INSTITUTE OF INFECTION AND GLOBAL HEALTH

LIVERPOOL

SURVIVAL OF *Campylobacter jejuni* IN THE ENVIRONMENT

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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ABSTRACT

Campylobacter jejuni is an emerging food borne pathogen and a successful human pathogen, with the infection mostly transmitted to humans through consumption of contaminated under-cooked poultry meat. However, the environment can also play a role in transmission either directly or indirectly to humans. The microorganism has reservoirs in water and various animals. Its survival outside the host is generally thought to be poor, but the organism survives well in poultry meat. Previous studies have suggested that the ability to survive may vary between different strain types of *C. jejuni*.

A number of survival experiments were conducted, based on the ability of different *C. jejuni* strains to retain culturability in sterile distilled water. These experiments demonstrated that survival varies between different strains of *C. jejuni* and that the retention of culturability was much better at low temperatures (4 °C) than higher temperatures (25 °C). Survival was also better in non-autoclaved natural water. One strain, *C. jejuni* M1, lost culturability more quickly at both temperatures than the others tested. However, cells remained viable in these samples, suggesting that the bacteria had entered into a viable but non-culturable (VBNC) state under stress conditions. These variations may contribute to the transmission of *C. jejuni* from the environment to humans or farm animals.

Using end-point and Q-PCR assays, a set of stress response genes, including genes implicated previously in the formation of VBNC cells, were targeted for different *C. jejuni* strains during survival in sterile distilled water. Differences in gene expression between different strains of *C. jejuni* were identified, including in key genes (*luxS*, *htrA*, *ppk1*), suggesting that these genes might have contributed to strain M1

switching to a VBNC state in response to starvation (sterile distilled water). This is the first report suggesting a role for the *C. jejuni luxS* (a gene likely to be involved in quorum sensing) in the formation of VBNC state and survival in water.

Co-existence with other microorganisms, such as *Pseudomonas* spp., is one of the suggested survival strategies of *C. jejuni* in the environment. In a small study using environmental PCR assays, it was demonstrated that when *C. jejuni* is present in the farm environment, *Pseudomonas* spp. are also always present. In preliminary *in vitro* experiments, we demonstrated that some fluorescent *Pseudomonas* spp. could secret proteinaceous products that enhance the growth of *Campylobacter*. In natural environments, it is likely that interactions with other species, such as *Pseudomonas*, play an important role in *C. jejuni* survival and subsequent transmission to humans or animals.

DECLARATION

This thesis is a presentation of my own research work. The material presented here has not been presented and is not being presented, either wholly or in part for any other degree or qualification. Some of the technical procedures were carried out in collaboration with other people and reference has been made to specific data from other colleagues where appropriate.

The research work was carried out under the guidance of Professor Craig Winstanley at the Institute of Infection and Global Health, Department of Clinical infection, Microbiology and Immunology, University of Liverpool.

Kasem Mustafa

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AI	Autoinducer
AP-PCR	Arbitrarily primed polymerase chain reaction amplification
BP	Base pair
°C	Celsius
CBA	Columbia Blood Agar
CCs	Clonal complexes
CDT	Cytolethal distending toxin
CFU	Colony forming unit
Cia	*Campylobacter* invasion antigens
CLOs	*Campylobacter*-like organisms
CN	Cetrimide and Sodium nalidixate
C_q	Quantification cycle values
CSF	Cerebrospinal fluid
DCs	Dendritic cells
DGGE	Denaturing gradient gel electrophoresis
DNase	Deoxyribonuclease
ED	Entner-Doudoroff
fla	Flagellin
FSA	Food Standards Agency
GBS	Guillain-Barré syndrome
H	Hour
HRM	High –resolution melting
IBD	Inflammatory bowel diseases

IBS	Irritable bowel syndrome
IgA	Immunoglobulin A
IID	Infectious intestinal disease
IL	Interleukin
ITS	Internal transcribed spacer
Kb	Kilobase
LB	Luria Bertani
LOSs	Lipooligosaccharides
mCCDA	Charcoal Cefoperazine Deoxycholate Agar
MFS	Miller Fisher syndrome
MHB	Muller Hinton Broth
MK	Menaquinone
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable number tandem repeat analysis
MST	Multispacer typing
N	Number of samples
PCR	Polymerase chain reaction
PFGE	Pulsed-Field gel electrophoresis
Q-PCR	Quantitative polymerase chain reaction
QS	Quorum sensing
REP-PCR	Repetitive sequencing-based PCR
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SDW	Sterile distilled water
STs	Sequence types

TBE	Tris borate EDTA
TLRs	Toll-like receptors
VBNC	Viable but non culturable
V/V	Volume/Volume

C h a p t e r 1

GENERAL INTRODUCTION

1.1 The Genus *Campylobacter*

The term *Campylobacter* is derived from the Greek word meaning curved rod. *Campylobacter* species are small Gram-negative rods, 0.2 to 0. 5 µm wide and 0.5 to 8 µm long characterised by S-shaped cells and microaerophilic in nature (Figure 1.1) (Halablab et al., 2008; Penner, 1988). They are generally flagellated with either bipolar flagella or a single polar flagellum enabling the organism to be motile with a characteristic corkscrew movement (Figure 1.2) (Smibert, 1978). Table 1.1 summarises currently recognized species and subspecies within the genus *Campylobacter*. The thermophilic species *C. jejuni* and *C. coli*, which grow best at 42 °C, are the most significant species in terms of food safety and are the major cause of *Campylobacter* infections in developed countries (Gillespie et al., 2002; Ketley, 1995; Man, 2011; Penner, 1988). As well as being microaerophilic, members of the genus *Campylobacter* have other fastidious growth requirements. They are unable to survive atmospheric oxygen levels and grow optimally in atmospheres containing 5% (v/v) oxygen and their optimal growth is at 42 °C, but they do not grow at temperatures below 30 °C. They are catalase positive, and previous studies suggested *Campylobacter* species are unable to ferment carbohydrates as a carbon source due to the lack of 6-phosphofructokinase, but they use amino acids or tricarboxylic acid cycle intermediates to obtain their energy (Adzitey and Nurul, 2011; Velayudhan and Kelly, 2002). However, one very recent study has revealed that some strains of *C. coli* (and *C. jejuni* subsp. *doylei*) are glycolytic and can utilise glucose via the pentose phosphate and Entner-Doudoroff (ED) pathway which is encoded by a genomic island. Hence, it

has been suggested that these glycolytic capabilities of some *C. coli* and *C. jejuni* subsp. *doylei* strains have been acquired via horizontal gene transfer (Vorwerk et al., 2015). Because of the fastidious growth requirements, these pathogens are unable to multiply during food processing or storage (Nachamkin et al., 2008; Park, 2002). *Campylobacter* infection is usually associated with a low infective dose of 500 organisms (Griffiths and Park, 1990). The bacterium can be found in the intestinal tract, reproductive organs, and oral cavity of humans and animals (Nachamkin et al., 2008).

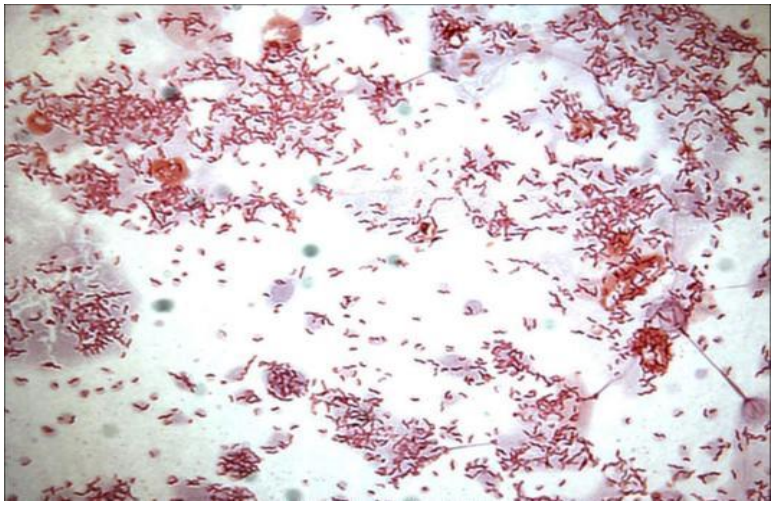


Figure 1.1 *C. jejuni* observed microscopically, recovered from a stool culture obtained from a one year old patient suffering from *Campylobacter* infection. Taken from http://www.microbelibrary.org.

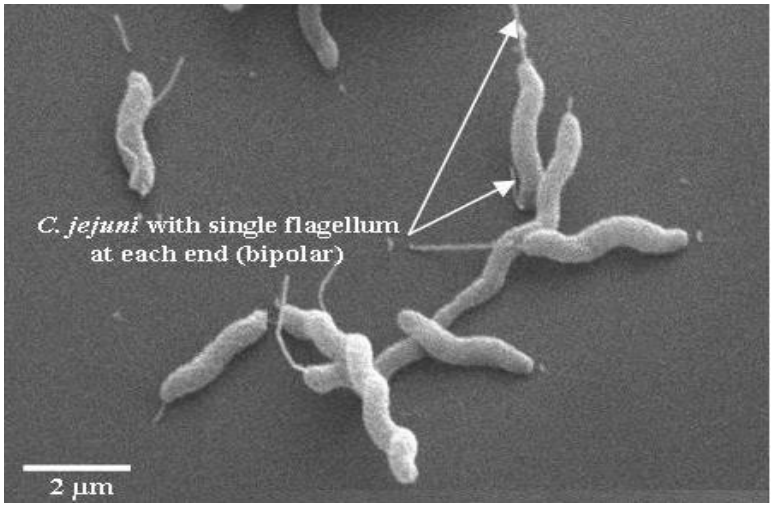


Figure 1.2 Image of unipolar and bipolar flagella and corkscrew shape of *C. jejuni* under electron microscope. Taken from http://www.microbelibrary.org.

Table 1.1 Currently recognized species and subspecies within the genus *Campylobacter* and isolation sites in humans and animals.

***Campylobacter* spp.**	**Isolation site in humans**	**Reference**
C. coli	Blood, faeces/intestinal tract, gallbladder, retroperitoneal abscess, cerebrospinal fluid (CSF)	(Blaser et al., 1986; Inglis et al., 2011; Petersen et al., 2007)
C. concisus	Blood, faeces/intestinal tract, oral cavity, duodenal biopsy, brain biopsy	(de Vries et al., 2008; Istivan et al., 2008; Macuch and Tanner, 2000; Vandamme et al., 1989; Zhang et al., 2009)
C. curvus	Faeces, oral cavity, alveolar abscess.	(Inglis et al., 2011; Macuch and Tanner, 2000; Petersen et al., 2007)
C. fetus subsp. *fetus*	Blood, faeces, gastric aspirate, CSF, vagina, liver, lungs, skin and spleen of an aborted foetus.	(Dronda et al., 1998; Ichiyama et al., 1998; Petersen et al., 2007; Sauerwein et al., 1993; Simor et al., 1986)
C. fetus subsp. *venerealis*	Blood	(Petersen et al., 2007)
C. fetus subsp. *testudinum*	Blood, Faeces, pleural fluid, hematoma, bile	(Fitzgerald et al., 2014; Patrick et al., 2013)
C. gracilis	Faeces, oral cavity, brain abscess	(de Vries et al., 2008; Inglis et al., 2011; Macuch and Tanner, 2000; Man et al., 2010)
C. hominis	Blood, faeces, intestinal tract	(Inglis et al., 2011; Linscott et al., 2005; Zhang et al., 2009)
C. helveticus	Faeces	(Inglis et al., 2011)

C. hyointestinalis		Blood, faeces	(Gorkiewicz et al., 2002; Lastovica, 1996)
C. insulaenigrae		Faeces	(Inglis et al., 2011)
C. jejuni subsp. *jejuni*		Blood, faeces, gastric biopsy, gallbladder, thoracic wall, peritoneal fluid, CSF, urine	(Blaser et al., 1986; Inglis et al., 2011; Petersen et al., 2007)
C. jejuni subsp. *doylei*		Gastric biopsy, bacteraemia, Faeces	(Parker et al., 2007; Steele and Owen, 1988)
C. lanienae		Faeces	(Logan et al., 2000)
C. lari subsp. *lari*		Blood, faeces, oral cavity	(Kumar et al., 2005; Morris et al., 1998; Petersen et al., 2007)
C. lari subsp. *concheus*		Faeces, bacteraemia	(Debruyne et al., 2009)
C. mucosalis		Faeces, oral cavity	(Inglis et al., 2011; Kumar et al., 2005)
C. peloridis		Faeces, dialysis fluid	(Debruyne et al., 2009)
C. rectus		Intestinal tract, oral cavity, vertebral abscesses	(de Vries et al., 2008; Macuch and Tanner, 2000; Man et al., 2010)
C. showae		Faeces/intestinal tract, intraorbital abscess, oral cavity	(de Vries et al., 2008; Inglis et al., 2011; Macuch and Tanner, 2000)
C. sputorum	Biovar *sputorum*	Blood, faeces, oral cavity, axillary abscess, pus (pressure sore)	(Lindblom et al., 1995; On et al., 1998; On et al., 1992; Petersen et al., 2007; Tee et al., 1998)
	Biovar *paraureolyticus*	Faeces	(On et al., 1998)

C. upsaliensis	Blood, faeces, breast abscesses	(Gaudreau and Lamothe, 1992; Lindblom et al., 1995; Patton et al., 1989)
C. ureolyticus	Faeces, intestinal tract, oral abscess, gangrenous lesions of lower limb, genital infections, genital abscess, soft tissue infections, amniotic fluids, urine	(Burgos-Portugal et al., 2012; Duerden et al., 1982; Petersen et al., 2007)

***Campylobacter* spp. that have not been found in humans**			
***Campylobacter* spp.**	**Source**	**Isolation site**	**Reference**
C. avium	poultry	Caeca	(Rossi et al., 2009)
C. canadensis	Whooping cranes	Cloaca	(Inglis et al., 2007)
C. corcagiensis	Captive lion	Faeces	(Koziel et al., 2014)
C. cuniculorum	Rabbits	Caeca	(Zanoni et al., 2009)
C. hyointestinalis subsp. *hyointestinalis*	Cattle, deer, pigs, hamsters	Faeces, small and large Intestine	(Gebhart et al., 1985; Hill et al., 1987; Petersen et al., 2007)
C. hyointestinalis subsp. *lawsonii*	Pigs	Stomach	(On et al., 1995)
C. subantarcticus	Wild birds in the sub-Antarctic area	Cloaca	(Debruyne et al., 2010a)
C. troglodytis	Chimpanzee	Faeces	**(**Kaur et al., 2011)
C. volucris	Black-headed gulls	Cloaca	(Debruyne et al., 2010b)
'*Campylobacter* sp. Dolphin DP' (provisional)	Dolphin	Oral cavity	(Goldman et al., 2011)
'*Campylobacter* sp. Prairie Dog' (provisional)	Prairie dogs	Intestine, liver	(Beisele et al., 2011)

Data adapted from (Kaakoush et al., 2015b; Man, 2011; On, 2013)

1.2 The history of *Campylobacter*

Campylobacter (previously named ''*Vibrio*'') was first isolated from aborted ovine foetuses by McFaydean and Stockman in 1913. A few years later the identical *Vibrio* was isolated from aborted bovine foetuses by Smith and Taylor in 1919, and from the blood of three pregnant women by Vincent et al in 1947 (Farrell and Harris, 1992; Skirrow, 2006; Zilbauer et al., 2008). Afterwards, the name *Campylobacter* was created by Sebald and Vernon in 1963, and the organism was recognised as a human pathogen causing bacterial gastroenteritis in man in the 1970s (Butzler, 2004; Nachamkin et al., 2008). In April 1982 in the Microbiology Department, Royal Perth Hospital, Western Australia, a culture of human gastric mucosa harvested a spiral bacterium with some characteristics of the genus *Campylobacter* and was named *Campylobacter pyloridis* (Itoh et al., 1987; Marshall et al., 1984). Afterwards, the name was changed to *C. pylori* (Marshall and Goodwin, 1987). Subsequently it was shown that *C. pylori* did not fit within the genus *Campylobacter* due to differences in their ribosomal RNA sequences, fatty acids, ultrastructural features, antibiotic susceptibilities and absence of methylated menaquinone 6 (MK-6) in *C. pylori* (Goodwin et al., 1989; Goodwin et al., 1986). Therefore, it was suggested to create a new genus, *Helicobacter,* and to transfer *C. pylori* associated with gastritis to this genus (Goodwin et al., 1989; Griffiths and Park, 1990). In the 1980s the interest in *Campylobacter* research was renewed. As a result, many *Campylobacter*-like organisms (CLOs) were isolated from different sources including humans, animals and the environment, and new species were reported (Benjamin et al., 1983; Fox et al., 1989; Gebhart et al., 1985; Marshall et al., 1984). Also, during the 1980s, bacterial phylogeny studies at the level of the rRNA cistron were established, and it was revealed that *Campylobacter* was heterogeneous. As a

consequence, bacterial classification schemes were revised (Nachamkin et al., 2008; Romaniuk et al., 1987).

In 1991, the taxonomy and nomenclature of the genus *Campylobacter* and related bacteria were completely revised by using DNA-rRNA hybridization by Vandamme *et al* (Vandamme and De Ley, 1991). The genera *Campylobacter*, *Arcobacter*, *Helicobacter*, *Wolinella*, and "*Flexispira*" belong to the phylogenetic lineage rRNA superfamily VI within the class *Proteobacteria* (which is now known as the *Epsilonproteobacteria*). This comprises three major rRNA homology groups: *Campylobacter* and *Bacteroides ureolyticus* constitute rRNA homology group I; *Arcobacter* are in rRNA homology group II; and *Helicobacter* and *Wollinella succinogenes* are assigned to rRNA homology group III. The genera *Campylobacter* and *Arcobacter* comprise the family *Campylobacteraceae* (Engberg, 2006; Nachamkin et al., 2008; Vandamme and De Ley, 1991) (Figure 1.3).

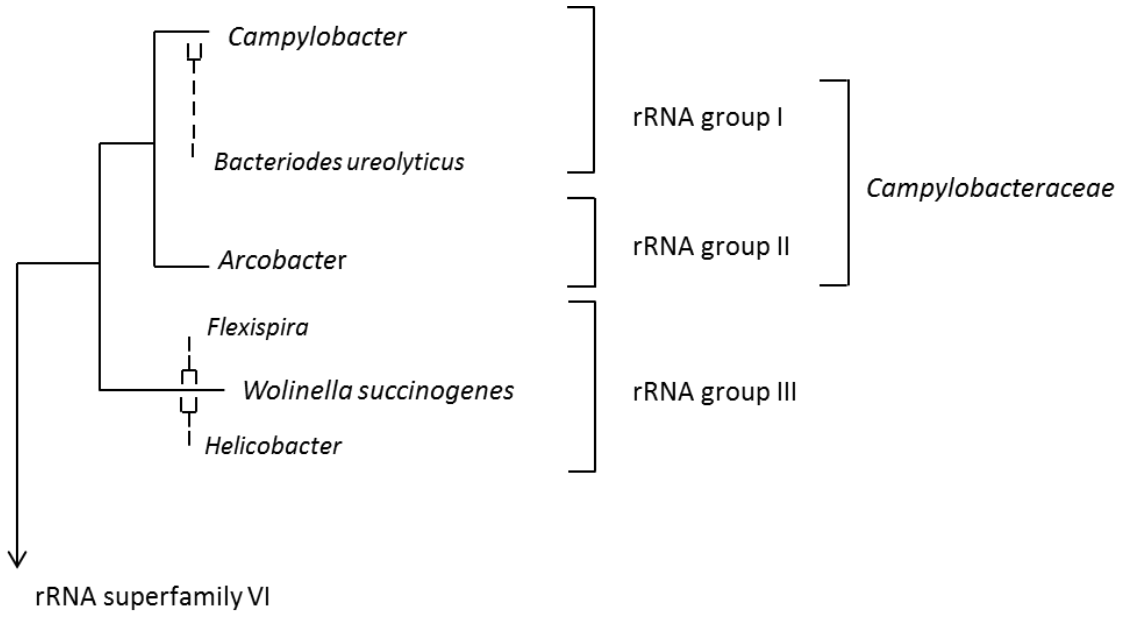


Figure 1.3 Phylogenetic tree of the family *Campylobacteraceae* and close relatives, based on similarity of 16S rRNA.

1.3 Diseases associated with *Campylobacter* infection

1.3.1 Campylobacteriosis in Humans

C. jejuni is the main bacterial cause of foodborne human intestinal disease worldwide, followed by *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157 (Acheson and Allos, 2001). Infection in humans is frequently associated with consumption of undercooked poultry meat, non-chlorinated water or unpasteurised milk (Shane, 2000). Campylobacteriosis is an acute diarrhoeal infection caused by members of the bacterial genus *Campylobacter*, most commonly *C. jejuni*. Clinical syndromes are similar to those of other acute bacterial enteritis including frequency of diarrhoea, blood in stools, vomiting, and abdominal pain (Coker et al., 2002; Nachamkin et al., 2008). The symptoms of campylobacteriosis induced by *C. coli* are clinically similar to that induced by *C. jejuni.* Usually, the incubation period for *C. jejuni* following ingestion is 24-72 h but longer incubation periods are possible, especially with low infectious doses. The peak of symptoms can last 24-48 h and may be associated with severe abdominal pain that mimics appendicitis (Blaser, 1997). Although campylobacteriosis rarely causes death, it may cause severe disabling sequelae including arthritis, autoimmune disorders [Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS)], and inflammatory bowel diseases (IBD), such as Crohn's Disease (Endtz et al., 2000; Lamhonwah et al., 2005; Wingstrand et al., 2006). It has also led to bacteraemia and /or meningitis of a newborn by the infected mother during the delivery process or shortly after birth, and may cause pancreatitis following *C. jejuni* enterocolitis (Peterson, 1994; Smith, 2002). Moreover, it has been suggested that *Campylobacter* species may play a role in driving the chronic esophageal inflammation that develops to cancer, and may be involved in colorectal cancer (Kaakoush et al., 2015a; Kaakoush

et al., 2015b). It has also been reported that *Campylobacter* species may cause extragastrointestinal symptoms such as bacteraemia, lung infections, brain abscesses and reactive arthritis (Kaakoush et al., 2015b). Figure 1.4 summarises clinical manifestations in humans associated with *Campylobacter* species.

In a one year study, it was estimated that *C .coli* contributed to 18.6% of human campylobacteriosis, with detection of a winter peak of human *C. coli* cases which may be attributable to pork and pork products (Gillespie et al., 2002; Gürtler et al., 2005). A comparison study of the characteristics of patients infected with *C. jejuni*, *C. coli*, and *C. fetus* demonstrated that *C. coli* was more prevalent in slightly older patients [34.6 years (mean age) for *C. coli* versus 27.5 years (mean age)] for *C. jejuni* and in those who had a history of a travel abroad, but less often in hot months than *C. jejuni*. *C. fetus* infection was found more often in much older patients than infection with *C. jejuni* and *C. coli* [68.4 years (mean age) for *C. fetus* versus 28.6 (mean age)] for *C. jejuni* and *C. coli* and those who had been hospitalised with a systemic disease (Bessède et al., 2014). *Campylobacter* infection in humans is likely to be a challenge in terms of global health for some time and it is considered to be of high public health significance (Kaakoush et al., 2015b).

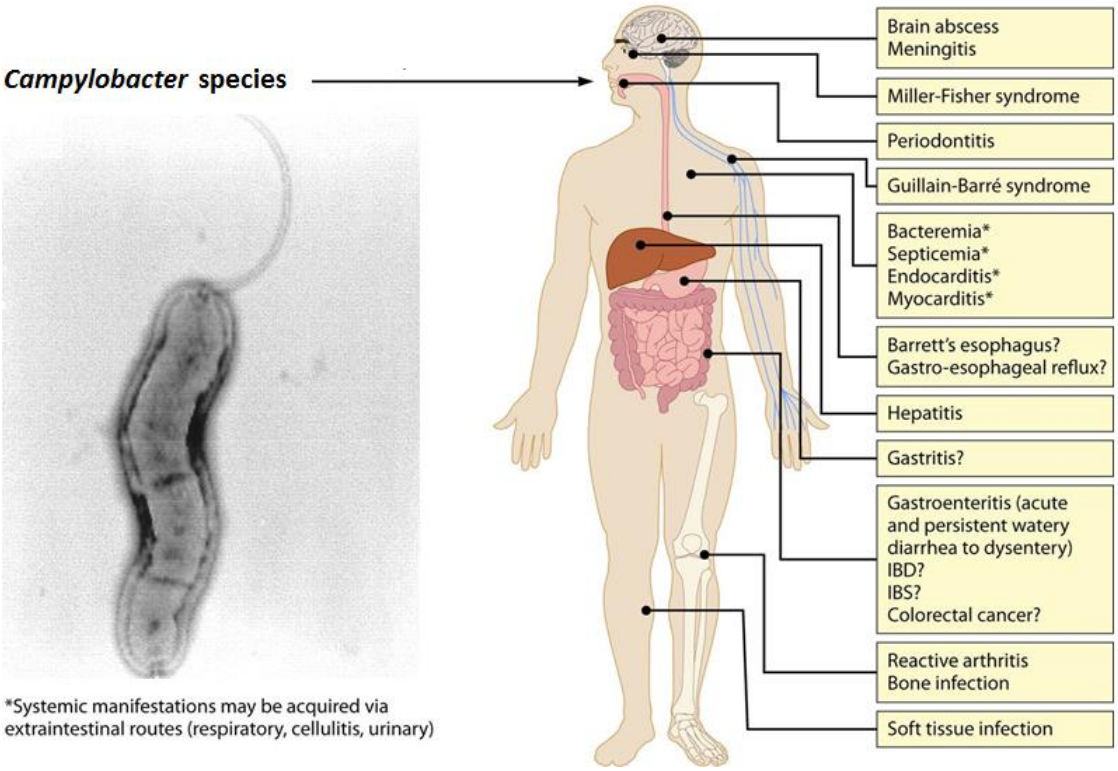


Figure 1.4 Clinical conditions associated with *Campylobacter* species in humans. Question marks: demonstrate conditions for which a role of *Campylobacter* is not confirmed. IBD: inflammatory bowel disease; IBS: irritable bowel syndrome.

Adapted from (Kaakoush et al., 2015b).

1.3.2 *Campylobacter* in poultry

Chickens are a prominent reservoir for thermotolerant *Campylobacter*, which colonises primarily the caecum and gastrointestinal tract, and chicken meat contaminated with *Campylobacter* is the primary source of human campylobacteriosis (Ghareeb et al., 2013; Hermans et al., 2011). Also, undercooked paté made with chicken liver has been considered as an important source of human campylobacteriosis in Europe and in the United States (Abid et al., 2013; O'Leary et al., 2009; Scott et al., 2015). Differential gene expression at two different temperatures (42 °C and 37 °C) may enable the organism to respond differentially to its chicken reservoir niche and the human host (Stintzi, 2003). Moreover, it has been shown that some *C. jejuni* isolates grown at 42 °C were more motile than C*. jejuni* grown at 37 °C, whereas some grown at 37 °C were more invasive into human T84 cells. *C. fetus* subsp. *fetus* was less able to grow and invade at 42 °C compared to at 37 °C (Aroori et al., 2013).

Previously, *Campylobacter* in poultry was considered to be a commensal organism because of its persistent caecal colonization in large numbers [10^6 -10^8 Colony Forming Unit (CFU)/g] with inefficient intestinal mucosal immune response to the bacterium (Hermans et al., 2012). However, studies have shown the induction of innate immune responses in the chicken gut and proliferation of heterophils in the caecum through recognition of *C. jejuni* by activated Toll-like receptors 4 (TLR4) and TLR21 in the chicken gut (de Zoete et al., 2010; Smith et al., 2008). However, post-mortem examination showed no caecal lesions despite heterophilia which clearly suggests that the immune responses did not lead to disease (Smith et al., 2008). This could be due to the differences between the immune systems of poultry and humans (Kaiser et al., 2005). Extra-intestinal *Campylobacter* spp. that are found in poultry livers may have

been implicated in extra-intestinal disease, such as vibrionic hepatitis of broiler chickens which is characterised by focal lesions in the liver and that adaptive T cell response (Jennings et al., 2011). Recently, it has been demonstrated that *C. jejuni* infections in some commercial broiler chickens can lead to disease characterised by pathological changes in the gut mucosa and diarrhoea, which in turn causes pododermatitis in flocks (Humphrey et al., 2014).

1.3.3 *Campylobacter* in other animals

It has been shown that both cattle and sheep can also act as a source of *Campylobacter* infections in humans. One study has estimated that cattle and sheep contribute to 35% and 4.3% of human cases of campylobacteriosis, respectively (Wilson et al., 2008).

The organism can be found in the digestive tract of healthy cattle (Atabay and Corry, 1998; Humphrey et al., 2007). Although cattle are considered an important source of human campylobacteriosis, unlike chicken, red meat is not massively contaminated at the point of sale. However, raw milk or possibly incompletely pasteurised milk can cause sporadic infections, or may cause outbreaks (Anand et al., 2015). It has been shown that *C. jejuni* is the most frequent species of *Campylobacter* detected in cattle followed by *C. coli* and other species including *C. fetus* subsp. *fetus, C. hyointestinalis* subsp. *hyointestinalis* and *C. lanienae* (Bae et al., 2005; Inglis and Kalischuk, 2003). It has been demonstrated that *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* (mostly in sheep) are two important pathogens in cattle and sheep affecting the reproductive ducts and gastrointestinal tract, respectively. The former disease is associated with

abortion due to early embryonic death (Campero et al., 2005; Truyers et al., 2014). Multilocus sequence typing (MLST) analysis was used to characterise *C. jejuni* isolates obtained in a 2-year longitudinal study of 15 dairy farms and 4 sheep farms in Lancashire, UK. The most prevalent clonal complexes (CCs) in cattle were ST-61, ST-21, ST-403 and ST-45 while in sheep the most prevalent clonal complexes were ST-42, ST-21, ST-48 and ST-52. The clonal complex ST-45, previously shown to be predominant during warm months in human cases, was also found to be more prevalent during warm months in ruminant samples (Grove-White et al., 2011).

A study in New Zealand has estimated the prevalence of *C. jejuni* in the faeces of wild birds and pets. The prevalence of *C. jejuni* in the faecal samples (n=906) was 20% in ducks, 18% in starlings (n=835), 9% in Canadian goose (n=23), 5% in dogs (n=498) and 7% in cats (n=82), and generally this prevalence was relatively higher during summer (Mohan, 2015). It has been shown that *C. upsaliensis* and *C. helveticus* are the most frequent species of *Campylobacter* found in dogs and cats, respectively (Baker et al., 1999; Moser et al., 2001; Parsons et al., 2010; Rossi et al., 2008). In a Danish longitudinal study (2-year study) of the prevalence of *Campylobacter* spp. in young pet dogs, of the 278 *Campylobacter* positive samples, 75% were positive for *C. upsaliensis*, 19.4% for *C. jejuni*, 2.1% for *C. lari*, 0.7% for *C. coli* and 2.8% only identified to *Campylobacter* spp. (Hald et al., 2004a). *Campylobacter* spp. may cause gastroenteritis associated with diarrhoea in dogs, but can also be found in the intestinal tracts without showing any gastroenteritis signs (Grøndalen et al., 2008). Using MLST analysis, it was reported that the majority of sequence types (STs) of *C. jejuni* isolates found in various populations of dogs (kennels and veterinary practices) were the same as those found in isolates from humans, including ST-45 and ST-21 (Parsons et al., 2009). Therefore,

Campylobacter carriage in pet dogs is considered a potential source for human campylobacteriosis (Procter et al., 2014; Rossi et al., 2008; Tenkate and Stafford, 2001). In a parallel study carried out in Liverpool, MLST analysis of isolates recovered from Cheshire bank voles revealed that all possess a novel, unique ST (ST-3704) (Williams et al., 2010). ST-3704 strains have now been isolated from bank voles inhabiting six different sites across Cheshire and Wirral. Furthermore, persistent ST-3704 strain infections have occurred in naturally-infected, captured bank voles and even their (captive-reared) F1 off-spring (Nicola Williams, unpublished data).

1.4 Pathogenesis

The mechanisms by which *Campylobacter* causes diseases are poorly understood (Dasti et al., 2010; Young et al., 2007). Following ingestion of a low infective dose, around 500 organisms, *Campylobacter* starts to adhere and colonise the intestinal mucosa aided by the flagellum, which also secretes invasive antigens named *Campylobacter* invasion antigens (Cia) (Dasti et al., 2010; Ketley, 1995; Nachamkin et al., 2008). It has been shown that the ability of the nonmotile *C. jejuni* (Δ*motAB*) mutant, possessing paralysed flagella, to invade Caco-2 cells was reduced. This mutant *C. jejuni* failed to colonize the caeca of chickens (Mertins et al., 2012). *Campylobacter* species are able to adhere to intestinal epithelial cells by secretion of the outer membrane protein CadF, which binds to fibronectin (Konkel et al., 1997). Unlike most bacteria, *C. jejuni* does not possess many classical virulence factors, but it is the only pathogen that carries out N-linked glycosylation of more than 30 proteins linked with adherence, colonization and invasion (Dasti et al., 2010). The pathogenicity of

Campylobacter may also be influenced by other microbes. A recent study has shown the role of the gut microbiota composition of humans in conferring resistance to enteropathogen colonization and that the colonization can change the composition of the human gut microbiota. For example, *Campylobacter*-positive abattoir workers were found to have significantly higher abundance of *Bacteroides*, *Escherichia* species, *Phascolarctobacterium* and *Streptococcus* in the gut microbiota than *Campylobacter*-negative workers, who had high proportions of *Clostridiales*, unclassified *Lachnospiraceae* and *Anaerovorax* (Dicksved et al., 2014). More recently, it has been shown that colonisation of *C. jejuni* in poultry leads to a change within the composition of the intestinal microbiota, associated with a higher water content of faecal samples, which indicates the beginning phase of diarrhoea (Sofka et al., 2015). The most important immunoglobulin secreted during infection with *Campylobacter* is IgA which can cross the gut wall, attenuate the organism and provide short term of immunity against the organism, while other immunoglobulins prevent bacteraemia (Wallis, 1994). Even though cell invasion may be responsible for the occurrence of clinical symptoms of campylobacteriosis, bacterial toxins might also aggravate the pathogenesis process (Ketley, 1995). *Campylobacter* produces a cytolethal distending toxin (CDT), which might play a role in disease pathogenesis; the toxin consists of three subunits CdtA, CdtB and CdtC (Ceelen et al., 2006; Johnson and Lior, 1988). The subunit CdtB acts as a Deoxyribonuclease (DNase) 1-like protein which causes cell cycle arrest, cytoplasm distension, chromatin disruption and cell death, whereas both subunits CdtA and CdtC are essential to deliver CdtB into the host cell (Lara-Tejero and Galán, 2000; Lara-Tejero and Galán, 2001). Hence, exposure of intestinal epithelial cells

to CDT triggers release of interleukin-8 (IL-8) which induces acute inflammatory response of the intestine (Hickey et al., 1999).

It has been shown that the autotransporter proteins comprise a group of outer membrane proteins of most Gram-negative bacteria that have unique structural properties and are often associated with virulence functions such as adhesion (Wells et al., 2007). For example, the autotransporter protein CapA of *C. jejuni* strain NCTC11168 may play an important role in adhesion to human epithelial cells and colonization in chickens. This was shown using the *capA* mutant of *C. jejuni* strain NCTC11168 demonstrated a significant decrease in its ability to invade Caco-2 cells and failed to colonize chicken gut (Ashgar et al., 2007).

Bacteria have evolved enzymes involved in the membrane disruption processes of host cell, in which phospholipids and proteins are the major chemical components, by hydrolysing these chemicals and haemolytic activity during invasion (Songer, 1997). For example, studies have indicated the role for the phospholipase A in the lysis of erythrocytes by *Campylobacter* species (Grant et al., 1997; Istivan et al., 2004). Moreover, it has been demonstrated that the *C. jejuni pldA*, which encodes phospholipase, mutant was shown reduced the ability in caecum colonization of chicken (Ziprin et al., 2001). It has been shown that the Peb protein is one of the most abundant periplasmic immunogenic proteins in *C. jejuni* which may play a key role in host-cell adhesion of eukaryotic cell membranes (Kervella et al., 1993) and in the utilization of amino acids, such as aspartate and glutamate, that are an essential carbon sources for the pathogen (Del Rocio Leon-Kempis et al., 2006).

1.5 Incidence and Epidemiology

Although the epidemiology of campylobacteriosis is poorly understood, poultry is considered the number one source of *Campylobacter* infections in humans. At the beginning, a few colonized broilers at the age of >2 weeks will infect the entire flock until the end of rearing (Hermans et al., 2012). The Food Standards Agency (FSA) has reported that the presence of *Campylobacter* in fresh chicken at retail in the UK is 65%, and its presence in chilled chicken is higher than in frozen chicken (at 48% and 14%, respectively). Additionally, a Scottish study estimated that poultry meat can contribute to up to 60-80% of human campylobacteriosis cases (FSA, 2009; Goddard et al., 2014). In recent years in many European countries, campylobacteriosis has been the most frequently reported zoonosis, followed by other infectious diseases such as salmonellosis (Eurosurveillance Editorial, 2012). In the United States in 2009, campylobacteriosis was the second most frequent cause of laboratory-confirmed cases of bacterial enteritis after salmonellosis, and the incidence was highest among the <4 years age group (Centers for Disease and Prevention, 2010). In population-based studies of infectious intestinal disease (IID) in the UK over 15 years, changes in incidence and etiology of pathogens associated with IID were examined in two studies (IID1 and IID2) covering different time periods (1993-1996 and 2008-2009, respectively). *Campylobacter* spp. was the most frequently identified bacterial cause of infectious intestinal disease in the IID1 and IID2 studies (Tam et al., 2012). The prevalence of salmonellosis cases reduced between IID1 and IID2, which reflects the success of *Salmonella* control strategy in both broilers and laying chickens in the UK (Tam et al., 2012). In developed countries, the incidence of campylobacteriosis is highest among infancy and age groups 15-44 years, with most infections occurring via

handling and consumption of chicken meat, whereas in developing countries the highest incidence of the disease is restricted to young children, and most infections are acquired by exposure to poorly treated water and farm animals (Butzler, 2004). It was shown that *Campylobacter* infections had risen to 1 million cases in England and Wales between 1989 and 2011, with the highest increase among age groups >50 years. (Nichols et al., 2012). Figure 1.5 shows the number of laboratory confirmed cases of *Campylobacter* infection in humans in the UK between 2000-2012. In general, salmonellosis continues to fall in the UK. Thus, the key aim of the FSA strategic plan 2010-2015 was to reduce campylobacteriosis in humans through decreasing *Campylobacter* levels in chicken (https://www.gov.uk /government /publications /zoonoses-report-uk-2012).

1.5.1 Seasonality

Cases of campylobacteriosis show distinct seasonality. In a long term study in England and Wales between 1989 and 2011, it was shown that constant seasonal increase in campylobacteriosis cases was observed between early May and early June across years, ages and regions, and was greater in children in rural areas. Seasonality could be due to a number of causes. It has been suggested that one cause may be attributed to flies depositing the organism onto food (Nichols, 2005; Nichols et al., 2012; Wingstrand et al., 2006).

1.5.2 Guillain-Barre syndrome (GBS)

Guillain-Barre syndrome (GBS) is the most critical postinfectious consequence of campylobacteriosis in human. GBS is an acute autoimmune disease which affects the peripheral nervous system with an incidence of <1 per 1000 infections. Lipo-oligosaccharides (LOSs) of *C. jejuni* activate TLR4 of dendritic cells (DCs) to release anti-ganglioside antibodies which react with self-glycolipids in peripheral nerves, characterised by ascending paralysis (Acheson and Allos, 2001; Huizinga et al., 2015; Yuki and Hartung, 2012).

1.5.3 Miller Fisher syndrome (MFS)

MFS is identified as a variant of GBS that is characterized by ophthalmoplegia (eye muscle weakness), hyporeflexia (absence of reflexes) and ataxia (abnormal muscle coordination). The incidence of MFS is estimated up to 5 % of all GBS cases in the west compared with up to 26 % in Asia (Ono et al., 2015).

1.5.4 Reactive arthritis (ReA)

ReA is a variety of rheumatologic phenomena characterized by a sterile inflammatory arthritis that may occur within 2-4 weeks of preceding remote bacterial infections of acute gastroenteritis such as *Salmonella, Shigella, Campylobacter*, and *Yersinia* infection (Hannu, 2011; Porter et al., 2013; Townes, 2010).

1.5.5 Inflammatory bowel disease (IBD)

IBD is considered as an emergent worldwide disease characterized by chronic inflammation of the gastrointestinal tract. The disease has two clinical subtypes, (1) Crohn's disease (CD) that can affect any part of the intestine, and (2) ulcerative colitis (UC) which is restricted to the colon and rectum. Although the exact causative agent of IBD is not known, it has been suggested that mucosa-associated bacteria, such as *Campylobacter* species, may play a key role in the development of IBD. The mechanism behind this could be immunopathogenetic, such as autoantibody generation (Castaño-Rodríguez et al., 2015; Kaakoush et al., 2014; Lamhonwah et al., 2005).

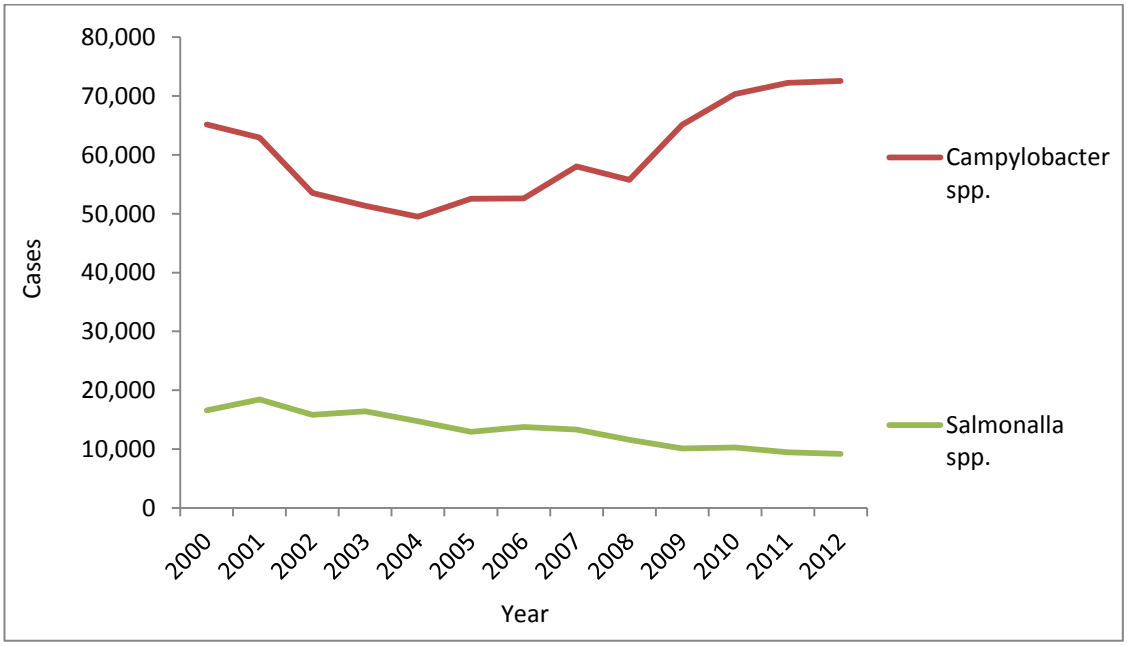


Figure 1.5 Number of laboratory confirmed cases of infectious intestinal diseases (campylobacteriosis and salmonellosis) in the UK between 2000-2012. Data from Food Standard Agency https://www.food.gov.uk/science/microbiology/fds/58736.

1.6 Treatment of campylobacteriosis

Human *Campylobacter* gastroenteritis is commonly self-limiting, and antibacterial treatment is not generally recommended in the United Kingdom, but ciprofloxacin (fluoroquinolone) and a macrolide are the drugs of choice in severe or prolonged cases. There has been a steady increase in the proportion of clinical ciprofloxacin-resistant *Campylobacter* isolates in the UK from 3% in 1991 to 37.5% in 2008 (Cody et al., 2010). Furthermore, some studies have shown a link between certain *C. jejuni* genotypes and resistance to ciprofloxacin, and correlation between chicken consumption and acquisition of resistance to ciprofloxacin (Habib et al., 2009; Kinana et al., 2006). Recently, it has been reported that a horizontally transferrable gene *erm*(B), encoding rRNA methylase, can confer macrolide resistance in *C. coli* (Wang et al., 2014).

1.7 Strain typing methods

Bacterial strain typing is the establishment of the relatedness of a group of bacterial isolates. It is a method for source tracing of bacterial strains that cause infections and for studying the epidemiology of diseases (Li et al., 2009; Tenover et al., 1995; Wassenaar and Newell, 2000). In general, the diversity within a bacterial species is caused by a combination of genetic events which include: mutation, horizontal gene transfer, gene loss, gene duplication and recombination (Fraser-Liggett, 2005).

Typing methods can be based on either phenotyping or genotyping. Phenotyping methods include morphology of colonies on culture media, serology, growth characteristics, biochemical tests, pathogenicity and antibiotic susceptibility. Bacterial

phenotypes are often not sufficiently variable to distinguish closely related strains. Because of their greater resolution, genotyping methods or DNA fingerprinting, based on the genetic structure of bacterial strains, have largely superseded phenotypic approaches, (Li et al., 2009). Table 1.2 summarises the wide range of bacterial genotyping methods that are available.

Table 1.2 Genotyping methods that have been used for bacterial strain typing.

Typing method	**Reference**
Arbitrarily primed polymerase chain reaction amplification (AP-PCR)	(Welsh and McClelland, 1990)
Amplified fragment length polymorphism (AFLP)	(Vos et al., 1995)
Denaturing gradient gel electrophoresis (DGGE)	(Muyzer et al., 1993)
DNA microarrays	(Schena et al., 1995)
Genome sequencing	(Fleischmann et al., 1995)
High -resolution melting (HRM) analysis	(Gundry et al., 2003)
Multilocus sequence typing (MLST)	(Maiden et al., 1998)
Multispacer typing (MST)	(Drancourt et al., 2004)
Multiple-locus variable number tandem repeat analysis (MLVA)	(Lupski and Weinstock, 1992; Vergnaud and Denoeud, 2000)
PCR-restriction fragment length polymorphism(RFLP)-based analysis (PCR-RFLP)	(Wichelhaus et al., 2001)
Pulsed-Field gel electrophoresis (PFGE)	(Schwartz and Cantor, 1984)
Repetitive sequencing-based PCR (REP-PCR)	(Koeuth et al., 1995; Stern et al., 1984)
16S-23S rRNA gene internal transcribed spacer (ITS)	(Gurtler and Stanisich, 1996)

1.7.1 Serotyping: is the most commonly used phenotypic method to characterise *Campylobacte*r strains despite the lack of discriminatory power. This method is based on the differences of surface structures (antigens) of bacteria which can be detected by antibodies and antisera. Using this approach strains can be differentiated by their different surface structures (Wiedmann, 2002).

Penner and Hennessy (1980) showed that bacteria, later classified as *C. fetus* subsp*. jejuni* could be serotyped on the basis of their thermostable antigens (penner scheme).Briefly, Bacterial antigens, *C. fetus* subsp*. jejuni* strains, were extracted from cell suspensions by heating at 100 °C in saline or by exposure to EDTA. The extracted thermostable antigens were diluted (1:10 in phosphate buffer saline) and incubated at 37 °C for 1 h with an equal amount of PBS + 1% sheep erythrocytes. The sensitized erythrocytes (adherence of antigens to the surface of erythrocytes) were washed and resuspended in phosphate buffer saline, and titrated against diluted rabbit antisera, for agglutination of sensitized erythrocytes with the presence of antibodies specific for lipopolysaccharides (LPSs) in antisera (Penner and Hennessy, 1980). The variability of the somatic "O" antigen of outer membrane of the *Campylobacter* LPS is believed to contribute to the antigenic basis of the Penner serotyping system (Shi et al., 2002). The scheme was used to identify the distinct antigenic specificities between *C. jejuni* and *C. coli* (Penner et al., 1983). The scheme has long been used as laboratory based epidemiologic method by researchers worldwide to study the transmission of *Campylobacter* infection from food, animal, and water to humans (Woodward and Rodgers, 2002). With the scheme, the strains are differentiated on the basis of heat-stable antigens presence on the bacterial surface (Moran and Penner, 1999).

1.7.2 Macro-restriction PFGE: Is a useful typing technique for many bacteria that can resolve large DNA molecules (20-200 kb). The organisms are embedded in agarose, lysed and the chromosomal DNA is digested with restrictions enzymes that cleave the bacterial DNA infrequently. The DNA fragments in the agarose (blocks) are loaded onto agarose gels for electrophoresis. The DNA fragments are resolved into discrete bands by electrophoresis in the gel where the direction of the electric field is changed or pulsed to allow resolution of very large DNA fragments (Tenover et al., 1995; Wassenaar and Newell, 2000). With *Campylobacter*, the technique was originally used for *C. jejuni*, and was later applied to *C. coli, C. hyointestinalis, C.fetus* and *C. upsaliensis* (Bourke et al., 1996; Fujita et al., 1995; Salama et al., 1992; Yan et al., 1991).

