0542	Cj0937	Cj1308	
Cj0596	Cj1427c	Cj0544	Cj0946	Cj1309c	
Cj0626	Cj1436c	Cj0551	Cj0955c	Cj1310c	
Cj0645	Cj1437c	Cj0555	Cj0956c	Cj1318	
Cj0651	Cj1440c	Cj0567	Cj0959c	Cj1322	
Cj0686	Cj1556	Cj0572	Cj0962	Cj1324	
Cj0729	Cj1568c	Cj0574	Cj0965c	Cj1325	
Cj0731	Cj1585c	Cj0575	Cj0968	Cj1384c	
Cj0759	Cj1599	Cj0577c	Cj0974	Cj1417c	
Cj0767c	Cj1606c	Cj0578c	Cj0989	Cj1423c	
Cj0786	Cj1638	Cj0581	Cj0991c	Cj1492c	

Appendix 3

C. coli RM2228 oligonucleotides present in *C. coli* C101, L101 and *C. jejuni* U101. Shaded colour represents the oligos which were common between strains

C101	U101	L101	C101	U101	L101	C101	U101
CCO0026	CCO0041	CCO0040	CCO0510	CCOA0136	CCOA0023	CCO1308	pCC31p38
CCO0035	CCO0347	CCO0102	CCO0511	CCOA0145	CCOA0075	CCO1309	pCC31p41
CCO0039	CCO0508	CCO0109	CCO0518	CCOA0146	CCOA0077	CCO1310	pCC31p42
CCO0040	CCO0509	CCO0137	CCO0534	CCOA0171	CCOA0105	CCO1311	pCC31p43
CCO0098	CCO0510	CCO0183	CCO0535	CCOA0175	CCOA0107	CCO1312	pCC31p48
CCO0104	CCO1215	CCO0184	CCO0536	CCOA0178	CCOA0115	CCO1325	RepA
CCO0105	CCO1303	CCO0185	CCO0601	CCOA0188	CCOA0117	CCO1326	
CCO0127	CCO1369	CCO0291	CCO0603	CCOA0189	CCOA0122	CCO1340	
CCO0128	CCO1446	CCO0347	CCO0654	CCOA0198	CCOA0131	CCO1341	
CCO0138	CCO1484	CCO0351	CCO0658	CCOA0199	CCOA0134	CCO1342	
CCO0182	CCO1541	CCO0354	CCO0815	CCOA0201	CCOA0137	CCO1514	
CCO0183	CCO1610	CCO0355	CCO0847	CCOA0205	CCOA0170	CCO1532	
CCO0184	CCO1656	CCO0412	CCO0917	CCOA0206	p3384_03	CCO1534	
CCO0185	CCO1662	CCO0535	CCO0919	CCOA0208	p3386_03	CCO1536	
CCO0187	CCO1738	CCO0601	CCO0921	p3384_02	pCC31p02	CCO1587	
CCO0190	CCO1785	CCO0603	CCO0926	pCC31p02	pCC31p03	CCO1651	
CCO0203	CCOA0013	CCO0658	CCO0955	pCC31p03	CCOA_CctmRNA2	CCO1658	
CCO0204	CCOA0017	CCO0764	CCO0970	pCC31p04	CCOA0049	CCO1662	
CCO0212	CCOA0018	CCO0921	CCO1005	pCC31p05	CCOA0053	CCO1667	
CCO0213	CCOA0020	CCO1040	CCO1041	pCC31p07	CCOA0062	CCO1669	
CCO0251	CCOA0027	CCO1076	CCO1049	pCC31p09	CCOA0064	CCO1688	
CCO0252	CCOA0055	CCO1143	CCO1076	pCC31p14	CCOA0078	CCO1689	
CCO0254	CCOA0058	CCO1280	CCO1077	pCC31p15	CCOA0109	CCO1694	
CCO0285	CCOA0060	CCO1281	CCO1078	pCC31p16	CCOA0122	CCO1707	
CCO0292	CCOA0061	CCO1310	CCO1142	pCC31p17	CCOA0130	CCO1708	
CCO0293	CCOA0062	CCO1484	CCO1170	pCC31p18	CCOA0131	CCO1738	
CCO0349	CCOA0072	CCO1587	CCO1171	pCC31p23	CCOA0147	CCO1740	
CCO0350	CCOA0074	CCO1654	CCO1218	pCC31p25	CCOA0184	CCO1758	
CCO0353	CCOA0076	CCO1668	CCO1277	pCC31p26	CCOA0203	CCO1784	
CCO0354	CCOA0077	CCO1707	CCO1280	pCC31p27	CCOA0206	CCO1785	
CCO0355	CCOA0078	CCO1708	CCO1298	pCC31p29	CCOA0207	CCO1786	
CCO0368	CCOA0081	CCO1758	CCO1299	pCC31p32		CCO1801	
CCO0384	CCOA0083	CCO1784	CCO1303	pCC31p34			
CCO0413	CCOA0105	CCO1785	CCO1306	pCC31p35			
CCO0509	CCOA0119	CCO1786	CCO1307	pCC31p36			