1.7.3 Flagellin typing (*fla* typing): The proteins of *Campylobacter* flagella are encoded by two highly homologous genes; a major flagellin gene (*flaA*) and a minor flagellin gene (*flaB*) (Guerry, 2007). Because both *flaA* and *flaB* genes have variable central regions and highly conserved flanking regions, they can be analysed using RFLP analysis of amplified PCR products. In *fla* typing, the *flaA* gene is amplified by PCR followed by digestion of the amplified DNA with a specific restriction enzyme to produce PCR product fragments which are separated according to their lengths by agarose gel electrophoresis (Eberle and Kiess, 2012; Fitzgerald et al., 2001). Although the technique has high discriminatory power, it is not the favoured method used in epidemiology studies (Eberle and Kiess, 2012). A database for *flaA* genotypes isolates is

available for scientists and publically accessible to share information on *flaA* gene typing (http://pubmlst.org/campylobacter/). However, in recent years MLST has superseded this approach and has become the gold standard for *Campylobacter* typing.

1.7.4 MLST

MLST was established in 1998. It is a molecular biological method for the characterization of bacterial isolates using the DNA sequences of internal fragments of housekeeping genes (commonly seven) located at various parts of the chromosome. MLST data are freely accessible over the World-Wide Web (www.mlst.net) and can be implemented for a wide range of bacterial species. Due to its ability to determine variation which is building up gradually within a population, MLST can be used to trace lineages in bacterial populations, enabling detailed studies of the epidemiology, evolution and pathogenicity of bacteria (Chan et al., 2001; Maiden, 2006; Maiden et al., 1998).

In the *Campylobacter* MLST system, the sequences of seven housekeeping genes (allele fragments), which are present in all isolates and are stabilized for conservation of metabolic function, are determined after amplification of fragments of 400-550 bp per gene from genomic DNA. For each individual gene, new allele fragment-sequences are assigned an allele number, with the first reported sequence being designated “1”. These numbers are obtained for each of the seven loci and are stored electronically in the ‘‘profile database’’ using web-sites such as mlst.net. These allele numbers are joined into sequence types (STs), which are also assigned an arbitrary number. STs are

grouped into ‘‘clonal complexes’’ (which can differ in allele number for up to two of the seven loci) and stored in the profiles database (Maiden, 2006; Nachamkin et al., 2008). This makes MLST a highly portable genotyping method (i.e., easily comparable data between laboratories). In addition, its use can decrease the risk of transporting live bacteria because killed bacterial suspensions, genomic DNA, or clinical material can be used to carry out nucleotide sequence determination from PCR products (Dingle et al., 2001). Table 1.3 summarises a number of studies where MLST has been applied to *Campylobacter* in different countries. Using MLST data, it has been found that *Campylobacter* is genetically highly diverse, with a weak clonal population structure, and has strong intra- and interspecies lateral genetic exchange (Dingle et al., 2001). Table 1.4 shows the seven housekeeping loci that were chosen for the *C. jejuni* MLST scheme with their protein products. These genes were chosen from multiple chromosomal locations encoding proteins responsible for intermediary metabolism and were suitable for primer design. The minimum distance between loci was 70 kb (Figure 1.6), which suggests that the coinheritance of any of the loci in a recombination event was unlikely. The MLST scheme was initially developed using 194 *C. jejuni* isolates from different sources including humans, animals and the environment. There were 155 STs identified which were assigned to 62 clonal complexes. The data indicated that there was horizontal gene exchange, including import of alleles from other *Campylobacter* species including *C. coli* (Dingle et al., 2001). Using this previously described MLST system for *C. jejuni,* a MLST scheme was established for *C. coli* (Dingle et al., 2005). There was an identity of approximately 86.5% between the two species, *C. jejuni* and *C. coli*, at the nucleotide sequence level within the MLST loci, with genetic exchange of the housekeeping genes only observed at a very low rate. In contrast, it

was shown that the *flaA* gene repeatedly exchanged between the two species (Dingle et al., 2005). Using MLST data, it has been indicated that *Campylobacter* isolates from bovine, ovine, poultry, pets, pigs and the environment are considered potential reservoirs of human campylobacteriosis. Associations between source and certain clonal complex have been demonstrated, suggesting niche adaptation of isolates (Hepworth et al., 2011; Manning et al., 2003; Williams et al., 2010). Figures 1.6 summarise the most common clonal complexes and sequence types, and their prevalence.

Table 1.3 Studies on *Campylobacte*r spp. MLST clonal complexes and sequence types in different countries. The table shows only the most common and more predominant MLST CCs than other CCs.

Country	**Year**	**Source**	**Number of isolates**		**Most common and more predominant CC**	**Reference**
			C. jejuni	***C. coli***		
Denmark	2002-2003	Humans	122 Gastro enteritis (96) Reactive arthritis (18) GBS (8)	-	ST-21 (4% reactive arthritis) ST-45 (4% reactive arthritis) ST-22 (4% GBS)	(Nielsen et al., 2010)
Finland	2012	Humans	95	-	ST-45 ST-283 ST-677	(Kovanen et al., 2014)
Finland	1998-2007	Humans (blood)	73	-	ST-677 ST-45 ST-21	(Feodoroff et al., 2013)
Italy	2009	Humans	11	7	ST-257 ST-206 ST-443 ST-21 ST-353 ST-828/*C.coli*	(Piccirillo et al., 2014)
		Chicken	23	16	ST-21 ST-446 ST-443 ST-828/*C.coli* ST-1150/*C.coli*	
Scotland	2005-2006	Pigs poultry Sheep Cattle gulls	443 (*C. jejuni* +*C.coli*)		ST-828 /*C. coli* (% of total dataset) 5-10 2.5-5 2.5-5 0.62-1.25 0.00-0.62	(Ogden et al., 2009)

		Pigeons gulls			ST-179 (% of total dataset) 5-10 0.00-0.62	
		Gulls Ducks pigeons			ST-1275 (% of total dataset) 5-10 062-1.25 0.00-0.62	
		Cattle Sheep Poultry birds			ST-61 (% of total dataset) 2.5-5% 1.25-2.5 1.25-2.5 0.00-0.62	
		Cattle Sheep Bird Ducks Poultry Pigeons			ST-21 (% of total dataset) 2.5-5% 1.25-2.5 0.62-1.25 0.62-1.25 0.62-1.25 0.00-0.62	
		Ducks Birds Gees Pigeons poultry Cattle Sheep gulls			ST-45 (% of total dataset) 2.5-5.00 1.25-2.5 1.25-2.5 1.25-2.5 1.25-2.5 0.00-0.62 0.00-0.62 0.00-0.62	
		Cattle Sheep Gulls			ST-42 (% of total dataset) 1.25-2.5 0.62-1.25 0.62-1.25	
		Cattle Sheep birds			ST-48 (% of total dataset) 0.62-1.25 0.62-1.25 0.00-0.62	

South Korea	2012	Duck	46	9	ST-21 ST-45 ST-828 /*C.coli*	(Wei et al., 2014)
UK	2006-2008	Cattle	849	-	ST-61 ST-21 ST-403 ST-45	(Grove-White et al., 2011)
		Sheep	154		ST-42 ST-2114 ST-4814 ST-52	
UK	2005-2008	Dogs	33	-	ST-45 (11) Rescue dog (6) Hunt dog (4) house hold dog (1) ST-21 (4) Hunt dog ST-508 (4) Boarding dog (2) hunt dog (1) vet visiting dog (1) ST-403 (3) Hunt dog (2) household dog (1)	(Parsons et al., 2009)
UK	2004-2006	Broilers flocks	226	8.5 %	ST-45 ST-21 ST-574 ST-443 ST-828(*C.coli*)	(Jorgensen et al., 2011)
UK	2003-2004	Humans	326	30	ST-21 ST-45 ST-257 *C. coli*	(Sopwith et al., 2006)
USA	2011-2012	Turkey	19	80	ST-353 ST-828 ST-828 /*C.coli*	(Kashoma et al., 2014)

Table 1.4 Seven housekeeping gene chosen for *C. jejuni* MLST scheme with their protein products.

Housekeeping gene	**product**
aspA	aspartase A
glnA	glutamine synthetase
gltA	citrate synthase
glyA	serine hydroxymethyl transferase
pgm	phosphoglucomutase
tkt	transketolase
uncA	ATP synthase α subunit

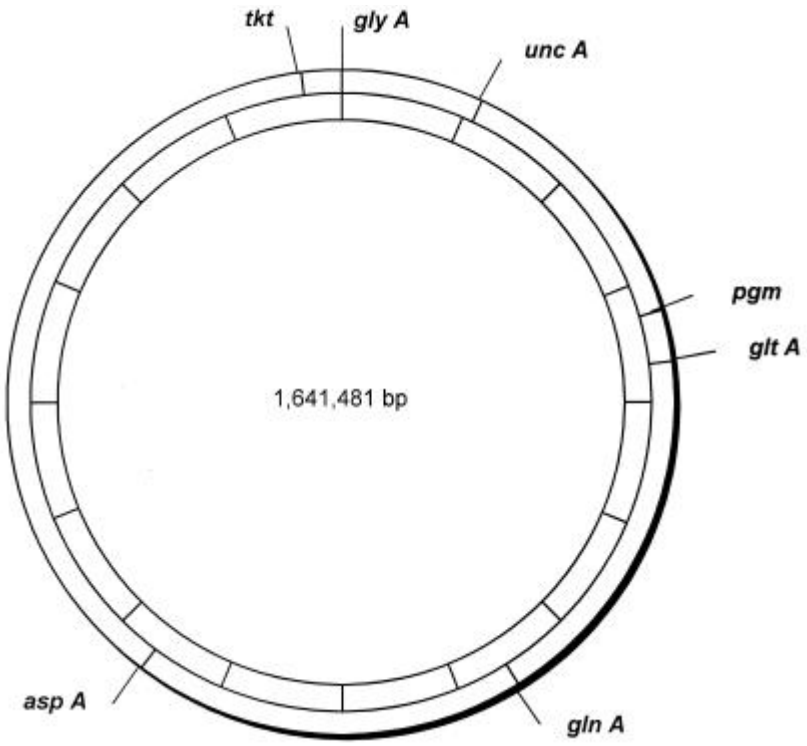


Figure 1.6 Chromosomal locations of MLST loci. The positions of the seven loci are shown on a map of the *C. jejuni* chromosome derived from the genome sequence of isolate NCTC11168 http://www.nature.com /nature /journal /v403 /n6770 /full /403665a0.html.

Data from (Dingle et al., 2001)

1.7.4.1 Common *C. jejuni* clonal complexes

Clonal complex ST-21 is the most common of the clonal complexes, comprising approximately 22% of all of the yielded isolates (Figures 1.6A and 1.6B-last accessed July 2015), and has a total of 667 different STs in it (Figure 1.6C-last accessed July 2015). It has been detected in multiple sources, including food-producing animals and humans, and is therefore considered to be a niche generalist (Dingle et al., 2001; http://pubmlst.org/campylobacter).

Contrary to the isolates of clonal complex ST-21, distributed among a wide range of hosts, it has been shown that certain clonal complexes are associated with particular farm animals. For example, one study, most of isolates were from the United Kingdom and the rest was from Northern Europe, reported that isolates from clonal complex ST-45 were the most prevalent in poultry, isolates from both clonal complexes ST-48 and ST-61 were the most prevalent in cattle, isolates from clonal complex ST-42 were overrepresented in sheep, and isolates from clonal complex ST-403 were predominant in pigs (Manning et al., 2003). Despite being poor survivors outside the body of their hosts, some *C. jejuni* have adapted to survive in environmental niches (Sopwith et al., 2008). For example, in one UK study using MLST analysis, the *C. jejuni* community isolated from faeces of livestock and wild animals, environmental water and soil samples in dairy cattle farmland in the UK was analysed. The clonal complex ST-45 isolates were overrepresented in wildlife faeces and environmental water, whereas the clonal complex ST-61 isolates were overrepresented in cattle faeces (French et al., 2005). A longitudinal study of *C. jejuni* in dairy cattle farms using MLST demonstrated predominance of three clonal complexes ST-61 (24.2%), ST-21 (23.6%) and ST-42 (20.5%) among the cattle isolates.

This indicates that there is an association between cattle and some genotypes of *C. jejuni* (Kwan et al., 2008b). A cross-sectional study of molecular epidemiology of *C. jejuni* in a dairy farm demonstrated that *C. jejuni* clonal complexes ST-21, ST-45, and ST-61, which have been commonly associated with human *Campylobacter* gastroenteritis disease, comprised the majority of genotypes isolated, again demonstrating potential host-associations (Kwan et al., 2008a). It has been suggested that the contaminated faeces of wild birds may contribute to the distribution of one of the most common genotypes (ST-45) in river water (Carter et al., 2009). It has been shown that livestock including poultry, cattle and sheep are principle sources of human campylobacteriosis. Using MLST studies in England and Scotland supplemented with statistical genetic approaches, it was revealed that 97% (1195) cases of sporadic campylobacteriosis in England could be attributed to the meat of farm animals and poultry, and that 76% cases of *Campylobacter* infections in humans in Scotland may be due to consumption of contaminated poultry meat. This indicates that applying strict biosecurity on farms could greatly reduce campylobacteriosis in humans (Sheppard et al., 2009; Wilson et al., 2008).

1.8 *Campylobacter* spp. in the environment

Campylobacter species are continually shed from all reservoirs into the environment (soil and water), which in turns plays a key role in transmission, either directly to humans or indirectly by livestock (Figure 1.7). Using MLST analyses, it has become clear that isolates of some clonal complexes, such as ST-45, are more frequently found in environmental water (surface water) and are associated with the late spring

incidence of human campylobacteriosis in north-western England, which indicates a link between the prevalence of this clonal complex in the environment and human campylobacteriosis (Bronowski et al., 2014; Ogden et al., 2009; Sopwith et al., 2008). Transmission of the organism from these reservoirs to humans is via different routes (Figure 1.7). In general, the transmission is associated with the consumption of contaminated chicken meat during carcass processing at slaughter, and cross contamination in the food preparation environment (Humphrey et al., 2007). It has also been shown that the organism can be transmitted to humans by eating contaminated raw vegetables (Carvalho et al., 2013). More recently, it has been suggested that *Campylobacter* survival is enhanced in water when there is interaction (gene product involvement) with the free-living protozoa *Acanthamoeba* (Vieira et al., 2015). It has also been shown that flies can contribute to *Campylobacter* transmission to humans and to chicken flocks during the summer (Hald et al., 2004b; Nichols, 2005). The possibility of *Campylobacter* contamination in the environment has been demonstrated in specific climatic conditions, which may play a role in *Campylobacter* outbreaks. For example, in June 2007 in British Columbia/Canada, one of the largest campylobacteriosis outbreaks was reported in wet muddy conditions. Among 537 bike racers (included in the study), 225 racers (42%) suffered diarrhoeal illness due to mud ingestion (Stuart et al., 2010). Moreover, it has been suggested that transmission can occur person-to-person (Domingues et al., 2012), despite the relatively lower rate of documentation compared to the total number of campylobacteriosis cases (Musher and Musher, 2004). For example, it has been suggested that the outbreaks of *Campylobacter* infections in families could be transmitted by faecal-oral route, via infected faeces of infants (Blaser et al., 1981).

Moreover, cases of *Campylobacter* infections have been reported in newborn babies, which could be transmitted by infected mothers (Youngs et al., 1985). It has also been reported that homosexual men are at higher risk for *Campylobacter* spp. and *Helicobacter* spp. infections (Gaudreau and Michaud, 2003; Laughon et al., 1988).

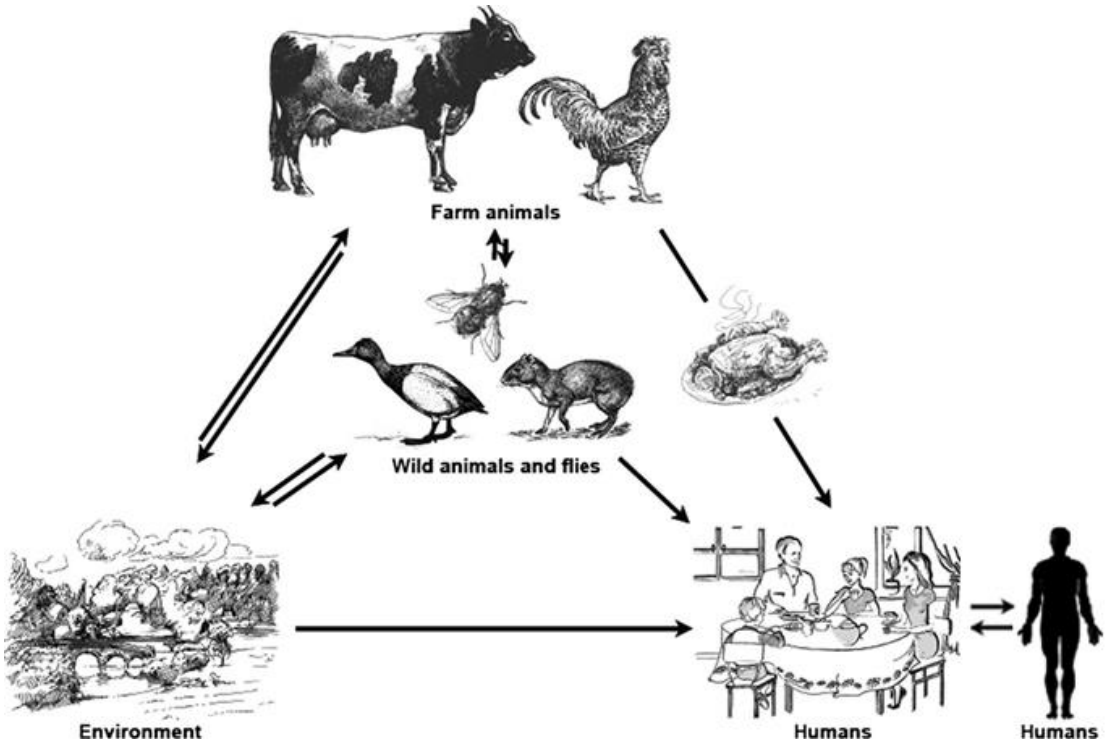


Figure 1.7 Transmission routes of *C. jejuni*. Adapted from (Bronowski et al., 2014)

1.9 Aims of this study

The survival of *C. jejuni* strains in the environment is likely to play a key role in the transmission of this pathogen, either directly or indirectly, to humans. The overall objective of this study was to increase our understanding of *Campylobacter* survival in natural environments, variations between *Campylobacter* genotypes, the genes that are important in survival, and interactions between *Campylobacter* and other species that might enhance survival. The specific aims were:

- To compare the survival of a diverse panel of *C. jejuni* strains from various sources and representing different MLSTs in sterilized water (and natural water) at selected time points [time 0, day 1 (at 25 °C) and day 3 (at 4 °C)] by measuring the ability to form colony forming units.
- To investigate the viable but non culturable (VBNC) state in *C. jejuni* strains (M1, 1336 and 414) in sterile distilled water (starvation conditions) at selected time points by detecting and counting viable cells of *C. jejuni* in sterile distilled water.
- To study gene expression variations of previously reported stress response genes between different *C. jejuni* strains (M1, 1336 and 414) during survival in water at selected time points by using both end-point PCR and real-time quantitative PCR (Q-PCR) assays for selected stress response genes.
- To investigate the effects of supernatants from *Pseudomonas* spp. cultures on the growth of *Campylobacter in vitro* and determine the co-existence of *Campylobacter* spp. with fluorescent *Pseudomonas* spp. in natural water by using environmental PCR assays.

C h a p t e r 2

MATERIALS AND METHODS

2.1 Bacterial strains used in this study

2.1.1 *Campylobacter* strains

The *Campylobacter jejuni* strains (n=27) used in this study were obtained from the University of Liverpool, Leahurst culture collection assembled and subjected to MLST during previous projects (French et al., 2005; Wilson et al., 2008; Wilson et al., 2009) (Table 2.1). The strains were chosen arbitrarily but to represent various sources and different clonal complexes. They comprised seven isolates from human sources, five from water sources, two from soil, six from cattle, four from chicken, one from a wild bird and one from bank-vole, as well *C. jejuni* isolate (ST-42). The strain panel included the widely studied strain M1 (Friis et al., 2010) and representatives of different clonal complexes (Table 2.1). All strains were stored at -80 °C on cryopreservation beads (Technical Service Consultants Ltd) according to manufacturer's instructions. When required, a bead was taken from the partially thawed stock to inoculate a blood agar plate.

Table 2.1 *C. jejuni* strains used in this study.

Leahurst Culture Collection Number	Source	ST	CC
11306	Human	21	21
12554	Human	21	21
11390	Human	45	45
12441	Human	45	45
11203	Human	61	61
12498	Human	61	61
M1	Human	137	45
2628	Water	21	21
2629	Water	21	21
1976	Water	38	48
1977	Water	38	48
1390	Water	42	42
708	Soil	45	45
709	Soil	45	45
504	Cattle	21	21
1107	Cattle	21	21
759	Cattle	45	45
2305	Cattle	45	45
549	Cattle	61	61
2245	Cattle	61	61
12609	Chicken	21	21
12692	Chicken	21	21
12769	Chicken	45	45
12814	Chicken	45	45
1336[a]	Bird	841	-
414[a]	Bank-Vole	3704	-
C. jejuni isolate[b]	Unknown	42	42

[a]further information about these two strains has been published previously (Hepworth et al., 2011; Williams et al., 2010).

[b]this isolate was originally thought to be NCTC11168, but MLST typing indicated that this was not the case. Subsequently, it will be referred to as *C. jejuni* isolate (ST-42).

ST/ sequence type

CC/ clonal complex

2.1.2 *Pseudomonas* strains

The bacterial species and strains of *Pseudomonas* (kindly provided by Dr Rob W. Jackson, University of Reading) are listed in Table 2.2. All strains were stored at -80 °C on cryopreservation beads according to manufacturer's instruction. When required, a bead was taken from partially thawed stock to inoculate an agar plate. *Pseudomonas* strains were grown on Luria Bertani agar (LA, SIGMA) overnight at 25 °C, Pseudomonas C-N selective Agar for 72 h at 30 °C or inoculated into Luria Bertani broth (LB) for 48 h at 30 °C.

Table 2.2 *Pseudomonas* spp. used in this study (all species are from environmental sources.

***Pseudomonas* spp.**	**Strain**	**References**
P. fluorescens	WCS365 pf01 pf5 F113 ATCC 17400	(O'Sullivan and O'Gara, 1992; Palleroni et al., 1973; Shanahan et al., 1992)
P. syringae	pv. *maculicola* pv. *tomato* DC3000 pv. *antirrhini* 152E pv. *coriandricola* pv. *syringae* B728a pv. *lachrymans* 789 pv. *glycinea* 49a/90 pv. *glycinea* 4180	(Arvanitis et al., 1995; Asakura et al., 2007; Chet et al., 1973; Hirano and Upper, 2000; Mitchell and Young, 1978; Parashar and Leben, 1972; Robert S et al., 1957; Young, 2010)
P. putida	PaW 340 KT 2440	(Dos Santos et al., 2004; Nelson et al., 2002)
P. avellanae	48	(Psallidas, 1987; Scortichini et al., 2000)
P.aeruginosa	159	R. W. Jackson strain collection
P. marginalis	247	R. W. Jackson strain collection
P. agarici	2472	R. W. Jackson strain collection
P. corrugata	2445	R. W. Jackson strain collection
P. entomophila	L 48	R. W. Jackson strain collection
P. cichorii	907	R. W. Jackson strain collection
P. tolaasii	2192T	R. W. Jackson strain collection
P. viridiflava	2848	R. W. Jackson strain collection

2.2 Growth conditions

The media and solutions used were sterilised by autoclaving at 121 °C for 15 min. *C. jejuni* strains (Table 2.1) were grown on Columbia Blood Agar Base (CBA, Oxoid) with 5% (v/v) defibrinated horse blood (Oxoid) at 37 °C for 48 h under microaerobic conditions (85% [v/v] N_2, 5% [v/v] O_2, and 10% [v/v] CO_2) by using anaerobic jars equipped with a Campygen gas pack (Oxoid) or in a Whitley VA500 Workstation incubator (Don Whitley Scientific Ltd). Details of the media used in this study are shown in Appendix 1 and details of solutions and buffers prepared in this study are shown in Appendix 2.

2.3 Experiments to test survival of *C. jejuni* in water

2.3.1 Preparation of cell suspensions for testing survival in sterile water

For survival experiments, bacteria were sub-cultured on CBA containing 5% (v/v) defibrinated horse blood for 24 h at 37 °C under microaerobic conditions. A loop-ful (STAR LAB) of 5 µL was taken, and suspended in 5 mL of Muller-Hinton Broth (MHB, Oxoid) supplemented with *Campylobacter* growth supplement (LAB M) in a 30 mL Universal tube (Ramboldi Ltd). Suspended bacterial samples in 30 mL Universal tubes were adjusted to a final optical density at 600 nm (OD_{600}) of 0.05 (3.8×10^7-3.5×10^8 CFU/mL) (Spectronic Biomate 5).

2.3.2 Preparation of sterile water sample

Filtered-tap water (PUR1TE SELECT) was collected, and autoclaved at 121 °C for 15 min. Aliquots of 99 mL (pH6.5) of autoclaved water sample were transferred into a 250 mL sterile borosilicate glass bottles with screw caps (Schott, Duran, Germany) in triplicate.

2.3.3 Inoculation of *C. jejuni* strains in sterile distilled water

250 mL sterile Borosilicate glass bottles (Schott) containing 99 mL sterile distilled water were inoculated with 1 mL from bacterial suspensions to a final concentration of 8×10^5-3.7×10^6 cells/mL. The inoculated samples were kept in the dark at 4 °C (for up to 10 days) or 25 °C (for up to 3 days). An un-inoculated sterile distilled water sample for each temperature was used as a control for the presence of contamination. All experiments were conducted using three independent technical replicates. For a subset of samples, three biological replicates were carried out at days 0 and 1 (for 25 °C), and 0 and 3 (for 4 °C) (strains M1, 1336 and 414). The error bars represented standard deviation calculated from three independent replicates.

Previous studies have shown that mean survival times in water were much longer at 4°C and 10°C than at 22°C and 37°C (Buswell et al., 1998). Two represent both of these scenarios, in this study the temperatures 4°C and 25°C were selected for convenience (easy availability of incubators at those temperatures) and to fit in with previous studies (Blaser et al., 1980; González and Hänninen, 2012; González et al., 2009).

2.3.4 Bacterial survival in sterile distilled water: determination of culturable cells counts

At days 0, 1, 2, and 3 (for 25 °C), and 0, 1, 2, 3, and 10 (for 4 °C), a 100 µL sample was taken and 10-fold dilutions were made in MHB supplemented with *Campylobacter* growth supplement. A 10 µL spot assay of appropriate dilutions was carried out on CBA plates in triplicate. The plates were incubated for 48 h at 37 °C under microaerobic conditions, and the survival was then determined by enumerating the CFU/ mL.

2.4. Survival of *C. jejuni* strains in natural water (Troughs)

2.4.1 Preparation of the inoculum

C. jejuni strains M1, 1336 and 414 (Table 2.1) were grown microaerobically on CBA for 48 h at 42 °C. The strains were sub-cultured microaerobically on CBA for 24 h at 42 °C. A loop-ful of 5 µL was harvested and inoculated into a 7 mL sterile bijou tube (Appleton Woods) containing 5 mL of MHB supplemented with *Campylobacter* growth supplement. The suspension was adjusted to a final optical density at 600 nm (OD_{600}) of 0.05 ($3.2x10^7$-$2.9x10^8$ CFU/mL).

2.4.2 Collection of trough water sample

Trough water samples were collected in sterile borosilicate glass bottles (1 litre, Schott) from field 6 at Leahurst (Figure 2.1). Samples were transported to the laboratory at ambient temperature within 1 h of sampling and half of the water sample was autoclaved at 121 °C for 15 min. Aliquots of 99 mL (pH7.5) of autoclaved and non-autoclaved water samples were transferred into a 250 mL sterile borosilicate glass bottles with screw caps in triplicate.

Figure 2.1 Sampling area of natural water at Leahurst

2.4.3 Inoculation of *C. jejuni* strains in natural (trough) water

250 mL sterile Borosilicate glass bottles containing 99 mL of autoclaved or non-autoclaved trough water were inoculated with 1 mL from bacterial suspensions so that the final concentration was 7.3×10^5-3×10^6 cells/mL. The inoculated samples were kept in the dark at 4 °C (for 3 days) and 25 °C (for 1 day). An un-inoculated water sample for each temperature was used as a control for the presence of contamination. The experiments were conducted in three independent replicates and the error bars represented standard deviation calculated from three independent replicates.

2.4.4 Bacterial survival in natural water

At days 0 and 1 (for 25 °C), and 0 and 3 (for 4 °C), a 100 µL sample was taken and 10-fold dilutions were made in MHB supplemented with *Campylobacter* growth supplement. A 10 µL spot assay of appropriate dilutions was carried out in triplicate on modified Charcoal Cefoperazine Deoxycholate Agar Base (mCCDA) supplemented with Tetracycline (30 µg/mL SIGMA). The plates were incubated for 48 h at 42 °C under microaerobic conditions, and survival was determined by enumerating of the CFU/mL.

2.5 Assay for viable but non culturable (VBNC) cells

2.5.1 Counting of VBNC cells of *C. jejuni* strains

At days 0, 1 (for 25 °C), and 0 and 3 (for 4 °C), inoculated samples in Borosilicate glass bottles for *C. jejuni* strains M1, 414 and 1336 were concentrated by centrifugation at 3893×g for 20 min (3-16PK, SIGMA) in a 50 mL Falcon tube (Corning, Appleton Woods). Supernatant was removed but 1 mL kept at the bottom of each Falcon tube and subsequently transferred into a 1.5 mL Eppendorf tube. Cells were pelleted by centrifugation at 5000×g for 20 min. The pellet was re-suspended in 1 mL of sterile distilled water. Using the LIVE/DEAD BacLight, Invitrogen kit, 3 µL of the mixture (SYTO 9 green-fluorescent and propidium iodide red-fluorescent) were added for each 1 mL of bacterial suspension and this mixture was incubated for 15 min at room temperature. 5 µL of the cell suspension was then placed on a clean microscope slide, covered with a 22×22 mm cover slip, sealed with nail polish and investigated with Fluorescence microscopy (Nikon ECLIPSE 80i). Enumeration of VBNC cells was carried out by using two nucleic acid stains, SYTO 9 dye that penetrates live cells (intact membranes) causing the cells to stain fluorescent green, and propidium iodide dye that penetrates only dead cells (damaged membranes) causing the cells to stain fluorescent red (LIVE/DEAD BacLight, Invitrogen kit) .

For each sample, three fields were enumerated at an average of 90-180 cells in each field. The percentage of viable cells was calculated as follows: % viable cells= [viable cell count (green cells)/total cell count (green cells +red cells)] ×100. The experiment was conducted in three independent replicates.

2.6 Genomic DNA preparation

2.6.1 Wizard Genomic DNA Purification Kit (Promega)

Genomic DNA was extracted from C*. jejuni* strains M1, 1336 and 414 by using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. Briefly, colonies of bacterial culture on CBA were mixed with 600 µL Nuclei Lysis Solution in a 1.5 mL Eppendorf tube (Appleton Woods). This suspension was incubated at 80 °C for 5 min and then cooled to room temperature. 3 µL of RNase (0.012 mg) solution was added and the mixture incubated for 30 min at 37 °C and then cooled to room temperature. 200 µL of protein precipitation solution was added and mixed well, after which the mixture was incubated on ice for 5 min and then centrifuged at maximum speed for 3 min. The supernatant was transferred to a clean 1.5 mL Eppendorf tube containing 600 µL of room temperature isopropanol, mixed gently and centrifuged for 2 min at maximum speed. The supernatant was discarded, 600 µL of room temperature 70% (v/v) ethanol was added, and the centrifugation was repeated at maximum speed for 2 min. The ethanol was aspirated and the DNA pellet was air-dried for 15 min and rehydrated in 100 µL of rehydration solution for 1 h at 65 °C. DNA concentrations were determined by using a NanoDrop-1000 spectrophotometer (Labtech) and the extracted DNA was stored at -20 °C.

2.6.2 Chelex Genomic DNA extraction method

Bacterial templates were prepared by re-suspending single colonies in 300 µL 20% (w/v) Chelex (BIORAD) in a 1.5 mL Eppendorf tube, heating to 95 °C for 10 min and centrifugation at 10, 000×g for 2 min. 50 µL of the supernatant was added into a fresh 1.5 mL Eppendorf tube containing 450 µL of sterile distilled water (SDW) and was used directly in PCR assays. DNA extracts were stored at -20 °C.

2.6.3 Preparation of bacterial DNA from boiled suspension

Bacterial templates of *C. jejuni* strains were prepared by suspending single colonies from CBA plates in 50 µL of SDW. The suspension was then mixed by vortexing, boiled for 5 min and was then used directly in PCR assays.

2.7 PCR protocol for amplification of bacterial genomic DNA

All oligonucleotide primers were obtained from SIGMA. All primers used in this study are listed in Table 2.3.

Table 2.3 Oligonucleotide Primers used in this study.

Name	Sequence (5'-3')	Amplicon size (bp)	Target gene	Temp * (°C)	Reference
C412F	GGATGACACTTT TCG GAGC	857	*16S rRNA*	58	(Katzav et al., 2008; Linton et al., 1996)
CampR2	GGCTTCATGCTC TCGAGTT				
q-rpoA-F	GCTTTAGATGCTTTCT TTAC	119	*rpoA*	55	(Phongsisay et al., 2007)

q-rpoA-R	ATTTGTCCATCAGTTGTTAC				
q-16S rRNA-F	GTCTCTTGTGAAATCTAATG	123	*16S rRNA*	55	(Phongsisay et al., 2007)
q-16S rRNA-R	GTATTCTTGGTGATATCTAC				
q-luxS-F	AAGTTATGAAAACACCTAAG	124	*luxS*	55	(Phongsisay et al., 2007)
q-luxS-R	ATAAATCCTGCGAATAAATG				
spoT-F	CTGTAAGCCAATCAAGACGAGG	182	*spoT*	55	Designed for this study
spoT-R	AGCCTTGCCTAARCAAAATGGT				
ppk1-F	TTGGCTTCGCTTTAATTCTCGT	167	*ppk1*	55	Designed for this study
ppk1-R	TACTGCTTGCATTAACTCCAGC				
amiA-F	TCAACTCCATCAGCAAYACCYT	150	*amiA*	55	Designed for this study
amiA-R	AAGAGAAGCACCTTTTTGGGTT				
q-htrB-F	TTATGCCTGATTGTATCTTG	125	*htrB*	55	(Phongsisay et al., 2007)
q-htrB-R	TTGAGTGTATTGAGGAAAAC				
htrA-F	CCACCTGAATTTCCTGGATTGA	141	*htrA*	55	Designed for this study
htrA-R	TGCACTTGGAAATCCTTTTGGT				
PS1	ATGAACAAAGTTCTGAAATTCTCTGCT	249	*oprl*	58	(DeVos et al., 1997)
PS2	CTTGCGGCTGGCTTTTTCCAG				

*Temp, refers to annealing temperature

2.7.1 Standard PCR protocol for the identification of *C. jejuni* strains

Modified Linton primers (Katzav et al., 2008; Linton et al., 1996) targeting the *16S rRNA* gene were used to confirm that bacteria were *C. jejuni*. PCR amplification was performed in 25 µL volumes. Each reaction contained 5 µL of the template from boiled suspension (section 2.6.3), 1.25 U of GoTaq polymerase (Promega), 5 mL of 5 x Taq buffer, 2.5 µM $MgCl_2$, 200 µM of nucleotides (dATP, dCTP, dGTP, dTTP), 12 pmol of each of the primers and 10.25 µL of distilled water. The PCR reaction was performed in a Thermal cycler (Labnet) using the following conditions:

One cycle of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30s and 72 °C for 1 min. The final elongation step lasted 10 min at 72 °C. The PCR product was visualised on a 1% (w/v) agarose gel (Bioline) which contained 0.2 µg/ mL of ethidium bromide. A DNA molecular weight marker [1kb plus ladder (Figure 2.4)], positive control (*C. jejuni*) and no template control (dH_2O) were included in each gel. 1 µL of 6x loading buffer (Thermo Scientific) was mixed with 5 µL of PCR product prior to loading. Amplicons were visualised under UV light (SYNGENE) and the presence of a product of 857 bp was considered positive.

2.7.2 PCR protocol for identification of *Campylobacter* isolates from environmental water samples

PCR amplification was performed in 25 µL volumes containing 2 µL of the template extracted by Chelex method, 22.5 µL of PCR master mix (1.1 x ReddyMix PCR master mixes with 1.5 mM $MgCl_2$, Thermo Scientific) and 12 pmol (0.25 µL) of each of the primers (Modified Linton primers, Table 2.3).

The PCR reaction was performed in a Thermal cycler (Applied Biosystems) using the following conditions: one cycle of 94 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The final stage carried out for 10 min at 72°C. The PCR products were visualised on a 1% (w/v) agarose gel (Alpha laboratories) which contained 6 µL peqgreen (peQlab). A DNA molecular mass marker (GeneRuler 100 bp ladder), a positive control (*C. jejuni*) and no template control (dH_2O) were included in each PCR run. Amplicons were visualised under UV light (Trasilluminator) and the presence of a single product of (857 bp) was considered as positive for *C. jejuni*.

2.7.3 PCR protocol for identification of *Pseudomonas* isolates from environmental water samples

PCR amplification was performed in 25 µL volumes containing 2 µL of the template extracted by the Chelex method, 22.2 µL of PCR master mix (1.1x ReddyMix PCR master mix with 1.5 mM $MgCl_2$, Thermo Scientific) and 4 pmol (0.4 µL) of each of the *Pseudomonas*-specific-primers (PS1 and PS2, Table 2.3). The PCR reaction was performed in a Thermal cycler using the following conditions:

One cycle of 94 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The final elongation step was carried out for 10 min at 72 °C. The PCR products were visualised on a 1% (w/v) agarose gel which contained 6 µL peqgreen (peQlab). A DNA molecular mass marker (GeneRuler 100 bp ladder), a positive control (*P. aeruginosa*) and a no template control (dH_2O) were included in

each PCR run. Amplicons were visualised under UV light and the presence of a single amplified product of (249 bp) was considered as positive for *Pseudomonas* spp.

2.7.4 Temperature gradient PCR

Gradient PCR (Mastercycler® thermal cycler) was used with a temperature gradient of 45.3 to 64.7 °C. The amplification was performed in a reaction volume of 25 µL. Each reaction contained 1 µL of DNA template (*C. jejuni* ST-42 strain) extracted by the Promega Wizard method (section 2.6.1), 1.25 U of GoTaq® DNA polymerase (Promega), 5 µL of 5 × GoTaq buffer, 2.5 µM $MgCl_2$, 200 µM of nucleotides (dATP, dCTP, dGTP, dTTP), 7.5 pmol of each of the oligonucleotide primers and 14.25 µL of distilled water.

The following PCR conditions were set: one cycle of 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, annealing temperature 45.3 to 64.7 °C for 1 min, and 72 °C for 2 min. The final elongation step was carried out for 10 min. The PCR product was visualised on a 1% (w/v) agarose gel which contained 0.2 µg/mL of ethidium bromide. A DNA molecular weight marker (1 Kb plus ladder) was included in each gel. The amplicons were visualised under UV light (SYNGENE).

2.7.5 PCR amplification of cDNA

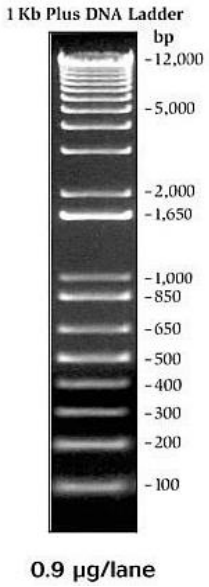
PCR amplification was performed at a reaction volume of 25 µL. Each reaction contained 5 µL of the cDNA template, 1.25 U of GoTaq®DNA polymerase (Promega), 5 µL of 5 × GoTaq buffer, 2.5 µM $MgCl_2$, 200 µM of nucleotides (dATP, dCTP, dGTP,

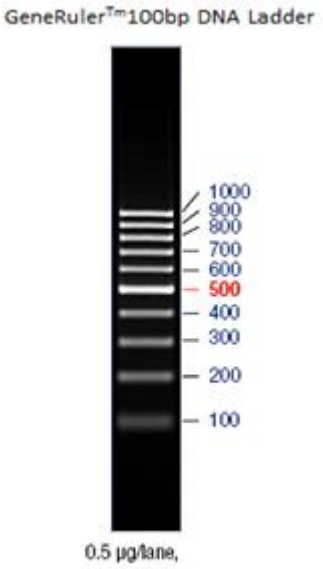
dTTP), 7.5 pmol of each of the oligonucleotide primers, and 10.25 µL of distilled water.

The following PCR conditions were set in a Thermal cycler: one cycle of 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The final elongation stage was carried out for 7 min at 72 °C. A DNA molecular marker (1kb plus ladder) was included in each gel. A negative template control (no reverse transcriptase enzyme) was included on each gel. The amplicons were loaded on 1% (w/v) agarose gel which contained 0.2 µg/mL of ethidium bromide and the gel was visualised under UV light (SYNGENE). Primer details are shown in Table 2.3.

2.8 Agarose gel electrophoresis

Gels were made by using agarose powder (Alpha laboratories). 1% (w/v) agarose gels were prepared by dissolving 1.5 g of agarose powder in 150 mL 0.5 x Tris-Borate-EDTA (TBE) buffer by boiling in a microwave oven. After the suspension was allowed to cool down to 50-55 °C, 0.2 µg/mL of ethidium bromide or 6 µL of peqgreen was added according to the manufacturer's instructions. The molecular markers 1 kb plus (Life Technologies) or GeneRuler 100 bp ladder (Thermo Scientific) were used (Figure 2.2). 5 µL of PCR product samples were mixed with 1 µL of 6 x DNA loading buffer (Thermo Scientific). Electrophoresis was carried out at 100 V for 55 min. Amplicons were photographed under UV light (SYNGENE or Transilluminator).





1 kb plus (Life Technologies)

Gen ruler 100 bp (Thermo Scientific)

Figure 2.2 DNA size markers used in this study

2.9 Gene expression analysis using Real Time Quantitative-PCR (Q-PCR) and RNA sequencing (RNAseq)

2.9.1 Oligonucleotide Primers

On arrival, the oligonucleotide primers were centrifuged at 10,000×g for 1 min and dissolved in DEPC-Treated Water (Ambion) at 1 nmol/µL for each primer. One µL of rehydrated primer was transferred into a 1.5 mL Eppendorf tube containing 99 µL of DEPC-Treated water (Ambion) and stored at -20 °C. The primers and designed primers, and target genes are listed in Table 2.3. New primer pairs (*amiA*, *ppk1*, *spoT* and *htrA*) were designed as follows: genome sequences for four strains (M1, NCTC11168, 1336, 414) were downloaded from NCBI using Geneious version 6.1.2 (Biomatters Ltd) and the genes of interest were aligned. Primer-3 which is integrated into Geneious was used to design primers specific to the genes of interest, using

default settings. Primers were designed with Q-PCR in mind, aiming for amplicon sizes of around 180 bp.

Artemis Comparison Tool (ACT) (https://www.sanger.ac.uk/resources/software/act) was used to identify the differences between the genomes of *C. jejuni* strains used in this study (M1, 1336, 414) and the strain NCTC11168.

The Primers were designed by Dr. Christina Bronowski, University of Liverpool.

2.9.2 RNA extraction from water survival experiments and cDNA synthesis

100 mL of inoculated water samples containing *C. jejuni* strains M1, 414 and 1336 (section 2.3.3) at days 0, 1 (for 25 °C) and 3 (for 4 °C), were concentrated by centrifugation at 3893×g (3-16 pk, SIGMA) for 15 min at 4 °C in Falcon tubes (Corning, Appleton Woods). Supernatants were removed but, for each sample, 1 mL was retained at the bottom of each Falcon tube and subsequently transferred into a 1.5 mL Eppendorf tube. Cells were pelleted by centrifugation (SIGMA) at 5000×g for 20 min. Total RNA of the *C. jejuni* strains was isolated by using the RiboPure-Bacteria Kit (Ambion) according to the manufacturer's instructions. Briefly, cell pellets were re-suspended in 350 µL RNAwiz and the suspension was vortexed vigorously for 15 s. Suspensions were then transferred to a tube containing 250 µL Zirconia Beads and exposed to beating by vortex for 10 min at room temperature. Zirconia Beads were pelleted by centrifugation at 16,000×g for 5 min at 4 °C and the bacterial lysate was transferred to a 1.5 mL Eppendorf tube. A 0.2 volume (v/v) of chloroform (typically 40 µL) was added, mixed well and incubated for 10 min at room temperature. The lysate

was separated into aqueous and organic phases by centrifugation at 16,000×g for 5 min at 4 °C, and the aqueous phase (top layer) was transferred to a 1.5 mL Eppendorf tube. 0.5 volumes (v/v) of 100% ethanol (500-750 µL) were added to the aqueous phase of each sample. Samples were passed through filter cartridges in 2 mL collection tubes for each sample. Samples were centrifuged at 16,000×g for 1 min at room temperature, after which the flow-through was discarded and the filter cartridge left in the tube.

The filter was washed with 700 µL wash solution 1, centrifuged at 16,000×g for 1 min at room temperature and the flow-through was discarded. The filter was washed twice with 500 µL wash solution 2/3, centrifuged at 16,000×g for 1 min at room temperature and the flow-through was discarded. The filter cartridge for each sample was centrifuged at 16,000×g for 1 min at room temperature to remove excess wash solution from the filter and then transferred to a fresh 2 mL collection tube. RNA was eluted with 50 µL preheated (100 °C) elution solution and centrifuged at 16,000×g for 1 min at room temperature. Maximum total RNA was yielded by repeating the elution step with a further 50 µL preheated elution solution into the same tube. Yielded RNA was purified from contaminated genomic DNA by adding $1/9^{th}$ (11 µL) volume (v/v) of 10 × DNase buffer, 4 µL (8 U) of DNase 1 (Ambion) and incubated for 30 min at 37 °C. A volume of DNase inactivation reagent equal to 20% (v/v) (20 µL) of the volume of RNA treated was added to each sample. Samples were mixed well, then left at room temperature for 2 min, before centrifugation for 1 min at maximum speed, after which the RNA solution was transferred to a new RNAase-free tube.

First-strand cDNA synthesis was carried out by using the SuperScriptVILO Kit (Invitrogen). The reaction for each sample was carried out in a volume of 20 µL

containing 14 µL (1.98-7.03 ng/µL) total RNA, 4 µL 5 × reaction mix and 2 µL 10 × superscript enzyme mix. The reactions were carried out at 25 °C for 10 min, 42 °C for 60 min and 85 °C for 5 min. Both extracted RNA and synthesised cDNA were quantified by using a NanoDrop-1000 spectrophotometer (Thermo Scientific).

2.9.3 Q-PCR

Q-PCR analysis using Two Standard Curve Method was carried out according to the instructions in the operator manual of the Rotor-Gene™ 6000 (Corbett Life Science). Briefly, the method depended on performing a standard curve for each gene. The expression of the gene of interest was then normalised with the internal control gene (for example, *16S rRNA*). Normalisation is the calculation of the ratios of the mRNA concentrations of the genes of interest to the concentrations of the internal control genes (Bustin et al., 2009). The standard curves were obtained by analysis using a 10-fold serial dilution of genomic DNA of *C. jejuni* (ST-42). The quantification cycle values (C_q) were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA). The C_q values determined by software analysis of the unknown samples were compared to the standard curve to determine the concentration of each unknown sample. The efficiency and the coefficient (R^2) of the standard curves were calculated automatically by the software for the Rotor-gene Q cycler.

Q-PCR was performed in a 72 well Rotor-Gene Q cycler (Qiagen), using a SYBR green PCR Kit (Qiagen). The PCR reaction was performed in a total volume of 12.5 µL in 0.1 mL PCR tubes with cap strips (Qiagen) containing 0.5 µL of the cDNA template and 12

µL of a PCR master mix (6.25 µL SYBR green, 12.5 pmol (1.25 µL) each of the primers and 3.25 µL dH_2O).

The following PCR conditions were set: an initial denaturation step of 95°C for 5 min followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 20 s. On each run, a no template control (dH_2O) was included. The amplification was carried out with three replicates of each dilution. Each specific amplicon was confirmed by the presence of a single melting peak. C_q values and data analysis were conducted using Rotor-Gene cycler software.

2.9.4 RNAseq

RNAseq or transcriptome sequencing is a newly developed technology to study transcriptome profiling that uses next-generation sequencing techniques, providing important information about internal conditions of cells. For example, RNAseq can be used to quantify and determine gene expression level changes under various conditions (Wang et al., 2009).

2.9.4.1 RNA extraction from water survival experiments

100 mL of inoculated water samples were prepared for *C. jejuni* M1 in triplicate as described previously at days 0, 1 (25 °C) and 3 (4 °C). Cells were concentrated by centrifugation at 3893×g (3-16pk, SIGMA) for 20 min at the corresponding temperature in 50 mL Falcon tubes (Corning, Appleton Woods). Supernatants were removed but, for each sample, 1 mL was retained at the bottom of each Falcon tube

and subsequently combined per sample and transferred into a 1.5 mL Eppendorf tube. Cells were pelleted by centrifugation at 5000 ×g for 10 min. A negative control was included using only sterile distilled water. Additionally a positive control of *C. jejuni* M1 grown in MHB was prepared. Cells were grown to approximately $1x10^7$ CFU/mL (OD_{600} of 0.05) in a 25 cm^2 cell culture flask with gas exchange lid (Corning) at 37 ^oC microaerophilic conditions for 18 h (exponential phase) . Cells were collected by centrifugation at 3000xg in a microcentrifuge (SIGMA). Once collected, cells were immediately re-suspended in TRIzol solution (3 times TRIzol volume to 1 volume of cells) (Ambion) and stored at -80 °C until further processing.

TRIzol samples were allowed to reach room temperature and cells were disrupted using vigorous vortexing. The samples were then incubated at room temperature for 5 min. RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research), following the manufacturer's instructions.

These experiments were carried out by Dr. Christina Bronowski, University of Liverpool.

2.9.4.2 Library construction and sequencing

rRNA was depleted from the total RNA sample using Ribozero (Epicentre), following the manufacturer's instructions. Libraries were prepared using the strand-specific ScriptSeq kit (Epicentre), following the manufacture's instruction. Sequencing was performed on the Illumina HiSeq 2500 using v4 chemistry (Illumina). This work was carried out at the Centre for Genomic Research, University of Liverpool.

2.9.4.3 RNAseq data analysis

The raw Fastq data files were trimmed for the presence of Illumina adapter sequences using Cutadapt (v1.2.1.) (Martin, 2011), using the -O3 option. The reads were further trimmed using Sickle (v1.200) (https://github.com/najoshi/sickle) with a minimum window quality score of 20. Reads shorter than 10 bp after trimming were removed. Sense and antisense overlaps between the annotation and mapped reads were counted using the HTSEQ package (Anders et al., 2015). Read counts were then normalised and Differential Expression calculated using EdgeR implemented in R (version 3.1.2 (2014-10-31), using Loess-style weighting to estimate the trended dispersion values.

For pairwise Differential Expression analysis between samples, the data was re-mapped to the *C. jejuni* M1 genome (Friis et al., 2010) using Bowtie2, and parsed using the BitSeq (Bayesian Inference of Transcripts from Sequencing data) pipeline (Glaus et al., 2012). BitSeq takes into account biological replicates and technical noise, and thereby calculates a posterior distribution of differential expression between samples.

Statistical Significance of BitSeq results was visualized in Artemis (Carver et al., 2008). Regions of Difference between the genomes of M1 (Friis et al., 2010), 1336, 414 (Hepworth et al., 2011) and NCTC11168 (Gundogdu et al., 2007; Parkhill et al., 2000) were derived through pairwise genome comparisons in ACT (Carver et al., 2005). Putative/Hypothetical genes were selected in Artemis and a putative function was derived from BLASTX searches

(http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

This work was carried out by Dr. Christina Bronowski, University of Liverpool.

2.10 Interaction between supernatants of cultures of *Pseudomonas* spp. and *C. jejuni* strains.

2.10.1 Bacterial strains and growth conditions

The bacterial species and strains of *Pseudomonas,* and *Campylobacter* used in this study are listed in Tables 2.1 and 2.2. *C. jejuni* isolate (ST-42) was streaked out from frozen stock (-80 °C) on CBA for 48 h at 37 °C. Cells were harvested by sub-culturing colonies microaerobically on CBA plate at 37 °C for 24 h. Aliquots of 5 mL of MHB supplemented with *Campylobacter* growth supplement were inoculated with a 5 µL loop -ful of *C. jejuni* isolate (ST-42) in 30 mL Universal tube and adjusted to an OD_{600} of 0.05 (Spectronic BioMate 5).

Bacterial cells of *Pseudomonas* spp. were harvested by streaking out from frozen stock (-80 °C) on Luria Agar (LA) and incubated aerobically at 25 °C for 24 h (Table 2.2).

2.10.2 Preparation of supernatants of *Pseudomonas* spp.

Aliquots of 5 mL of (LB) broth were inoculated with a 5 µL loop of *Pseudomonas* spp. in 30 mL Universal tube (Ramboldi Ltd) for each strain of *Pseudomonas* spp. (24 strains) and incubated aerobically at 25 °C while shaking at 180 rpm (Stuart) for 24 h.

Each culture was adjusted to OD_{600} of 0.5 (Spectronic BioMate 5) using LB Broth. The cultures were filtered using a 0.2 µm filter (Minisart) and the supernatant was stored at 4 °C.

2.10.3 Interaction assay

Aliquots of 180 µL of *Campylobacter* culture (OD_{600} of 0.05) were mixed with 20 µL of supernatant of *Pseudomonas* spp. (24 strains) in 72 wells of a sterile 96 well plate (Costar) in triplicate (for each strain). The following controls were also carried out: 6 replicates of *C. jejuni* culture mixed with LB (180 µL+20 µL respectively) and 6 replicates of *C. jejuni* only (200 µL). The plate was incubated microaerobically at 25 °C, 30 °C or 37 °C for 72 h. At 24 h intervals the OD_{600} was measured inside a Microplate reader (FLUOstar Omega). Each experiment was conducted in three independent replicates.

2.10.4 Treatment of supernatants by boiling or proteinase K

Aliquots of 180 µL of *Campylobacter* culture (OD_{600} of 0.05) were inoculated into 60 wells of a sterile 96 well plate. The following were added: aliquots of 20 µL of supernatant of *P. syringae* pv. *glycinea* 49a/90 in 12 replicates, aliquots of 20 µL of boiled supernatant of *P. syringae* pv. *glycinea* 49a/90 (boiled at 95 °C for 5 min) in 12 replicates, aliquots of 20 µL of supernatant of *P. syringae* pv. *glycinea* 49a/90 treated with proteinase K (Applied Biosystems) in 12 replicates, aliquots of 20 µL of LB treated with proteinase k in 12 replicates and aliquots of 20 µL of LB in 12 replicates as a control. The plate was incubated microaerobically at 37°C for 72 h. At 24 h

intervals the OD_{600} was measured inside Microplate reader (FLUOstar Omega). Proteinase K was added at 1 µL (0.02 mg)/200 µL sample and incubated at 37 °C for 30 min. After incubation, protease inhibiter cocktail (MERCK MILLIPORE) was added at 0.5 µL/200 µL sample. The experiment was conducted in triplicate.

2.11 Experiments to asses co-existence of *C. jejuni* and *Pseudomonas* spp. in environmental samples

2.11.1 Collection of environmental water samples

Environmental water samples were collected from troughs in Leahurst fields 1, 3, 6, 8 and 12 (n=5), ditches in fields 1, 3 and cuckoo lane (n=3), ponds in field 9 (n=1) and permanent puddles in fields 6 and 7 (n=2) (Figure 2.1). Samples (30 mL for each one) were collected from Leahurst fields in sterile disposable syringes (50 mL, TERUMO) and transferred to the laboratory at ambient temperature within 1 h of sampling. All samples were tested within 1 h of sampling.

2.11.2 Enrichment of collected water samples

To isolate *C. jejuni* strains, aliquots of 10 mL of water sample were mixed with 10 mL of Campylobacter Enrichment Broth (Double Exeter Broth, appendix 2) containing 5% (v/v) lysed horse blood (Southern group laboratories) in 30 mL Universal tube. Enrichment cultures were incubated for 48 h at 42 °C under microaerobic conditions. After incubation, aliquots of 50-100 µL of enrichment culture broth were spread with a sterile loop on to a sterile filter paper 0.45 µm pore size (Sartorius), which had been previously placed on mCCDA plate. Plates were left on the bench for 30 min and were

then incubated micoaerobically for 24-48 h at 42 °C after aseptically removing the filter papers. Using 5 µL sterile plastic loops, each mCCDA plate positive for grown colonies was sub-cultured on two CBA plates with the same inoculum and incubated for 48 h at both 30 °C (aerobically) and 42 °C (microaerobically). CBA Plates positive for grown colonies at both 30 °C (aerobically) and 42 °C (microaerobically) were suspected *Arcobacter*, whereas colonies present when there was growth on CBA plates only at 42 °C (microaerobically) were considered as likely to be *Campylobacter* isolates. The suspensions of these colonies were stored at -80 °C on beads (Microbank).

To isolate *Pseudomonas* spp., aliquots of 10 mL of water samples were mixed with 10 mL of LB broth (appendix 2) in 30 mL Universal tubes. Enrichment cultures were incubated aerobically for 48 h at 30°C. After incubation, aliquots of 50-100 µL of enrichment culture broth were plated out on Pseudomonas C-N Selective Agar (Oxoid) containing Cetrimide and Sodium nalidixate (CN). Plates were incubated aerobically at 30 °C for 48-72 h.

2.11.3 Extraction of bacterial DNA from environmental water samples

DNA was extracted directly from enrichment broth cultures (Double Exeter Broth culture) after incubation microaerobically for 48 h at 42 °C or from LB broth cultures after aerobic incubation for 48-72 h at 30 °C (section 2.11.2) by using the Chelex method (section 2.6.2). DNA samples were stored at 4 °C or at -20 °C for long term storage.

Chapter 3

SURVIVAL OF *C. jejuni* STRAINS IN STERILE DISTILLED WATER AND NATURAL WATER

3.1 Introduction

3.1.1 Survival of *Campylobacter* spp. in water

Although campylobacteriosis is primarily considered to be a zoonotic infection, with the infection mostly transmitted to humans through consumption of contaminated food, especially under-cooked poultry meat, it is clear that the environment can also play a role in transmission either directly or indirectly to humans. For example, *Campylobacter* infections can be acquired directly via un-chlorinated drinking water (Olson et al., 2008). In addition, the environment potentially plays an important role in transmission of *Campylobacter* to livestock (Stanley and Jones, 2003).

Little is known about the survival mechanisms of *Campylobacter* outside the host body compared to the survival of other enteric pathogens such *as Salmonella* spp. and *Escherichia coli.* The genome sequence of *C. jejuni* NCTC 11168 and other strains suggested that the capability of the organism for regulating gene expression in response to stresses associated with environmental survival may be limited compared to other bacteria (Murphy et al., 2006).

However, it is clear that *Campylobacter,* despite its requirement for microaerophilic conditions in the laboratory, and its apparently limited ability to respond to stress, can survive in the environment. Water-borne outbreaks of *Campylobacter* have been reported worldwide due to contamination of untreated water with *Campylobacter* spp. (Clark et al., 2003). For instance, *Campylobacter* outbreaks have been linked with faecal contamination of drinking water in the Scandinavian countries of Finland (Miettinen et al., 2001), Norway (Kapperud et al., 1992) and Sweden (Martin et al.,

2006). *C. jejuni* was the causative agent of seven reported waterborne epidemics in Finland between 1998 and 2001, with most outbreaks happening in July, August, September or October (Hänninen and Kärenlampi, 2004). More recently, whole genome sequencing of *C. jejuni* was used to analyse isolates from a waterborne outbreak in Finland (Revez et al., 2014). Furthermore, outbreaks of contaminated drinking water have been reported in England and Wales (Said et al., 2003; Smith et al., 2006), Scotland (Jones and Roworth, 1996) and the United States (Taylor et al., 2013). Tap water consumption has also caused outbreaks of campylobacteriosis in Wales due to contamination with surface water of pasture land (Richardson et al., 2007).

Isolation of indistinguishable strains of *C. jejuni* from groundwater and a dairy farm suggested that the source of contamination of groundwater could be from the dairy farm within the drainage or shed area (Jones, 2001). Indeed contaminated groundwater is considered to be a potential source of transmission of *Campylobacter* to poultry flocks and broiler chickens through the drinking water system (Pearson et al., 1993; Van de Giessen et al., 1996). It has also been suggested that these organisms can survive in natural water in a VBNC state after environmental water contamination by animal hosts (Guillou et al., 2008). Moreover, drinking bottled water has been a public health concern due to the ability of *Campylobacter* to survive in water for several months during storage before consumption (Evans et al., 2003; Gillespie et al., 2002). Thus, water is considered as one of the important transmission routes of campylobacteriosis in humans, either directly or indirectly, following transmission to livestock.

Previous studies have revealed that survival of *Campylobacter* in natural water samples or ground water is highly temperature and strain origin-dependent, as well as varying according to the salinity and pH value of the water. Low temperatures (around 4 °C) enhanced the survival of *Campylobacter*, whereas at increased temperatures (20-25 °C) viability declined rapidly (Cools et al., 2003; Thomas et al., 2002). Previous studies have determined that survival times for *C. jejuni* in water can vary from a few days at ambient temperatures up to 4 months at 4 °C (Buswell et al., 1998; Rollins and Colwell, 1986). It has been postulated that strain variations contribute to these differences in survival times. Various factors could contribute to *Campylobacter* survival within surface aquifers, including low oxidative stress, lack of oxygen in deeper zones, protection from UV light and lower temperature (Jones, 2001).

In the farming environment, water types potentially play an important role in the epidemiology of *Campylobacter* spp., with *C. jejuni* the most frequently species found in trough and running water, whereas *C. coli* is the most common species found in standing water (Kemp et al., 2005), highlighting the variations within the genus.

However, previous comparisons have not addressed variations based on strain genotyping. Yet previous epidemiological studies have suggested that some strain types are more commonly found in the environment. Using MLST it has been shown that *C. jejuni* ST-61, associated with cattle faecal isolates, is only rarely isolated from the environment, whereas the ST-45 complex is not only commonly found amongst livestock and wildlife faecal isolates, but also amongst water isolates (Colles et al., 2003; French et al., 2005). In addition, there are particular MLSTs found exclusively amongst wildlife or environmental sources (Hepworth et al., 2011). There are

significant gaps in our knowledge concerning the survival of different *Campylobacter* genotypes in the environment. In order to address the food safety and public health concerns on gastroenteritis in the community, a better understanding of the occurrence and behaviour of *Campylobacter* in contaminated water sources, and the mechanisms underlying variations, is needed (Van Dyke et al., 2010).

3.1.2 VBNC state

Although the concept of the VBNC state remains controversial, it has a long history and can be a useful measure of physiological change by bacteria in response to stress. It is a state in which bacterial cells remain viable according to various measures of metabolic activity but are unable to grow on the routine culture media, on which the bacteria would normally be able to grow and form colonies (Rollins and Colwell, 1986). It has been demonstrated that bacteria in this state can remain capable of causing infections or colonising a host and can be returned to a state of culturability (Chaisowwong et al., 2012).

Rollins and Colwell were the first to describe the phenomenon of the VBNC state in *C. jejuni* in 1986. In water microcosms, *Campylobacter* forms VBNC coccoid-shape cells with an intact cell membrane when exposed to stresses such as low nutrient conditions (Rollins and Colwell, 1986). Further studies have since described the influence of a harsh environment on survival of *Campylobacter*. Combinations of low temperature and starvation, and pH stress, are factors that can drive *C. jejuni* to enter into a VBNC state (Baffone et al., 2006; Chaveerach et al., 2003). Furthermore, high temperature and oxidative stress can also drive *Campylobacter* cells to form a VBNC

state (Klančnik et al., 2006). It has also been demonstrated that bactericidal processes for bacteria such as pasteurization of milk (Gunasekera et al., 2002) and chlorination of waste water (Oliver, 2005) may result in formation of the VBNC state.

Bacterial cells in a VBNC state generally show dwarfing or coccoid shape, and during this stage significant metabolic changes take place, such as decreasing nutrient transport, respiration rate and the levels of DNA, RNA and protein synthesis (Oliver, 2000; Porter et al., 1995). Starvation and cold shock proteins are synthesized during this period (McGovern and Oliver, 1995; Morton and Oliver, 1994). It has also been shown that due to the low metabolic activity of flagellated coccoid cells in the VBNC state, these cells are non-motile even with the presence of flagella (Moore, 2001).

Campylobacter species in the VBNC state have been detected in a farm's water supply by immunofluorescence microscopy; chickens became colonised with the organism after drinking this contaminated water (Pearson et al., 1993). The cells in VBNC state may play an important role in the pathogenicity of *C. jejuni*; for example, the expression of the virulence gene *cadF,* encoding an outer-membrane protein of *C. jejuni* involved in adhesion to intestinal fibronectin was still detected at high levels up to the third week of entering a VBNC state (Patrone et al., 2013). Moreover, it has been shown that the coccoid cells of *C. jejuni* are capable of adhering to chicken skin under aerobic conditions (Jang et al., 2007), and are also capable of invading Caco-2 human epithelial cells *in vitro* (Chaisowwong et al., 2012). Therefore the bacteria in this state are believed to be a potential concern for both food safety and public health (Chaisowwong et al., 2012).

It has been shown that detection rates of *Campylobacter* based on culture methods within a watershed were much lower than prevalence rates based on the use of Q-

PCR assays. This difference in detection levels was attributed to the *Campylobacter* entering the VBNC state (Van Dyke et al., 2010). Furthermore, despite the high number of waterborne outbreaks of *Campylobacter*, it is often difficult to detect *Campylobacter* in drinking water samples using culture. This may be due to using poor methods to recover the cells or because the cells in the environment are often presence in the form of a VBNC state (Percival et al., 2013). Although the mechanisms underlying a switch to the VBNC state are not well understood, some genes have been implicated in the process in *Campylobacter*. A mutant *C. jejuni* (Δ*ppk1*) showed decreased ability to enter VBNC state due to lack in poly-P synthesis Poly-P, a source of energy and an essential molecule for survival during starvation, is synthesized by mediating the key enzyme polyphosphate kinase 1 (ppk1) (Gangaiah et al., 2009; Kassem and Rajashekara, 2011). Inorganic polyphosphate (poly-P) is a linear polymer of phosphate residues linked by phosphoanhydride bonds which provide a high energy to the cell (Kornberg, 1999).

Reverse transcription-PCR (RT-PCR) methods have been employed to monitor continued expression of genes of cells in the VBNC state (del Mar Lleò et al., 2000). Furthermore, Detection of bacterial mRNAs in VBNC cells has been suggested as an indicator for cell viability considering the short half-life of mRNA inside the cell (del Mar Lleò et al., 2000; Sheridan et al., 1998)

3.1.3 Aims of this chapter

The aims of this chapter are:

1. To compare the survival of a diverse panel of *C. jejuni* strains from various sources and representing different MLSTs in sterile distilled water at two different temperatures (4 °C and 25 °C) by measuring the ability to form colony forming units.

2. To investigate the VBNC state in *C. jejuni* strains under starvation conditions at two different temperatures (4 °C and 25 °C) by detecting and counting viable cells of *C. jejuni* in sterile distilled water.

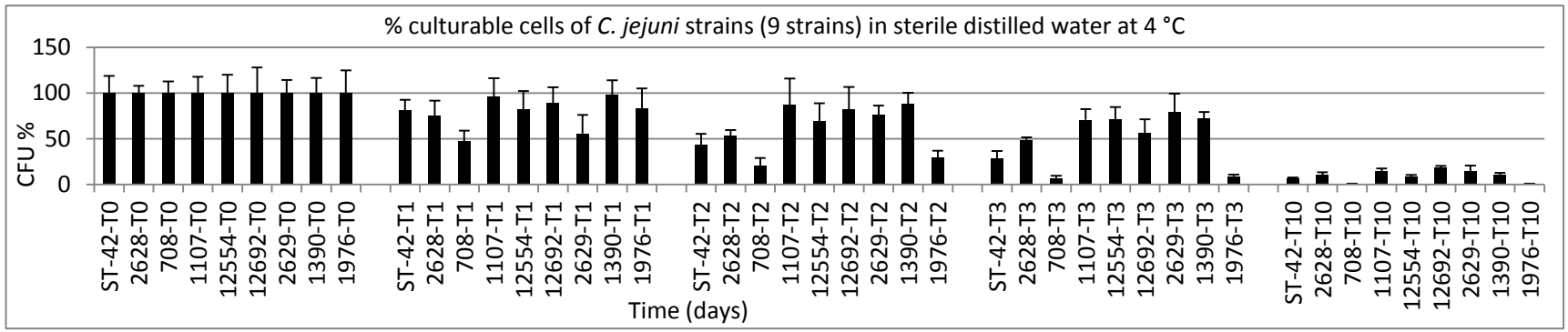
3. To compare the survival of *C. jejuni* strains in autoclaved and non-autoclaved natural water at two different temperatures (4 °C and 25 °C) by measuring the ability to form colony forming units.

3.2 Results

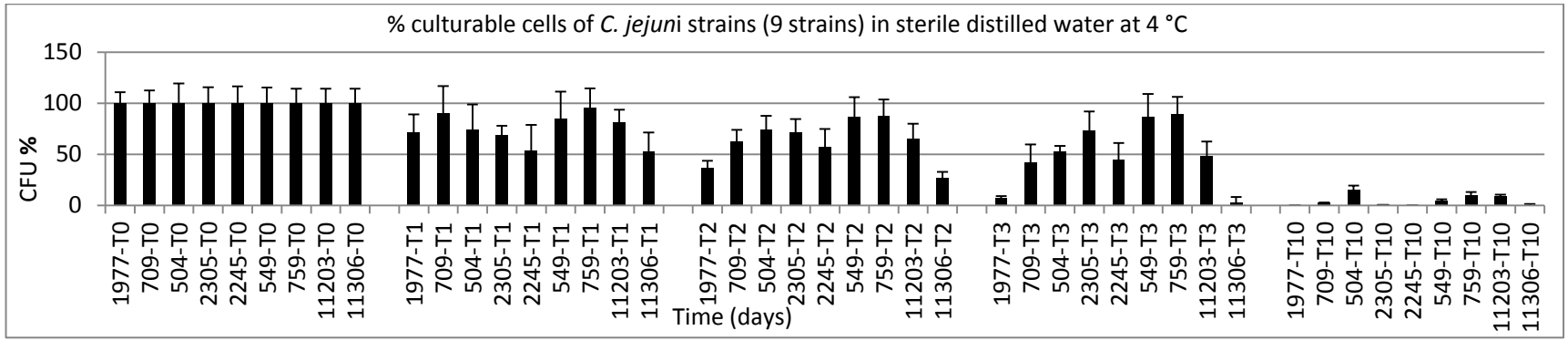
3.2.1 Survival in sterile distilled water and classification of *C. jejuni* strains into groups

27 strains of *C. jejuni* (Table 2.1), chosen to represent a diverse range of sources and MLST clonal complexes, were screened for survival based on the retention of culturability in sterile distilled water at two different temperatures (4 °C and 25 °C) in the dark. This rapid screen (Figure 3.1A, 3.1B 3.1C and 3.1D), based on a single biological replicate, was used to classify the strains into four groups (>75%, 50 -75%, 10-50% and <10%) on the basis of maintenance of CFU/mL on CBA plates at 4 °C (for day 3) and at 25 °C (for day 1). Maintenance of culturability at greater than 75% of the inoculum CFU/mL was considered as a high maintenance of CFU/mL, 10-75% CFU/mL was considered as an intermediate maintenance of CFU/mL, and less than 10% CFU/mL was considered as a low maintenance of CFU/mL (Table 3.1). The distribution of strain types according to MLST and source is shown in tables 3.2 and 3.3.

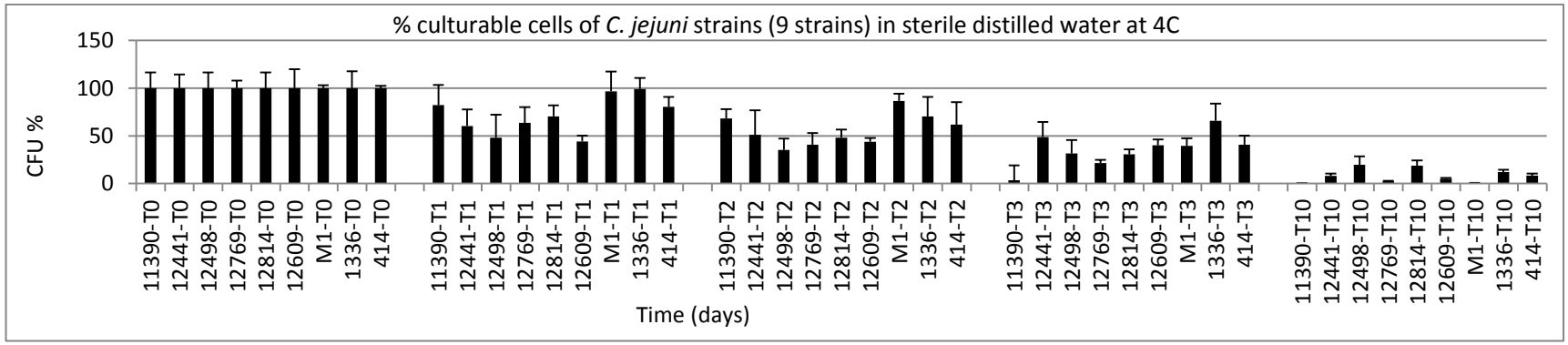
A


% culturable cells of C. jejuni strains (9 strains) in sterile distilled water at 4 °C
CFU %
150
100
50
0
ST-42-T0
2628-T0
708-T0
1107-T0
12554-T0
12692-T0
2629-T0
1390-T0
1976-T0
ST-42-T1
2628-T1
708-T1
1107-T1
12554-T1
12692-T1
2629-T1
1390-T1
1976-T1
ST-42-T2
2628-T2
708-T2
1107-T2
12554-T2
12692-T2
2629-T2
1390-T2
1976-T2
ST-42-T3
2628-T3
708-T3
1107-T3
12554-T3
12692-T3
2629-T3
1390-T3
1976-T3
ST-42-T10
2628-T10
708-T10
1107-T10
12554-T10
12692-T10
2629-T10
1390-T10
1976-T10
Time (days)

A


% culturable cells of C. jejuni strains (9 strains) in sterile distilled water at 4 °C
CFU %
150
100
50
0
1977-T0
709-T0
504-T0
2305-T0
2245-T0
549-T0
759-T0
11203-T0
11306-T0
1977-T1
709-T1
504-T1
2305-T1
2245-T1
549-T1
759-T1
11203-T1
11306-T1
1977-T2
709-T2
504-T2
2305-T2
2245-T2
549-T2
759-T2
11203-T2
11306-T2
1977-T3
709-T3
504-T3
2305-T3
2245-T3
549-T3
759-T3
11203-T3
11306-T3
1977-T10
709-T10
504-T10
2305-T10
2245-T10
549-T10
759-T10
11203-T10
11306-T10
Time (days)

A


% culturable cells of C. jejuni strains (9 strains) in sterile distilled water at 4C
CFU %
150
100
50
0
11390-T0
12441-T0
12498-T0
12769-T0
12814-T0
12609-T0
M1-T0
1336-T0
414-T0
11390-T1
12441-T1
12498-T1
12769-T1
12814-T1
12609-T1
M1-T1
1336-T1
414-T1
11390-T2
12441-T2
12498-T2
12769-T2
12814-T2
12609-T2
M1-T2
1336-T2
414-T2
11390-T3
12441-T3
12498-T3
12769-T3
12814-T3
12609-T3
M1-T3
1336-T3
414-T3
11390-T10
12441-T10
12498-T10
12769-T10
12814-T10
12609-T10
M1-T10
1336-T10
414-T10
Time (days)

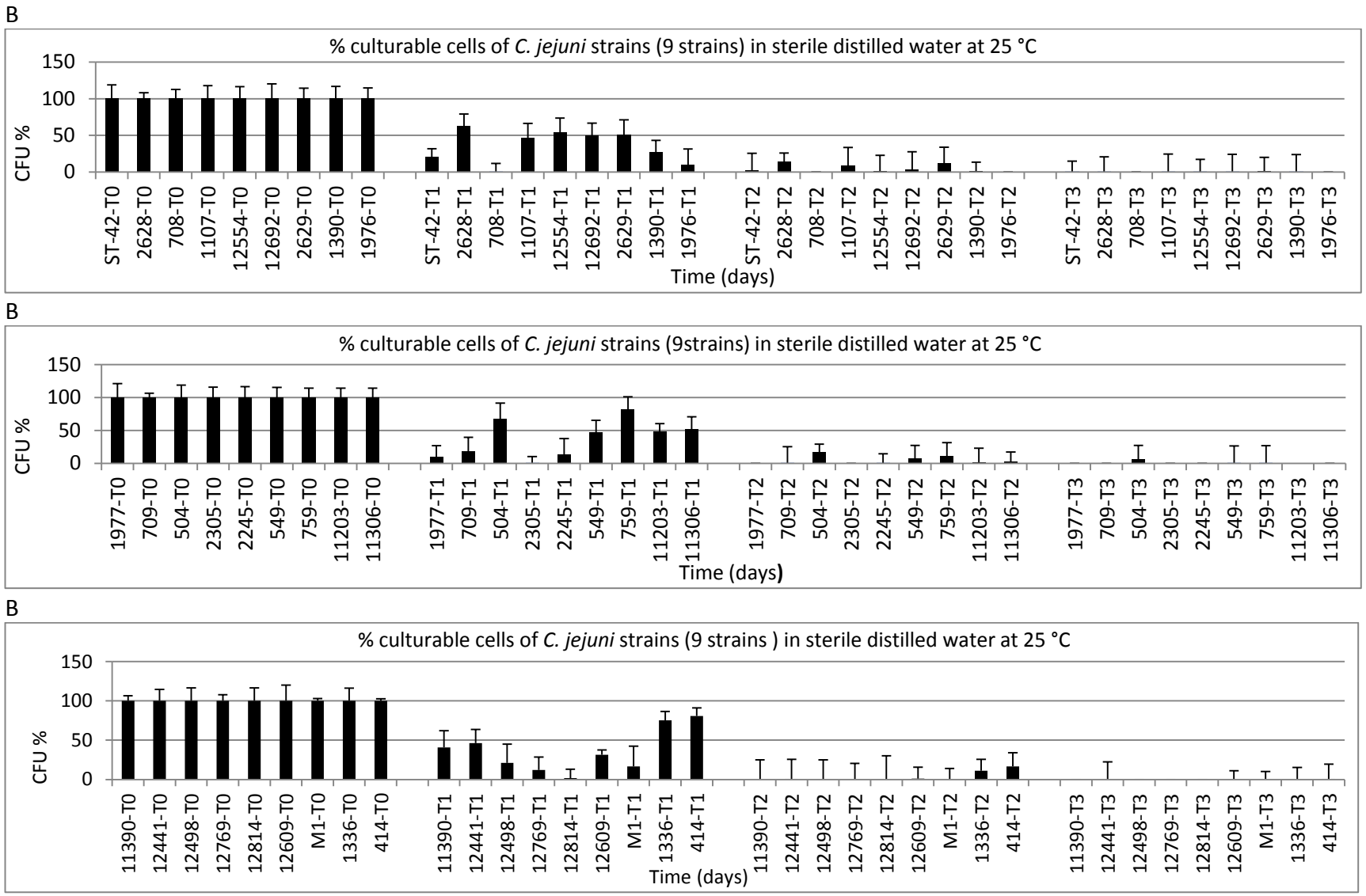


Figure 3.1 Rapid screen for culturability of *C. jejuni* cells (27 strains) during incubation periods in sterile distilled water. The percentage of the original inoculum retaining culurability is shown for (A) 0, 1, 2, 3 and 10 days at 4 °C, and (B) 0, 1, 2 and 3 days at 25 °C. The error bars represent standard deviation calculated from three technical replicate

C

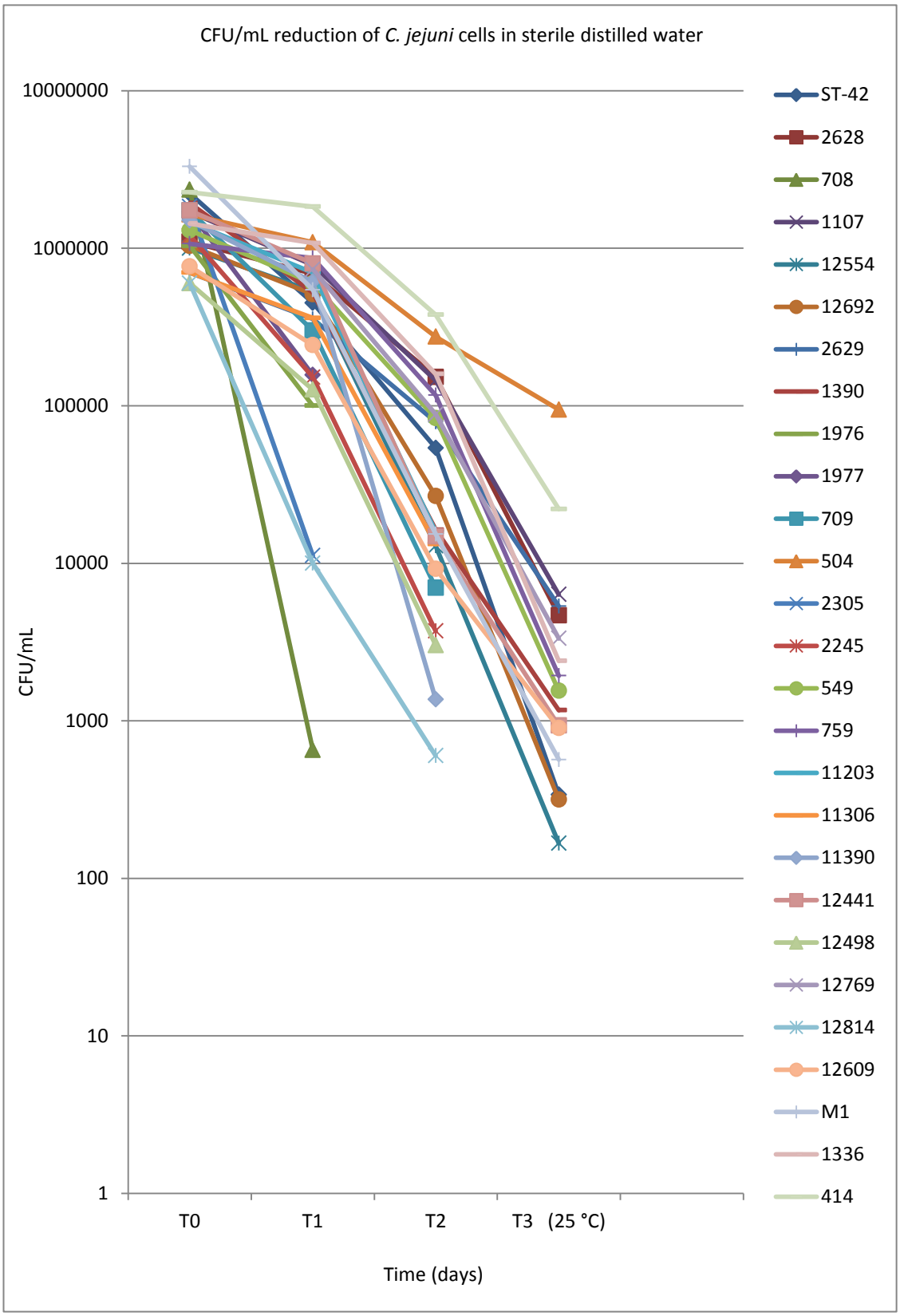


Figure 3.1C CFU/mL reduction of *C. jejuni* cells (27 strains) during incubation periods in sterile distilled water at 25 °C. These data represent three technical replicates.

D

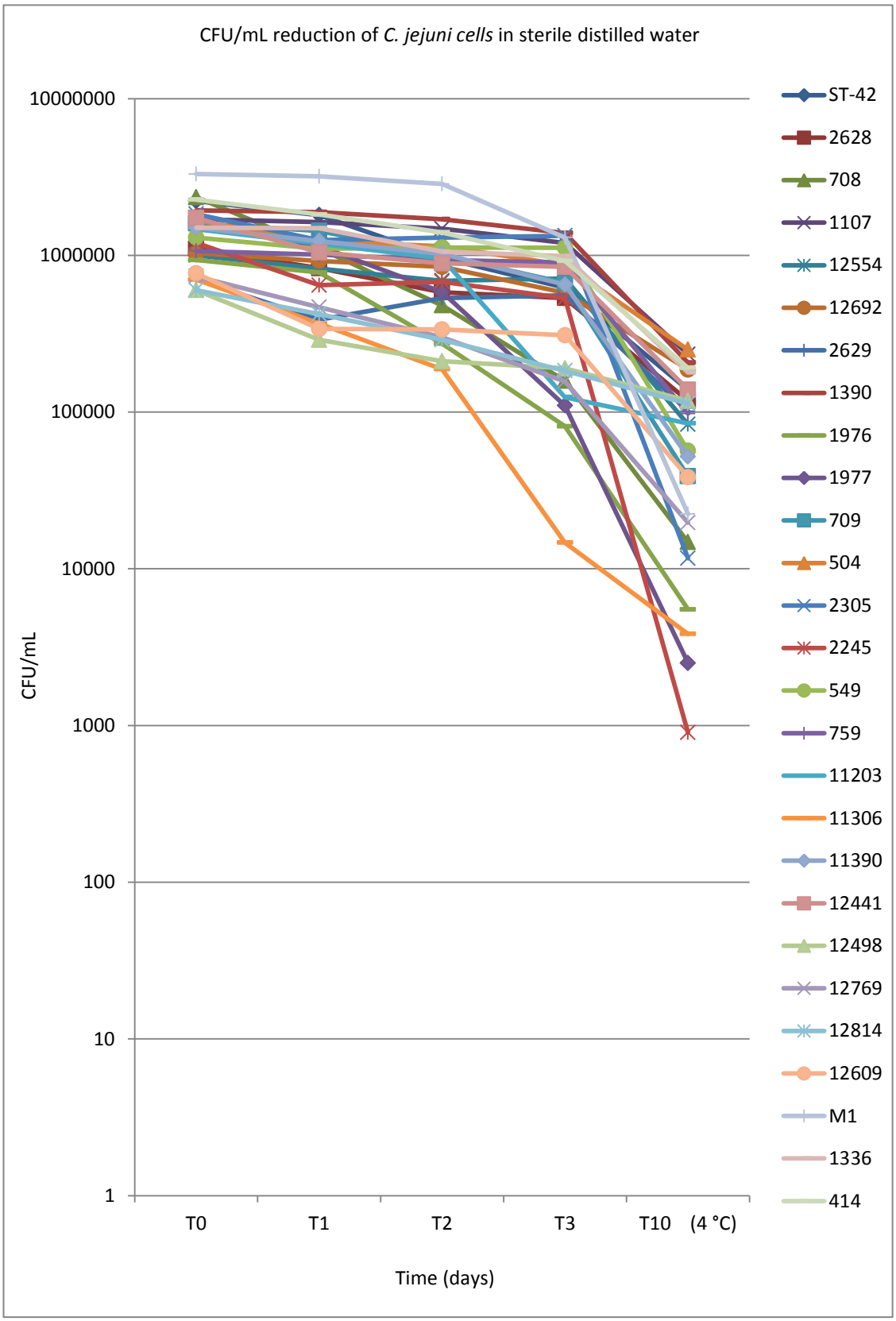


Figure 3.1D CFU/mL reduction of *C. jejuni* cells (27 strains) during incubation periods in sterile distilled water at 4 °C. These data represent three technical replicates.

Table 3.1 Classification of *C. jejuni* strains (27 strains) on the basis of maintenance of CFU/mL in sterile distilled water at two different temperatures (initial screen).

High >75% CFU/mL		**Intermediate 1 50-75% CFU/mL**		**Intermediate 2 10-50% CFU/mL**		**Low <10% CFU/mL**	
day 3 (4 °C)	**day 1 (25 °C)**	**day 3 (4 °C)**	**day 1 (25 °C)**	**day 3 (4 °C)**	**day 1 (25 °C)**	**day 3 (4 °C)**	**day 1 (25 °C)**
Strain /CC[a]	**Strain /CC**	**Strain /CC**	**Strain /CC**	**Strain /CC**	**Strain /CC**	**Strain /CC**	**Strain /CC**
12554 (21)	1336 (841)	11306 (21)	11306 (21)	12441 (45)	12441 (45)	11203 (61)	M1 (45)
11390 (45)	414 (3704)	1390 (42)	12554 (21)	12498 (61)	12498 (61)	1976 (48)	1976 (48)
2629 (21)		1336 (841)	2628 (21)	M1 (45)	11203 (61)	1977 (48)	1977 (48)
759 (45)	759 (45)		2629 (21)	2628 (21)	11390 (45)	708 (45)	708 (45)
549 (61)		504 (21)	504 (21)	709 (45)	709 (45)		12814 (45)
1107 (21)		2305 (45)		414 (3704)	1390 (42)		
		12692 (21)		2245 (61)	2245 (61)		
		C. jejuni			549 (61)		
		(ST-42)			2305 (45)		
					1107 (21)		
				12769 (45)	12769 (45)		
				12609 (21)	12609 (21)		
				12814 (45)	12692 (21)		
					C. jejuni		
					(ST- 42)		

[a] clonal complex

High: maitenance of culturability at greater than 75% of the inoculum CFU/mL
Intermediate 1: maitenance of culturability at 50-75% of the inoculum CFU/mL
Intermediate 2: maitenance of culturability at 10-50% of the inoculum CFU/mL
Low: maitenance of culturability at less than 10% of the inoculum CFU/mL

Red=human source, blue=water/soil source, green=cattle source, purple=wildlife source, brown=chicken source.

Table 3.2 Distribution of *C. jejuni* strains (27 strains) from different clonal complex groups amongst survival groups (initial screen) in sterile disilled water at two different temperatures

MLST (CC)	High >75% CFU/mL		Intermediate 1 50 – 75% CFU/mL		Intermediate 2 10 – 50 CFU/mL		Low <10% CFU/mL	
	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)
ST-21	3	0	3	5	2	3	0	0
ST-42	0	0	2	0	0	2	0	0
ST-45	2	1	1	0	5	5	1	3
ST-61	1	0	0	0	2	4	1	0
ST-48	0	0	0	0	0	0	2	2
Wildlife	0	2	1	0	1	0	0	0

High: maitenance of culturability at greater than 75% of the inoculum CFU/mL
Intermediate 1: maitenance of culturability at 50-75% of the inoculum CFU/mL
Intermediate 2: maitenance of culturability at 10-50% of the inoculum CFU/mL
Low: maitenance of culturability at less than 10% of the inoculum CFU/mL

Table 3.3 Distribution of *C. jejuni* strains (27 strains) from different source groups amongst survival groups (initial screen) in sterile distilled water at two different temperatures

MLST (CC) source	High >75% CFU/mL		Intermediate 1 50 – 75% CFU/mL		Intermediate 2 10 – 50 CFU/mL		Low <10% CFU/mL	
	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)	Day 3 (4 °C)	Day 1 (25 °C)
Human	2	0	1	2	3	4	1	1
Water/Soil	1	0	1	2	2	2	3	3
Cattle	3	1	2	1	1	4	0	0
Chicken	0	0	1	0	3	3	0	1
Wildlife	0	2	1	0	1	0	0	0

High: maitenance of culturability at greater than 75% of the inoculum CFU/mL
Intermediate 1: maitenance of culturability at 50-75% of the inoculum CFU/mL
Intermediate 2: maitenance of culturability at 10-50% of the inoculum CFU/mL
Low: maitenance of culturability at less than 10% of the inoculum CFU/mL

The present study demonstrated that some strains that survived well at 4 °C also survived well at 25 °C such as strains 759, 504 and 11306 (High, Table 3.1). Some strains that poorly survived at 4 °C poorly survived at 25 °C such as strains 1976, 1977 and 708 (Low, Table 3.1). Some strains that survived well at 4°C poorly survived at 25 °C such as strains 1107, 11390 and 549 (Table 3.1). Others have reported differences in survival between the lower and higher temperatures (Cools et al., 2003; Guillou et al., 2008). The prolonged survival of *C. jejuni* strains at low temperatures (4 °C and 10 °C) has been attributed to Polynucleotide phosphorylase (PNPase), encoded by *pnp* gene, despite the mechanism by which PNPase prolongs the cold survival of *Campylobacter* strains is poorly understood. It was shown that mutant strains of *C. jejuni* ((Δ*pnp*) at low temperatures (4 °C and 10 °C) demonstrated a difference of 3 log CFU/mL compared to the wild-type strains, whereas survival rates of the strains at higher temperatures (37 °C or 42 °C) were similar (Haddad et al., 2009).

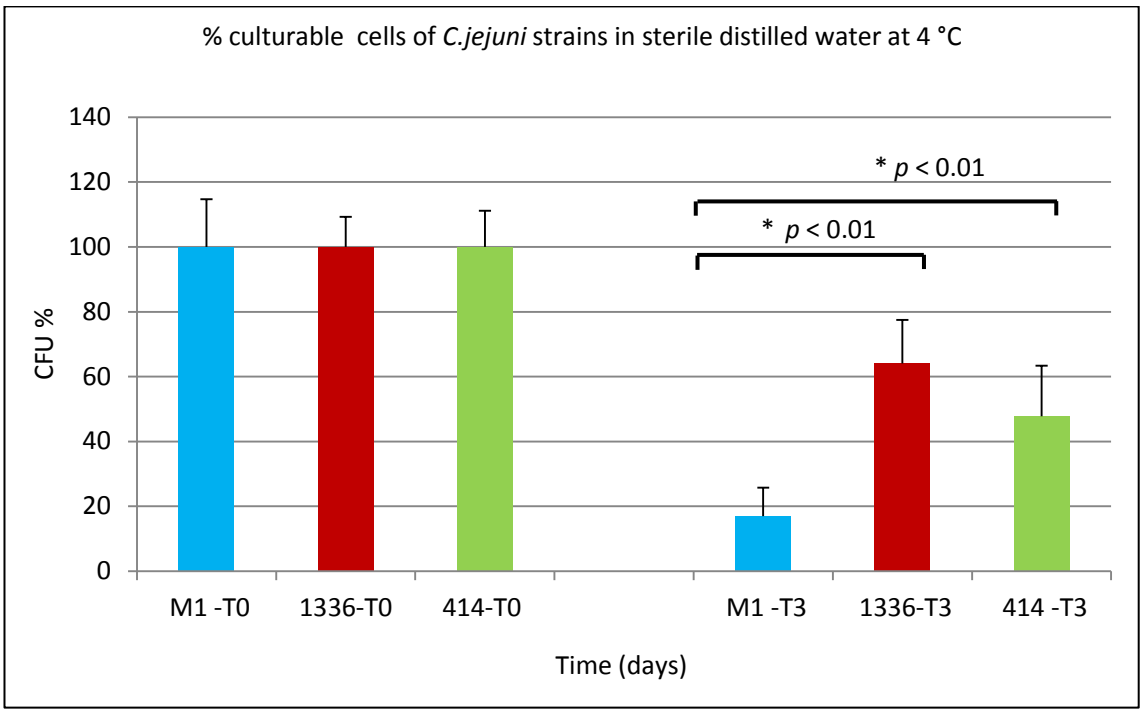
3.2.2 Survival of selected strains in sterile distilled water using three biological replicates

Three strains of *C. jejuni* strains were selected from among the 27 strains to compare the survival assay using biological replicates (in triplicate) in sterile distilled water. The strains selected M1, 1336 and 414 were isolated from different sources (human, wild birds and bank vole respectively), and were chosen to represent, based on the initial screen, different survival abilities at the two temperatures (Table 3.1). Strain M1 is a widely studied human isolate (Friis et al., 2010), 1336 is a representative of a

previously reported water/wild-life group of strains (Hepworth et al., 2011) and 414 is a niche specialist found associated with the bank vole (Hepworth et al., 2011; Williams et al., 2010). All three have been genome sequenced (Friis et al., 2010; Hepworth et al., 2011).

The results from the initial screen (based on 1 replicate) differed a little from the results obtained when the experiments were done in triplicate. Figures 3.2A, 3.2B, and 3.2C show the results obtained for % survival and CFU/mL reduction for the three selected strains of *C. jejuni* based on culturability throughout incubation in sterile distilled water held at 4 °C or 25 °C (100% represents the number of viable cells at time zero). The results confirmed that the culturability of the cells varied between the different strains of *C. jejuni*. At 4 °C the greatest variability between strains was observed at day 3; whereas only approximately 17% of strain M1 cells were still culturable after 3 days at 4 °C, for strains 1336 and 414 much higher levels (64% and 48% respectively) remained culturable. Indeed, the survival (based on culture) of strain M1 was significantly different from survival of either of the other two strains ($p < 0.01$). At 25 °C the greatest variability between strains was observed at day 1. Whereas only approximately 1.2% of strain M1 cells remained culturable after 1 day at 25 °C, for strains 1336 and 414 approximately 71% and 82% respectively remained culturable. The survival of strain M1 was significantly different from survival of either of the other two strains ($P < 0.01$).