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	C101, U101, L101		C101, L101		L101, U101		C101, U101		Unique to each strain

Appendix 4

C. coli C101 RM1221 probes 0.8 to 1.2	
RM1221 Locus Tag	**Match**
CJE_tRNA-Ala-4	CJE_tRNA-Ala-4
CJE1550	hypothetical protein
CJE0555	hypothetical protein
CJE0581	hypothetical protein
CJE1732	arsenate reductase
CJE0559	hypothetical protein
CJE1527	hypothetical protein
CJE0575	hypothetical protein
CJE0588	hypothetical protein
CJE1733	arsenical-resistance protein, putative
CJE1721	RloC protein, putative
CJE0598	hypothetical protein
CJE1724	type I restriction-modification system, M subunit
CJE1425	hypothetical protein
CJE1428	hypothetical protein

C. coli C101 NCTC 11168 probes 0.8 to 1.2	
Cj0171	hypothetical protein
Cj0417	hypothetical protein
Cj0974	hypothetical protein
Cj0987c	putative MFS (Major Facilitator Superfamily) transport protein
Cj1427c	putative sugar-nucleotide epimerase/dehydratease
Cj1441c	UDP-glucose 6-dehydrogenase
Cjp47	-
Cjr03	5S ribosomal RNA
Cjr09	5S ribosomal RNA
pTet_30	hypothetical protein

Appendix 4 (continued)

C. coli C101 RM2228 probes 0.8 to 1.2	
CCO_Cc5SD	5S ribosomal RNA
CCO_Cc5SE	5S ribosomal RNA
CCO0371	phosphoglycerate transporter protein pgtP
CCO0956	conserved hypothetical protein
CCO1339	ISCco1, transposase
CCO1401	conserved hypothetical protein
CCO1444	flagellin (flaA)
CCO1467	toxin-like outer membrane protein, putative
CCO1529	Domain of unknown function (DUF386) superfamily
CCO1549	capsule biosynthesis protein, putative
CCO1676	arsenite efflux transporter
CCOA0050	hypothetical protein
CCOA0065	mobilization/transfer protein
CCOA0084	Domain of unknown function (DUF332) superfamily
CCOA0103	conserved hypothetical protein
CCOA0170	conserved hypothetical protein
CCOA0205	conserved hypothetical protein
pCC31p26	putative DNA invertase
pCC31p39	putative type IV secretion system component

U101 RM1221 probes between 0.8 to 1.2 flags	
RM1221.locus tag	**RM1221.description**
CJE_CjtmRNA1	sRNA
CJE_tRNA-Ala-4	tRNA-Ala
CJE0013	pseudogene
CJE0030	hypothetical protein
CJE0205	hypothetical protein
CJE0207	hypothetical protein
CJE0213	hypothetical protein
CJE0214	hypothetical protein
CJE0224	hypothetical protein
CJE0225	hypothetical protein
CJE0226	phage major tail tube protein, putative
CJE0227	major tail sheath protein
CJE0229	hypothetical protein
CJE0230	hypothetical protein
CJE0231	tail fiber protein H, putative
CJE0233	baseplate assembly protein J, putative

Appendix 4 (continued)