A



B

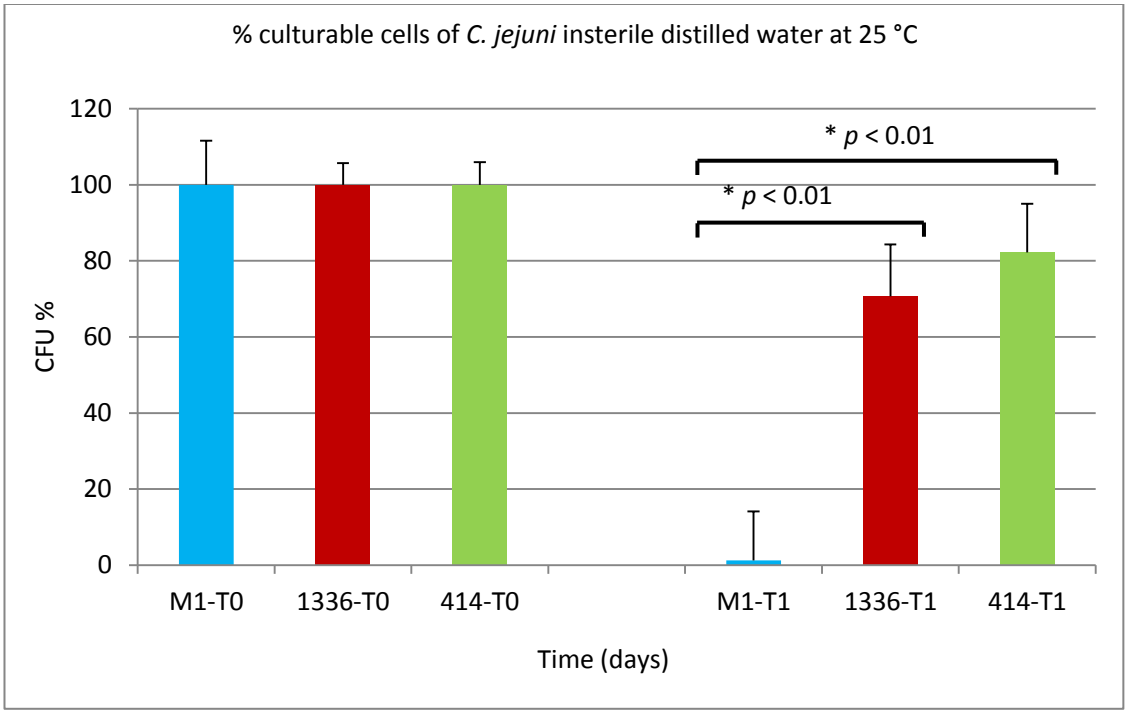


Figure 3.2 Culturability of *C. jejuni* cells (M1, 1336, 414) during inoculation periods in sterile distilled water. The percentage of the original inoculum retaining culturability is shown for (A) 0 and 3 days at 4 °C, and (B) 0 and 1 day at 25 °C. The error bars represent standard deviation calculated from three independent biological replicates.

C

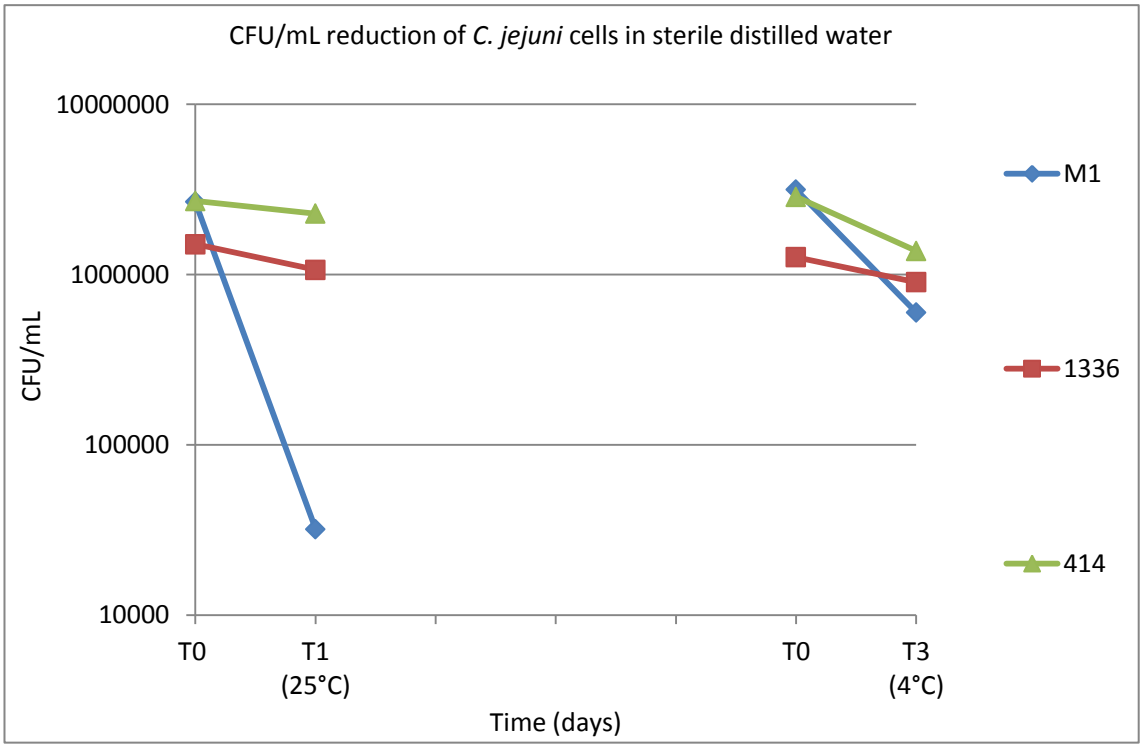


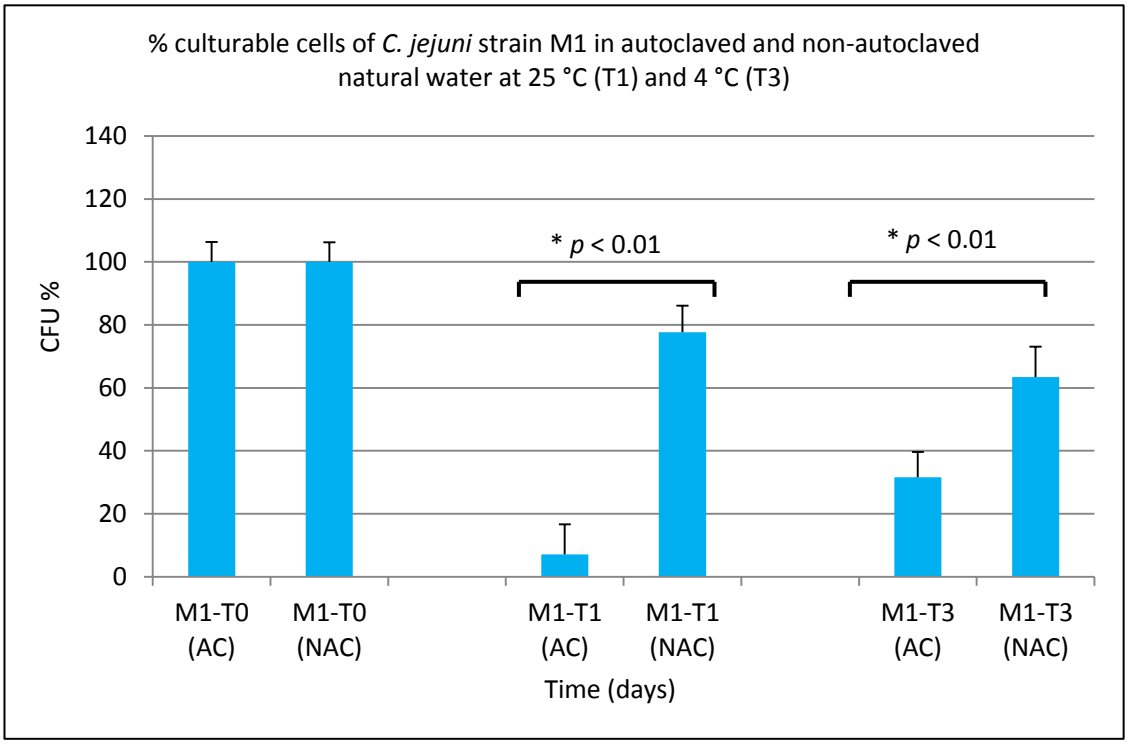
Figure 3.2C CFU/mL reduction of *C. jejuni* cells during incubation periods in sterile distilled water at 25 °C (T1) and 4 °C (T3). These data represent three independent biological replicates.

3.2.3 Survival of selected strains in natural water using three biological replicates

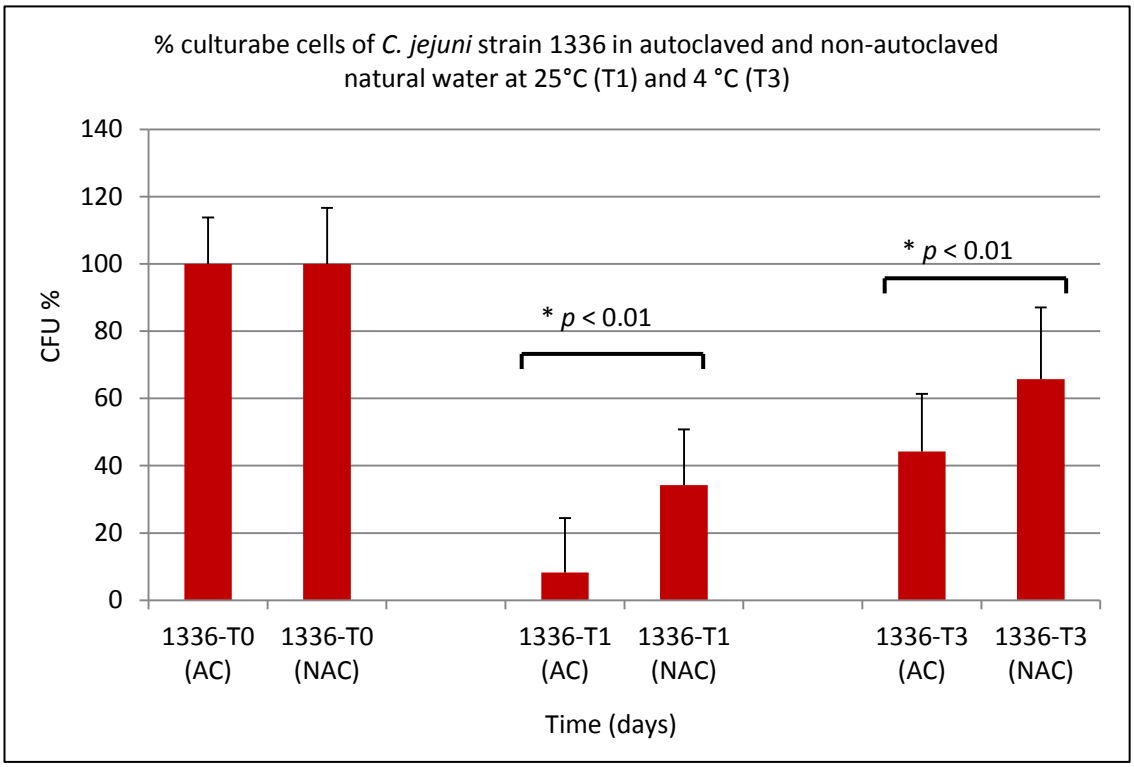
Figures 3.3 and 3.4 show the results obtained for % survival and CFU/mL reduction for the three selected strains of *C. jejuni* based on culturability throughout incubation in both autoclaved and non-autoclaved natural water held at 4 °C or 25 °C (100% represents the number of viable cells at time zero). The results confirmed that the survival (based on culture) of all strains was enhanced in non-autoclaved water compared to autoclaved water at both temperatures. At 25 °C and 4 °C in autoclaved natural water, whereas only approximately 7% and 31.5% respectively of strain M1 cells were still culturable after 1 day and 3 days respectively, for non-autoclaved natural water much higher levels (77.5% and 63.5% respectively) remained culturable. For strain 1336, at 25 °C and 4 °C in autoclaved natural water, whereas

only approximately 8% and 44% respectively of the cells were still culturable after 1 day and 3 days respectively, for non-autoclaved natural water much higher levels (34% and 65.5% respectively) remained culturable. For strain 414, comparing in autoclaved natural water there was enhanced survival in non-autoclaved natural water at 25 °C (59%) and slight enhanced survival at 4 °C (62.6%). Table 3.4 shows the results obtained for % survival for the three selected strains of *C. jejuni* based on culturability throughout incubation in sterile distilled water, autoclaved natural water and non-autoclaved natural water held at 4 °C or 25 °C.

A



B



C

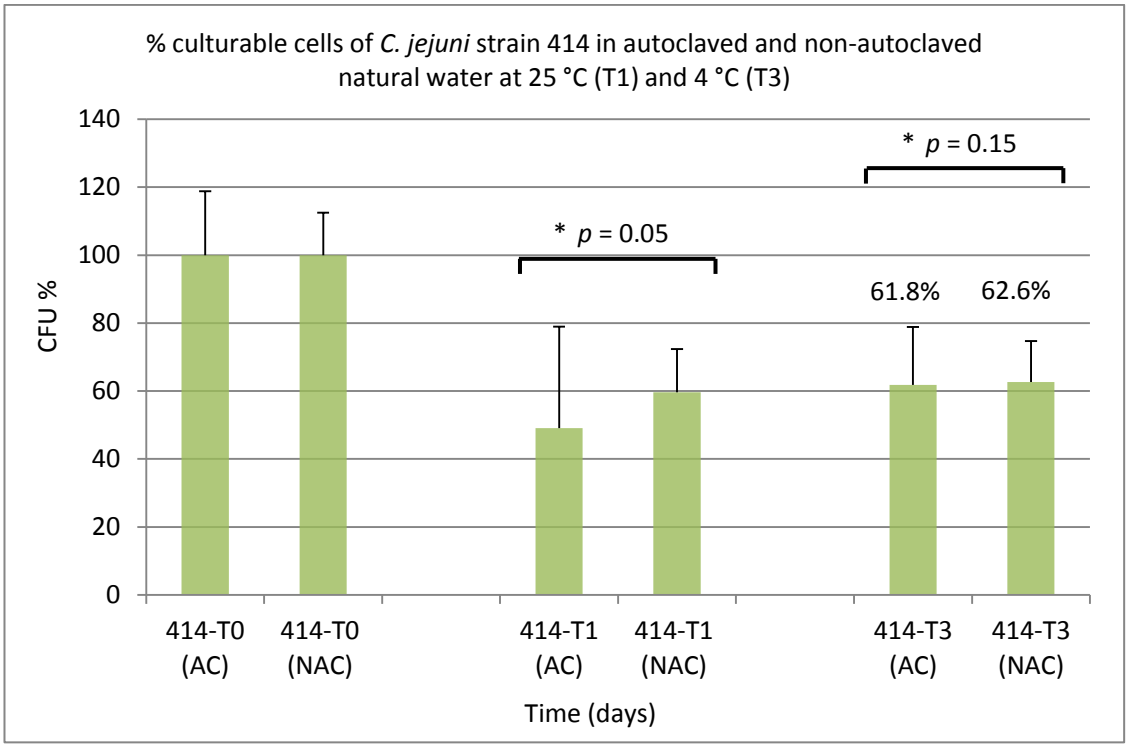


Figure 3.3 Culturability of *C. jejuni* cells (M1, 1336, 414) during incubation periods in autoclaved and non-autoclaved natural water. The percentage of the original inoculum retaining culturability is shown for (A, B and C) 0 and 1 days at 25 °C, and 0 and 3 days at 4 °C. The error bars represent standard deviation calculated from three independent biological replicates.

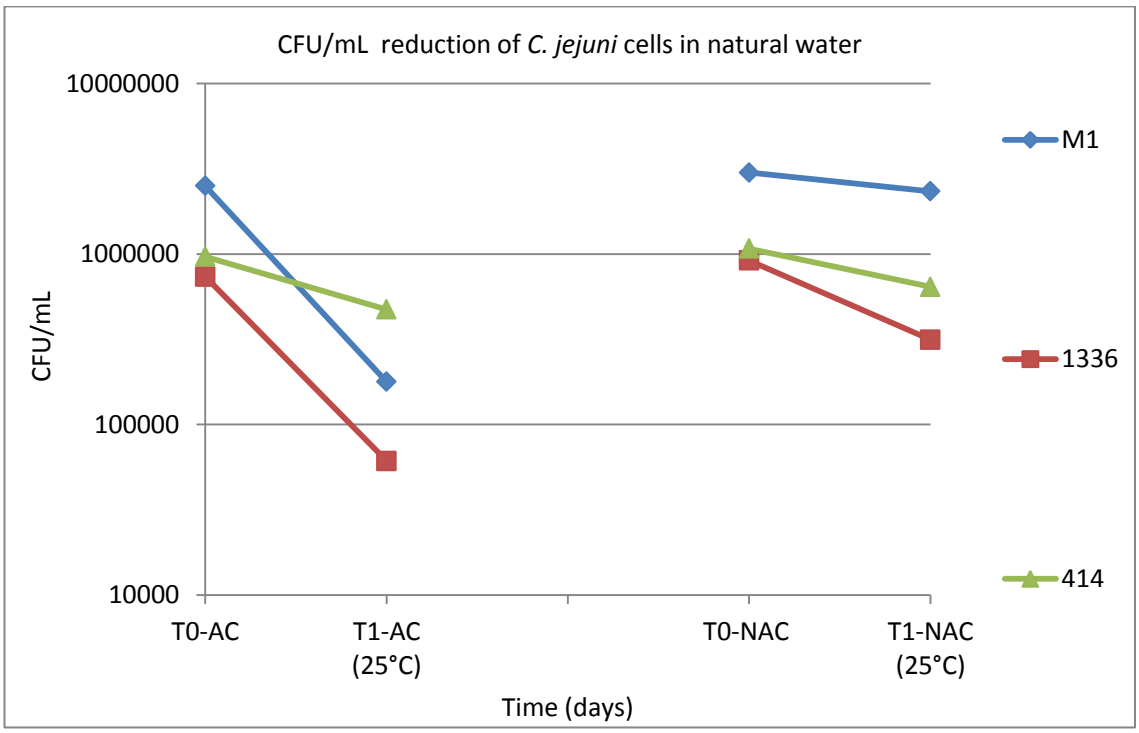
AC / autoclaved

NAC/non-autoclaved

Table 3.4 % Culturable cells of *C. jejuni* (M1, 1336, 414) during incubation periods in sterile distilled water, sterile natural water and natural water at 4 °C and 25 °C.

Strain	**% Survival in sterile distilled water**		**% Survival in sterile natural water**		**% Survival in natural water**	
	4°C	25°C	4°C	25°C	4°C	25°C
M1	17%	1.2%	31%	7%	63%	77.5%
1336	64%	71%	44%	8%	65.5%	34%
414	48%	82%	61.5%	49%	62.8%	59%

A



B

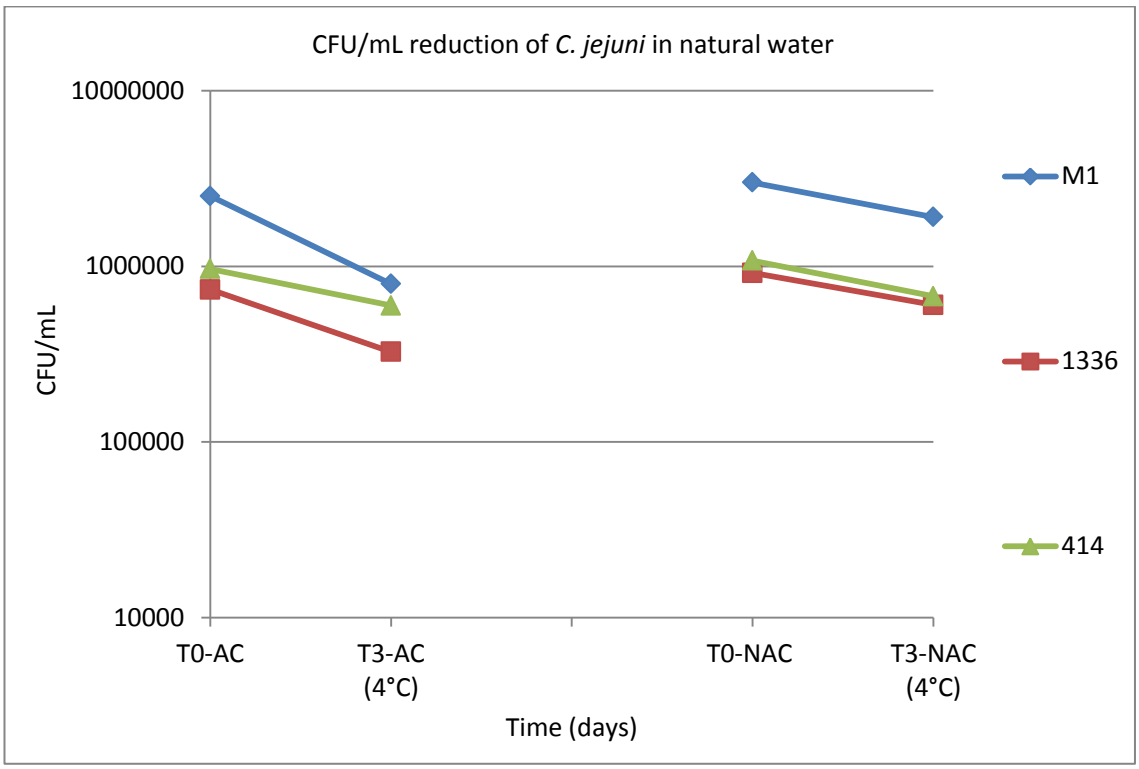


Figure 3.4 CFU/mL reductions of *C. jejuni* (M1, 1336 and 414) cells during incubation periods in autoclaved (AC) and non-autoclaved (NAC) natural water at (A) 25 °C (T1) and (B) 4 °C (T3). These data represent three independent biological replicates.

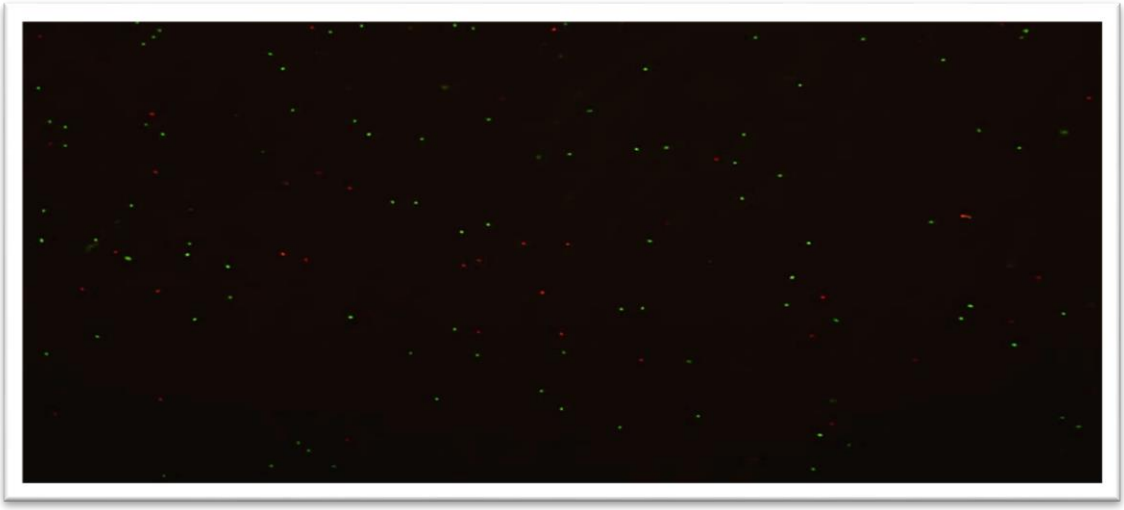
3.2.4 Direct viable cell counts

Considerable variations were observed between the strains with respect to the retention of culturability, with strain M1 demonstrating much quicker loss of culturability than the other two strains. In order to determine whether the VBNC state may play a role, sterile distilled water survival experiment samples were analysed using viable cell counts, deliberately targeting time-points where the greatest variations between strains were evident. Figure 3.5 shows examples of total viable cell counts for strain M1 at three different time points: day 0 (room

temperature), day 1 (at 25 °C) and day 3 (at 4 °C) using fluorescence microscopy. Whereas the prevalence of culturable cells on CBA media for these strains declined rapidly, especially for strain M1, at both 4 °C and 25 °C (Figure 3.2C), the numbers of viable cells (green cells) remained constant throughout the experiment at both temperatures ($p < 0.001$) (Figure 3.5 and 3.6).

Strain M1-T0

Strain M1-T1



Strain M1-T3

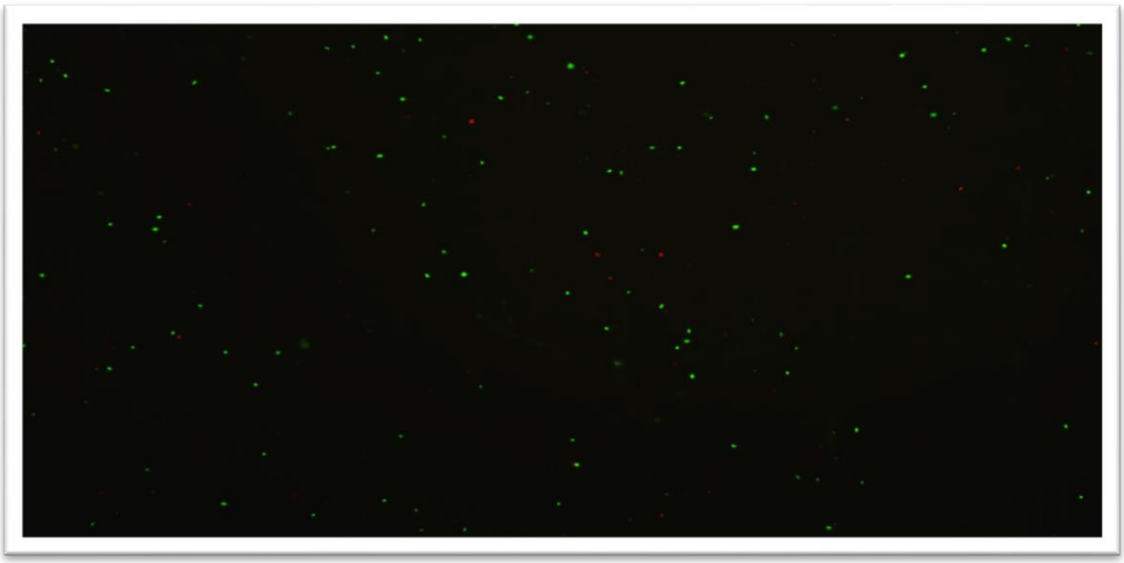


Figure 3.5 Total viable cells of *C. jejuni* strain M1 at three time points visualized with fluorescence microscopy. Field of view is 1100 µm at the magnification of 20 ×.

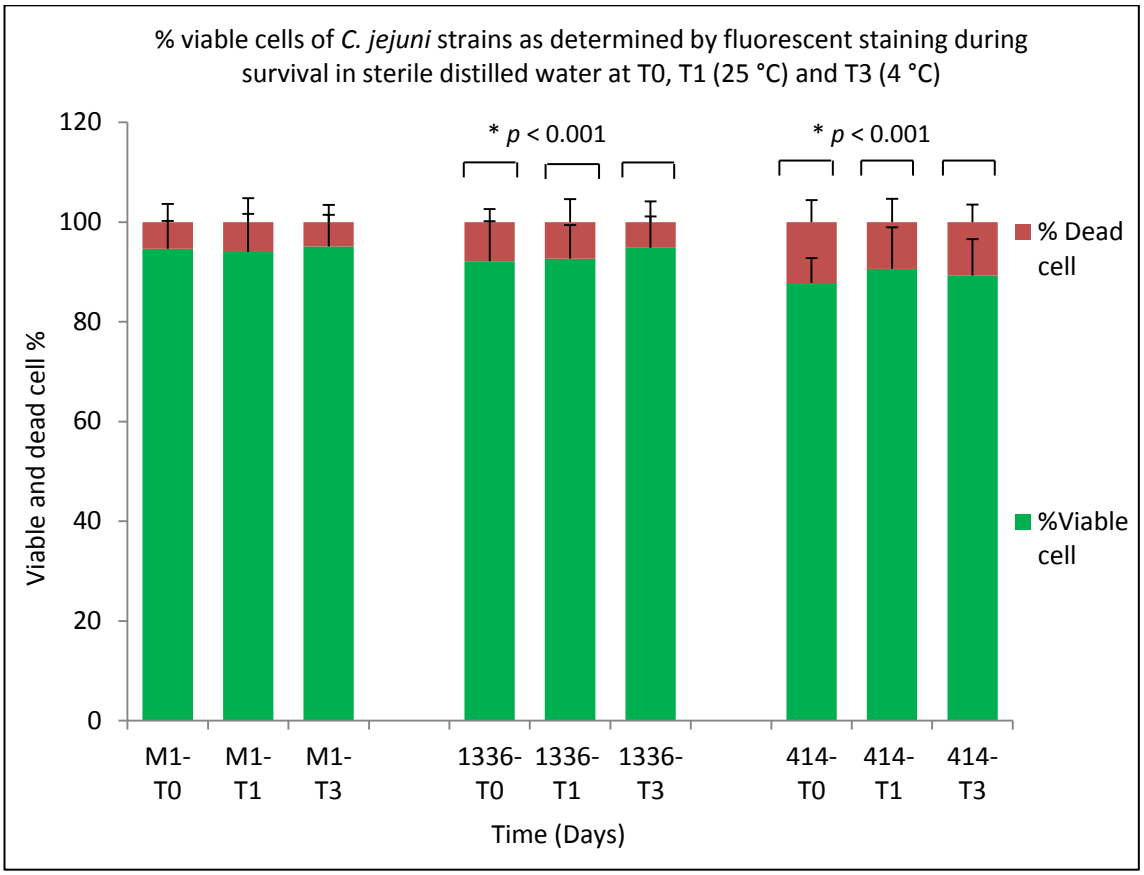


Figure 3.6 Percentage of viable and dead cells determined by LIVE/DEAD staining using fluorescence microscopy. Error bars represent the mean of standard deviation of three independent replicates (viable cells and dead cells).

3.3 Discussion

Although *C. jejuni* has been isolated from diverse animal and environmental sources, the factors influencing the ability of *C. jejuni* to colonise specific hosts or survive in the environment are poorly understood. However, there is now a persuasive body of evidence to support the hypothesis that *C. jejuni* strains have adapted to exploit specific ecological niches, suggesting that survival may vary. Several studies have sought to determine clonal prevalence amongst isolates from diverse sources by applying MLST (Colles et al., 2003; Dingle et al., 2005; Dingle et al., 2001; French et al., 2005; Kärenlampi et al., 2007; Manning et al., 2003; McCarthy et al., 2007; Sails et al., 2003). Whilst some MLST clonal complexes, such as the ST-21 complex, are widespread, others have a more restricted provenance (Colles et al., 2003; French et al., 2005). For example, members of the ST-45 complex (which are commonly associated with human infection) are encountered significantly more frequently in wildlife and water than other common clonal complexes and may be associated with environmental exposure, particularly in rural areas (Sopwith et al., 2006). The introduction of full allelic profiling provided further evidence for an association between specific *C. jejuni* genotypes and particular host species, and has given insight into the genetic mechanisms that permit adaptation to specific niches (McCarthy et al., 2007).

In previous work carried out at the University of Liverpool, MLST was applied to a large number of *C. jejuni* isolates obtained from a wide range of animal and environmental sources sampled in a structured way during a narrow spatiotemporal window (French et al., 2005; Kwan et al., 2008a). In these studies most wildlife and water isolates possessed unusual or novel STs, unassigned to existing clonal

complexes and never found amongst isolates from humans or livestock (Hepworth et al., 2011).

Gene flow analysis has revealed that although most isolates from wildlife (albeit represented mainly by wild birds) and environmental sources represent a genetically indistinguishable population, there is only very limited genetic exchange between this *C. jejuni* population and strains associated with ruminant livestock (Kwan et al., 2008a). These findings are consistent with the existence of restricted transmission pathways for different *C. jejuni* ''ecotypes'' that have adapted to exploit the specific niches they occupy. Those isolates solely associated with wildlife and water sources have been referred to as the water-wildlife group and include strain 1336 (Hepworth et al., 2011). DNA microarray technology (comparative phylogenomics) was employed to differentiate *C. jejuni* into 'livestock' and 'nonlivestock' clades (Champion et al., 2005; Stabler et al., 2013) and subsequently expanded to multiplex PCR assays (mPCR) enabling predictive analysis to investigate if human campylobacteriosis cases derived from domesticated (livestock) or water and wildlife sources (Stabler et al., 2013). The specific association between ST-3704 and Cheshire bank voles appears to be an extreme example of the well-recognised host-association for some *C. jejuni* STs/CCs. Strain 414 is a representative of this group.

In this study, a selection of isolates mostly taken from the previous large surveys carried out at the University of Liverpool was screened for variations in the ability to survive in water. The isolates were chosen to include widespread MLST clonal complexes (such as ST-21), including clonal complexes more often isolated from the environment (ST-45), clonal complexes found amongst human isolates but with evidence of host restriction (such as the cattle associated ST-61), and representatives

of water/wild-life specialists (1336 and the bank vole specialist 414). The isolates were also chosen to represent a variety of sources (human, cattle, poultry, environment, and wild-life). In the initial screen, there was no clear association between the ability to retain culturability in the water (at either 4 °C or 25 °C) and either MLST clonal complex or source, though there was a tendency for the ST-45 isolates to be amongst the poor or lower-intermediate survivors, based on culture. However, the numbers representing each group were insufficient to draw many conclusions from this comparison, which was also only based on one biological replicate per strain. The initial screen did however enable the assignment of strains into various categories based on their ability to retain culturability, enabling the selection of three isolates for closer study, M1, 1336 and 414, representing different survival abilities.

The results of this study demonstrated that the survival time (based on culture) in sterile distilled water varies significantly between different strains of *C. jejuni*. Strain M1, a representative of the ST-45 clonal complex (Friis et al., 2010), underwent the most rapid decline in culturability of the three strains at both temperatures tested. It has been reported previously that *Campylobacter* population decline occurs more rapidly at higher temperatures (such as >20 °C) (Cools et al., 2003; Guillou et al., 2008; Thomas et al., 1999) and this was the case for each of the three strains analysed in detail.

Experiments were carried out in both autoclaved and non-autoclaved natural water to compare the same three strains. In general, the results obtained in autoclaved natural water resembled those obtained in sterile distilled water. In particular, differences between the strains were observed, with strain 414 better able to retain

culturability than the other two strains. However, it was notable, especially for strain M1, that culturability was better retained in non-autoclaved natural water, suggesting that some component of the natural water system that is sensitive to autoclaving can help strain M1 to retain culturability. This study was not designed to identify the active component, but possible candidates are other microorganisms or temperature-sensitive secreted products from other microorganisms. This observation does however support the idea that bacteria may behave very differently in complex ecological systems than they do in simplified laboratory systems.

It has been shown that survival times of *Campylobacter* in water microcosms, representing environmental water, were temperature-dependant and origin-dependant. They are generally able to survive much better at low temperatures (4 and 10 °C), untreated natural water and are able to survive for long time in well water (Buswell et al., 1998; Cools et al., 2003; González and Hänninen, 2012; Korhonen and Martikalnon, 1991). These studies are compatible with the concept of strain discrimination on the basis of survival in water. Some strains of *C. jejuni* appear to be adapted to survive well in environmental water and able to cause a risk of human campylobacteriosis through the consumption of this contaminated water.

Strain M1 is ST-137, a member of the ST-45 clonal complex, and was isolated from a human infection associated with transmission from chicken (Friis et al., 2010). It has also been used in experiments to highlight strain to strain variations in the infection ecology of *C. jejuni* in chickens (Chaloner et al., 2014). Given that the ST-45 clonal complex is more often associated with isolation from the environment (French et al., 2005), it is perhaps surprising that strain M1, the ST-45 isolate used in this study, was apparently the least able to survive (least culturable). However, it should also be

noted that considerable genomic divergence can occur within clonal complexes (Revez et al., 2014), and that adaptation to a new host can lead to alterations in phenotype (Hanel et al., 2009; Leblanc-Maridor et al., 2011). *Campylobacter* genomes contain numerous contingency loci that can facilitate such adaptation (Jerome et al., 2011; Thomas et al., 2014). In addition, other adaptations can also occur (Lefébure and Stanhope, 2009). Indeed, phenotypic instability due to high frequency phase variations makes *Campylobacter* spp. notoriously difficult to work with (Bayliss et al., 2012). Hence, the lower ability of strain M1, a human isolate that had also passed through chicken, could have been due to adaptive changes. Strain 1336 and 414 had not been exposed to the same host environments. However, more rapid loss of culturability amongst members of the ST-45 complex was also found in the initial screen on a larger selection of isolates from different sources, where the ST-45 isolates were generally poor or lower intermediate survivors (Table.3.1 and 3.2).

The result of the LIVE/DEAD staining (fluorescent microscopy)(Fakruddin et al., 2013) experiments indicated that the viability of the *C. jejuni* cells remained constant, suggesting that the loss of culturability may not indicate poor survival, but could be due to the ability of strain M1 to enter more quickly into a VBNC state in nutrient-poor environment such as sterile distilled water. Previous studies (Federighi et al., 1998; Guillou et al., 2008; Uyttendaele et al., 2001) demonstrated this phenomenon in microcosm water. Even though the time-points at which strain variations based on culture were most evident were selected [4 °C (for day 3) and at 25 °C (for day 1)], variations in the percentage of viable cells were minor (Figure 3.6). Hence, it is feasible that some strains (such as strain M1) might actually enhance their survival in the environment by being able to enter the VBNC state more rapidly than others.

Further work is needed to fully characterise the extent of variations between different strains of *C. jejuni*, and to better determine whether there are clear associations between particular genotypes (such as clonal complexes) and the rate of loss of culturabilty, and whether this correlates with observations about the relative prevalence in environmental samples. However, it should also be noted that most environmental surveys rely on culture to characterise the *Campylobacter* populations present. This does not take into account the issue of whether VBNC cells are present. However, the ST-45 complex is well represented amongst isolates from the environment, suggesting that loss of culturability is not an issue that leads to the ST-45 complex being undetectable in natural samples. Clearly, the situation is more complex in natural environments. However, the work presented here does suggest that there are variations between strains and that such variations could play a role in the better survival (or better culturability) of some strains in natural environmental samples.

Although the nature of the VBNC state remains controversial, the present study is in agreement with findings suggesting that *Campylobacter* can enter a VBNC state under stress conditions such as in nutrient-poor water (Baffone et al., 2006; Chaveerach et al., 2003; Rollins and Colwell, 1986; Tholozan et al., 1999). It also supports previous observations that the capability of *C. jejuni* to enter the VBNC state varies between different strains (Cools et al., 2003). It has been shown previously that resuscitation from a VBNC state to cause infections can also vary (Beumer et al., 1992; Mederma et al., 1992) and that the physiology of the VBNC cells can vary according to temperature (Hazeleger et al., 1995). Other studies contradict the idea of resuscitation to cause infection and conclude that it is doubtful that the VBNC state plays an important role

in *Campylobacter* epidemiology (Hald et al., 2001; Ziprin et al., 2003; Ziprin and Harvey, 2004). However, given that there are evident variations between strains, and that the VBNC state itself may vary depending upon the conditions used to induce it, there may be no clear single answer to the question of how important the VBNC state is.

The survival experiments of *Campylobacter* in water were conducted in the dark because it has been reported previously that survival time could be affected when bacteria are exposed to the light due to the lethal effect of Ultraviolet (UV) radiation (Obiri-Danso et al., 2001). We used a non-selective medium (CBA) for recovery and counting CFUs of *C. jejuni* strains in sterile distilled water because the recovery of stressed bacterial cells, due to starvation, on selective media is lower (Cools et al., 2003; Uyttendaele et al., 2001). Furthermore, our results confirm the studies showing that refrigerated temperatures support the survival of *C. jejuni* in drinking water including tap water, and bottled water (Cools et al., 2003; Evans et al., 2003; González et al., 2009; Guillou et al., 2008).

This study provides clear evidence that there are variations between strains in terms of the ability to retain culturability in water, and the rate at which *C. jejuni* can enter a VBNC state. However, it will be necessary to screen far larger numbers of isolates from different sources and representing different clonal complex, to identify any clear links between these variations and the epidemiology of *Campylobacter* infections.

3.4 Conclusions

Survival in water, based on culturability, varies between different strains of *C. jejuni*, with potential implications for strain transmission. However, LIVE/DEAD staining suggests that cells remain viable in samples where culturability has dropped to very low levels. The retention of culturability is prolonged at low temperatures as well as in untreated natural water. These variations may contribute to the fact that some strains of *C. jejuni* are better adapted to survive in aquatic environments, contributing to the transmission of *Campylobacter* species from the environment, directly or indirectly to humans, constituting public health threat. Our observations are consistent with the notion that *Campylobacter* can enter into a VBNC state in response to starvation (nutrient-poor environment) such as water, but that this ability varies between strains, and may be reduced in more complex natural environments.

C h a p t e r 4

GENE EXPRESSION DURING SURVIVAL IN STERILE DISTILLED WATER

4.1 Introduction

In chapter 3, clear variations between strains with respect to the ability to retain culturability were demonstrated. In order to survive outside the host body, *C. jejuni* must confront and survive conditions where they are under stress. Despite the lack of several common stress response genes that exist in other bacteria such as *Escherichia coli*, *C. jejuni* are able to overcome harsh environmental conditions during transmission (Gaynor et al., 2005; Kassem and Rajashekara, 2011). Results that have been obtained from PCR amplification and DNA sequencing in previous studies indicate that the *C. jejuni* genome contains a *luxS* gene which encodes autoinducer-2 (AI-2), known as a quorum sensing (QS) signal molecule (Cloak et al., 2002). Additionally, this gene may play an important role in response to oxidative stress, ability in colonisation, expression of CDT, expression of flagellar genes and regulation of motility (Plummer, 2012). Studies have shown that *C. jejuni* is able to use the stringent response to control various stress survival and virulence characteristics such as survival in stationary phase, survival under high oxygen rate/low carbon dioxide rate conditions and resisting rifampicin. This involves the key regulator gene *spoT* (Gaynor et al., 2005). *C. jejuni* needs to adapt to starvation and survive in nutrient-poor environments such as water. It was shown that a mutant *C. jejuni* (Δ*ppk1*) was deficient in poly-P (energy and phosphate store) accumulation, associated with a decreased ability to form VBNC cells under stress. In addition, this gene might contribute to each of natural transmission, antibiotic and bile acid resistance and regulation of stress starvation via synthesis of poly-P (Gangaiah et al., 2009). Although

there have been few studies focusing on the coccoid form of *Campylobacter,* it has been suggested that the *C. jejuni amiA* gene is involved in transforming the cells into coccoid form (Ikeda and Karlyshev, 2012; Nachamkin et al., 2008). It has also been indicated by using Q-PCR that expression of the *C. jejuni htrB* gene may be critical for survival and stress responses such as heat, acid, osmotic and oxidative stresses (Phongsisay et al., 2007). A mutant *C. jejuni* (Δ*htrA*) showed defective growth at 44 °C and other characteristics, suggesting that the *htrA* gene is essential for *C. jejuni* to resist heat, protect periplasmic proteins from misfolding, survive at high oxygen tensions and to invade epithelial cells in human gut (Andersen et al., 2005; Baek et al., 2011a; Brøndsted et al., 2005). It is essential for *C. jejuni* during transmission to counter atmospheric oxygen tensions and the toxicity of reactive oxygen species (ROS). These toxic products of the metabolism of oxygen can cause oxidative damage to the nucleic acid of the organism. It has been shown that the expression of the catalase (*katA*) gene but not the superoxide dismutase (*sodB*) gene, the main antioxidant defence of most organisms, of *C. jejuni* increased after exposure to oxidative stress (Garénaux et al., 2008), despite the important role for *sodB* gene of *C. jejuni* in intracellular survival (Pesci et al., 1994). Additionally, it has been suggested that catalase in *C. jejuni* might play an important role in intracellular survival (Day et al., 2000). Moreover, an ankyrin-containing protein (Cj1386) has recently been identified in *C. jejuni*, encoded by a gene based directly downstream of the *katA* gene, and is thought to be involved in the same detoxification pathway as catalase (Flint et al., 2012). Amongst three classified types of superoxide dismutases (SODs) based on their metal cofactors, all possessed by *Escherichia coli, C. jejuni* only possess one type (SodB) (Bakshi et al., 2006; Purdy et al., 1999).

Unlike *E. coli*, in which alkyl hydroxide reductase (Ahp) consists of the catalytic site (AhpC) and flavoprotein (AhpF) subunit, *C. jejuni* only possess the catalytic site (AhpC) of Ahp. This protein may play a role in oxidative stress resistance in *C. jejuni* (Atack and Kelly, 2009; Baillon et al., 1999; Poole et al., 2000). Furthermore, *C. jejuni* is the first Gram-negative organism to be described in which the expression of *katA* and *ahpC* genes are regulated by ‘‘peroxide stress regulator’’ (PerR) but not oxidative stress regulator (OxyR) (Van Vliet et al., 1999). It has been shown that in *C. jejuni*, methionine sulphoxide reductases (MsrA and MsrB) appear to be encoded by two genes (Cj0637c and Cj1112c) and may play a role in repairing the oxidized methionine residues (MetO) in proteins (Atack and Kelly, 2008; Moskovitz, 2005). *C. jejuni* is able to avoid the damaging effect of iron-mediated oxidative stress on the cells by acquisition of the iron-binding proteins ferritin and Dps, which contribute to iron assimilation and protection from ROS by sequestering intracellular free iron (Ishikawa et al., 2003; Wai et al., 1996). For *C. jejuni* to colonise its host in the human gut (37 °C) and the chicken gut (42 °C), the organism may up-regulate the expression of various genes at these two temperatures. For instance, it has been shown that the temperature change can alter expression of up to 336 genes in *C. jejuni* (Stintzi, 2003).

In general, *C. jejuni* as a respiratory microorganism is unable to utilize sugars as a carbon source due to the lack of the glycolytic enzyme phosphofructokinase (Parkhill et al., 2000; Sellars et al., 2002; Velayudhan and Kelly, 2002). Therefore, *C. jejuni* depend on the availability of amino acids such as serine, glutamine, aspartate and proline for its growth (Guccione et al., 2008; Hofreuter et al., 2008). The immunogenic protein (Cj0917) in *C. jejuni*, is homologous to carbon starvation protein

A (CstA) in *E. coli*, and is thought to play a role in *in vivo* peptide uptake. It is the main up-regulated gene during starvation (Rasmussen et al., 2013).

For the purpose of this study, a sub-set of stress response genes (Table 4.1 and Figure 4.1), highlighted in the literature as important for survival in stressful conditions, was selected for analysis using Q-PCR assays during survival of *C. jejuni* strains in sterile distilled water at two different temperatures .

Table 4.1 Selected stress response and other genes in *C. jejuni*.

Stress response genes	Environmental stress	Reference
luxS	AI-2/QS/oxidative stress/ expression of CDT/regulation of motility	(Cloak et al., 2002; Plummer, 2012)
spoT	oxidative stress/stringent response	(Gaynor et al., 2005)
ppk1	starvation/ antibiotic and bile acid resistance	(Gangaiah et al., 2009)
amiA	Morphological change	(Ikeda and Karlyshev, 2012; Nachamkin et al., 2008)
htrB	Low pH/Heat shock/Oxidative/Osmotic	(Klančnik et al., 2006)
htrA	Heat shock/Aerobic tolerance	(Baek et al., 2011a; Brøndsted et al., 2005)
16SrRNA	Reference gene (internal control)	(Phongsisay et al., 2007)
rpoA	Alpha subunit of RNA polymerase (reference gene)	(Hyytiäinen et al., 2012; Phongsisay et al., 2007; Ritz et al., 2009)

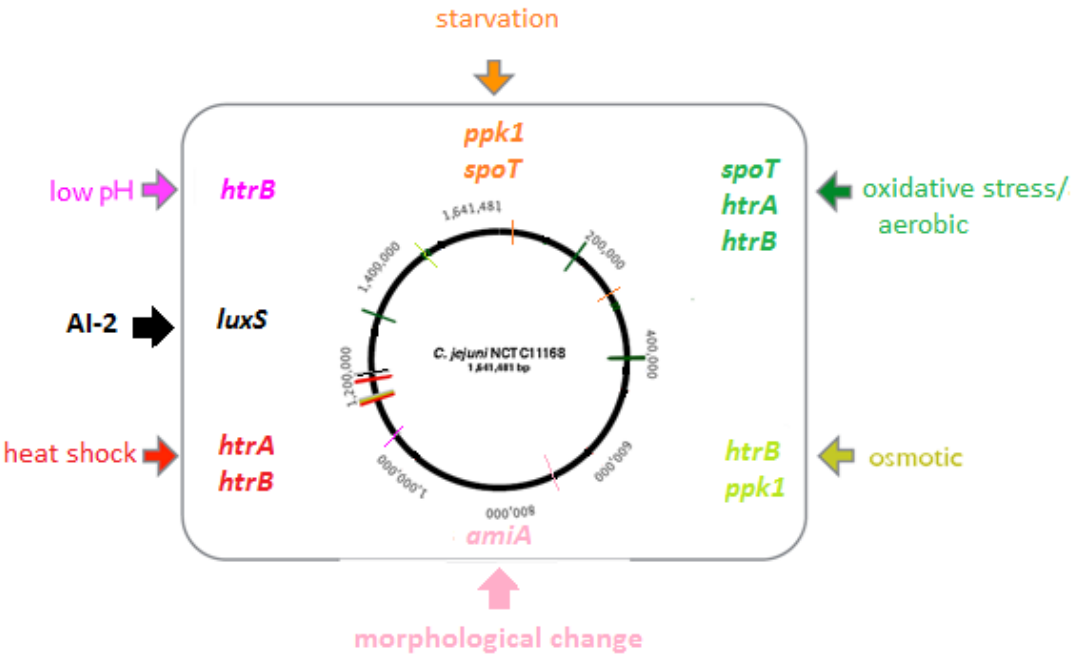


Figure 4.1 (A sub set of stress response genes in *C. jejuni*. The black circle represents the chromosome of *C. jejuni* NCTC11168).

Adapted from (Bronowski et al., 2014)

4.1.2 Aims of this chapter

The overall aim of this chapter was to identify genes that might play an important role in the differences observed between strains in the ability to retain culturability during survival in water, as described in the previous chapter. This will be achieved by:

1. At selected time-points, in both 4 °C and 25 °C water survival experiments, determining differences in gene expression of a panel of previously reported stress response genes between the different *C. jejuni* strains (M1, 1336 and 414) using end-point PCR assays.

2. Developing Q-PCR assays for selected response genes.

3. At selected time-points, in both 4 °C and 25 °C water survival experiments, using Q-PCR assays to determine variations in gene expression of stress response genes between the *C. jejuni* strains

4.2 Results

4.2.1 Temperature gradient PCR to determine optimal annealing temperatures for end-point PCR assays

We used Q-PCR to look for variations in the expression of 8 selected stress response genes (Table 4.1) highlighted in the literature as important for survival in low nutrient or stressful conditions, or the formation of biofilm/VBNC forms. For targeting these genes, four oligonucleotide primer pairs were designed (section 2.9.1) for the purpose of this study and the other four primer pairs (published previously) were purchased from SIGMA (Table 2.3).

Temperature gradient PCR was performed to determine the optimal annealing temperatures of the oligonucleotide primers used in this study. For each primer pair there were a range of temperatures at which single amplicons of the correct size were obtained. The temperature (55 °C) was selected as the annealing temperature for all of the primer pairs.

To test the ability of the primers to amplify from each of the strains used in this study using the annealing temperature 55 °C, the primer pairs were next tested on *C. jejuni* strains M1, 1336, 414. The result showed that the amplification primers were effective at annealing temperature 55 °C. Furthermore, non-specific PCR products were not detected from the no template control. Interestingly, this experiment revealed the absence of *luxS* gene from the genome of *C. jejuni* strain 1336 (Figure 4.2).

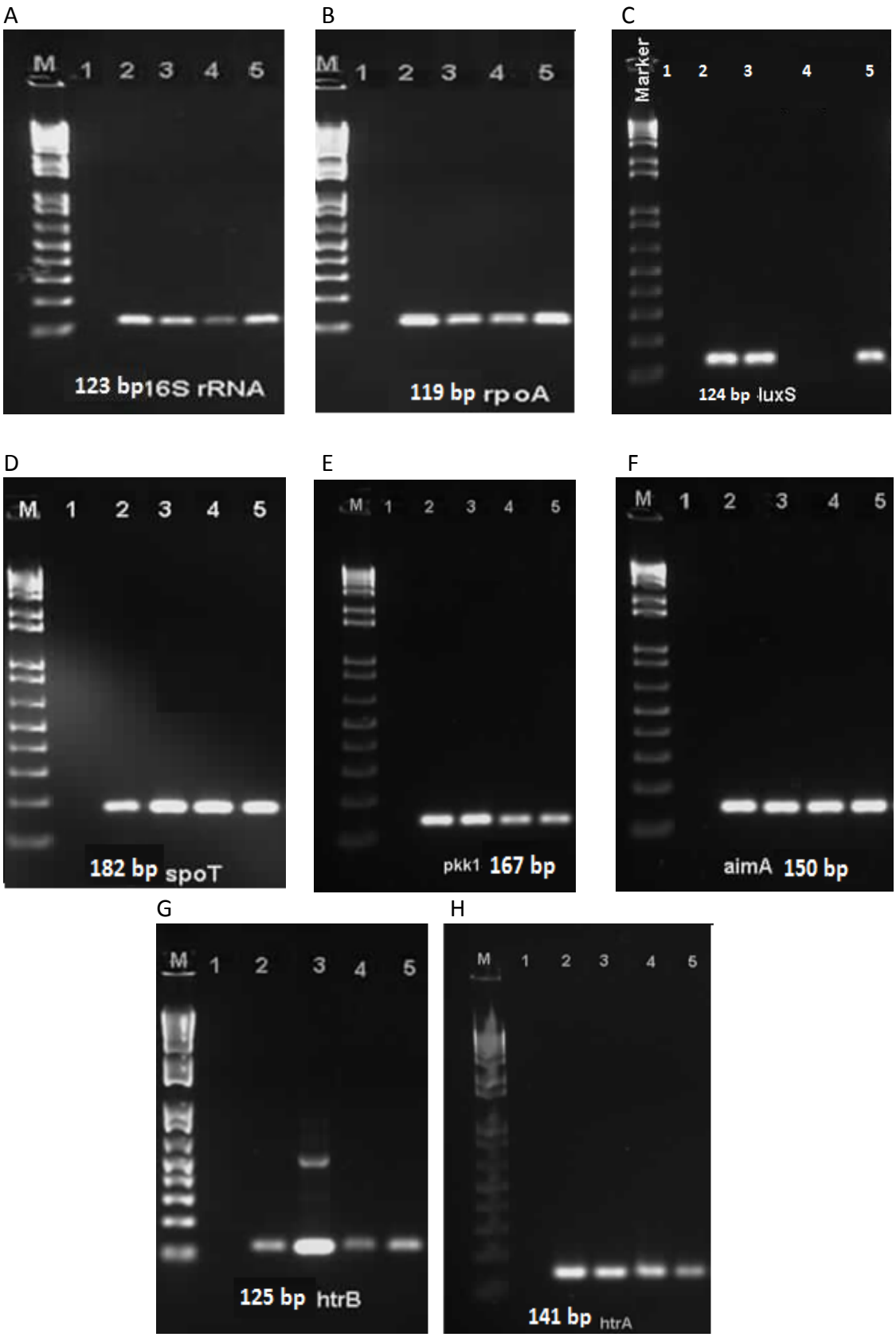


Figure 4.2 PCR amplification using primer pairs at annealing temperature of 55 °C. M: 1 kb-DNA molecular weight Marker; Lane 1: no template control (dH_2O); Lane 2: positive control (*C. jejuni* ST-42 strain); Lane 3, 4 and 5: amplicons size of 123, 119, 124, 182, 167, 150, 125 and 141 bp of the 8 selected genes respectively (A, B, C, D, E, F, G and H) of templates *C. jejuni* strains M1 (lane 3), 1336 (lane 4) and 414 (lane 5).

4.2.2 RNA extraction and conversion to cDNA

At T0 (day 0 at room temperature) and for the days where we identified greatest variation between strains (M1, 1336, 414) with respect to maintenance of culturability, T1 (day 1 at 25 °C) and T3 (day 3 at 4 °C), RNA was extracted from the water survival experiment and the amounts and the purity of extracted RNA and cDNA of *C. jejuni* strains were determined by using a NanoDrop-1000 spectrophotometer (Thermo Scientific). The absorbance was measured at 260 nm (A_{260}) and the purity was determined by measuring A_{260}/A_{280} ratio (Table 4.2A and Table 4.2B).

4.2.3 PCR amplification of cDNA

In order to determine whether expression of various stress genes was detectable at the chosen time points in the water survival experiments, a series of end-point PCR assays were carried out on the cDNA samples.

Figures 4.3-4.10 show the results of end point PCR amplification of cDNA targeting specific stress response genes and control genes during survival of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at days 0, 1 (for 25 °C) and 3 (for 4 °C). Expression of gene-specific cDNA levels of the stress response genes was evaluated and is summarised in Table 4.3.

The *rpoA* gene was chosen as the reference (control) gene for this study because it had been reported to be "the most suitable internal control to study stress response in *C. jejuni* by RT-qPCR" (Ritz et al., 2009). However, because detection of expression of the *rpoA* gene was not consistent, 16S rRNA was chosen as the reference (control) gene for subsequent Q-PCR assays rather than *rpoA*.

Table 4.2A RNA quantity and quality measurements of *C. jejuni* strains (M1, 1336, 414) at three time points [T0, T1 (at 25 °C), T3 (at 4 °C)].

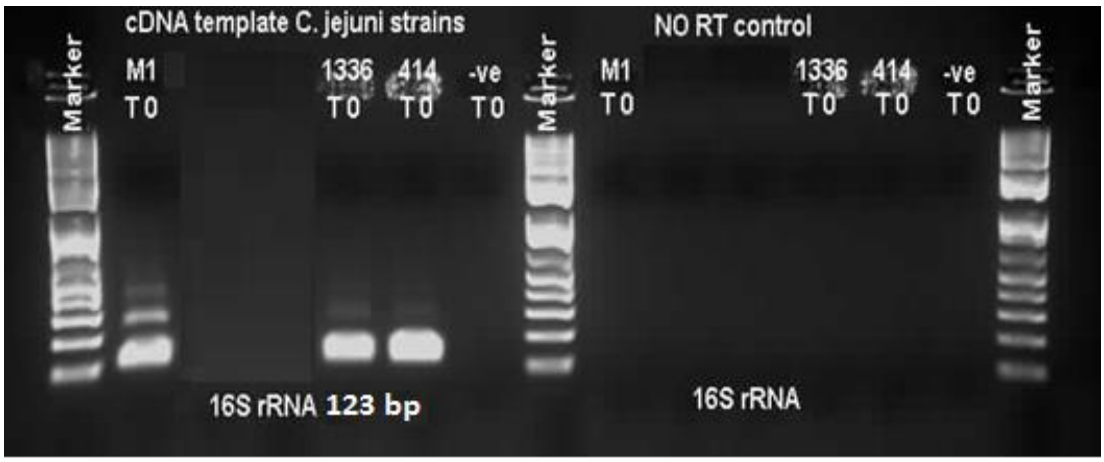
Sample ID	Total volume(µL)	ng/µl	260/280	260/230	Total (ng)
M1-T0	100	7.03	1.37	0.43	703
1336-T0	100	4.29	1.36	0.22	429
414-T0	100	4.17	1.51	0.28	417
M1-T1	100	5.16	1.6	0.34	516
1336-T1	100	2.87	1.28	0.18	287
414-T1	100	3.67	1.29	0.27	367
M1-T3	100	3	1.19	0.05	300
1336-T3	100	1.98	2.57	0.11	198
414-T3	100	2.14	1.02	0.11	214

Table 4.2B cDNA quantity and quality measurements of *C. jejuni* strains (M1, 1336, 414) at three time points [T0, T1 (at 25 °C), T3 (at 4 °C)].

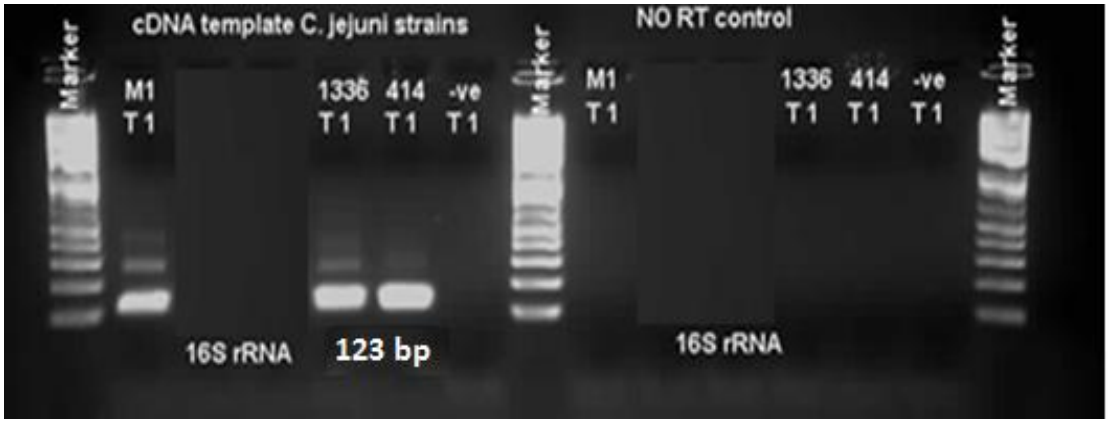
Sample ID	Total volume(µl)	ng/µl	260/280	260/230	Total(µg)
M1- T0	20	1274	1.86	1.99	25.5
1336T0	20	1325	1.86	2.12	26.5
414-T0	20	1319	1.87	2.16	26.4
M1-T1	20	1139.8	1.83	1.94	22.8
1336-T1	20	1302.3	1.86	2.12	26
414-T1	20	1904.5	1.82	2.1	38.1
M1-T3	20	1177.4	1.86	2.12	23.5
1336-T3	20	1097.9	1.86	2.11	22
414-T3	20	1702.3	1.83	2.11	34

The purity of DNA and RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. Generally, a ratio of ~1.8 is considered as "pure" for DNA, and a ratio of ~2.0 is considered as "pure" for RNA. Lower ratio in either case indicates the presence of contaminants of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The secondary measure of nucleic acid purity is assessed by the ratio of absorbance at 260 nm and 230 nm, the 260/230 values are usually higher than the 260/280 values and are often in the range of 2.0-2.2.

A



B



C

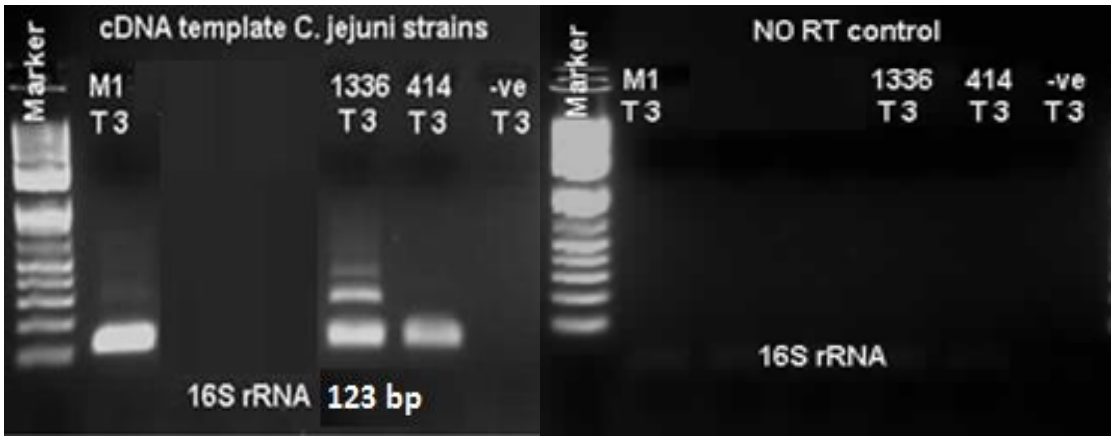
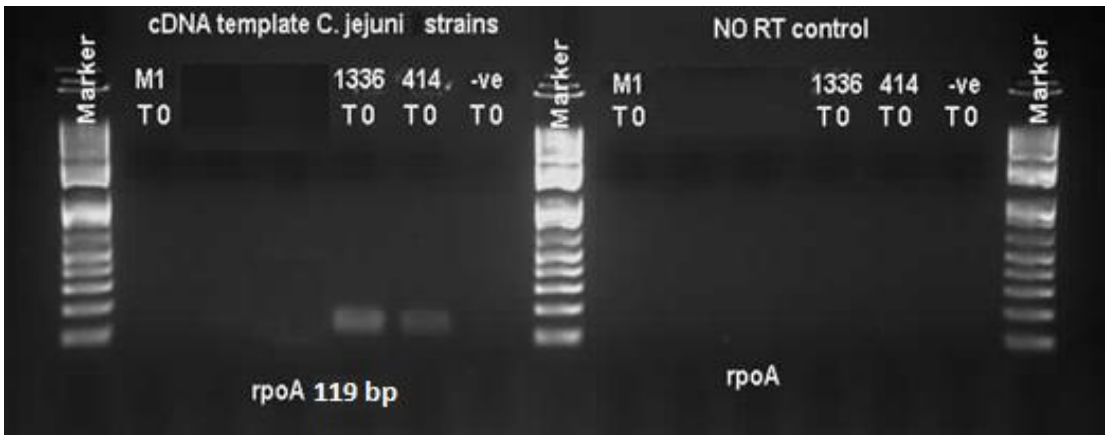
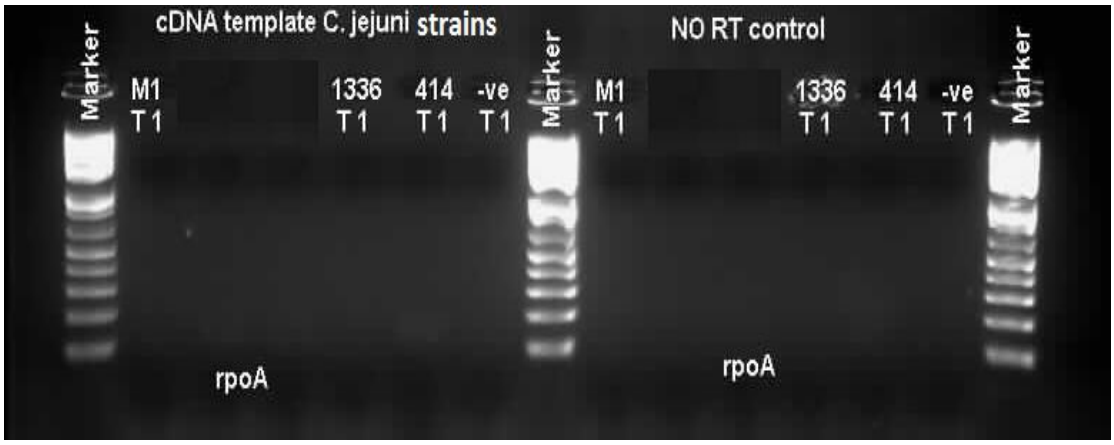


Figure 4.3 PCR amplification of segments of 16S rRNA gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 123 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



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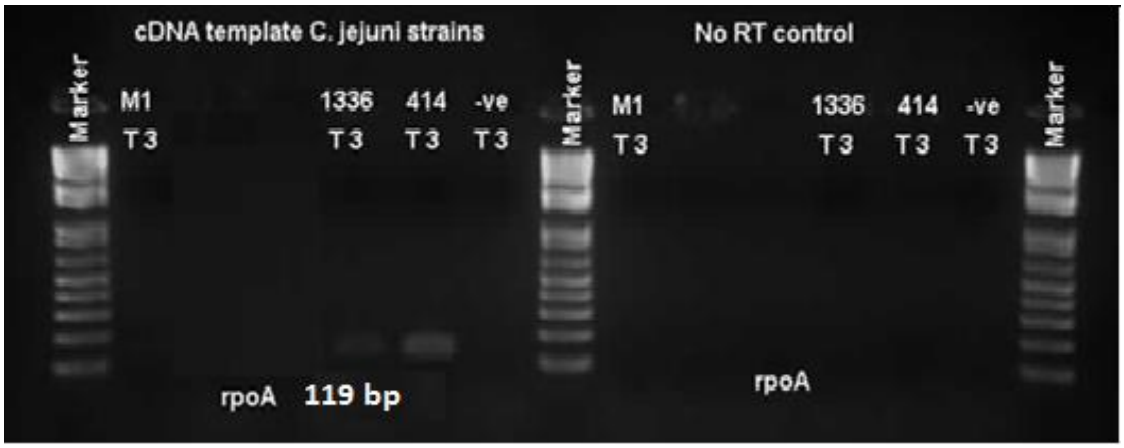
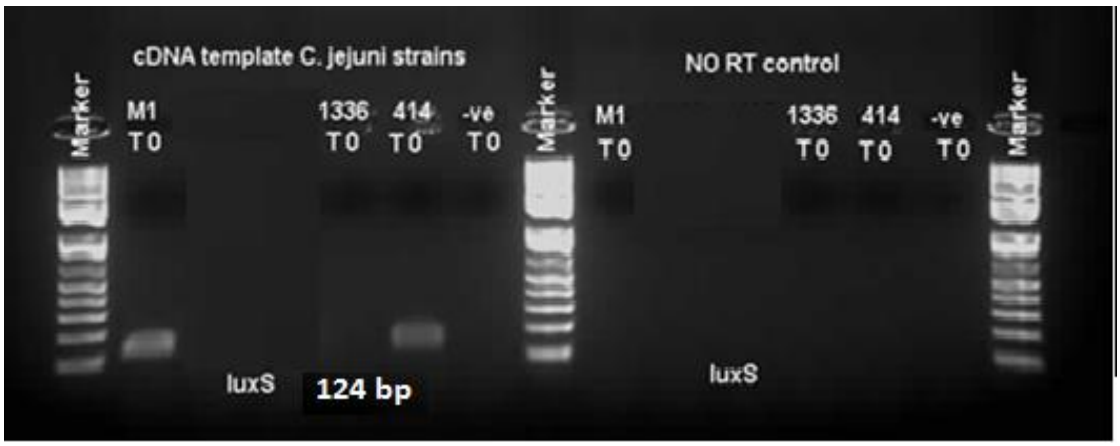
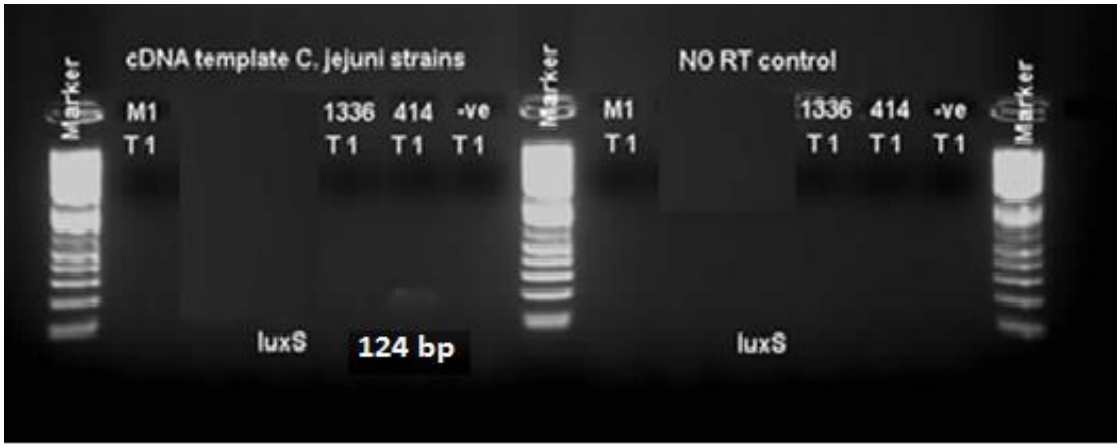


Figure 4.4 PCR amplification of segments of *rpoA* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 119 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

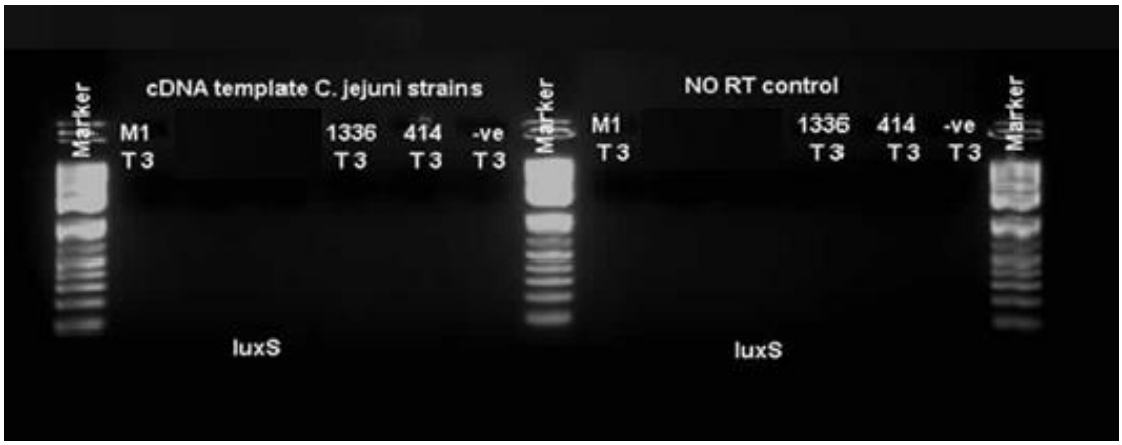
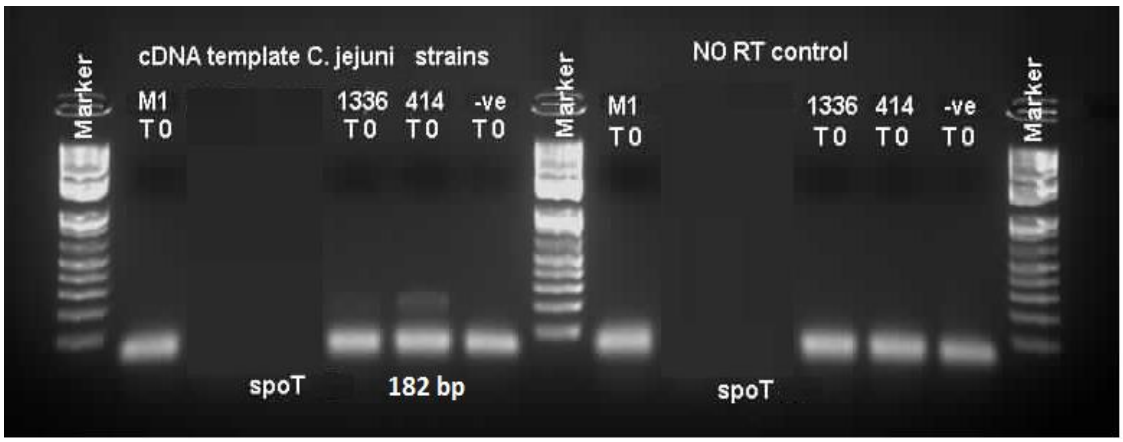
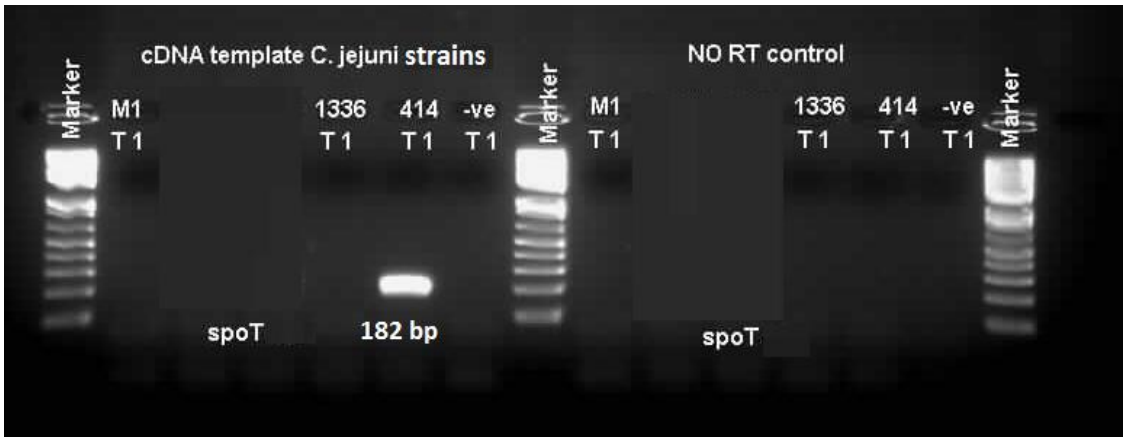


Figure 4.5 PCR amplification of segments of *luxS* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 124 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

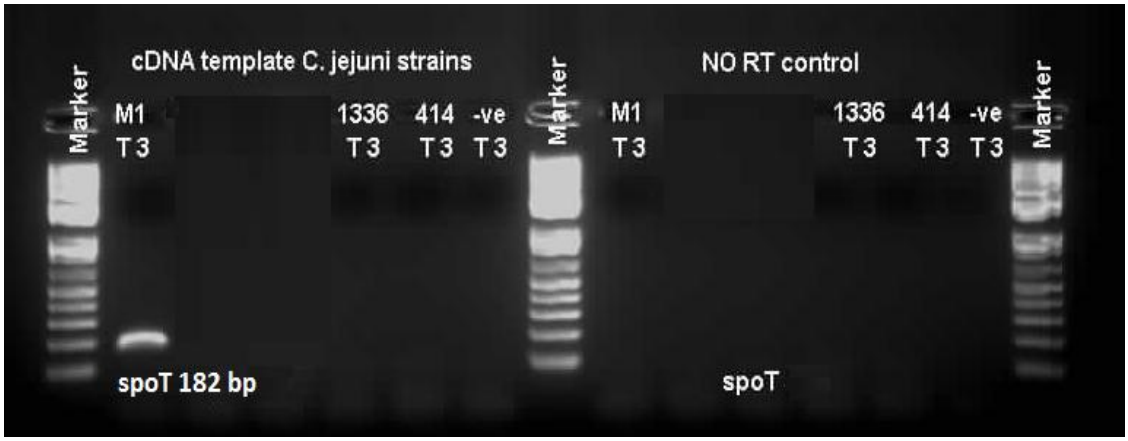
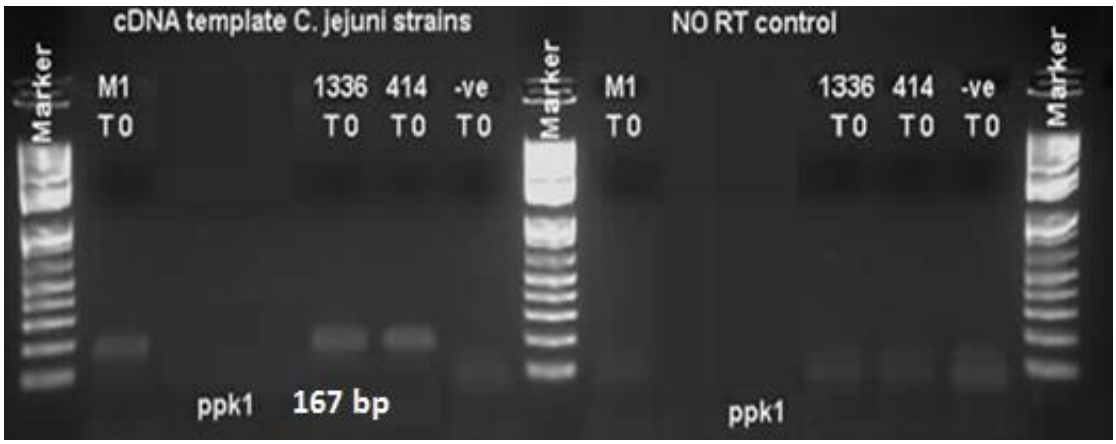
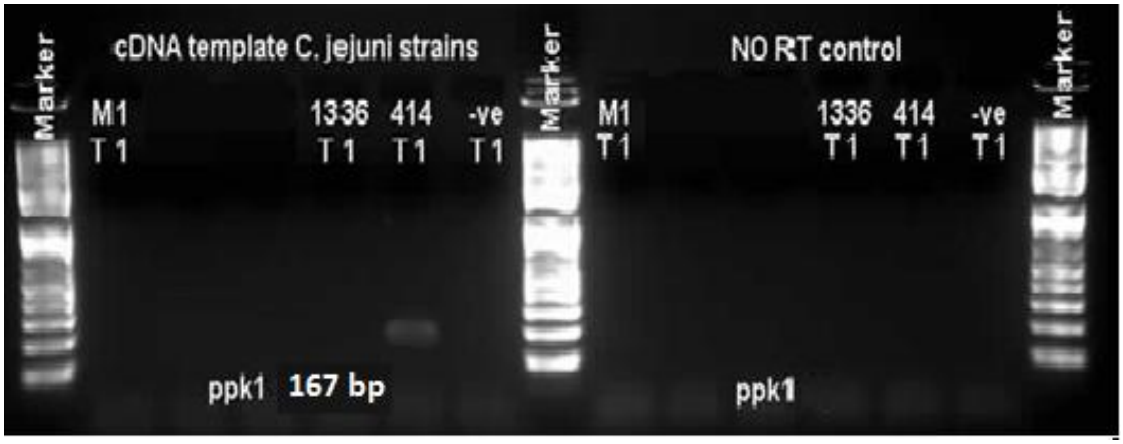


Figure 4.6 PCR amplification of segments of *spoT* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 182 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

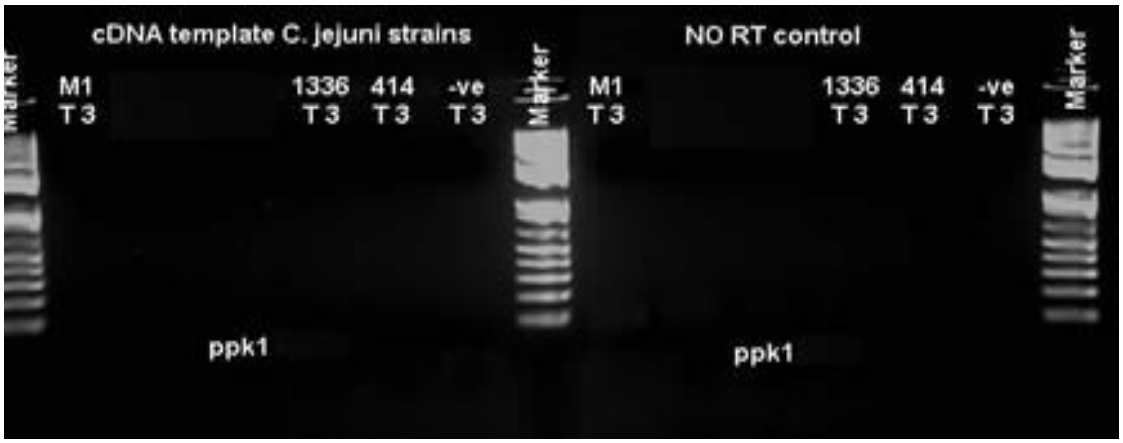
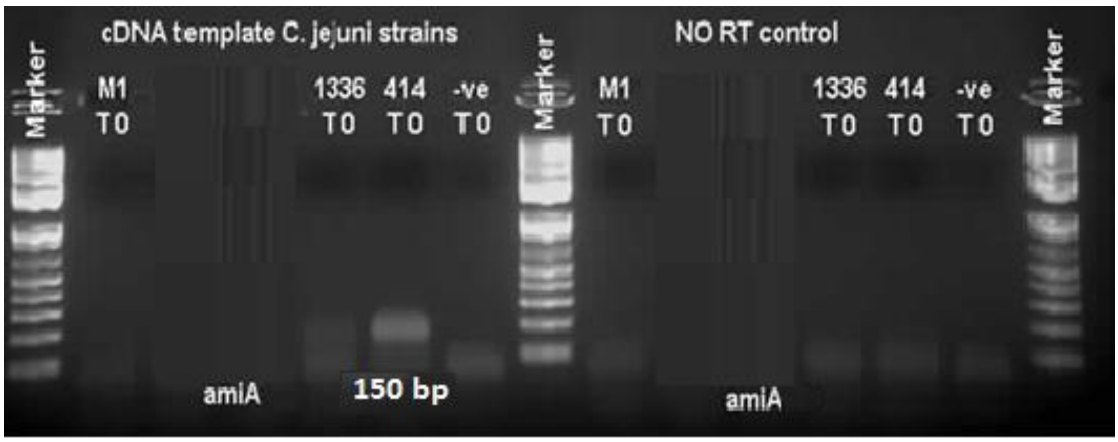
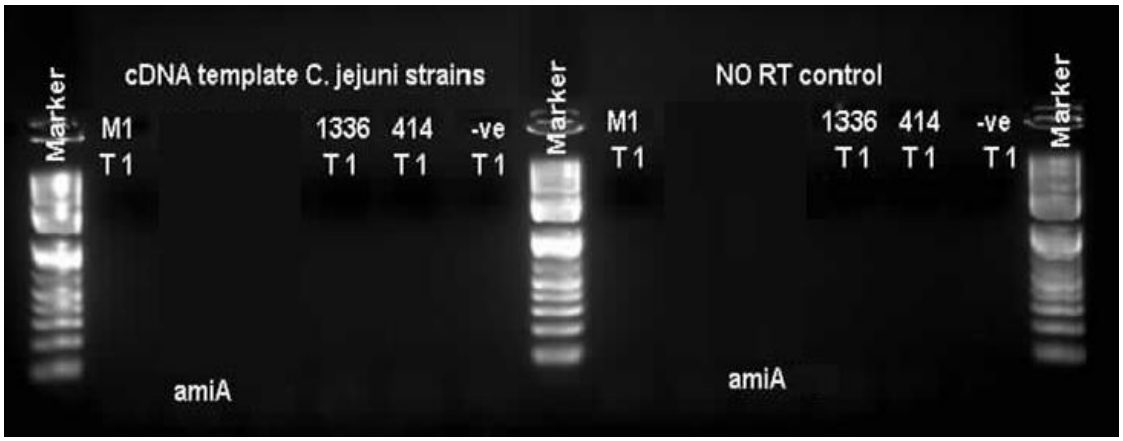


Figure 4.7 PCR amplification of segments of *ppk1* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 167 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

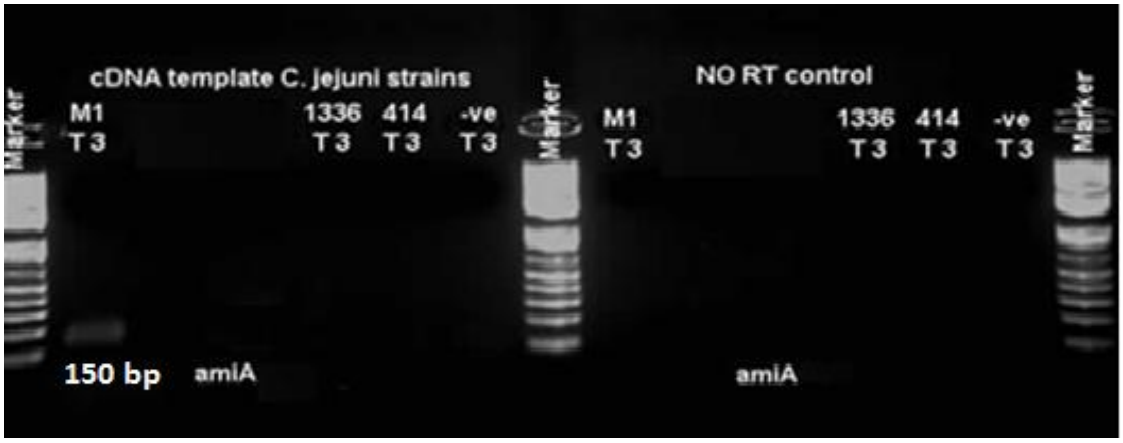
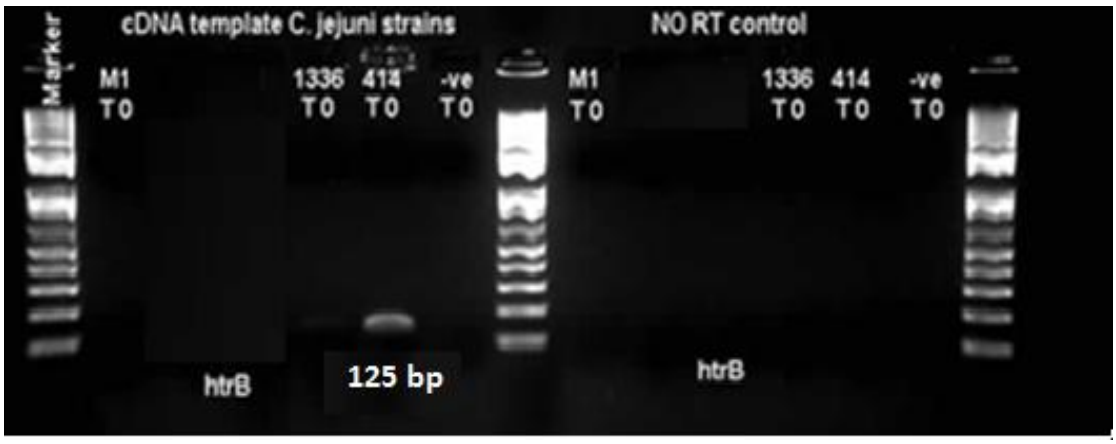
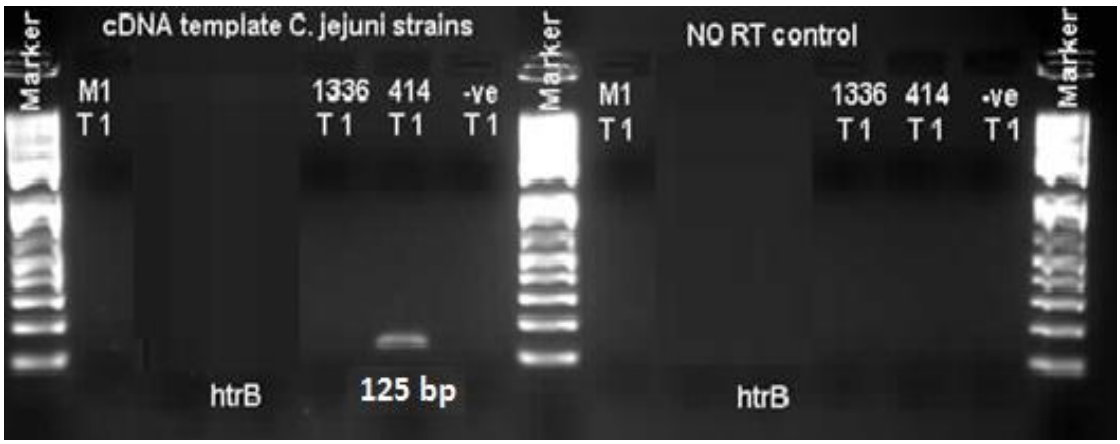


Figure 4.8 PCR amplification of segments of *amiA* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 150 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

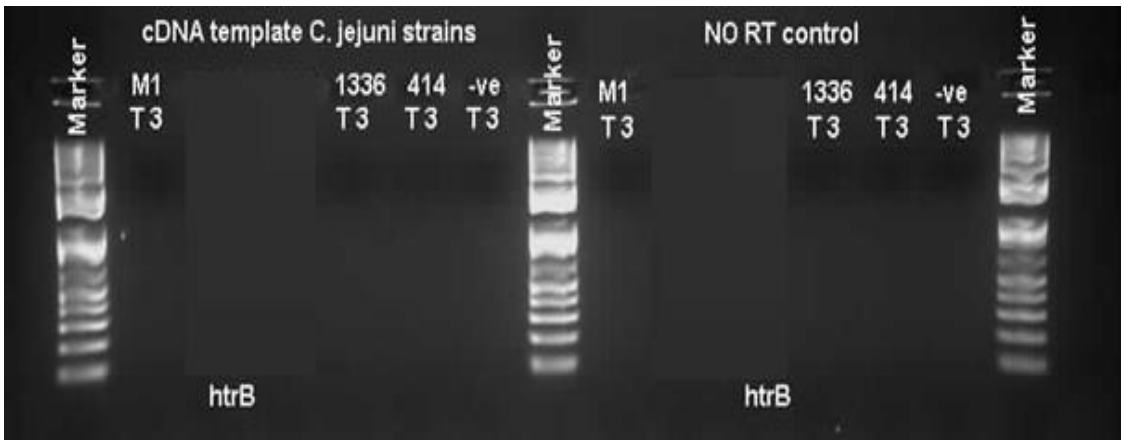
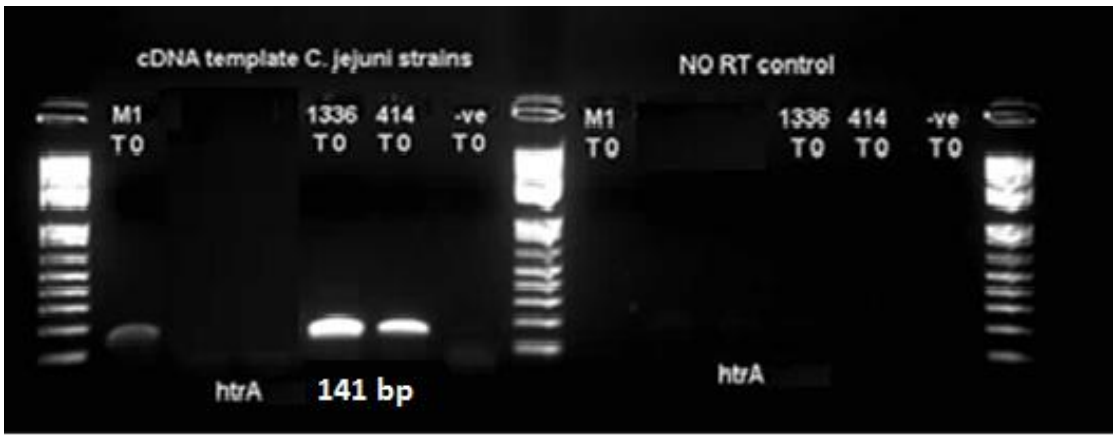
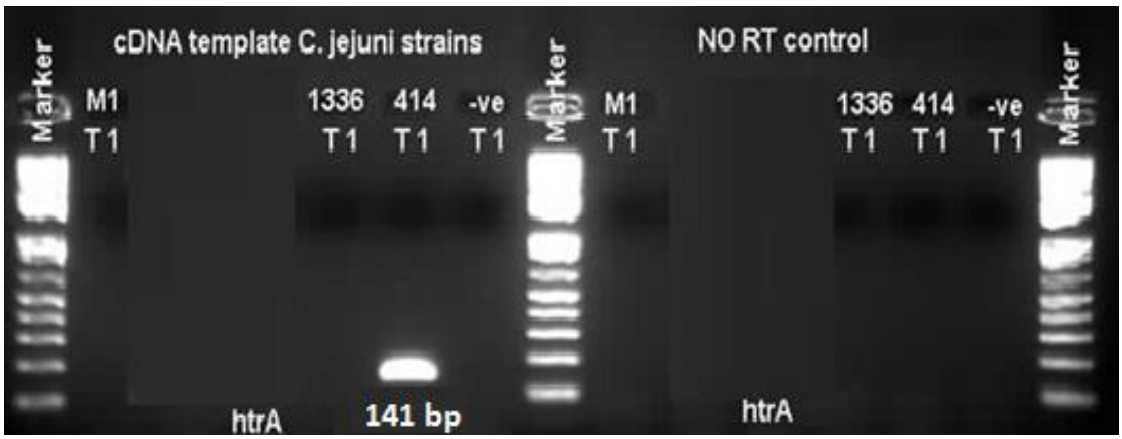


Figure 4.9 PCR amplification of segments of *htrB* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 125 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

A



B



C

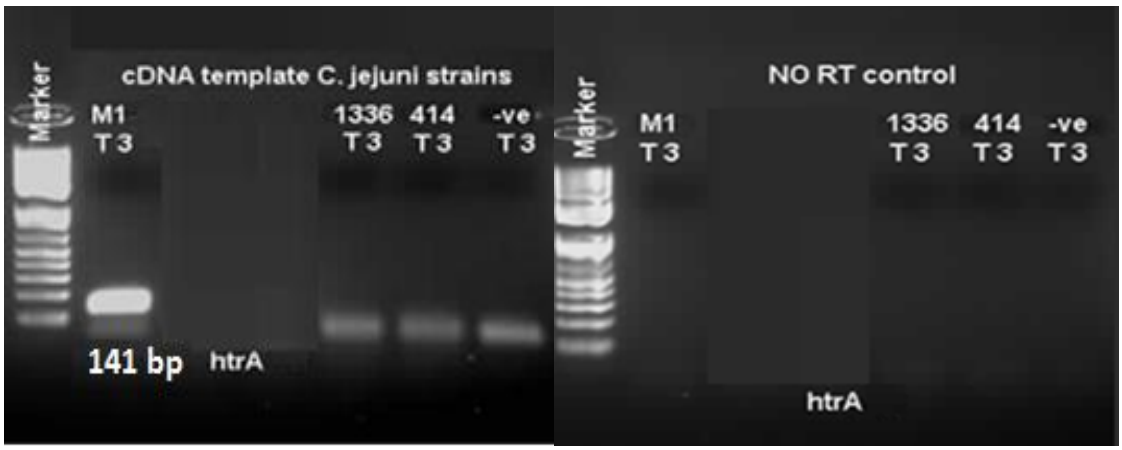


Figure 4.10 PCR amplification of segments of *htrA* gene during survival test of *C. jejuni* strains M1, 1336 and 414 in sterile distilled water at day 0 (A), day 1 at 25 °C (B) and day 3 at 4 °C (C). On gel, Marker: 1 kb DNA molecular weight marker; amplicon size: 141 bp; -ve: no template control (dH_2O); NO RT control: no reverse transcriptase enzyme.