U101 RM1221 probes between 0.8 to 1.2 flags (continued)	
RM1221.locus tag	**RM1221.description**
CJE0237	hypothetical protein
CJE0240	hypothetical protein
CJE0241	hypothetical protein
CJE0244	Mu-like prophage I protein, putative
CJE0246	hypothetical protein
CJE0247	hypothetical protein
CJE0250	hypothetical protein
CJE0251	prophage MuSo1, F protein, putative
CJE0253	tail protein X, putative
CJE0257	hypothetical protein
CJE0261	hypothetical protein
CJE0264	hypothetical protein
CJE0268	hypothetical protein
CJE0269	bacteriophage DNA transposition protein B, putative
CJE0465	hypothetical protein
CJE0466	hypothetical protein
CJE0487	hypothetical protein
CJE0489	succinate dehydrogenase, iron-sulfur protein (*sdh*B)
CJE0602	hypothetical protein
CJE0671	hypothetical protein
CJE0721	hypothetical protein
CJE0757	di-/tripeptide transporter
CJE0776	potassium-transporting ATPase, C subunit, putative
CJE0777	Pseudogene
CJE0789	hypothetical protein
CJE0836	Pseudogene
CJE1048	hypothetical protein
CJE1049	hypothetical protein
CJE1050	hypothetical protein
CJE1097	hypothetical protein
CJE1108	hypothetical protein
CJE1121	Pseudogene
CJE1148	hypothetical protein
CJE1304	hypothetical protein
CJE1432	hypothetical protein
CJE1551	hypothetical protein
CJE1603	capsular polysaccharide biosynthesis protein, putative
CJE1608	capsular biosynthesis nucleotidyltransferase, putative
CJE1674	hypothetical protein
CJE1694	CRISPR-associated Cas2 family protein (*cas*2)
CJE1723	MloA protein, putative
CJE1726	hypothetical protein
CJE1877	hypothetical protein

Appendix 4 (continued)

C. jejuni U101 NCTC 11168 flags 0.8 to 1.2 flags	
NCTC 11168Locus Tag	**Match**
Cj0259	dihydroorotase (pyrC)
Cj0416	hypothetical protein
Cj0973	hypothetical protein
Cj1055c	putative sulfatase family protein
Cj1136	putative glycosyltransferase
Cj1143	two-domain bifunctional protein (neuA1)
Cj1254	hypothetical protein
Cj1332	flagellin modification protein A (ptmA)
Cj1429c	hypothetical protein
Cj1432c	putative sugar transferase
Cj1435c	putative phosphatase
Cj1441c	UDP-glucose 6-dehydrogenase (kfiD)
Cj1633	putative ATP-binding protein

C. jejuni U101 RM2228 probes 0.8 to 1.2	
RM 2228 Locus Tag	**RM2228 gene description**
CCO0257	glcG protein
CCO_Cc5SF	5S ribosomal RNA
CCO_CcrnpB1	sRNA
CCO0072	probable integral membrane protein Cj0033
CCO0186	hypothetical protein
CCO0281	hypothetical protein
CCO0356	hypothetical protein
CCO0804	hypothetical protein
CCO0866	probable oxidoreductase Cj0807
CCO0968	conserved hypothetical protein
CCO1077	hypothetical protein
CCO1142	conserved hypothetical protein
CCO1216	hypothetical protein
CCO1326	hypothetical protein
CCO1412	acetyltransferase, GNAT family family
CCO1443	flagellin Cj1338c
CCO1467	toxin-like outer membrane protein, putative
CCO1532	D-3-phosphoglycerate dehydrogenase (serA)
CCO1533	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase
CCO1588	conserved hypothetical protein
CCO1801	hypothetical protein

Appendix 4 (continued)

C. jejuni U101 RM2228 probes 0.8 to 1.2(continued)	
RM 2228 Locus Tag	**RM2228 gene description**
CCOA0010	helicase, Snf2 family
CCOA0035	hypothetical protein
CCOA0050	hypothetical protein
CCOA0113	hypothetical protein
CCOA0124	hypothetical protein
CCOA0138	conserved hypothetical protein
CCOA0159	DNA primase, putative
CCOA0160	hypothetical protein
CCOA0173	conserved hypothetical protein

L101 RM1221 probes between 0.8 to 1.2 flags	
RM1221 Locus Tag	**Gene description**
CJE0844	Pseudogene
CJE_CjtmRNA1	sRNA
CJE_tRNA-Ala-4	CJE_tRNA-Ala-4
CJE_tRNA-Thr-2	CJE_tRNA-Thr-2
CJE0232	phage tail protein, putative
CJE0465	hypothetical protein
CJE0496	Pseudo
CJE0546	pentapeptide repeat-containing protein
CJE0551	hypothetical protein
CJE0560	hypothetical protein
CJE0573	hypothetical protein
CJE0576	hypothetical protein
CJE0581	hypothetical protein
CJE0588	hypothetical protein
CJE0589	hypothetical protein
CJE0731	type III restriction/modification enzyme, methylase subunit
CJE0732	type III restriction-modification enzyme
CJE0846	hypothetical protein
CJE1095	hypothetical protein
CJE1104	hypothetical protein
CJE1108	hypothetical protein
CJE1116	hypothetical protein
CJE1132	hypothetical protein
CJE1136	hypothetical protein
CJE1148	hypothetical protein
CJE1439	hypothetical protein