Table 4.3 End point PCR assays for stress response gene expression. The table indicates the presence (+) or absence (-) of amplicons derived from cDNA of *C. jejuni* stress response genes tested in sterile distilled water at different time points. *16S rRNA* gene was used a positive control for the cDNA. No PCR products were detected from the negative control (no reverse transcriptase enzyme) indicating the absence of DNA contamination in the RNA samples.

			Alpha subunit of RNA polymerase	Positive control	QS	Oxidative/ starvation	Osmotic stress/ starvation	Morphological change	low pH/ Heat shock/ Oxidative/ Osmotic	Aerobic tolerance/ Heat shock
	Target gene		*rpoA*	*16S rRNA*	*luxS*	*spoT*	*ppk1*	*amiA*	*htrB*	*htrA*
Strain	**Day**	**°C**								
M1	**0**	**RT**	-	+	+	-	+	-	-	+
	1	**25**	-	+	-	-	-	-	-	-
	3	**4**	-	+	-	+	-	+	-	+
1336	**0**	**RT**	+	+	-	+	+	+	-	+
	1	**25**	-	+	-	-	-	-	-	-
	3	**4**	+	+	-	-	-	-	-	-
414	**0**	**RT**	+	+	+	+	+	+	+	+
	1	**25**	+	+	+	+	+	-	+	+
	3	**4**	+	+	-	-	-	-	-	-

4.2.4 Development of Q-PCR assays

Primer pairs targeting four genes (*amiA*, *ppk1*, *spoT* and *htrA*) were designed for the purpose of this study as follows: genome sequences for four strains (M1, NCTC11168, 1336, 414) were downloaded from NCBI using Geneious version 6.1.2 (Biomathes Ltd) and the genes of interest were aligned (Figures 4.11-4.14). Primer-3, which is integrated into Geneious, was used to design primers specific to the genes of interest, using default settings. Primers were designed with Q-PCR in mind, aiming for amplicon sizes at around 180 bp. Initially, temperature gradient PCR was performed to determine optimal annealing temperature of the primers used in this study (Table 2.3). The temperature (55 °C) was selected as optimal annealing temperature for all of the primer pairs. All of the primer pairs were next tested on DNA from *C. jejuni* strains M1, 1336, 414 for amplification at annealing temperature 55 °C. Standard curves were obtained from a 10-fold dilution series of genomic DNA of *C. jejuni* (ST-42). The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA. The C_q values determined by software analysis of the unknown samples were compared to the standard curve to determine the concentration of each unknown sample. The efficiency and the coefficient (R^2) of the standard curves were calculated automatically by the software for the Rotor-gene Q cycler.

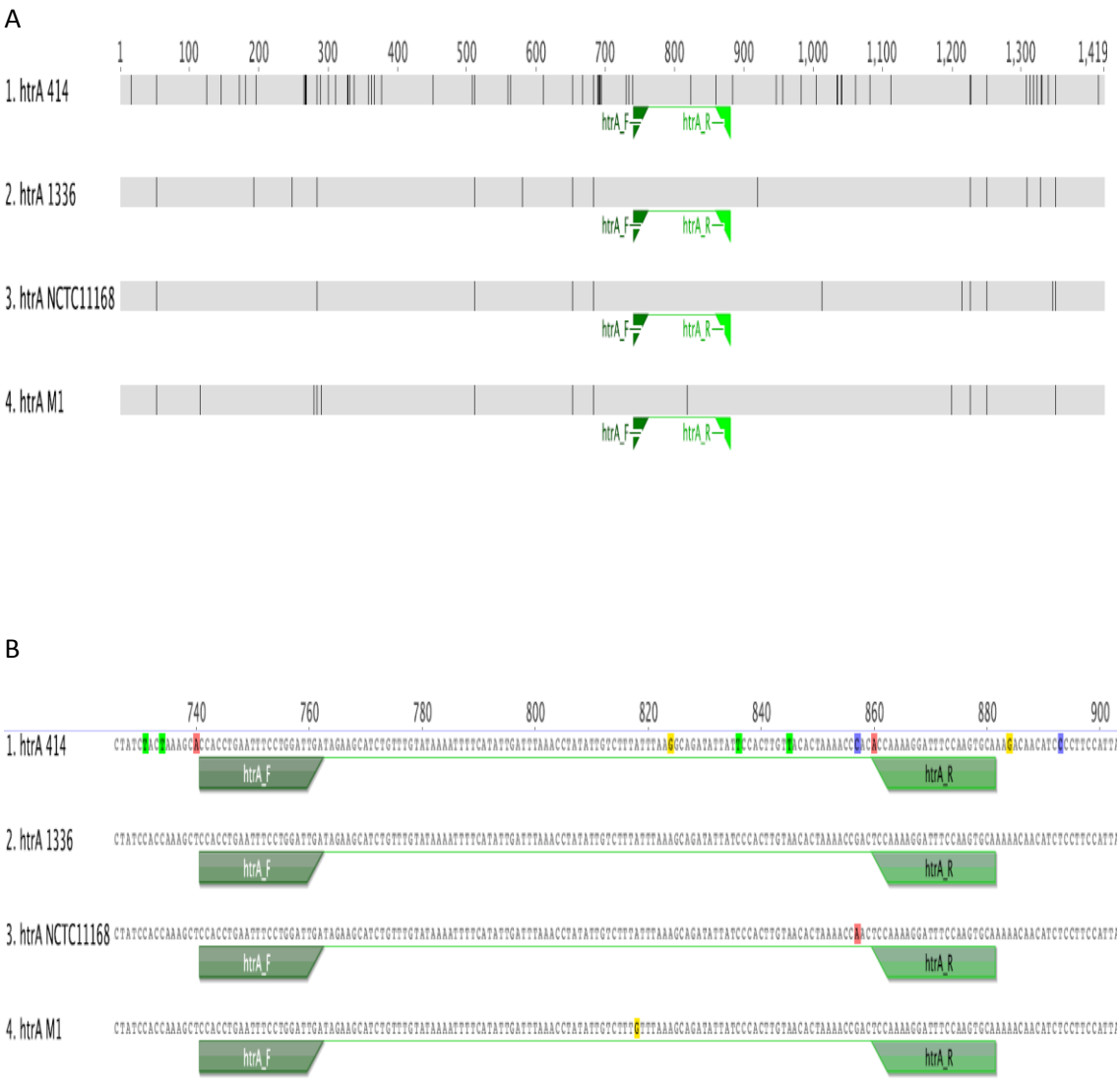


Figure 4.11 Alignments of the genomes of *C. jejuni* strains 414, 1336, 11168 and M1 in Mauve (https://asap.genetics.wisc.edu/software/mauve/). (A) The primer binding sites for *htrA* primers [141 bp (amplicon size)] are indicated by green triangles. Single nucleotide polymorphisms (SNPs) are indicated by vertical grey lines and numbers across the top indicate nucleotide position of the *htrA* gene (1419 bp). (B) Close up of the *htrA* gene section that htrA forward (F) and reverse (R) primers bind to. Coloured letters indicate SNPs.

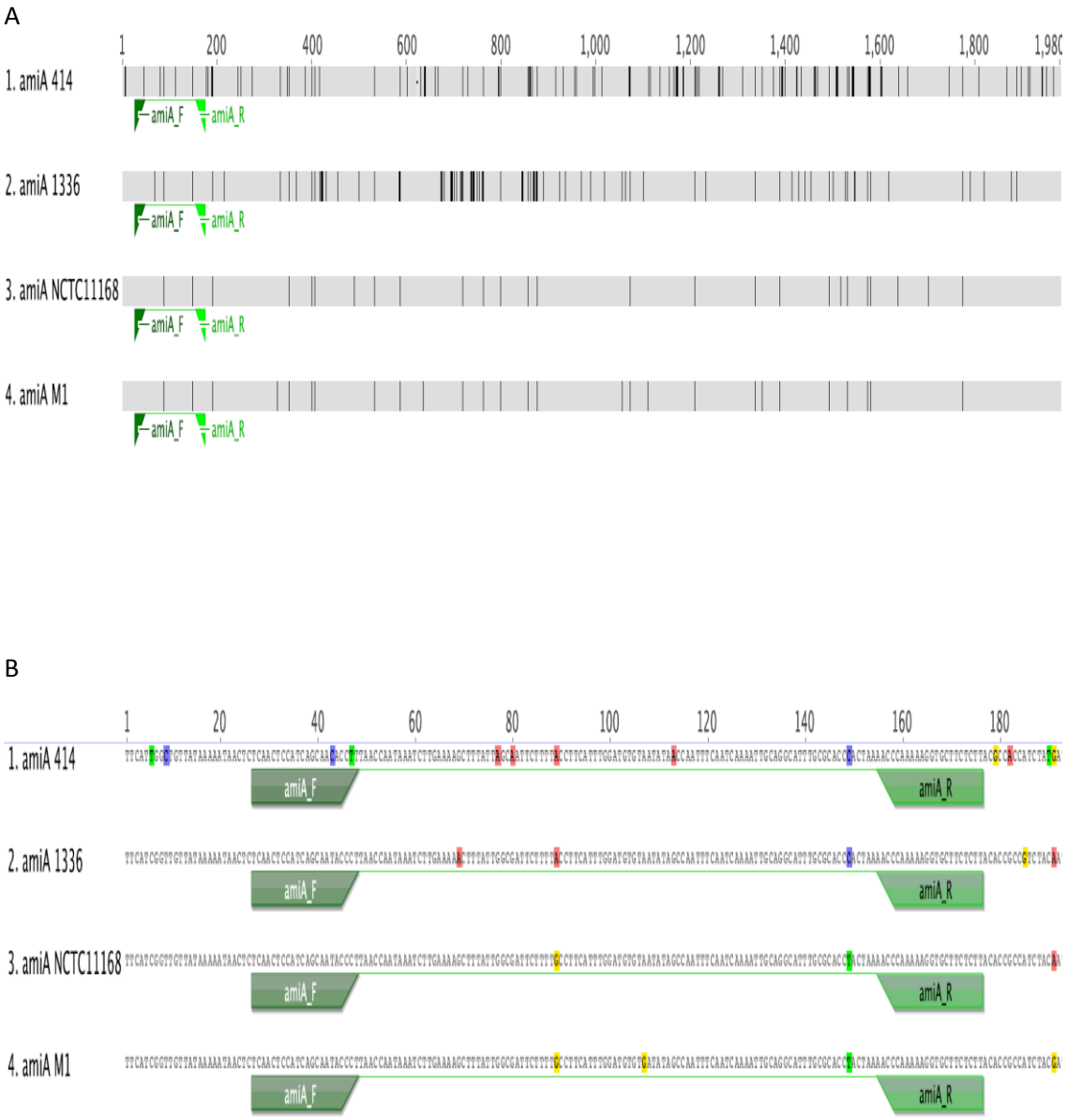


Figure 4.12 Alignments of the genomes of *C. jejuni* strains 414, 1336, 11168 and M1 in Mauve (*https://asap.genetics.wisc.edu/software/mauve/*). (A) The primer binding sites for *amiA* primers [150 bp (amplicon size)] are indicated by green triangles. Single nucleotide polymorphisms (SNPs) are indicated by vertical grey lines and numbers across the top indicate nucleotide position of the *amiA* gene (1980 bp). (B) Close up of the *amiA* gene section that amiA forward (F) and reverse (R) primers bind to. Coloured letters indicate SNPs.

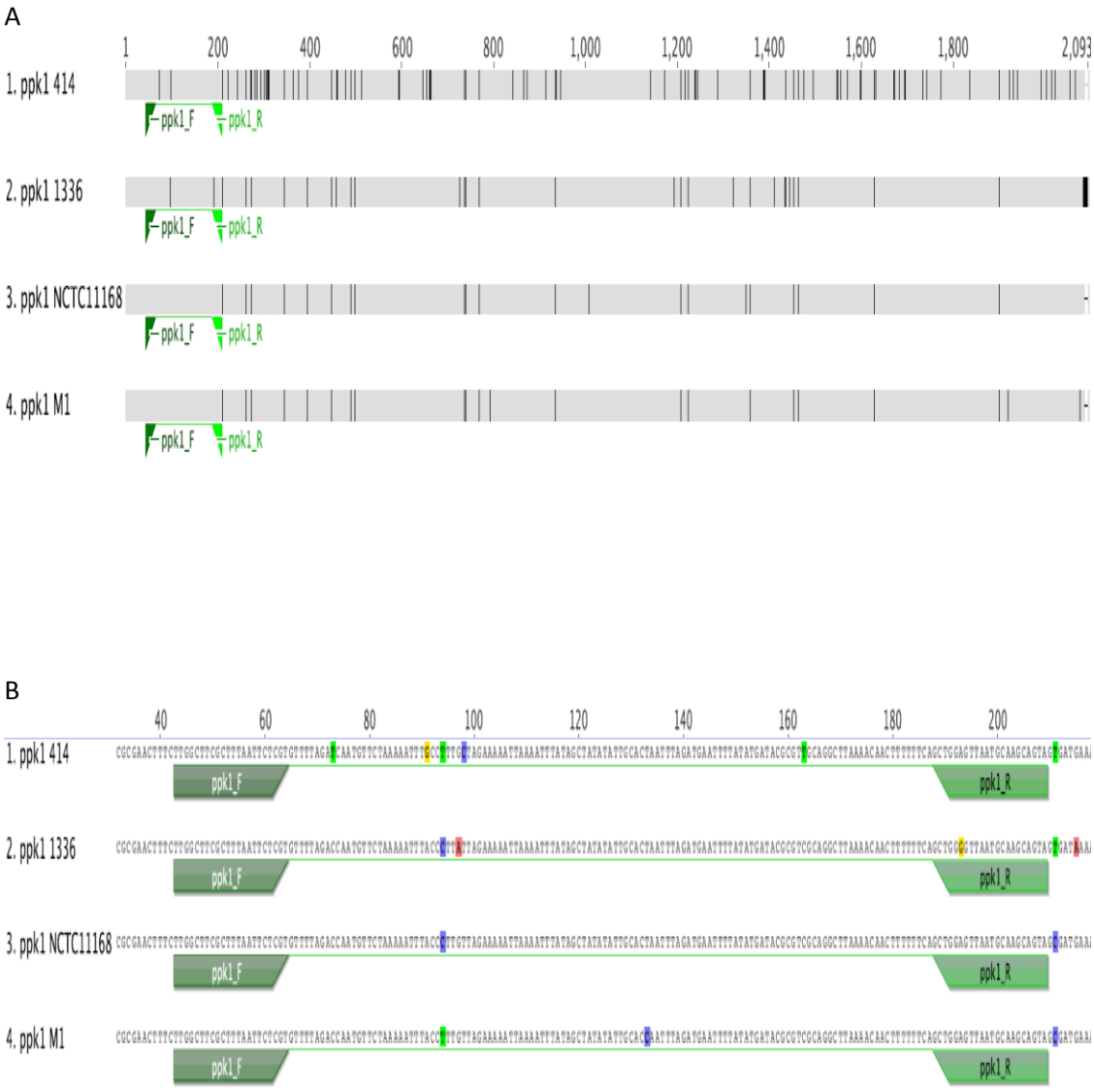


Figure 4.13 Alignments of the genomes of *C. jejuni* strains 414, 1336, 11168 and M1 in Mauve (*https://asap.genetics.wisc.edu/software/mauve/*). (A) The primer binding sites for *ppk1* primers [167 bp (amplicon size)] are indicated by green triangles. Single nucleotide polymorphisms (SNPs) are indicated by vertical grey lines and numbers across the top indicate nucleotide position of the *ppk1* gene (2093 bp). (B) Close up of the *ppk1* gene section that ppk1 forward (F) and reverse (R) primers bind to. Coloured letters indicate SNPs.

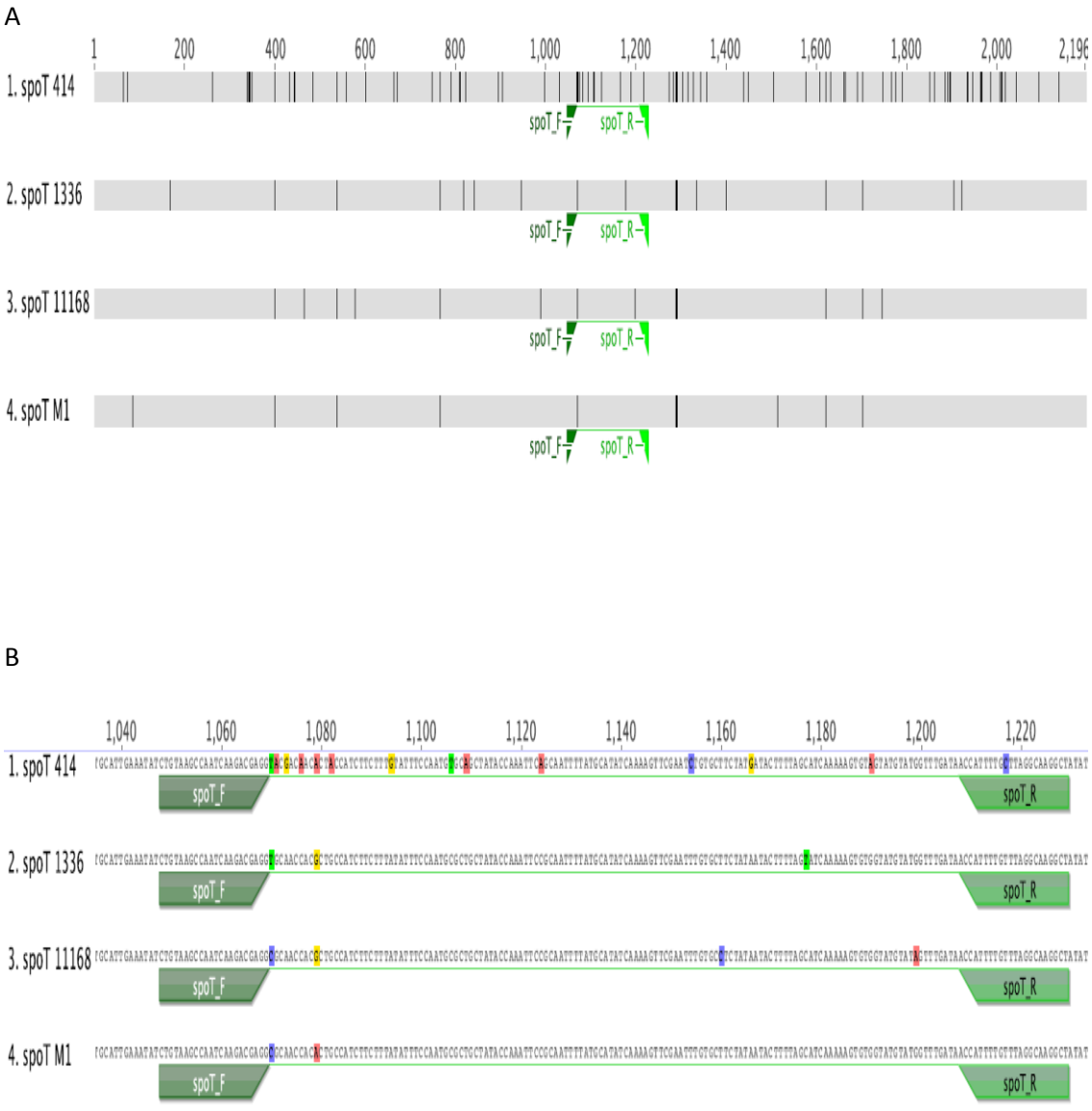


Figure 4.14 Alignments of the genomes of *C. jejuni* strains 414, 1336, 11168 and M1 in Mauve (*https://asap.genetics.wisc.edu/software/mauve/*). (A) The primer binding sites for *spoT* primers [182 bp (amplicon size)] are indicated by green triangles. Single nucleotide polymorphisms (SNPs) are indicated by vertical grey lines and numbers across the top indicate nucleotide position of the *spoT* gene (2196 bp). (B) Close up of the *spoT* gene section that spoT forward (F) and reverse (R) primers bind to. Coloured letters indicate SNPs.

4.2.5 Gene expression analysis using Q-PCR

Given the limitations of end-point PCR with respect to quantification, further investigations were carried out using Q-PCR assays.

For the days where we identified greatest variation between C. *jejuni* strains with respect to maintenance of culturability (Chapter 3), we used 2-step Q-PCR to look for variations in the expression of our chosen panel of stress response genes highlighted in the literature as important for survival in low nutrient environment or stressful conditions, or the formation of biofilm/VBNC forms (Table 4.1). The assay included two steps of (I) reverse transcription reaction and (II) Q-PCR method. The method was used to quantify the relative concentration of each stress response genes in *C. jejuni* strains, expressed during survival in sterile distilled water at two different temperatures 4 °C (day 3) and 25 °C (day 1). Amplification efficiency, R^2 value and detection limit were determined (Table 4.4 and Figures 4.15-4.22). The 16S rRNA gene was used as an internal control to normalise the expression of gene of interest and the results obtained are shown in Figures 4.25. Statistical analyses of normalised data of the Q-PCR in Table 4.5.

From the normalised data, notable findings were that (i) *luxS* expression was up-regulated in strain M1 compared to strain 414 at both the day 1 (25 °C) and day 3 (4 °C) time-points, and compared to M1 at time zero; (ii) the *htrA* gene was up-regulated in strain 414 compared to either of the other strains at both the day 1 (25 °C) and day 3 (4 °C) time-points and at time zero; (iii) the *ppk1* gene was up-regulated in strain M1 compared to either of the other strains at day 1 (25 °C) and compared to time zero; (iv) only very low levels of gene expression were detectable in strain 1336.

Table 4.4 Summary of Q-PCR assays for stress response genes used in this study.

Target	Strain	R^2	M (slope)	Efficiency	Detection limit (ng/µL)	Cq value of negative control
rpoA	M1	0.99542	-3.863	0.81	0.0001	> 40
	1336	0.99262	- 3.680	0.87	0.0001	> 40
	414	0.98288	- 3.645	0.88	0.0001	> 40
luxS	M1	0.99375	- 3.511	0.93	0.0001	> 40
	1336	0.99062	- 3.820	0.83	0.0001	> 40
	414	0.99696	- 3.454	0.95	0.0001	> 35
spoT	M1	0.99809	- 3.659	0.88	0.0001	> 40
	1336	0.98184	- 3.726	0.86	0.001	> 35
	414	0.98834	- 3.654	0.88	0.0001	> 40
ppk1	M1	0.99759	- 3.582	0.90	0.0001	> 40
	1336	0.99892	- 3.796	0.83	0.0001	> 34
	414	0.99606	- 3.410	0.96	0.0001	> 37
amiA	M1	0.99607	- 3.823	0.83	0.001	> 40
	1336	0.99649	- 3.329	1.00	0.0001	> 40
	414	0.99190	- 3.827	0.83	0.0001	> 34
htrB	M1	0.99524	- 3.490	0.93	0.0001	> 32
	1336	0.99696	- 3.54	0.85	0.0001	> 36
	414	0.98274	- 3.114	1.09	0.0001	> 35
htrA	M1	0.98940	- 3.308	1.01	0.0001	> 34
	1336	0.99505	- 3.523	0.92	0.0001	> 40
	414	0.99588	- 3.417	0.96	0.0001	> 40
16S rRNA	M1	0.99824	- 3.4442	0.95	0.01	> 40
	1336	0.99899	- 3.366	0.98	0.01	> 40
	414	0.99824	- 3.442	0.95	0.01	> 40

R^2: is the coefficient of correlation obtained for the standard curve, the R^2 value reflects the linearity of the standard curve. Ideally, R2 = 1, although 0.980 is generally the maximum value.

M: is the slope of the standard curve calculated automaticaly, the optimal value is -3.32.

Efficiency: Amplification efficiency (E) of a PCR, calculated from the slope of the standard curve using the formula $E = 10^{-1/slope}$. Ideally sould be 100%, meaning that for each cycle the amount of product doubles (E=2).

C_q=quantification cycle: the cycle number at which the fluorescence signal crosses threshold (background signal).

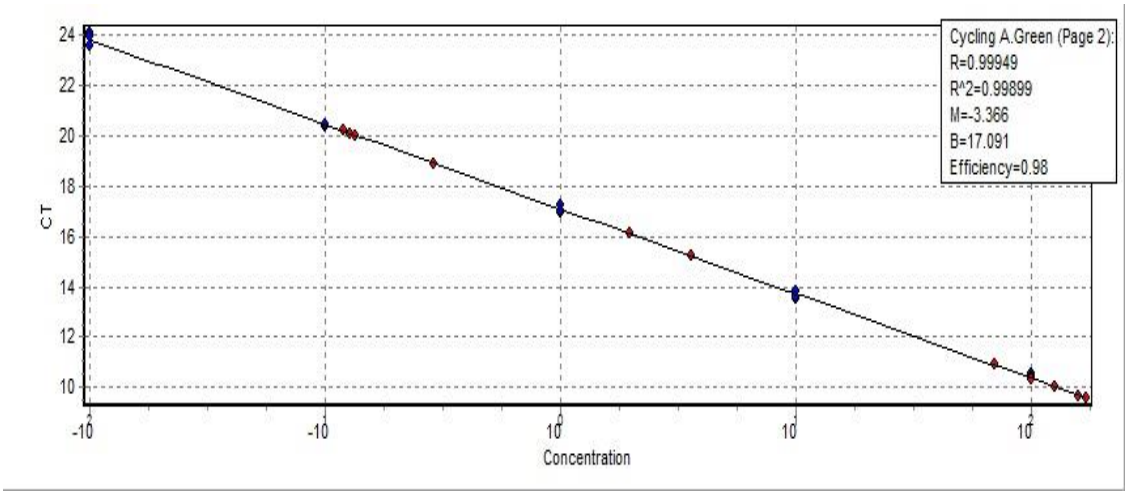


Figure 4.15 Standard curves for Q-PCR for the *16S rRNA*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

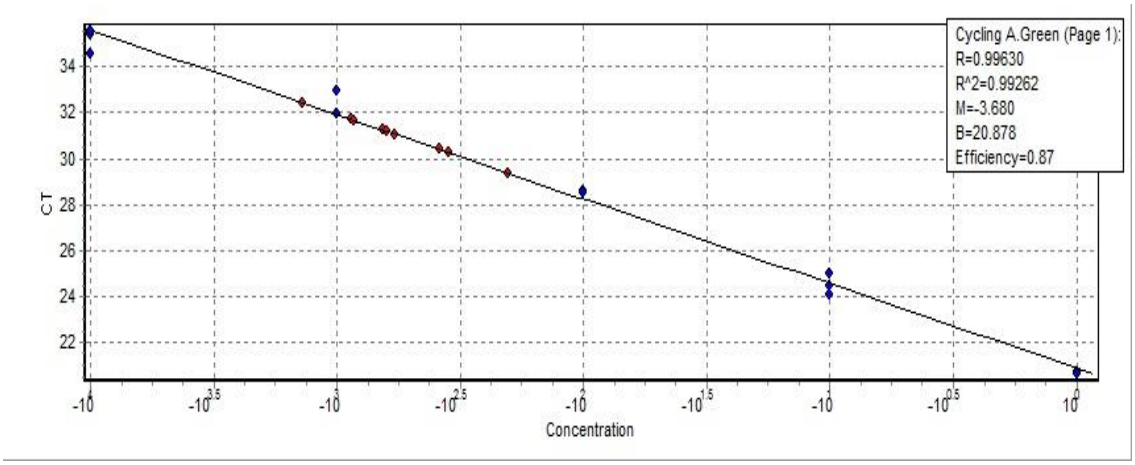


Figure 4.16 Standard curves for Q-PCR for the *rpoA*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

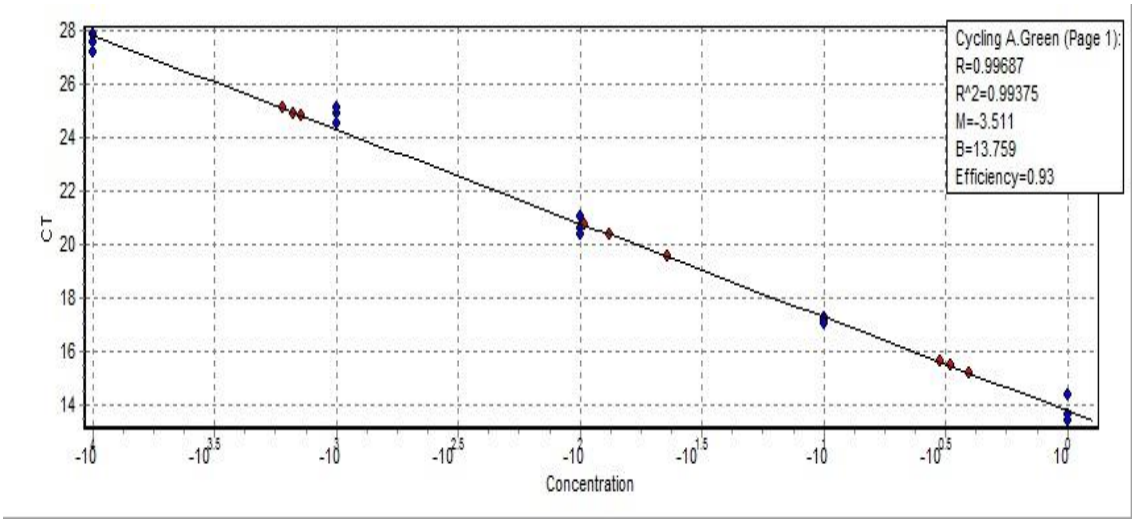


Figure 4.17 Standard curves for Q-PCR for the *luxS*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

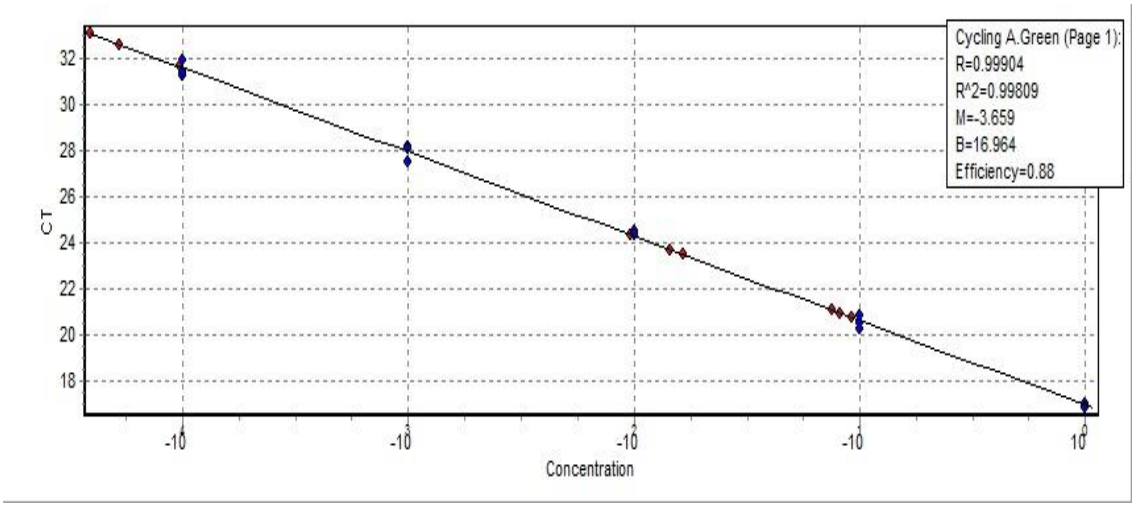


Figure 4.18 Standard curves for Q-PCR for the *spoT*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

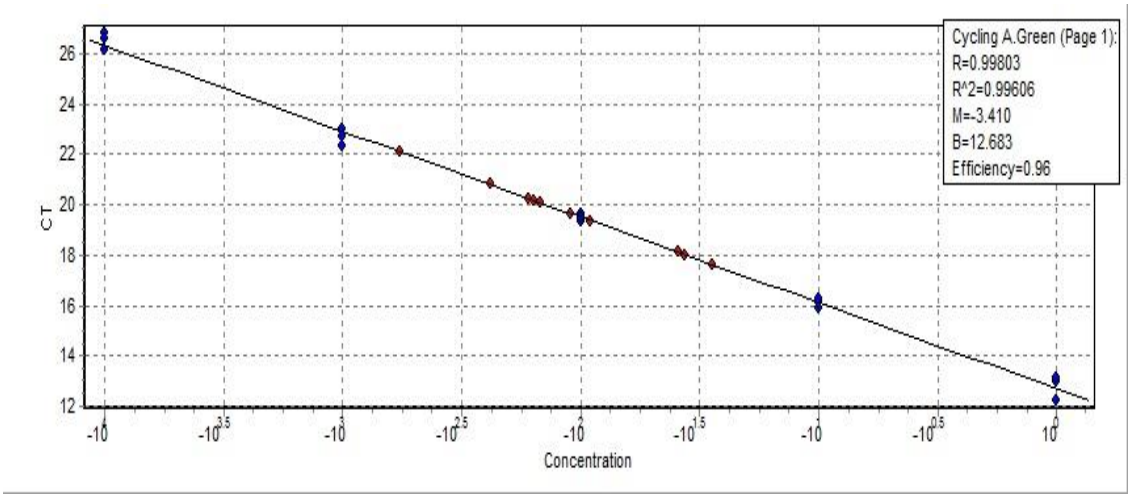


Figure 4.19 Standard curves for Q-PCR for the *ppk1*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

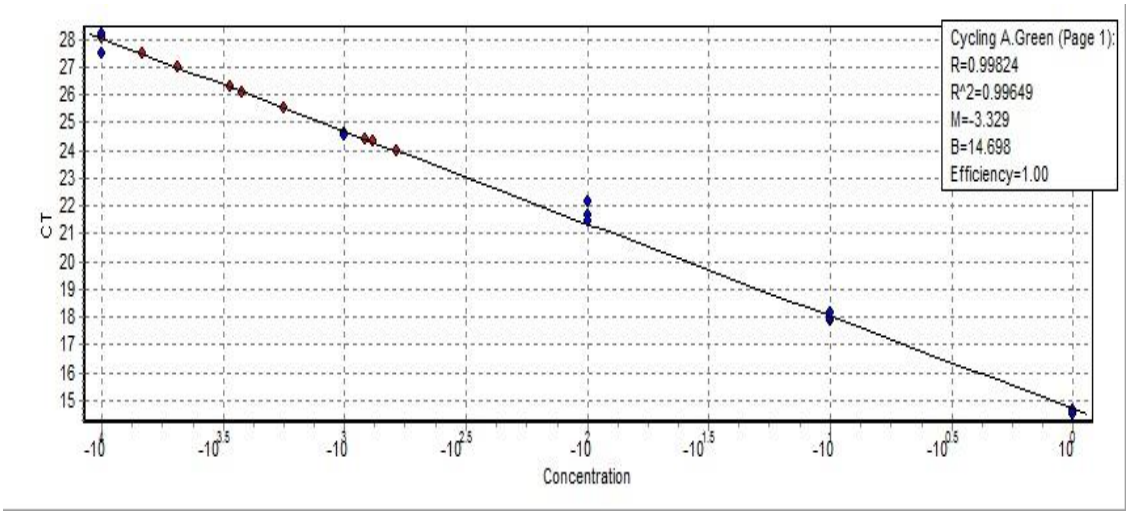


Figure 4.20 Standard curves for Q-PCR for the *amiA*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

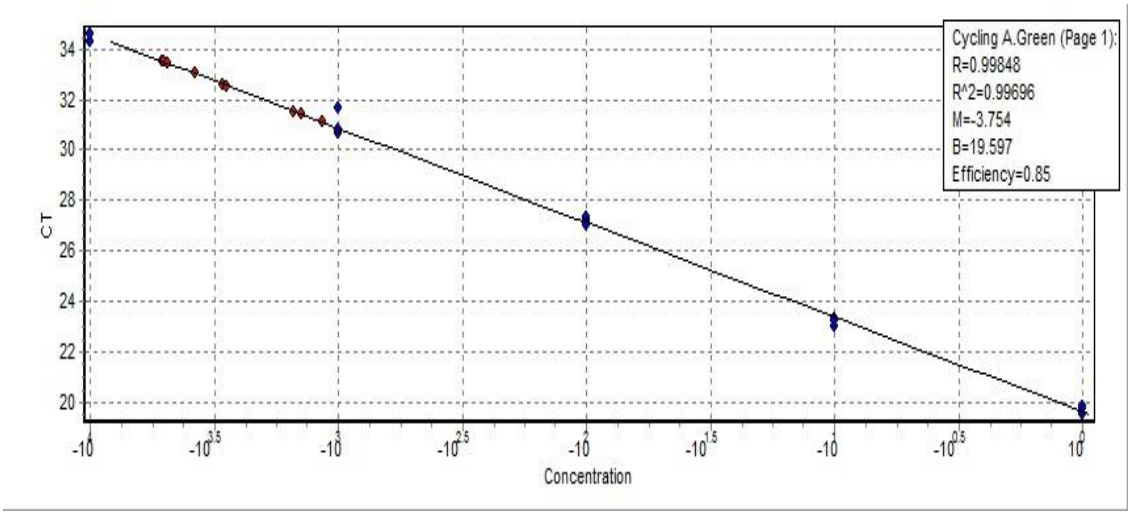


Figure 4.21 Standard curves for Q-PCR for the *htrB*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

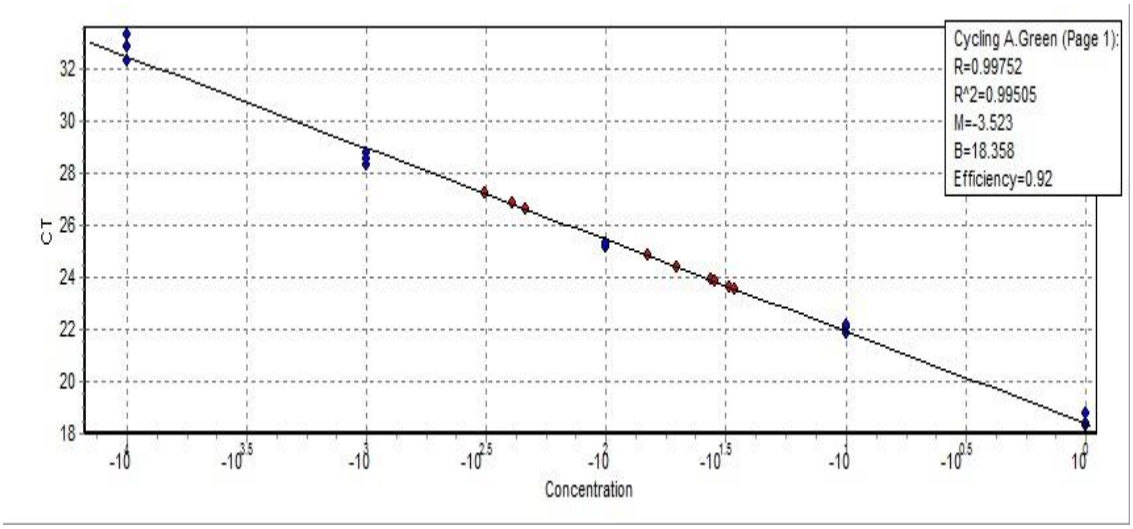
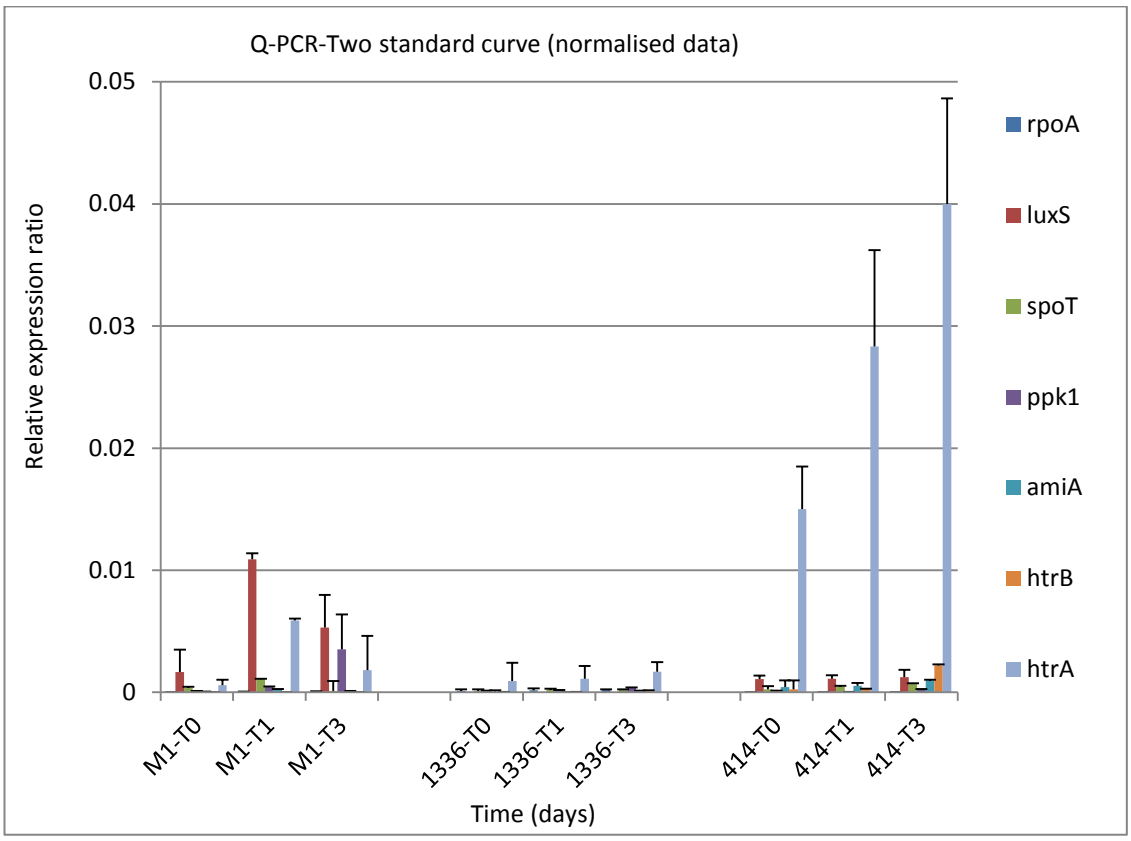
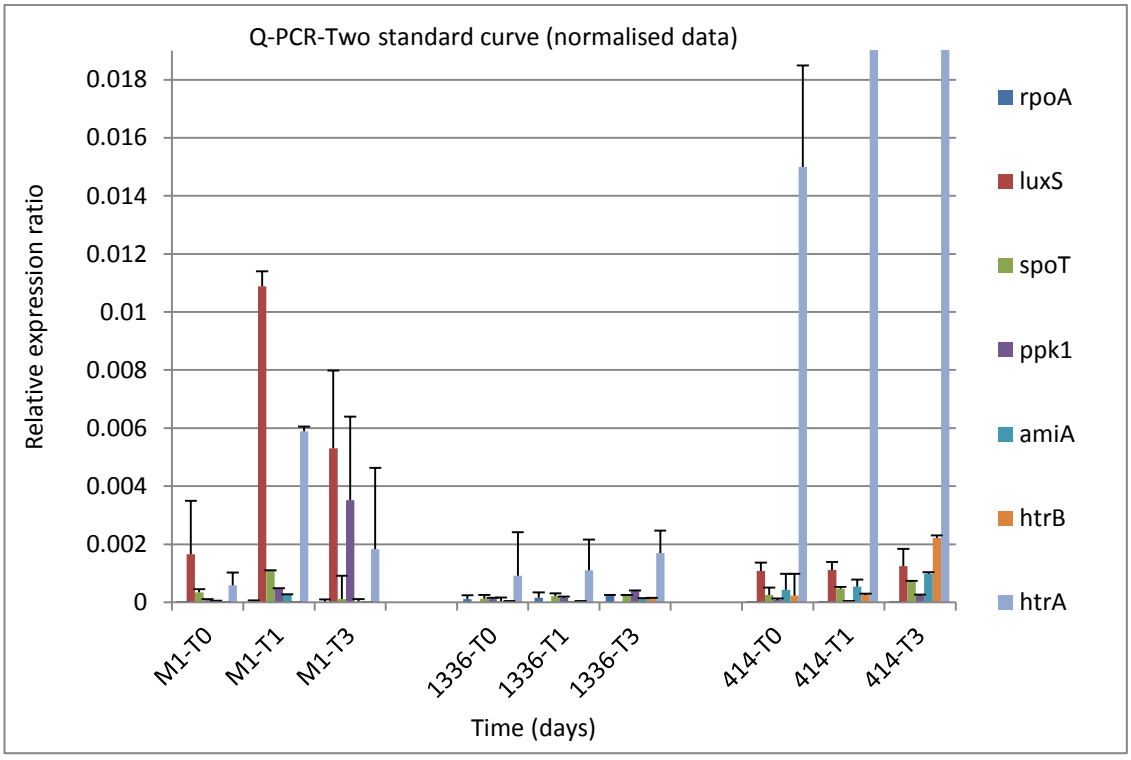


Figure 4.22 Standard curves for Q-PCR for the *htrA*. The C_q values were plotted against $\log_{10}$ concentration of the genomic DNA (standard curve of a 10-fold serial dilution of genomic DNA of strain ST-42).

A



B



Figures 4.23 Relative expressions data analysis [(A) higher y axis values and (B) lower y axis values] of stress response genes of *C. jejuni* during survival in sterile distilled water at 4 °C (day 3) and at 25 °C (day 1) tested by Q-PCR. Error bars represent standard deviation calculated from three independent replicates.

4.2.6 RNAseq analysis of strain M1 gene expression

The RNAseq method was applied to study the expression of genes in strain M1 under a number of different conditions. These included growth in MHB at 37 °C to OD_{600} of 0.05. Henceforth, I will refer to this as the control sample. The other conditions used were the same as used for the Q-PCR assays in this study, namely strain M1 in sterile distilled water at time zero (T0), at day 1 (T1) at 25 °C, and at day 3 (T3) at 4 °C. The RNAseq data were generated by Dr. Christina Bronowski, University of Liverpool.

Using these data, analysis was carried out to look at variations in the expression of genes which from previous work were potentially associated with survival in water. Figure 4.24 shows the RNAseq data obtained for the genes used in the Q-PCR analysis in this study, comparing each of these three time points in water survival experiments with gene expression in the control (grown in MHB). Table 4.6 provides a summary of these data and identifies statistically significant variations. In addition, data for a far wider selection of genes implicated in stress responses, survival, biofilm formation or the VBNC form are included in Table 4.6.

Figure 4.24 and Table 4.6 indicate that the *luxS* gene is up-regulated compared to the control at all three time-points. "In agreement with the Q-PCR findings, *luxS* was up-regulated in strain M1 at day 1 (T1) at 25 °C, and at day 3 (T3) at 4 °C compared to time zero". Other genes significantly up-regulated ($p < 0.05$) during survival in water compared to growth in broth include *katA* (all three time-points), *fliA* (T0 and T3 only), *sodB* (all three time-points), *ahpC* (all three time-points), *clpB* (all three time-points), *hrcA* (T0 and T3 only), *grpE* (all three time-points), *groS* (all three time-points), *groL* (all three time-points), and *yjiY* (all three time-points). The *peb1c* gene was also up-regulated at all three time-points (p range 0.005 – 0.055).

It has been demonstrated that CosR (*Campylobacter* oxidative stress regulator; Cj0355c) may play a role in regulation of many oxidative stress genes especially *sodB* by binding to the promoter regions of *sodB* and *ahpC* and regulates their transcriptions (Hwang et al., 2011).

Amongst genes significantly down-regulated during survival in water ($p < 0.05$) were *rpoA*, *spoT*, *ppk1*, *amiA* (T1 and T3 only), *htrB* (T1 and T3 only), *nrfA*, *racS* (T1 and T3 only), *dcuA*, *clpP*, *cheA, fdxA* (T1 and T3 only), *sdhA*, *sdhB*, *sdhC*, *hslU*, *lon* (T1 and T3 only), *clpA* (T1 and T3 only) (Table 4.6).

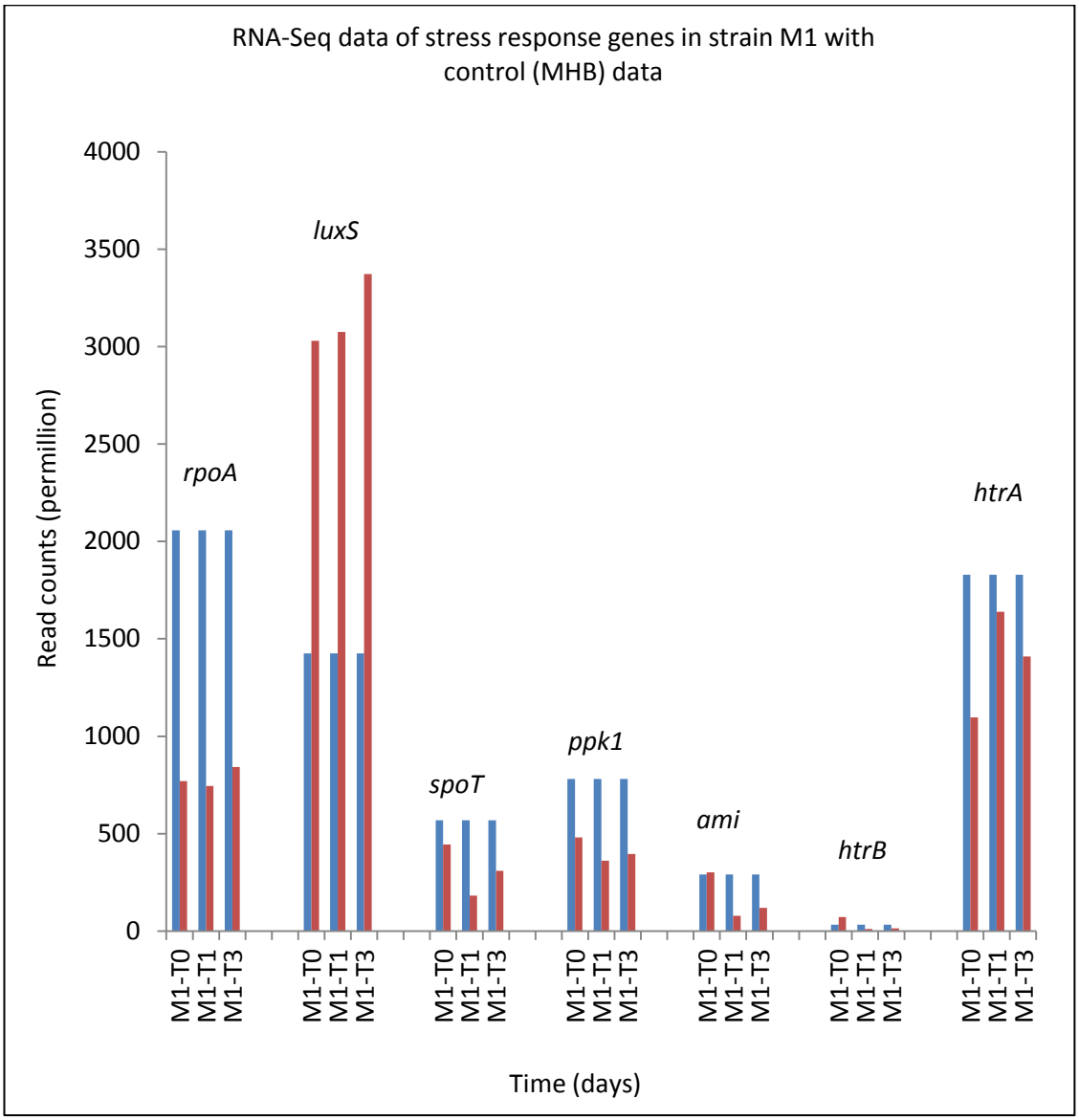


Figure 4.24 RNAseq data of selected stress response genes (red) in strain M1 compared with control (MHB) data (blue). Read counts alongside those obtained from the control data are shown for stress response genes of strain M1 during survival in sterile distilled water at time zero [T0], at day 1 (25 °C) [T1] and at day 3 (4 °C) [T3].

Table 4.5 Statistical analyses [p-value, using One-Way ANOVA] of normalised data for *htrA*, *luxS* and *ppk1* in *C. jejuni* strains M1, 1336 and 414. Statistics are testing for row headers being up-regulated compared to column headers.

	414-T0	414-T1	414-T3	1336-T0	1336-T1	1336-T3	M1-T0	M1-T1	M1-T3
htrA (414-T0)				0.001			0.001		
htrA (414-T1)	0.143				0.003			0.008	
htrA (414-T3)	0.018					0.001			0.001
htrA (M1-T3)								0.015	
luxS (M1-T0)	0.409								
luxS (M1-T1)		0.001					0.001		
luxS (M1-T3)			0.005				0.015		
ppk1 (M1-T0)	0.522			0.184					
ppk1 (M1-T1)		0.001			0.005		0.001		
ppk1 (M1-T3)			0.039			0.046	0.085		

Table 4.6 RNAseq data for strain M1 during survival in sterile distilled water at different time points. The average reads per million is obtained from 3 technical replicates per time point per gene at each time point; the fold changes are compared to the control grown in MHB at 37 °C (a negative value indicates down-regulation of the gene, while a positive value indicates up-regulation); the statistical analyses are shown as a p-value, using 2-tailed Student's T-Test.

Gene	Function	Control	T0			T1 (25 °C)			T3 (4 °C)		
		average Reads/ million	average Reads/ million	Fold change to control	p-value	average reads/ million	Fold change to control	p-value	average reads/ million	Fold change to control	p-value
rpoA	DNA-directed RNA polymerase subunit alpha	2056.46	769.96	-1.42	0.019	745.4	-0.68	0.018	842.66	-0.98	0.022
luxS	S-ribosylhomocysteine lyase	1424.63	3029.86	0.65	0.006	3076.1	1.62	0.0009	3372.7	1.34	0.001
spoT	Penta-phosphate guanosine-3'-pyrophosphohydrolase (SpoT)	569.06	443.7	-0.37	0.03	181.3	-0.88	0.0005	309	-0.58	0.002
Ppk1	Polyphosphate kinase	780.83	480.2	-0.72	0.025	360.93	-0.34	0.001	406.6	-0.65	0.003
amiA	N-acetylmuramoyl-L-alanine amidase	290.86	301.43	0.04	0.402	77.86	-1.15	0.0005	119	-1	0.0004
htrB	Putative lipid A biosynthesis acyltransferase	31.96	71.8	1.15	0.015	10.43	-0.86	0.007	15.03	-0.81	0.015
htrA	Protease do	1829.43	1095.96	-0.75	0.018	1639.1	0.61	0.334	1409.53	-0.08	0.064
katA	Catalase	99.9	1138.5	3.52	0.0002	758.66	3.72	0.0005	678.23	3.08	0.0002
dps	DNA protection during starvation protein	2942.23	3666.06	0.29	0.207	2552.1	0.57	0.020	3333.9	0.47	0.112
ytcD/cj1546	Uncharacterized HTH-type transcriptional regulator ytcD	16.93	71.633	2.06	0.005	15	0.59	0.461	29.26	1.08	0.026

cgb	Putative bacterial hemoglobin	12.16	41.466	1.77	0.007	10.96	0.62	0.213	10.16	0.01	0.173
flaA	Flagellin A	896.76	1436.3	0.67	0.021	782.4	0.58	0.247	954.16	0.39	0.370
flaB	Flagellin B	12502.86	11924.0	-0.08	0.454	17782.16	1.28	0.006	14222.7	0.48	0.093
nrfA	cytochrome c552	4838.26	538.33	-3.18	0.004	1192.23	-1.24	0.005	894.4	-2.13	0.005
dnaJ	Chaperone protein dnaJ	44.76	147.03	1.69	0.026	39.06	0.55	0.440	48.06	0.37	0.695
hspR	Transcriptional regulator, MerR family	80.6	99.9	0.29	0.012	46.9	-0.02	0.004	115.5	0.81	0.022
racR	DNA-binding response regulator	238.16	162.83	-0.55	0.095	133.86	-0.05	0.050	227.8	0.24	0.693
racS	Sensor protein	134.03	181.56	0.42	0.048	30.86	-1.39	0.014	70.06	-0.66	0.047
fliA	RNA polymerase sigma factor	139.06	676.23	2.27	0.001	136.7	0.74	0.769	614.4	2.44	0.0008
dcuA	Anaerobic C4-dicarboxylate transporter dcuA	2856.03	1247.93	-1.2	0.033	1042.03	-0.67	0.015	946.83	-1.29	0.015
sodB	superoxide dismutase	1389.03	2489.46	0.84	0.0005	3451.23	2.1	0.006	3035.4	1.44	0.015
clpP	ATP-dependent Clp protease proteolytic subunit	839.73	445.86	-0.93	0.007	381.2	-0.37	0.002	624.6	-0.13	0.026
cheA	Chemotaxis protein CheA	5691.7	1336.06	-2.1	0.004	2383.86	-0.48	0.009	2389.36	-0.95	0.010
perR	transcriptional regulator Fur family	76.53	56	-0.02	0.304	52	-0.56	0.407	52.2	-0.26	0.022
fdxA	Ferredoxin, 4Fe-4S	103.66	78.166	-0.41	0.108	22.4	-1.43	0.008	52.4	-0.68	0.028
ahpC/tsaA	Probable peroxiredoxin	1668.93	4793.53	1.51	0.005	5599.4	2.52	0.0009	4475.93	1.72	0.01
sdhA	succinate dehydrogenase flavoprotein subunit	2424.56	547.5	-2.16	0.001	496.53	-1.52	0.001	554.76	-1.83	0.003

sdhB	succinate dehydrogenase iron-sulfur protein	913.3	217.73	-2.09	0.0009	300.36	-0.83	0.0008	232.26	-1.68	0.002
sdhC	Succinate dehydrogenase, subunit C	742.3	150.46	-2.31	0.002	275.76	-0.66	0.003	195.16	-1.63	0.004
nssR	Transcriptional regulator, putative	78.1	199.7	1.34	0.031	39.86	-0.2	0.013	194.86	1.62	0.009
clpB	Chaperone protein clpB	321.76	1139.66	1.81	0.0007	1053.06	2.49	0.0002	934.8	1.84	0.0001
hslU	ATP-dependent hsl protease ATP-binding subunit	1841.63	1291.6	-0.53	0.023	1247.7	0.21	0.033	1206.76	-0.31	0.014
hrcA	Heat-inducible transcription repressor hrcA	152.23	572.36	1.91	0.006	237.93	1.42	0.092	670.2	2.45	0.0004
grpE	Protein grpE	49.43	102.53	1.04	0.001	118.53	2.03	0.010	102.36	1.35	0.005
dnaK	Chaperone protein dnaK	1537.7	1732.9	0.16	0.34	2096.56	1.22	0.108	1705.46	0.45	0.048
peb1c	Probable ABC transporter ATP-binding protein	55.56	81	0.53	0.013	42.83	0.39	0.005	47.96	0.08	0.055
lon	ATP-dependent protease La	868.76	737.03	-0.25	0.218	277.96	-0.87	0.006	470.76	-0.59	0.013
clpA	ATP-dependent Clp protease, ATP-binding subunit	802.1	575.23	-0.49	0.075	118.36	-1.99	0.008	250.73	-1.37	0.012
groS	10 kDa chaperonin	205.2	380.16	0.87	0.009	830.63	2.79	0.0002	530.73	1.67	0.001
groL	60 kDa chaperonin	3801.3	4776.5	0.31	0.03	8249.4	1.89	0.003	5659.36	0.87	0.004
yjiY (*cstA*)	starvation response and peptide uptake	462.6	1423.06	1.61	0.0043	2420.96	3.16	0.002	842.66	2.54	0.007

4.3 Discussion

In Chapter 3, evidence was presented suggesting that strains of *C. jejuni* vary in their ability to retain culturability in water, with some strains, such as M1, rapidly losing colony forming ability without a corresponding loss of viability, whereas others such as 1336 and 414, retain far higher levels of culturability. We hypothesized that these variations between different strains of *C. jejuni* during survival in water might be attributed to differences in expression in genes reported previously as being important in the response to harsh environments, including genes implicated in the formation of VBNC cells. In order to test this hypothesis, and with the aim of elucidating the mechanisms underlying these variations, a set of stress response genes (Table 4.1) was targeted in end-point and Q-PCR assays at specific time points during survival in water where variations were observed (Chapter 3).

Initial experiments using end-point PCR amplification targeting the cDNA template of selected genes indicated considerable variation in expression between strains, genes and time points (Table 4.3). Although it is hard to draw firm conclusions from these data, expression of the targeted genes (*luxS*, *spoT*, *ppk1*, *amiA*, *htrB*, *htrA*) was undetectable at the day 3 (4 °C) time point for strains 1336 and 414, whereas expression of some genes (*spoT*, *amiA*, *htrA*) was detectable for strain M1. This did correspond with a difference between strains that retained culturability better (1336 and 414) compared to a strain that did not (M1). However, at the day 1 (25 °C) time point, the biggest variation was seen with the lack of expression of genes in strains M1 and 1336, in contrast to strain 414. In terms of retaining culturability, strains 1336 and 414 were similar, whereas strain M1 was associated with rapid loss. Hence, there is no clear pattern to the data. Perhaps the most surprising result was the inability to

detect expression of *rpoA* in all of the samples. This gene was included along with 16S rRNA as a potential control/reference gene because of a previous study suggesting that it was the best choice as an internal control to study stress response in *C. jejuni* using (Q-PCR) (Ritz et al., 2009). In this previous study, Ritz *et al.* (2009) set out to identify stable housekeeping genes by studying gene expression variations in different growth phases and under different stress conditions for six target genes: *gyrA*, *ilvC*, *rpoA*, *slyD*, *thiC*, and *rrs* (16S rRNA). The variations tested were exponential phase, stationary phase, cold shock, oxidative stress and a combination of cold shock and oxidative stress. The *rpoA* gene, encoding the alpha subunit of RNA polymerase, was recommended because it had the least variation in gene expression when all of these conditions were compared. It is worth noting, however, that the authors also saw very little variation with respect to the 16S rRNA gene expression. They chose to reject this gene in favour of *rpoA* because of the fact that inevitably levels of 16S rRNA transcripts are much higher than for other genes. In this study, the 16S rRNA gene expression could be detected in all samples, whereas *rpoA* expression could not be detected in some. Hence, the decision was made to use 16S rRNA as the control gene for Q-PCR assays. It is not clear why the *rpoA* expression levels are low, especially in strain M1, but this observation was confirmed by subsequent inclusion in the Q-PCR assays, where expression relative to 16S rRNA was low to undetectable. The RNAseq data confirmed that the expression of this gene was variable under the conditions used in this study.

It is worth noting that others have since suggested that *rpoA* gene expression is not constitutive and is highly sensitive to environmental change. For example, it varies during cell processing procedures prior to RNA isolation (Hyytiäinen et al., 2012). The

authors in this 2012 study also selected 16S rRNA in their study as an internal control for Q-PCR, suggesting that *rpoA* is not an appropriate choice, and others have also the 16S rRNA in similar studies (Bui et al., 2012).

Real-time fluorescence-based Q-PCR is considered as a model for nucleic acid quantification due to its specificity, sensitivity and speed (Bustin et al., 2009). Q-PCR analysis demonstrated that there were differences in gene expression levels during survival in sterile distilled water between the different strains of *C. jejuni*.

When the Q-PCR assays were applied to study of gene expression variations between strains at key time points in the water survival experiments, a number of differences were observed. In strain 414, which retains culturability at the key time points, levels of expression of *htrA* were significantly higher (Table 4.5) when compared to the other two strains at all-time points. When normalised data were used (expression relative to 16S rRNA), expression was also significantly higher (Table 4.5) at the day 3 (4 °C) time point than at time zero for strain 414. It should be noted that during the preparation of the inoculum for the water survival experiments, there is inevitably a period of exposure of the *C. jejuni* cells to the stresses associated with being removed from nutrient-rich to nutrient-poor conditions. Hence, it seems likely that some of the stress responses have already occurred at the time zero point in these experiments. This perhaps explains why there were not more differences between the Day 1 or Day 3 sample times and the Day zero sample time.

The *htrA* gene product, HtrA, is required for both heat and oxygen tolerance (Brøndsted et al., 2005). HtrA displays both chaperone and protease properties, both implicated in the ability to tolerate stress, though the chaperone activity was identified as more important for resistance to oxidative stress (Baek et al., 2011b).

HtrA is a periplasmic serine protease that can contribute to host cell binding (Baek et al., 2011a), and has been implicated in acute ulcerative enterocolitis and driving immune responses during infection of mice (Heimesaat et al., 2014). In the context of this study, it seems likely that a potential role in the response to oxidative stress is more relevant than properties as a potential virulence factor. HtrA is important for stress tolerance and survival of Gram-negative bacteria generally (Laskowska et al., 1996; Li et al., 1996). However, it has been reported previously that *htrA* is down-regulated in *C. jejuni* in response to low nutrient and especially oxidative stress (Bui et al., 2012). This appears to contradict the findings in our study but probably reflects the considerable variations between strains. In the study of Bui *et al*. (2012), they used strain NCTC11168. They also used very specific oxidative stress conditions (exposure to 10 mM hydrogen peroxide for 15 min) which differ considerably from those used in this study. It is possible that the enhanced levels of expression of *htrA* in strain 414 contribute to its ability to retain culturability better than strain M1, where levels are significantly lower. However, the fact that levels were also lower in strain 1336, which can retain culturability, would indicate that if *htrA* does play a role, it may be strain-specific, and not a mechanism that can be universally applied to *C. jejuni*.

Of the three strains compared, strain M1 was clearly different in terms of its inability to retain culturability under the experimental conditions used. The Q-PCR assays strongly suggest that gene expression is occurring in the day 3 (4 °C) and day 1 (25 °C) samples despite the low colony forming unit counts for M1, which supports the conclusion from staining experiments presented in Chapter 3 that M1 is entering a VBNC state. Clearly, the strain with lowest detectable expression levels for the genes

selected is strain 1336 (Figure 4.23) rather than M1. Hence, it was particularly interesting to ascertain whether there were gene expression changes specific to strain M1. The most notable of these related to the *luxS* gene, which was significantly up-regulated (relative to 16S rRNA) in M1 compared to strain 414 in both the day 3 (4 °C) and day 1 (25 °C) samples, and which was significantly up-regulated in these samples in comparison to the day zero sample (Table 4.5). RNAseq analysis confirmed that *luxS* is expressed at high levels during survival of strain M1 in water. These observations are consistent with a role for *luxS* during the conversion of M1 to the VBNC state. *luxS* is responsible for encoding an autoinducer (AI-2 or LuxS) in the *C. jejuni* QS system (Cloak et al., 2002). The QS system has been implicated previously in a variety of important physiological pathways in response to cell density. These include motility/flagellar gene expression, oxidative stress, ability to colonise animals, expression of CDT and virulence in an animal model (Plummer, 2012). However, different groups have reported varying observations (Elvers and Park, 2002; He et al., 2008; Quiñones et al., 2009). As with the studies of most genes in *C. jejuni*, or indeed in other common pathogens, conclusions have been drawn based on the analysis of one or two strains, with the assumption that there is general applicability to the whole species. In one study, *luxS* mutants constructed in two different strains, NCTC11168 and 81176, were compared and differing phenotypes depending on the strain background were reported, suggesting that this assumption may be flawed (Adler et al., 2014).

It has been suggested that *Campylobacter* spp. generally possess *luxS* gene, but *C. lari* is an exception to this (Tazumi et al., 2011). Indeed, it has become clear that not only is *luxS* not carried by all *Campylobacter* species (Gölz et al., 2012), but that not all *C.*

jejuni carry *luxS* gene (Hepworth et al., 2011) and we found this to be the case for strain 1336.

However, although the *luxS* gene has been reported as absent from some water/wild-life-associated strains (Hepworth et al., 2011), it is generally present in isolates from human sources. In a study analysing the genomes of 442 *C. jejuni* UK human isolates from two large surveys of pathogens associated with intestinal infectious disease [the IID1 and IID2 studies; (Tam et al., 2012)], *luxS* was present in all but six strains, five of which shared the same ST (ST-433). In all there were six ST-433 isolates amongst the IID1 and IID2 isolates, with one carrying the *luxS* gene (unpublished data, C. Winstanley, University of Liverpool). Therefore this gene potentially helps *C. jejuni* to survive in the environment, and is carried by the vast majority of strains that cause infections in humans.

Strain 1336, isolated from a wild bird, was previously reported as a representative of a diverse group of *C. jejuni* water/wild-life isolates that do not tend to be found amongst human isolates or isolates from farm animals and are hence considered to be uncharacteristic of human food-chain associated isolates (Hepworth et al., 2011). Strain 414 is a representative of ST-3704, associated with bank voles. The genomes of both strains 1336 and 414 lack regions that are normally carried by *C. jejuni* associated with the human food chain. However, despite lacking a number of genetic regions associated with the ability to colonise chickens, and lacking the *luxS* gene, strain 1336, unlike strain 414, was able to colonise chickens (Hepworth et al., 2011). Clearly further work is needed in order to resolve the role of the QS system in the variations seen in strain M1 compared to strain 414 with respect to loss of culturability and a switch to the VBNC state. The results of this study are consistent

with the idea that there is expression of *luxS* in VBNC populations of *C. jejuni*. However, it should be noted that there may be a small proportion of vegetative cells in which *luxS* could, in theory, be highly up-regulated. This study also emphasises the likely importance of variations between strains.

There were also some significant differences in the expression of *ppk1* between strain M1 and the other two strains (Figure 4.23). At both time-points day 3 (4 °C) and day 1 (25 °C), levels of expression of *ppk1* (relative to 16S rRNA) were significantly higher (Table 4.5) in strain M1 than in either strain 1336 or strain 414. For day 1 (25 °C), the increase in expression of *ppk1* in M1 reached significance compared to the day zero value. At the day 3 (4 °C) time-point, expression was also higher compared to day zero, but the change did not reach statistical significance (p =0.085). *ppk1* encodes polyphosphate kinase-1, a key enzyme in the synthesis of polyphosphate, which is an essential molecule for survival, mediating stress responses and host interactions in a number of bacterial pathogens (Kornberg, 1999). It has been demonstrated that a *ppk1* mutant of *C. jejuni* not only had defective polyphosphate accumulation, but also had decreased ability to form the VBNC state under acid stress conditions (Gangaiah et al., 2009). The authors were able to complement the mutation and restore previous phenotypes. Given that strain M1 is seen to enter the VBNC state at the chosen time-points in the experiments presented in this chapter, the enhanced expression of *ppk1* is consistent with the role of polyphosphate accumulation in the formation of the VBNC state suggested by this previous study (Candon et al., 2007; Gangaiah et al., 2009).

The low values for gene expression achieved across the board for strain 1336 suggest that genes other than the ones targeted here are important in the survival of this

strain in water, and further emphasize the importance of variations between strains. However, it should be noted that using end-point PCR assays, expression of most genes was detectable at the zero time-point. The lack of expression of most of the selected genes beyond the time zero point, was also observed for strain 1336 in the end-point PCR assays.

However, overall, there was poor agreement between the end-point PCR assays and the Q-PCR assays. The reasons behind this are unclear. For the end-point PCR assays, only one replicate based on single RNA extractions for each assay was used. The later observations based on Q-PCR assays suggest that this was inadequate to ensure detection of gene expression. In particular, for strain 414, gene expression was not detected by end-point PCR for any of the target genes in the Day 3 (4 °C) sample, whereas Q-PCR clearly identified expression of some genes, especially *htrA*. Similarly, for strain M1 the end-point PCR proved to be inadequate at detecting expression of several genes in the Day 3 (4 °C) that were subsequently detected using Q-PCR.

The RNAseq data, obtained for strain M1 only, highlighted a number of genes up-regulated in water compared to growth in laboratory media. Most notably, *luxS* was again identified, along with genes implicated in oxidative stress (*katA*, *sodB*, *ahpC*). Although the design of the RNAseq and Q-PCR experiments are not the same, the RNAseq data are consistent with the idea that *luxS* plays an important role during survival in water. Given that expression in strain M1 was higher than for other strains, and that under the chosen conditions the M1 strain enters a VBNC state, these observations implicate *luxS* in a role during this process. Further analysis of the global transcriptome of strain M1 during survival in water is ongoing in a parallel project, but it is clear that genes are being actively expressed, confirming the fact that

although strain M1 is losing culturability, it remains VBNC. Previous studies have shown using RNAseq that gene expression can vary considerably between strains of *Campylobacter* (Dugar et al., 2013). The key to understanding important phenotypic variations, such as different survival behaviour in environmental samples, is likely to lie within the detail of the strain-specific transcriptome profiles. In this study, some of these variations have been identified, but further work is needed to fully characterise the mechanisms underlying the differences between strains.

4.4 Conclusions

Gene expression differences implicated in variations in the survival behaviour in sterile distilled water of different strains of *C. jejuni* were identified. In particular, variations in the expression of key genes *(luxS*, *ppk1, htrA*) were implicated in the conversion of strain M1 to a VBNC state. This is the first report implicating the *C. jejuni* QS system (*luxS*) in a role in survival in water.

Chapter 5

CO-EXISTENCE OF *C. jejuni* AND *Pseudomonas* spp. IN THE ENVIRONMENT, AND INTERACTIONS BETWEEN THEM

5.1 Introduction

C. jejuni is a successful human pathogen and has reservoirs in water and various animals but its survival outside the host is poor (Altekruse et al., 1999; Bronowski et al., 2014; Young et al., 2007). Despite this, *C. jejuni* can often be isolated from the environment, and apparently survives well on chicken meat, suggesting that it does have strategies enabling it to survive. Interaction of *C. jejuni* with other microorganisms is one of the suggested survival strategies of this microorganism in the environment (Hilbert et al., 2010) and during the contamination of food. Various bacterial species are found on chicken meat and some of these bacteria are known as spoilage bacteria. For example, *Pseudomonas* species such as *P. fluorescens*, *P. frangi*, *P. chlororaphis* and *P. putida* are found on chicken meat in levels of up to 10^3 to 10^4 CFU/g (Ghafir et al., 2008; Hilbert et al., 2010; Mead et al., 1993; Russell et al., 1995). It has also been shown that the numbers of *Campylobacter* on chicken meat surface are higher than that of internal contamination (Luber and Bartelt, 2007). *Pseudomonas* species are also commonly found in a wide range of natural environmental niches (Silby et al., 2011). It has been proposed that *C. jejuni* may interact with aerobic *Pseudomonas* spp. found on food or in other environments in order to counteract the toxic effects of atmospheric oxygen (Hilbert et al., 2010). Little is understood about the mechanism by which *Pseudomonas* spp. enhance the aerotolerance of *C. jejuni*. However, the concept of bacteria benefitting from co-existence with other species is not a new one. *In vitro* experiments have shown that

an anaerobic microorganism such as *Porphyromonas gingivalis*, which causes periodontitis in humans, is able to grow in the presence of oxygen when co-cultured with *Fusobacterium nucleatum*. This is possibly due to metabolizing of both oxygen and hydrogen peroxide molecules and the creation of a capnophilic environment by *F. nucleatum*, thus supporting growth of *P. gingivalis* (Diaz et al., 2002; Kuramitsu et al., 2007). In certain situations some individual organisms are found to be antibiotic sensitive but are turned resistant within mixed infections. For example studies *in vitro* have demonstrated the protection of a penicillin-sensitive strain of *F*. *neccrophorum* from penicillin therapy by penicillin-resistant strains of *Bacteroides fragilis*, pigmented *Prevotella* and *Porphyromonas* spp., and *Prevotella oralis* (Hackman and Wilkins, 1976). In animal model experiments it has been demonstrated that combinations of aerobic and anaerobic bacteria promote levels of disease that could not be produced by individual organism. For example, mono and mixed intra-abdominal infections with *B*. *fragilis* and *E.coli* in a rat fibrin clot model demonstrated that only with mixed infection there was an increase of *B. fragilis* numbers about 6 h after initial decrease, and only mixed infections resulted in abscesses lesions. It was suggested that the presence of *E.coli* enabled *B. fragilis* to survive by changing the pH, excreting nutrients or using up the oxygen (Verweij et al., 1991). Thus, it is possible that aerobic organisms such as *Pseudomonas* spp. could play key role in the ability of *Campylobacter* species to survive in the environment.

5.1.1 The genus *Pseudomonas*

The genus *Pseudomonas* was first reported by the Botanist Migula in 1894. It consists of a group of bacteria with large genomes that have considerable metabolic and physiological versatility. Their adaptability allows them to colonise diverse aquatic and terrestrial habitats as well as causing various infections (of humans, animals and plants) and exhibiting biochemical properties that can be exploited for biotechnological applications (Peix et al., 2009; Silby et al., 2011). Over the years, the taxonomy of *Pseudomonas* has been revised. The advent of molecular methods for studying phylogeny revealed that organisms previously categorised as *Pseudomonas* were actually phylogentically diverse and the various species (including newly described genera) were separated on the basis of rRNA homology into different groups (O'Sullivan and O'Gara, 1992; Palleroni et al., 1973; Palleroni and Moore, 2004; Ramos, 2011). *Pseudomonas sensu stricto* lie in the *Gamma* sub-division of *Proteobacteria*, in what is known as rRNA homology group I. More recently, whole genome sequencing has enabled much closer analysis of the relationships between *Pseudomonas* species (Silby et al., 2011).

Phenotypically, *Pseudomonas* species are classified into fluorescent species and non-fluorescent species. *P. stutzeri* is the most intensely studied species of the non-fluorescent species (Palleroni, 2008). The fluorescent *Pseudomonads* are composed of a distinct group of bacteria that have the ability to produce a yellow–green pigment. They are Gram-negative, polar flagellated motile rods, chemoheterotrophic and are grouped in rRNA homology group I. Many members of species of this group are psychrotrophic, involved in food spoilage. The most common fluorescent species in this group include *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. chlororaphis*, *P.*

syringae and *P. cichorii* (Arnaut-Rollier et al., 1999a; Franzetti and Scarpellini, 2007). It has also been shown that these spoilage bacteria have been isolated from both fresh and refrigerated poultry skin (Arnaut-Rollier et al., 1999b). Previous studies confirmed that pigmented and non-pigmented strains of *Pseudomonas* were the most frequently bacteria found amongst the microorganisms on poultry skin (Barnes and Thornley, 1966; Kraft, 1992; Russell et al., 1995).

5.1.2 Bacterial secretome

Bacterial Pathogens have evolved a number of complex secretion systems to encounter hostile environments or to increase bacterial pathogenicity while infecting their hosts. The majority of these systems depend on a large number of proteins which administer virulence factors either into the environment or into the host cell (Bleves et al., 2010). There are two major pathways in bacteria to secrete proteins across the cytoplasmic membrane. The general secretion (Sec) pathway by which most of unfolded proteins are secreted, and twin-arginine translocation (Tat) pathway by which folded proteins are secreted (Bleves et al., 2010; Natale et al., 2008) Natale, paol 2008).

The secretion systems are classified as type I secretion system (T1SS) up to type VI secretion system (T6SS) (Bleves et al., 2010; Sankarasubramanian et al., 2016). The T1SS is comprised of 3 proteins, a pore-forming outer membrane protein (OMP), a membrane fusion protein (MFP), and an inner membrane ATP-binding cassette (ABC) protein, the latter two proteins are cytoplasmic membrane proteins. Various sizes of proteins are secreted via this widespread pathway (Delepelaire, 2004). The T2SS is involved in the transport of cellulases, pectinases, phospholipases, lipases and toxins

from the periplasim to the outer membrane. Furthermore, these proteins can increase the virulence of human, animal and plant bacterial pathogens (Cianciotto, 2005; Sandkvist, 2001). The T3SS is homologous to the bacterila flagellum, involved in direct injection of proteins into the host cells (Bergeron et al., 2016).

The T4SS is related to conjugation system, which transports DNA or proteins from donor to target cells (Bhatty et al., 2013). The T4SS plays an important role in bacterial pathogenesis of several bacterial pathogens (Zechner et al., 2012). The T5SS is the simplest protein secretion system in which the proteins are secreted either via the autotransporter system (type Va), two partner secretion pathway (Vb), or type Vc system (Henderson et al., 2004). The T6SS is an important virulence factor for several important bacterial pathogens such as *Vibrio cholerae*, which can translocate proteins directly into the host cells. Protein expression is controlled at both transcriptional and post-transcriptional levels (Bingle et al., 2008).

5.1.3 *Pseudomonas* secretome

The opportunistic human pathogen *Pseudomonas aeruginosa*, which is implicated in infection of the respiratory tract of cystic fibrosis (CF) cases, possesses five (excluding the T4SS) of the six secretion systems (Table 5.1).

Table 5.1 secretion systems and scretomes in *Pseudomonas aeruginosa* PAO1 strain

PA no.	Secretion type	Gene	protein	Function	Reference
PA1249	T1SS	*aprA*	AprA	Alkaline protease	Duong, F, L 1996; Guzzo et al 1991
PA1245	T1SS	*aprX*	AprX	unknown (Hypothetical protein)	(Duong et al., 2001)
PA3407	T1SS	*hasAp*	HasAp	Heme acquisition protein	(Létoffé et al., 1998)
PA3724	T2SS	*lasB*	LasB	Elastase	(Braun et al., 1998)
PA1871	T2SS	*lasA*	LasA	Staphylolytic and elastolytic activity	(Braun et al., 1998)
PA0844	T2SS	*plcH*	PlcH	Hemolytic phospholipase C	(Voulhoux et al., 2001)
PA3319	T2SS	*plcN*	PlcN	Non hemolytic phospholipase C	(Voulhoux et al., 2001)
PA0026	T2SS	*plcB*	PlcB	Phospholipase C specific of phosphatidyl-ethanolamine	(Barker et al., 2004)
PA0852	T2SS	*cbpD*	CbpD	Chitin-binding protein	(Folders et al., 2000)
PA1948	T2SS	*toxA*	ToxA	Exotoxin A, ADP-ribosyl transferase	(Lu et al., 1993)
PA0572	T2SS	*pmpA*	PmpA	Putative metalloprotease	(Bleves et al., 2010)
PA4175	T2SS	*prpL*	PrpL	Lysine specific endopeptidase (Protease IV)	(Fox et al., 2008)
PA2862	T2SS	*lipA*	LipA	Triacyl glycerol acyl hydrolase	(Jaeger et al., 1994)
PA4813	T2SS	*lipC*	LipC	Lipase	(Martínez et al., 1999)
PA3296	T2SS	*phoA*	PhoA	Alkaline phosphatase	(Filloux et al., 1988)
PA2939	T2SS	*paAP*	PaAP	Aminopeptidase	(Braun et al., 1998)
PA0688	T2SS	*lapA*	LapA	Alkaline phosphatase	(Ball et al., 2002)
PA3841	T3SS	*exoS*	ExoS	ADP-ribosyl transferase)	(Wehmhöner et al., 2003)
PA0044	T3SS	*exoT*	ExoT	ADP-ribosyl transferase	(Wehmhöner et al., 2003)
PA2191	T3SS	*exoY*	ExoY	Adenylate cyclase	(Yahr et al., 1998)
PA5112	T5SS	*estA*	EstA	Esterase	(Wilhelm et al., 1999)
PA4540	T5SS	*lepA*	LepA	Exoprotease	(Kida et al., 2008)
PA4082	T5SS	*cupB5*	CupB5	Hemagglutinin-like	(Ruer et al., 2008)
PA0085	T6SS	*hcp1*	Hcp1	Nanotubes formation	(Mougous et al., 2006)

Adapted from (Bleves et al., 2010)

5.1.4 Bacterial QS systems

Chemical signal molecules are used in bacteria to communicate between cells. In QS, hormone-like molecules, which are termed autoinducers, are produced and released by bacteria, and detected by other cells in the population. When the concentration of the autoinducer reaches a certain threshold, because there are sufficient members of the community producing the signal molecule, then the bacteria respond *en masse* to change their gene expression and alter their behaviour as a bacterial population (Waters and Bassler, 2005). Hence, this is considered to be a density-dependent control system. The bacterium *Vibrio fischeri*, in which the phenomenon of QS was first described, is considered the paradigm for QS in most Gram-negative bacteria (Nealson and Hastings, 1979).

In *V. fischeri*, the luminescence genes are arranged in a single operon, *luxCDABE*, which exists as part of *luxICDABE* operon. The fatty acid reductase complex, the product of *luxCDE,* is involved in synthesis of fatty aldehyde substrate which is catalysed by the luciferase LuxAB subunits leading to light production (Eberhard et al., 1981; Meighen, 1991; Miller and Bassler, 2001). The expression of *LuxICDABE* is controlled by the proteins LuxI and LuxR. LuxI, the product of the *luxI* gene, synthesises the *Vibrio* autoinducer 3OC6-homoserine lactone, which freely diffuses into the environment. When the autoinducer molecule reaches the threshold concentration, the signal is transduced via the LuxR protein which acts as a regulator to activate transcription of the *lux* genes encoding luminescence (Eberhard et al., 1981; Engebrecht et al., 1983; Engebrecht and Silverman, 1984; Kaplan and Greenberg, 1985; Stevens et al., 1994). QS systems have since been reported in a wide range of Gram negative and Gram positive bacteria (Sifri, 2008; Williams, 2007).

5.1.5 *Pseudomonas* QS systems

The opportunistic human pathogen *Pseudomonas aeruginosa* is among a number of Gram-negative bacteria in which homologous QS systems have been discovered (Pesci and Iglewski, 1997). The pathogenicity of *P. aeruginosa* is increased through QS control of the expression of many virulence genes. *P. aeruginosa* possesses two QS systems *las* and *rhl*. The *las* system consists of the transcriptional activator protein LasR, a homolog of a LuxR, and LasI, a homolog of a LuxI, which controls the synthesis of the *Pseudomonas* autoinducer [PAI-1; (*N*-(3-oxododecanoyl)-L-homoserine lactone]. When the extracellular autoinducer (PAI-1) reaches a critical threshold concentration, it leads to activation of LasR, a regulatory protein (Venturi, 2006). Likewise, the *rhI* system comprises the transcriptional activator protein RhlR and RhlI, which controls production of a second *Pseudomonas* autoinducer [PAI-2; (*N*-butanoyl-L-homoserine lactone]. *P. aeruginosa* also contains a third signalling system (the quinolone signal system) which interacts with both the *las* and *rhl* systems (Venturi, 2006). Together these QS systems regulate the expression of numerous genes, including those controlling the secretion of virulence factors such as elastase, alkaline protease, pyocyanin, and hydrogen cyanide, and genes involved in biofilm formation (McGrath et al., 2004; Venturi, 2006). Similar QS systems have also been described in a number of other species of *Pseudomonas*, including *P. syringae*, *P. fluorescens*, *P. putida*, *P. aureofaciens* and *P. chlororaphils* (Table 5.2) (Venturi, 2006).

Table 5.2 QS systems in the different *Pseudomonas* species.

***Pseudomonas* sp.**	**Genes of QS**	**AHL signal molecule**	**QS-regulated functions**	**References**
P. aeruginosa	*lasI, lasR rhlI, rhlR*	3-oxo-C12-AHL C4-AHL	elastase, alkaline protease, Biofilm development, exotoxin A, lipase, Xcp secretion, lectins, hydrogen cyanide, twitching, pyocyanin, swarming rhamnolipids, virulence, other cellular functions	(Hentzer et al., 2003; Juhas et al., 2005; Schuster et al., 2003; Smith and Iglewski, 2003; Wagner et al., 2003; Whiteley et al., 1999)
P. syringae pv. *syringae* B728a	*ahlI, ahl R*	3-oxo-C6-AHL	Cell aggregation, epiphytic fitness	(Dumenyo et al., 1998; Quiñones et al., 2004)
P. aureofaciens	*phzI, phzR csaI, csaR*	C6-AHL currently unknown	Phenazine antibiotics cell-surface components rhizosphere colonization	(Pierson et al., 1994; Wood et al., 1997; Zhang and Pierson, 2001)
P. chlororaphis	*phzI, phzR*	C6-AHL	Phenazine-1-carboxamide biosynthesis	(Chin-A-Woeng et al., 2001; Chin-A-Woeng et al., 2005)
P. putida	*ppuI, ppuR*	3-oxo-C12-AHL	Biofilm formation	(Bertani and Venturi, 2004; Steidle et al., 2002)
P. fluorescens NCIMB 10586	*mupI, mupR*	Long acyl-chain-AHL	Mupirocin biosynthesis	(El-Sayed et al., 2001)
P. fluorescens F113	*hdtS*	3-OH-C14:1-AHL	Presently unknown	(Laue et al., 2000)

5.1.6 Cross talk between bacterial species via secreted products

There has been considerable interest in the possibility that bacteria in mixed species communities may communicate via signalling systems such as QS (Williams, 2007). A number of interactions between *P. aeruginosa* and other bacterial species, both positive and negative, have been described (Tashiro et al., 2013). It has been demonstrated previously that there can be cross-talk between the QS systems of *P. aeruginosa* and other bacterial species. For example, it has been shown both in mixed biofilms cultures and in a murine chronic infection model, that *Burkholderia cenocepacia* can recognise and respond to *P. aeruginosa* autoinducer molecules, though this communication is not reciprocated (Riedel et al., 2001). It has also been shown that signal molecules produced by oropharyngeal flora can lead to up-regulation of *P. aeruginosa* genes (Duan et al., 2003). There has also been reported cross-talk between *P. aeruginosa* and the Gram-positive*, Staphylococcus aureus*, whereby QS signal molecules from *P. aeruginosa* cause inhibition of the *S. aureus* virulence-related *agr* QS system (Chan et al., 2004). In *Campylobacter*, the *luxS* gene of *Campylobacter* is responsible for the production of *S*-ribosylhomocysteinase (LuxS) which cleaves *S*-ribosylhomocysteine (SRH), yielding unstable (*S*)-4, 5-dihydroxy-2,3-pentanedione (DPD) and leading to a final product of AI-2, the suggested signal for QS (De Keersmaecker et al., 2005; Rajan et al., 2005; Winzer et al., 2002). The receptor (LuxR equivalent) is not known but it has been demonstrated that a *luxS* mutant of *C. jejuni* (NCTC11168) had decreased significantly in CFU after six days of incubation in chicken meat juice at 5°C, comparing with wild type in which the CFU remained unchanged for 27 days (Ligowska et al., 2011). This suggests that LuxS, a QS molecule in *Campylobacter*, plays a role in adaptation to cold temperature and meat juice, and

is important for survival in the environment. In the previous chapter, evidence was presented suggesting that *luxS* might also play an important role in the survival of *C. jejuni* in water.

5.1.7 Aims of this chapter

Given the evidence that *Pseudomonas* spp. could play a key role in the ability of *Campylobacter* species to survival in the environment, and previous studies showing that *Pseudomonas* can interact with other species, preliminary experiments were carried out:

1. To determine the effects of supernatants (secreted products) from *Pseudomonas* spp. cultures on the growth of *Campylobacter in vitro*.
2. To use environmental PCR assays to establish the extent to which co-existence of *Campylobacter* spp. and fluorescent *Pseudomonas* spp. occurs in natural water.

5.2 Results

5.2.1 Laboratory interaction assay between *C. jejuni* and *Pseudomonas* spp.

Initially the supernatants of 24 strains of *Pseudomonas* spp. (Table 2.2) were tested to determine their ability to enhance (or inhibit) the growth of *Campylobacter* under microaerobic growth conditions at 37 °C. The results are shown in Figure 5.1. The experiment indicated that most of the supernatants of the *Pseudomonas* strains significantly enhanced the growth of *C. jejuni* ST-42 (72 h), and the greatest effect was shown with the supernatants from *P. syringae* pv. *glycinea* 49a/90, *P. avellanae* 48, *P. syringae* pv. *antirrhini* 152E, *P. fluorescence* F113 and *P. syringae* pv. *lachrymans* 789 respectively (Table 5.3). Therefore, these strains along with *P. putida* KT 2440 were chosen for further analysis by performing the interaction assay with *C. jejuni* ST-42 culture microaerobically at two further temperatures: 30 °C and 25 °C (Figures 5.2 and Figure 5.3, respectively) in triplicate. The results showed that *C. jejuni* growth was significantly enhanced when incubated at 30 °C (72 h) with the supernatants from *P. syringae* pv. *glycinea* 49a/90, *P. syringae* pv. *antirrhini* 152E, *P. syringae* pv. *lachrymans* 789, and *P. avellanae* 48 respectively (Figure 5.2), whereas significantly enhanced growth of *C. jejuni* at 25 °C (72 h) was only seen in the presence of the supernatant of *P. syringae* pv. *glycinea* 49a/90 (Figure 5.3).

The strain ST-42 was originally thought to be NCTC11168 which was selected as a reference strain in this study for being genome sequenced, has been a widely used laboratory strain for studying *C. jejuini* pathogenesis and is considered as a good colonizer of chickens (Day et al., 2009; Gaynor et al., 2004). As preliminary test to determine whether *Pseudomonas* supernatants could have an impact on the growth of *Campylobacter,* experiments were conducted at temperatures that are optimal for

growth of *Campylobacter*. This would be difficult to detect at lower temperatures because of poor growth rates. Hence, the conditions used here do not mimic environmental conditions.

5.2.2 The effect of pre-treated supernatant on growth

In order to try and understand more about the component of *Pseudomonas* supernatants that might be responsible for the enhanced growth shown by *C. jejuni* ST-42, the species with the greatest effect (*P. syringae* pv. *glycinea* 49a/90) was selected for further experiments. To determine whether the component might be a protein, the supernatant of *P. syringae* pv. *glycinea* 49a/90 was treated by boiling or proteinase K prior to being added to the *C. jejuni* ST-42 culture (Figure 5.4). The figure shows that the growth enhancement of *C. jejuni* ST-42 compared with the control (*C. jejuni* ST-42+LB) did not occur if the supernatant of *P. syringae* pv. *glycinea* 49a/90 was pre-treated either by boiling or with proteinase K. At 72 h the growth of *C. jejuni* at 37 °C in the presence of *P. syringae* pv. *glycinea* 49a/90 untreated supernatant was significantly higher than either the controls or when the pre-treated (boiling or proteinase K) supernatants were present.

Although the reason for the suppression of *Campylobacter* growth after the addition of proteinase K is not known, it could be due to inhibitory products being formed as a result of the digestion of proteinaceous components of the growth medium.