Appendix 4 (continued)

L101 RM1221 probes between 0.8 to 1.2 flags(continued)	
RM1221 Locus Tag	**Gene description**
CJE1527	hypothetical protein
CJE1611	GDP-mannose 4,6-dehydratase (wcbK)
CJE1695	CRISPR-associated Cas1 family protein (cas1)
CJE1732	arsenate reductase (arsC)
CJE1733	arsenical-resistance protein, putative
CJE1763	50S ribosomal protein L36 (rpmJ)
CJE1877	hypothetical protein

C. coli L101 RM2228 probes 0.8 to 1.2	
RM2228 locus tag	**RM2228 gene description**
CCO_Cc5SE	5S ribosomal RNA
CCO_Cc5SF	5S ribosomal RNA
CCO_CctmRNA1	sRNA
CCO_tRNA-Ala-2	tRNA-Ala
CCO_tRNA-Asp-2	tRNA-Asp
CCO_tRNA-Gly-2	tRNA-Gly
CCO0001	similar to 50S ribosomal protein L3
CCO0072	probable integral membrane protein Cj0033
CCO0107	conserved hypothetical protein
CCO0280	methyl-accepting chemotaxis protein (tlpA)
CCO0349	carboxyphosphonoenolpyruvate phosphonomutase
CCO0429	membrane protein, putative
CCO0430	membrane protein, putative
CCO0804	hypothetical protein
CCO0925	hypothetical protein
CCO1126	Pseudogene
CCO1211	glycosyl transferase, group 1 family protein
CCO1349	membrane protein, putative
CCO1409	conserved hypothetical protein
CCO1444	flagellin (flaA)
CCO1467	toxin-like outer membrane protein, putative
CCO1468	vacuolating cytotoxin precursor, putative
CCO1530	Putative cyclase superfamily
CCO1548	minor teichoic acids biosynthesis protein ggab
CCO1663	Helicase conserved C-terminal domain protein
CCO1675	arsenite efflux transporter
CCOA_CctmRNA2	sRNA
CCOA0001	replication protein
CCOA0006	conserved hypothetical protein

Appendix 4 (continued)

C. coli L101 RM2228 probes 0.8 to 1.2(continued)	
RM2228 locus tag	**RM2228 gene description**
CCOA0024	hypothetical protein
CCOA0028	hypothetical protein
CCOA0029	hypothetical protein
CCOA0067	aminoglycoside 3'-phosphotransferase
CCOA0072	TnpV
CCOA0079	hypothetical protein
CCOA0109	conserved hypothetical protein
CCOA0120	hypothetical protein
CCOA0127	conserved hypothetical protein
CCOA0145	Pseudogene
CCOA0171	conserved hypothetical protein
CCOA0179	hypothetical protein
CCOA0186	type IV secretion system protein, putative
CCOA0196	conserved hypothetical protein
CCOA0203	ISCcol, transposase orfA
CCOA0208	hypothetical protein
p3386_02	putative Rep
pCC31p01	tetracycline resistance
pCC31p10	cpp10
pCC31p12	cpp12
pCC31p13	cpp13
pCC31p18	cpp18
pCC31p22	cpp23
pCC31p30	putative type IV secretion system component
pCC31p36	putative type IV secretion system component
pCC31p43	cpp44
pCC31p49	cpp50

C. coli L101 NCTC 11168 oligos with Cy5/Cy3 between 0.8 to 1.2	
NCTC 11168 locus tag	**NCTC 11168 gene description**
Cj0171	hypothetical protein
Cj0417	hypothetical protein
Cj0974	hypothetical protein
Cj0987c	putative MFS (Major Facilitator Superfamily) transport protein
Cj1427c	putative sugar-nucleotide epimerase/dehydratease
Cj1441c	UDP-glucose 6-dehydrogenase (*kfi*D)
Cjp47	hypothetical protein
Cjr03	5S ribosomal RNA
Cjr09	5S ribosomal RNA
pTet_30	hypothetical protein