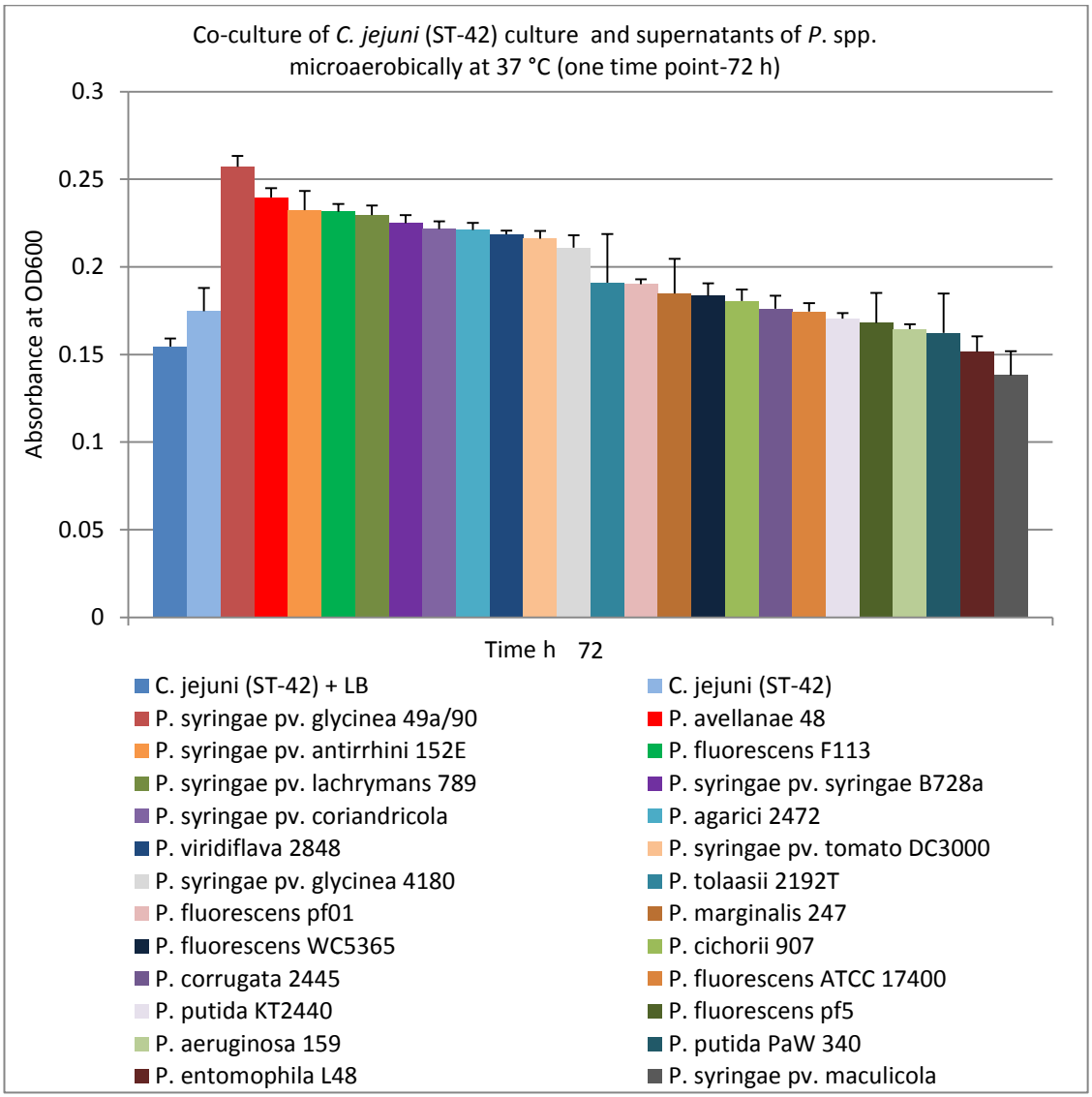
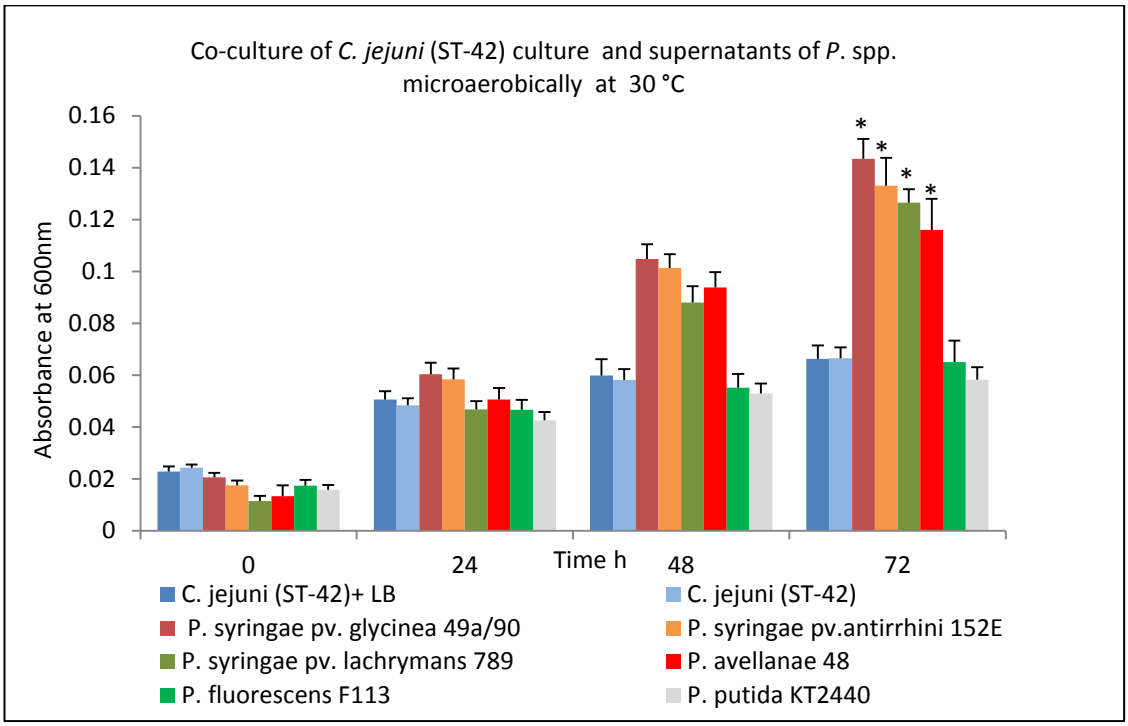
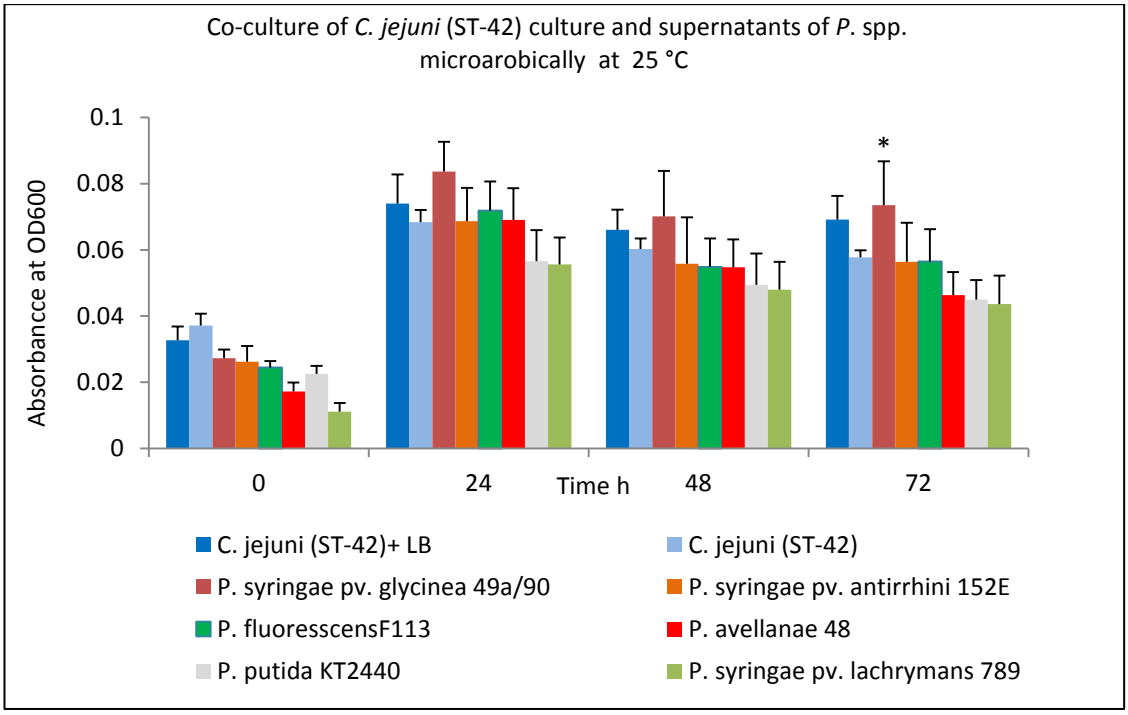


Figure 5.1 Growth of *C. jejuni* (ST-42) cultures under microaerobic conditions at 37 °C (one time point-72 h) in the presence of supernatants of 24 strains of *Pseudomonas* spp.. The error bars represent standard error of three replicates. *C. jejuni* (ST-42) +LB indicates that LB only (rather than LB from a culture of *Pseudomonas* supernatant) was added.



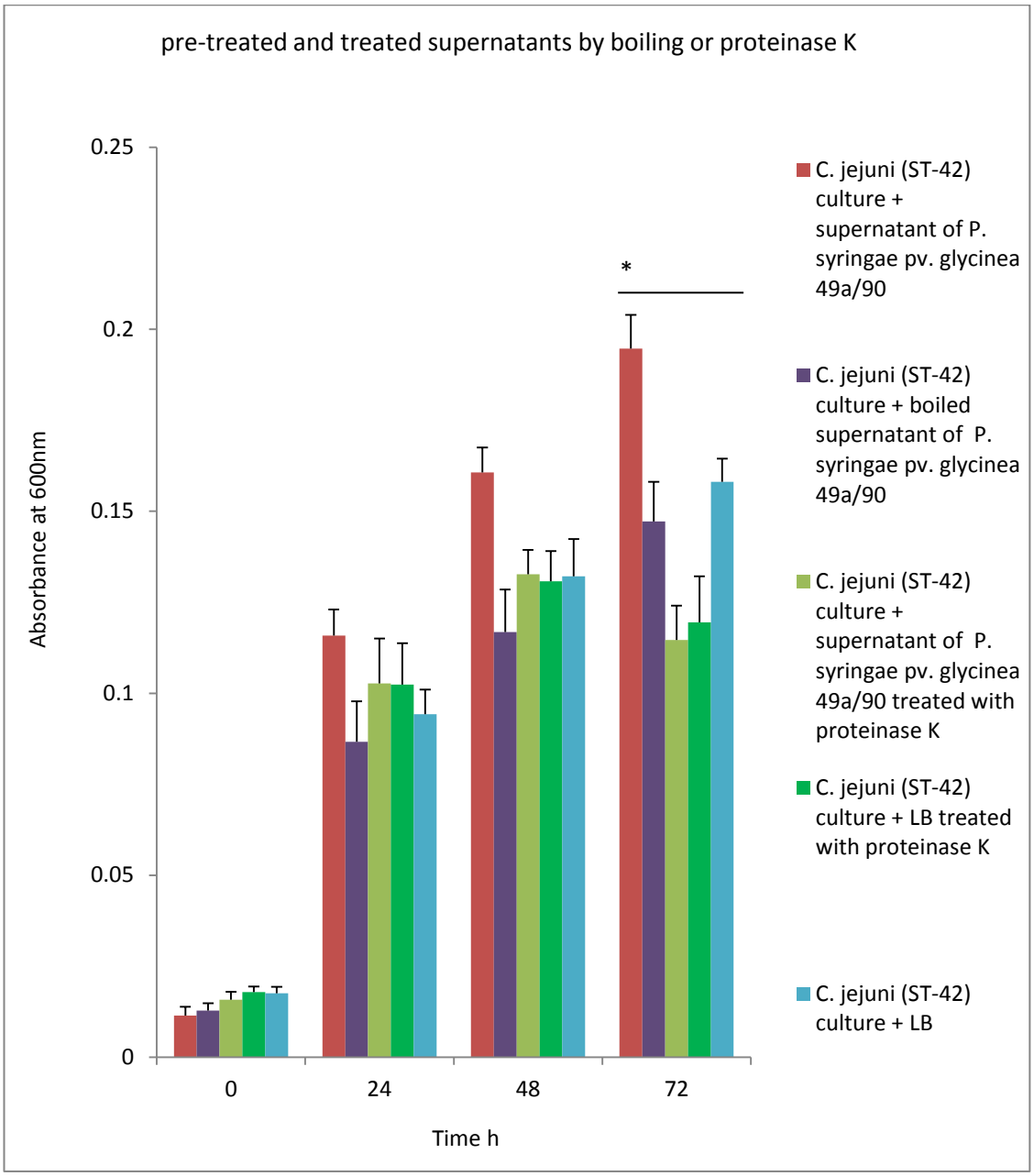
*$p < 0.01$ compared to *C. jejuni* (ST-42) +LB and *C. jejuni* (ST-42)

Figure 5.2 Growth of *C. jejuni* (ST-42) cultures under microaerobic conditions at 30 °C in the presence of supernatants of 6 strains of *Pseudomonas* spp.. The error bars represent standard error of three replicates. *C. jejuni* (ST-42) +LB indicates that LB only (rather than LB from a culture of *Pseudomonas* supernatant) was added.



*$p < 0.01$ compared to *C. jejuni* (ST-42)

Figure 5.3 Growth of *C. jejuni* (ST-42) cultures under microaerobic conditions at 25 °C in the presence of supernatants of 6 strains of *Pseudomonas* spp.. The error bars represent standard error of three replicates. *C. jejuni* (ST-42) +LB indicates that LB only (rather than LB from a culture of *Pseudomonas* supernatant) was added.



*$p < 0.01$ compared to *C. jejuni* (ST-42) culture+boiled supernatant of *P. syringae* pv. *glycinea* 49a/90, *C. jejuni* (ST-42) culture+supernatant of *P. syringae* pv. *glycinea* 49a/90 treated with proteinase K, *C. jejuni* (ST-42)culture+LB treated with proteinase K and *C. jejuni* (ST-42) culture+LB.

Figure 5.4 Effects of supernatants (treated by boiling or proteinase K) of *P. syringae* pv. *glycinea* 49a/90 on growth of *C. jejuni* (ST-42) culture under microaerobic growth conditions at 37 °C. The error bars represent standard error of three replicates.

Table 5.3 Statistical analyses [p-value, using One-Way ANOVA] for 72 h of Co-culture of *C. jejuni* (ST-42) culture and supernatants of *P*. spp. microaerobically at 37 °C. Statistics are testing for the growth of row headers being significantly enhanced compared to column headers.

Co-culture of *C. jejuni* (ST-42) culture and supernatants of *P*. spp.	***C. jejuni* (ST-42) culture**	***C. jejuni* (ST-42) culture+LB**
C. jejuni culture+supernatant of *P. syringae* pv. *glycinea* 49a/90	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. avellanae* 48	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *antirrhini* 152E	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. fluorescens* F113	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *lachrymans* 789	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *syringae* B728a	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *coriandricola*	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. agarici* 2472	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. viridiflava* 2848	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *tomato* DC3000	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. syringae* pv. *glycinea* 4180	$p < 0.01$	$p < 0.01$
C. jejuni culture+supernatant of *P. tolaasii* 2192T	0.062	$p < 0.01$
C. jejuni culture+supernatant of *P. fluorescens* pf01	0.076	$p < 0.01$
C. jejuni culture+supernatant of *P. marginalis* 247	0.247	$p < 0.01$
C. jejuni culture+supernatant of *P. fluorescens* WC5365	0.307	$p < 0.01$
C. jejuni culture+supernatant of *P. cichorii* 907	0.495	$p < 0.01$
C. jejuni culture+supernatant of *P. corrugata* 2445	0.882	0.014
C. jejuni culture+supernatant of *P. fluorescens* ATCC 17400	0.984	0.021
C. jejuni culture+supernatant of*P. putida* KT2440	0.607	0.067
C. jejuni culture+supernatant of *P. fluorescens* pf5	0.427	0.118
C. jejuni culture+supernatant of *P. aeruginosa* 159	0.229	0.247
C. jejuni culture+supernatant of *P. putida* PaW 340	0.147	0.365
C. jejuni culture+supernatant of *P. entomophila* L 48	$p < 0.01$	0.734
C. jejuni culture+supernatant of *P. syringae* pv. *maculicola*	$p < 0.01$	0.058

5.2.3 Environmental PCR assays to assess co-existence of *C. jejuni* and *Pseudomonas* spp. in environmental samples.

To determine co-existence of *C. jejuni* and *Pseudomonas* spp. in environmental water samples, eleven environmental water samples (section 2.11.1), including samples from troughs, ditches, ponds and puddles were tested for PCR amplification of the gene *oprI* and a region of the 16S rRNA gene, specific for rRNA group I *Pseudomonas* spp. and *Campylobacter* respectively. The oligonucleotide primers C412F GGATGACACTTT TCG GAGC and CampR2 GGCTTCATGCTC TCGAGTT were used to amplify a 857 bp fragment of the 16S rRNA gene-specific to *Campylobacter* species. The oligonucleotide primers PS1 ATGAACAAAGTTCTGAAATTCTCTGCT and PS2 CTTGCGGCTGGCTTTTTCCAG were used to amplify a 249 bp region of the *oprI* gene-specific to fluorescent *Pseudomonas* species. Furthermore, non-specific PCR products were not detected from template controls (Figure 5.5 and Figure 5.6). Amplification results for environmental PCR assays [section 2.11.2 (DNA extracted from plate culture) and section 2.11.3 (DNA extracted directly from enriched broth cultures)] for detection *C. jejuni* and *Pseudomonas* spp. are presented in Table 5.4. Amongst the 11 environmental water samples (section 2.11.1), 8 samples were found to be positive for *Campylobacter* spp. and 11 samples were found to be positive for fluorescent *Pseudomonas* spp. (Figure 5.6).

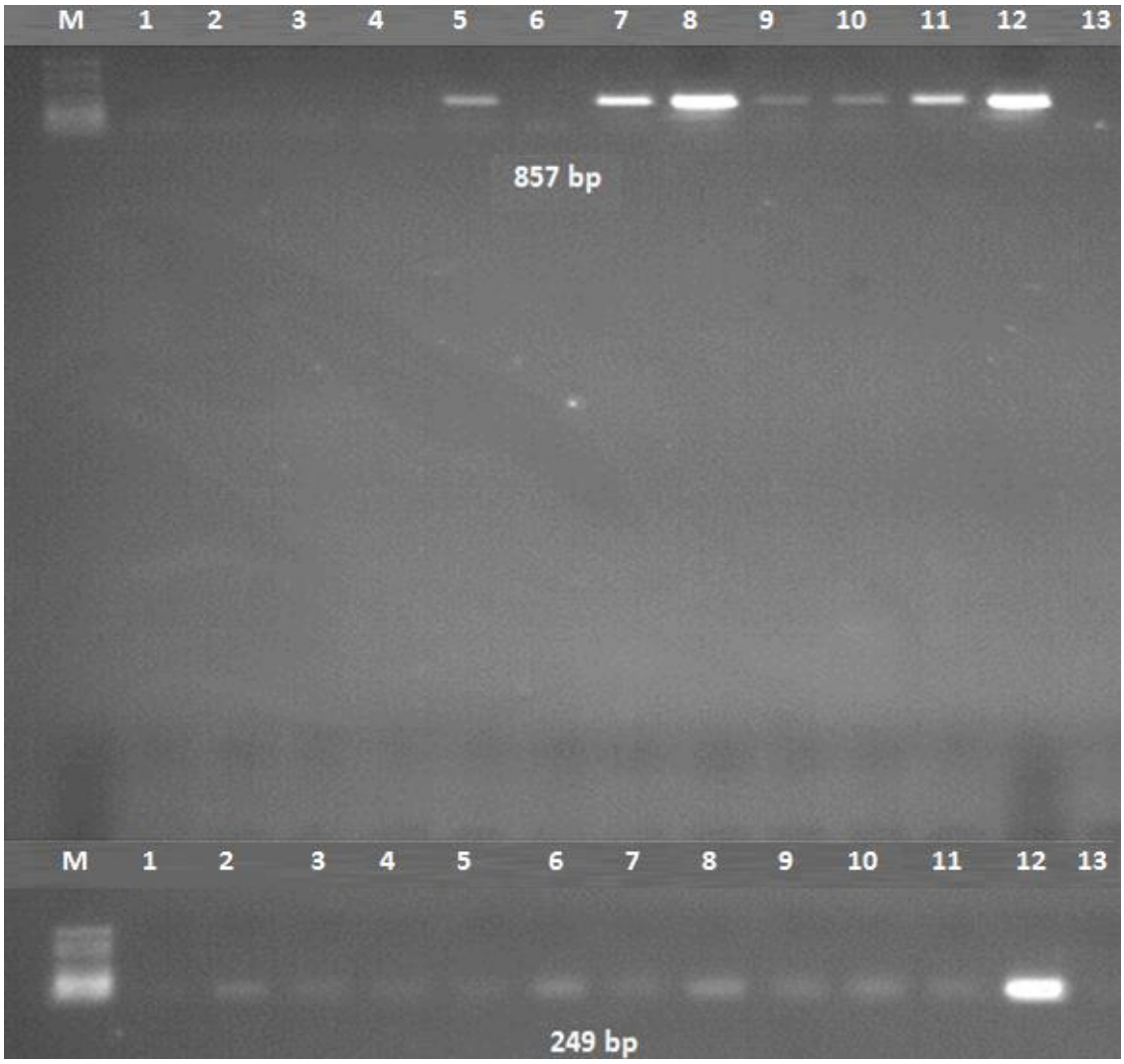


Figure 5.5 PCR amplification results (broth culture PCR assay) for detection *C. jejuni* and *Pseudomonas* spp.

M: 100 bp-DNA molecular weight; upper lanes 5, 7, 8, 9, 10, and 11: products of 857 bp of *C. jejuni* 16S rRNA gene; lane 12: positive control (*C. jejuni*); lane 13: no template control (dsH_2O).

Lower lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11: products of 249 bp of *Pseudomonas* spp. *oprI* gene; lane 12: positive control (*Pseudomonas aeruginosa*); lane 13: no template control (dsH_2O).

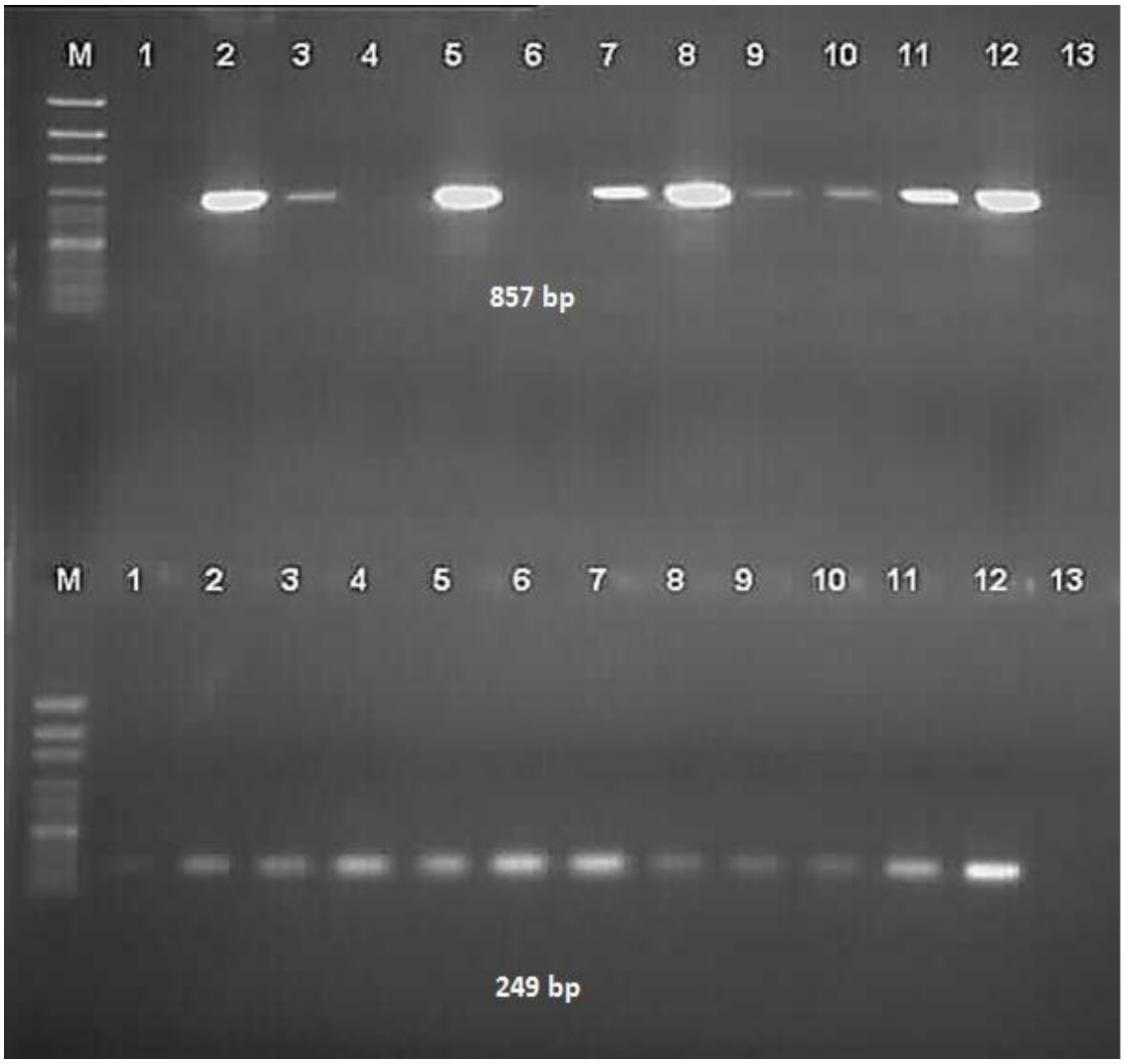


Figure 5.6 Amplification results of both the environmental and plate culture PCR for detection of *C. jejuni* and *Pseudomonas* spp.
M: 100 bp-DNA molecular weight; upper lanes, 2, 3, 5, 7, 8, 9, 10, and 11: products of 857 bp of 16S rRNA gene from *C. jejuni* strains; lane 12: positive control (*C. jejuni*); lane 13: no template control (dsH_2O).
Lower lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11: products of 249 bp of *Pseudomonas* spp. *oprI*; lane 12: positive control (*Pseudomonas aeruginosa*); lane 13: no template control (dsH_2O).

Table 5.4 Amplification results of the environmental PCR assays (plate culture PCR assay and broth culture PCR assay) for detection *C. jejuni* and *P.* spp.

Sample type (section 2.11.1)	**Plate culture result (section 2.11.2)**		**PCR result (section 2.11.3)**	
	C. jejuni (CBA)	*Pseudomonas* spp. (C-N agar)	*C. jejuni* (857 bp band)	*Pseudomonas* spp. (249 bp band)
Trough 1	-	+	-	+
Trough 3	+	+	-	+
Trough 6	-	+	-	+
Trough 8	-	+	-	+
Trough 12	+	+	+	+
Ditch 1	-	+	-	+
Ditch 3	+	+	+	+
Cuckoo lane	+	+	+	+
Pond 9	-	+	+	+
Puddle 7	-	+	+	+
Puddle 6	+	+	+	+

5.3 Discussion

This was a limited preliminary study aimed at looking for evidence that molecules secreted by *Pseudomonas* spp. could modulate the behaviour of *C. jejuni*. Enhanced growth in laboratory media of *C. jejuni* (ST-42) was found in the presence of supernatants from some species of *Pseudomonas*, but the effects were species specific. In a previous study, Hilbert *et al* demonstrated that strains of *C. jejuni* survived longer under aerobic conditions when co-incubated with spoilage bacteria (*P. putida*), and that survival varied depending on both the strain of *Campylobacter* and the strain of *Pseudomonas* used (Hilbert et al., 2010). Here, we demonstrated effects that varied according to the species of *Pseudomonas* used, but only one strain of *C. jejuni* was tested. In order to determine whether this interaction is generally

applicable, it would be necessary to test a wider range of *C. jejuni* strains, representing different sources and clonal complexes. Given the work presented in previous chapters, where variations between strains of *C. jejuni* with respect to survival in water were evident, it is likely that different strains would also exhibit different responses with respect to this interaction with *Pseudomonas*.

The greatest effect was seen with the supernatant of *P. syringae* pv. *glycinea* 49a/90 isolated from field grown plants of soybean cv. Maple Arrow at Dornburg near Jena, Germany in 1990 (Ullrich et al., 1993). This organism causes bacterial blight of soybean and can produce a phytotoxin called coronatine. Effects were also seen with other pathovars of *P. syringae*, such as *lachrymans*, *avellanae* and *antirrhini*, but were not so clear with some of the more common environmental species of *Pseudomonas* that are not as closely associated with plants, such as *P. putida* or *P. fluorescens*. Hence, the significance of the effect on *Campylobacter* is not clear. It would be important to test a wider range of *Pseudomonas* isolates, including some found in environmental samples where *Campylobacter* are also present. Here, we demonstrated that, in the environmental samples tested, wherever *Campylobacter* was identified, *Pseudomonas* species were also present, consistent with the idea that co-existence with *Pseudomonas* may act as a survival strategy for *Campylobacter* outside a host body (Hilbert et al., 2010).

The work presented in this chapter suggests that the active component secreted by *P. syringae* pv. *glycinea* 49a/90 is proteinaceous, given that it is sensitive both to boiling and to proteinase K treatment. Little is known about the secretome of this organism, beyond the non-protein phytotoxin coronatine. However, various *P. syringae* pathovars do carry the Hrp type III secretion system implicated in plant pathogenicity

(O'Brien et al., 2011). It has been shown that secretion of virulence factors in several plant pathogenic bacteria is thermoregulated. For example, Coronatin toxin in *P. syringae* pathovars acts as a virulence factor, most likely by enhancing ethylene biothensis in plant (Kenyon and Turner, 1992). The yield of coronatine was increased after a 7-day incubation of *P. syringae* pv. *glycinea* broth cultures at 18°C, and the toxin yields were reduced to minimal at higher temperatures (30 °C) which could be due to reduced synthesis of the enzymes involved in the synthesis of the toxin (Palmer and Bender, 1993). Similarly, it has been demonstrated that secretion systems play an important role in the survival of *P. aeruginosa*, human opportunistic pathogen, at lower temperatures (below 37 °C) by decreasing the channel size of the major outer membrane porin (OprF) at suboptimal temperatures (Jaouen et al., 2004). Moreover, this thermoadaptation is possibly specific for proteins secreted by the T1SS (APrP), T2SS (PrpL), or T6SS (HcpB) pathways and thus facilitate nosocomial infection at room temperature (25 °C) (Termine and Michel, 2010)

Indeed the *P. avellanae* secretome has been characterised (O'Brien et al., 2012). However, induction of this system is tightly controlled and it should not be active under the conditions used here to grow the strains. Although *P. syringae* do have QS systems (Table 5.2), the signal molecule is not a protein. Therefore, it seems unlikely that the interaction is due to cross talk between the *Pseudomonas* QS system and the *Campylobacter* LuxS QS system. To resolve the nature of the active protein component, it would be necessary to purify protein from the *Pseudomonas* supernatants, repeating the assay on extracts to confirm that the active component is still present. This was beyond the scope of the work here, but would be an interesting avenue to follow. If the active protein was identified, its effect on other

Campylobacter phenotypes could be characterised, including potential effects on survival in model systems more reflective of the real environment in which the two organisms co-exist.

For detection of *Pseudomonas* bacteria in the environmental samples, oligonucleotide primers were used to amplify a 249 bp of the *oprI* gene-specific to fluorescent *Pseudomonas* spp. The *oprI* gene encodes lipoprotein I (OprI) which is one of the abundant proteins present in the outer member of fluorescent *Pseudomonas* spp., has a low molecular-weight (8 kDa) and may only be expressed in rRNA group I *Pseudomonas*. It can be used in phylogeny of the genus *Pseudomonas* (De Vos et al., 1998; De Vos et al., 1993; Duchêne et al., 1989). However, further studies would be needed in order to characterise the species of *Pseudomonas* present, either by using more specific PCR assays, or by sequencing *oprI* amplicons and screening against sequence data from known species in the database.

In this study, *Pseudomonas* spp. were detected in all environmental water samples, suggesting, as has been reported, that they are ubiquitous in the environment (Witholt et al., 1992), whereas *Campylobacter* spp. were detectable only in some samples. Amongst the 11 environmental water samples, 8 samples were found to be positive for *Campylobacter* spp. and all 11 samples were found to be positive for fluorescent *Pseudomonas* spp. (Table 5.4 and Figure 5.6). Indeed, several culture-negative samples [Table 5.4 and Figure 5.6; Lane 9 (puddle 9) and lane 10 (puddle 7)] were positive for the PCR test. This may be because of the presence of dead cells in the samples, or because *Campylobacter* were present, but not culturable on the media used. As was demonstrated earlier in this thesis, different *C. jejuni* strains exhibited variations with respect to the retention of culturability in environmental

conditions. In addition, one culture-positive sample [Table 5.4 and Figure 5.6; Lane 2 (trough 3)] for *Campylobacter* was not positive by the PCR test [Table 5.4 and Figure 5.5; Lane 2 (trough 3)]. In this case, the culture-positive status was based on the presence of a single colony on the mCCDA plate, whereas generally multiple colonies were found. This anomalous result could have been due to contamination on the plate.

Previous studies have shown that contaminated poultry meat has been considered as the main source of campylobacteriosis in humans (Nauta et al., 2007). *Campylobacter* as a microaerophilic microorganism has to survive atmospheric oxygen tension on the poultry meat. Co-existence with Pseudomonas spp. is thought to be one of the strategies that is followed by *C. jejuni* to survive atmospheric oxygen tension on chicken meat (Hilbert et al., 2010). It would be interesting to carry out more comprehensive studies, not only on a wider range of environmental samples, but in the poultry meat environment, to better characterise the co-existence of *Pseudomonas* and *Campylobacter*, and to select more relevant model strains for study of the interactions between them.

5.4 Conclusions

In a limited preliminary study, it was demonstrated that *Campylobacter* spp. co-exist with *Pseudomonas* species in the environment (natural water), supporting the notion that interactions between *Campylobacter* with and other microorganisms may enhance its survival in the atmospheric oxygen tensions of the environment. Some *Pseudomonas* strains secrete products that can enhance the growth of *Campylobacter* under laboratory conditions, and the activity was sensitive to boiling and proteinase K treatment, suggesting that the active component may be a protein (or proteins). Further work is needed to characterise the nature of this interaction, the variations that might occur when different strains of *C. jejuni* or fluorescent *Pseudomonas* spp. are tested, and the relevance to interactions that might occur in natural environments.

C h a p t e r 6

GENERAL DISCUSSION

Campylobacter jejuni remains an important and increasing cause of food related illness worldwide, imposing a considerable burden on global economics and posing public health concerns. In the context of the UK in particular, the pathogen remains a key priority, especially in relation to contaminated poultry products as a source of infection. Compared to other enteric bacteria such as *Salmonella* spp. and *E. coli*, less is known about the metabolism, pathogenicity and environmental survival mechanisms of *Campylobacter* (Duffy et al., 2008; Murphy et al., 2006; Young et al., 2007). Yet, there are far more cases of infectious intestinal disease associated with *Campylobacter* than with the other better known bacterial agents of disease.

Various studies have reported the survival characteristics of the pathogen *E.coli* in water. It has been reported that the organism may survive for two months in lakes, troughs, rivers and faecal contaminated puddles. The bacterium can survive for long times at cold temperatures (<10 °C), and can enter a VBNC state when exposed to stresses such as starvation (Avery et al., 2008; Wang and Doyle, 1998). It has also been suggested that water is one of the main reservoirs of spreading *Salmonella* spp. in the environment, where the microorganism can survive long enough to be transmitted (Gorski et al., 2011). Hence, the potential role for the environment in the transmission of zoonotic pathogens is well established.

Although *C. jejuni* is a fastidious bacterium that requires microaerobic conditions for its growth, the organism is widespread in the environment, processed meats and animal reservoirs. It is likely that the environment plays an important role in transmission of *Campylobacter* infections to humans, either directly through

contaminated water, or indirectly by facilitating contamination of livestock, with subsequent transmission to humans (Clark et al., 2003; Murphy et al., 2006; Olson et al., 2008; Park, 2002; Stanley and Jones, 2003). Compared to other bacteria, the relatively small genome of *C. jejuni* lacks many of the genes that are essential for regulating gene expression in response to adverse environmental conditions (Table 6.1), and this has further restricted our knowledge of the organisms, because many of the widespread mechanisms considered important in other foodborne bacterial pathogens, simply do not apply to *Campylobacter*. However, it is clear that *Campylobacter* can survive in the environment and can cause bacterial food borne diseases in humans (Murphy et al., 2006). It has been considered that untreated water is one of the important routes of transmission of the pathogen to humans, and can contaminate drinking water from which water-borne outbreaks may emerge (Clark et al., 2003). It has also been suggested that contaminated surface water of pastures can be a source of contamination of both tap and ground water which may lead to waterborne outbreaks in humans and transmission of *Campylobacter* to poultry flocks via drinking water systems (Jones, 2001; Pearson et al., 1993; Richardson et al., 2007; Van de Giessen et al., 1996). It has been hypothesized that *Campylobacter* follow diverse mechanisms to survive in the environment, such as formation of biofilm, entering a VBNC state or coexistence with other microorganisms (Hilbert et al., 2010). However, our understanding of these processes remains at a relatively low level.

C. jejuni has been isolated from diverse animal and environmental sources where it has adapted to specific ecological niches. Using MLST analysis, it has been shown that whilst some MLST clonal complexes (CCs), such as ST-21 complex, are broadly

distributed, some others such as members of ST-45 complex, which are frequently isolated from human campylobacteriosis, are more predominant in wildlife and environmental water than other common clonal complexes (Colles et al., 2003; Dingle et al., 2005; Dingle et al., 2001; French et al., 2005; Kärenlampi et al., 2007; Manning et al., 2003; McCarthy et al., 2007; Sails et al., 2003; Sopwith et al., 2006).

In this study, a diverse panel of *C. jejuni* strains (from the University of Liverpool) from various sources including human, cattle, poultry, environment, and wildlife, was screened for variations in the ability to survive in sterile distilled water based on culture at 4 °C and 25 °C. The isolates represented different MLST groups, including the broadly distributed MLST clonal complexes (ST-21 and ST-45), clonal complexes found amongst human isolates which are adapted to specific host (cattle associated ST-61), and representatives of water/wild-life isolates (1336) and the bank vole isolates (414). The initial screen which was only based on one biological replicate assigned the strains into various categories based on retaining culturability by enumerating CFU/mL. Representatives of different survival abilities, M1, 1336 and 414 were selected for closer study. This study demonstrated that the survival time in sterile distilled water, based on the ability to retain culturabilty, varies significantly between various strains of *C. jejuni*. Strain M1 is a member of ST-45 clonal complex, which is widespread and is highly prevalent in the environment (Friis et al., 2010). Of the three strains tested, strain M1 declined most rapidly in culturability at both temperatures (4 °C and 25 °C) tested. Each of the three strains underwent rapid decline at 25 °C which is in agreement with other findings (Colles et al., 2003; Guillou et al., 2008). The study also demonstrated that *C. jejuni* strains were able to survive much better at low temperature (4 °C) than warm temperature (25 °C), which is in

agreement with other findings (Buswell et al., 1998; Cools et al., 2003; González and Hänninen, 2012; Korhonen and Martikalnon, 1991).

Experiments were carried out for comparing the same three strains in both autoclaved and non-autoclaved natural water. The results demonstrated that the culturability was better retained in non-autoclaved natural water, which may be due to some component of the natural water system that is degraded by autoclaving, but emphasizes the risks of drawing conclusions from less natural systems.

The result of the LIVE/DEAD staining experiments showed that the rapid loss of culturability might not indicate poor survival, but could be due to the capability of strain M1 to enter more quickly into a VBNC state. This result is in agreement with findings of other studies that suggest *Campylobacter* can enter a VBNC state under stress conditions such as starvation (nutrient-poor water), and this capability varies between different strains (Baffone et al., 2006; Chaveerach et al., 2003; Cools et al., 2003; Rollins and Colwell, 1986; Tholozan et al., 1999).

One key conclusion from these studies is that it is dangerous to assume that the properties exhibited by one strain of a bacterial species apply to all members of that species. For example, it has been shown that the ability of different strains of *C. jejuni* varies in formation of the VBNC state, and that some strains of *C. jejuni* are more frequently isolated from environmental sources (Cools et al., 2003; Hald et al., 2001; Ziprin et al., 2003; Ziprin and Harvey, 2004). Moreover, the variation has also been noted in the capability of these cells to resuscitate and to cause infections (Beumer et al., 1992; Mederma et al., 1992). In addition, the behaviour variation between strains of *C. jejuni* has been noted in biofilm formation. For example, most *C. jejuni* strains possess the *luxS* gene, which encodes AI-2, implicated in formation of biofilms, but

some strains of *C. jejuni* (such as strain 1336) lack the *luxS* gene (Buswell et al., 1998; Hepworth et al., 2011; Plummer, 2012). Hence, any potential role for *luxS* may be strain-specific, rather than applicable to the whole species.

This emphasizes the need to ensure that reported phenotypes or mechanisms described in one strain of *C. jejuni* are in fact shared more generally, before assuming that a property is applicable to all strains of *C. jejuni*. Even the same strain, for example the widely studied NCTC11168, can be subject to considerable variations, leading to phenotypic changes (Gaynor et al., 2004; Revez et al., 2012; Thomas et al., 2014). *C. jejuni* are especially adept at adaptive change because of the existence of contingency loci (Jerome et al., 2011), which can cause significant phase variations and hence phenotypic changes. Indeed, *C. jejuni* is notoriously difficult to work with because it often hard to get consistent replicates. This genomic flexibility and the capacity for rapid adaptive changes, emphasize the need for caution when assuming that observations based on a single strain are widely applicable.

This study postulated that variations in the survival behaviour in sterile distilled water between different strains of *C. jejuni* could de due to differences in expression of stress response genes in the response to adverse environment, including genes involved in the induction of VBNC state. For elucidating the mechanisms underlying the variations between different strains of *C. jejuni* in the ability to retain culturability in water, end-point PCR and Q-PCR assays were applied to target a set of stress response genes, reported previously, at specific time points during survival in sterile distilled water. Q-PCR analysis indicated that there were differences in gene expression levels during survival in sterile distilled water between the different strains of *C. jejuni*. Interestingly, although there were low colony forming unit counts for

strain M1 recovered from water, the *luxS* gene was significantly up-regulated in this strain compared to strain 414 in both the day 3 (4 °C) and day 1 (25 °C) samples.

RNAseq analysis has confirmed the high levels of *luxS* expression during survival of strain M1 in sterile distilled water. These observations are consistent with a role for *luxS* during the conversion of M1 to the VBNC state, and this study reveals for the first time implicating the *C. jejuni* QS system (*luxS*) in a role in survival in water. Given that QS is considered to be a cell density dependent phenomenon, this is an interesting finding. The bacteria are actually diluted during the process of introducing them into the water, yet the QS-related gene is up-regulated. As yet the partner regulatory gene associated with the putative signal molecule that LuxS is responsible for synthesising has not been identified. Various roles for LuxS have been proposed, in oxidative stress, flagellar gene expression, ability to colonise animals, CDT expression and virulence in an animal model (Plummer, 2012). However, our observations suggest that LuxS may not be part of a conventional QS system, or may have other characteristics. A role for QS in survival has been reported for *Vibrio cholerae* in relation to biofilms exposed to seawater (Joelsson et al., 2006). More recently, it has been reported that QS signal molecules can revive *V. cholerae* from a dormant state in water (i.e., recovery from VBNC state) http://www.ncbi.nlm.nih.gov/pubmed/23716683. QS has also been implicated in biofilm formation by *Burkholderia*, *Vibrio* and *Listeria* pathogens (Belval et al., 2006; Engebrecht et al., 1983; Riedel et al., 2001). An interesting interaction between LuxS, flagella and autoagglutination in *C. jejuni* has been reported (Jeon et al., 2003). This implied that the LuxS molecule may play a role in the regulation of motility and agglutination or the formation of surface structures in *C. jejuni*. Our study, coupled to parallel RNAseq

data indicating the up-regulation of flagellar genes (C. Bronowski and C. Winstanley, unpublished) suggests that, LuxS plays a role in the survival of strain M1 in water, and that this may be due to interactions with flagella and autoagglutination activity. However, further work is needed to resolve the precise mechanisms underlying our observations.

Co-existence of *C. jejuni* and *Pseudomonas* species is one of the survival strategies of *C. jejuni* outside the host body and during food contamination (Hilbert et al., 2010). *Pseudomonas* species such as *P. fluorescens*, *P. franji*, *P. chlororaphis* and *P.putida* can be found on poultry meat, and are known spoilage bacteria that are generally distributed in a wide range of environmental niches (Ghafir et al., 2008; Hilbert et al., 2010; Mead et al., 1993; Russell et al., 1995; Silby et al., 2011). In order to overcome the atmospheric oxygen stress and to enhance the aerotolerance of *C. jejuni*, this organism interacts with aerobic *Pseudomonas* species found in natural environmental niches or on food (Hilbert et al., 2010).

In a limited preliminary study of *in vitro* interaction of *C. jejuni* (ST-42) with supernatants of *Pseudomonas* spp., we demonstrated that molecules secreted from some species of *Pseudomonas* could enhance the growth of *C. jejuni*, and the effects were species-dependant. Although only one strain of *C. jejuni* was investigated, the survival varied according to the species of *Pseudomonas* used. In a previous study of interaction between *C. jejuni* strains with species of *Pseudomonas* under aerobic conditions, Hilbert *et al*. reported the survival variations were dependant on the strain of *Campylobacter* and the strain of *Pseudomonas* used (Hilbert et al., 2010). In this thesis the growth of *C. jejuni* ST-42 was significantly enhanced by the effect of most of the supernatants of the *Pseudomonas* strains, and the greatest effect was

evident with the supernatant of *P. syringae* pv. *glycinea* 49a/90. Environmental PCR assays demonstrated co-existence of *Campylobacter* spp. and fluorescent *Pseudomonas* spp., which occurs in natural water, and showed that wherever *Campylobacter* was found, *Pseudomonas* species were also present. These results are compatible with the hypothesis that co-existence with *Pseudomonas* may act as a survival strategy for *Campylobacter* in the environment (Hilbert et al., 2010). This experiment demonstrated that the molecules secreted from *P. syringae* pv. *glycinea* 49a/90 and involved in the interaction with *C. jejuni* were sensitive both to boiling and to proteinase K treatment, suggesting that the active component in the secretome was proteinaceous. However, although little is known about the secretome of this bacterium, it is unlikely that interaction is due to cross talk between the *Pseudomonas* QS system and the *Campylobacter* LuxS QS system, given that the signal molecule is not a protein.

Table 6.1 Key regulators of the stress defence system found in *E. coli* are absent in *C. jejuni.*

Gene	**Function**	**Presence in**	
		E. coli	***C. jejuni***
Oxidative stress gene			
soxRS	superoxide stress regulator	+	-
oxyR	superoxide stress regulator	+	-
sodA	Encodes Manganese SOD	+	-
katG	Encodes catalase, hydroperoxidase I (HPI)	+	-
Osmoregulator gene			
proU or *opuC*	Induced by high osmolarity due to the excluded solutes by the cell membrane	+	-
otsAB	Involved in trehalose synthesis for osmoregulation	+	-
betAB or *gbsAB*	Encode enzymes for converting choline to glycine betaine for osmoregulation	+	-
Stationary phase / starvation gene			
rpoS	Encodes a sigma factor required for the stationary phase stress resistance and for starvation survival in Gram-negative bacteria	+	-
Heat and cold shock gene			
rpoH	Encodes sigma factor which involved in heat shock response	+	-
cspA	Encodes protein essential for cold shock response	+	-
Global regulation gene			
lrp	Encodes transcriptional regulators for expression of a group of genes to regulate metabolic pathways in response to the availability of amino acids and nitrogen	+	-

Data adapted from (Park, 2002).

Future work

The present study suggests that survival in water varies significantly between different strains of *C. jejuni.* However, more work is needed in this area (survival of *C. jejuni* strains in sterile distilled water and natural water) in order to fully characterise the extent of survival variations between different strains of *C. jejuni*, and to better establish whether there are clear associations between particular clonal complexes and the rate of loss of culturabilty, and whether this correlates with observations about the relative prevalence in environmental samples. Also, it is important to screen further numbers of isolates from different sources and representing different clonal complex, to identify any clear relationships between these survival variations in the environment and the epidemiology of *Campylobacter* infections in humans.

This study provides clear evidence for expression of *luxS* in VBNC populations of *C. jejuni* (strain M1). However, further work is necessary in the area of gene expression during survival in sterile distilled water, in order to indicate the role of other stress response genes that may play a role in oxidative stress in *C. jejuni* such as *katA*, *sodB* and *msrA/B,* and to determine the precise role of the QS system in the variations observed in strain M1 compared to strain 414 with regard to loss of culturability, survival strategies and the conversion to the VBNC state. Here, some of these variations have been established, but further work is needed to fully identify the mechanisms underlying the differences between strains.

Some of this work is already being carried out. Indeed a comprehensive set of RNAseq data for the M1 survival experiments is currently being analysed in order to better

understand the mechanisms involved and to design follow up experiments to test the hypotheses generated

This study provides evidence for laboratory-based interaction between *C. jejuni* (ST-42) and *Pseudomonas* species in which the effects were species specific. Therefore, in order to determine general applicability and to characterise the nature of this interaction, future work is necessary to repeat the experiment of laboratory interaction assay between *C. jejuni* and *Pseudomonas* spp. under aerobic conditions at 4 °C and 20 °C, to test different strains of *C. jejuni* representing different sources and clonal complexes such as M1 (ST-45), 11168 (ST-21), 1336 (wild bird isolate) and strain 1107 (cattle isolate). Strains from poultry meat surfaces could also be identified rapidly using hippurate hydrolysis, according to the methods of Hwang and Ederer (Hilbert et al., 2010; Hwang and Ederer, 1975) and further characterized by PCR analysis of the hippuricase gene *hip* (Linton et al., 1997). The identified isolates, which are more relevant model strains, can be selected for the interaction assay with the *Pseudomonas* spp. that are found on chicken meat surfaces. The *Pseudomonas* spp. can be identified by plating the diluted chicken exudate on *Pseudomonas* selective agar and incubated under aerobic conditions at 25°C for 48h. Additionally, the colonies are identified by PCR amplification of the gene *oprI* specific for fluorescent *Pseudomonas* spp.

In order to identify the nature of the molecules (proteins) secreted from fluorescent *Pseudomonas* spp. that influence *C. jejuni* behaviour, further work is needed to isolate the relevant protein from the *Pseudomonas* supernatants, for example by repeating the assay on size-fractionated extracts in order to characterise the active protein component. It would also be important to test the potential effects on

survival in model systems more reflective of the real environment in which the two organisms co-exist. For example, co-cultures could be studied in biofilm systems. However, to characterise the species of *Pseudomonas* present in this study, further studies are needed including more specific PCR assays, or by sequencing *oprI* amplicons and screening against sequence data from known species in the database.

In conclusion, it is evident that the environment plays an important role in the transmission of *Campylobacter*, but that there are important strain variations that are poorly understood. We have just started to identify some of the genes that might be important in the various mechanisms underlying *Campylobacter* survival strategies but much more work needs to be done in order to resolve the underlying processes and potentially exploit our knowledge to design better interventions.

APPENDIX 1 (MEDIA)

Medium	Company	Components	
		Formula	**gm/litre**
Muller-Hinton Broth	Oxid	Beef, dehydrated infusion from Casein hydrolysate Starch	300.0 17.5 1.5
Columbia Blood Agar	Oxoid	Special peptone Starch Sodium chloride agar	23.0 1.0 5.0 10.0
Campylobacter growth supplement	LAB M	Sodium pyruvate Sodium metabisulphate Ferrus sulphate	0.125 g 0.125 g 0.125 g
Campylobacter Blood – Free Selective Agar Base	Oxoid	Nutrient BrothNo.2 Bacteriological charcoal Casein hydrolysate Sodium desoxycholate Ferrous sulphate Sodium pyruvate Agar	25.0 4.0 3.0 1.0 0.25 0.25 12.0
Nutrient Broth NO.2	Oxoid	Lab-Lemco powder Peptone Sodium chloride	10.0 10.0 5.0
Pseudomonas Agar Base	Oxoid	Gelatin peptone Casein hydrolysate Potassium sulphate Magnesium chloride Agar	16.0 10.0 10.0 1.4 11.0
Pseudomonas CN selective supplement	Oxoid	 Cetrimide Sodium nalidixate	**Per vial** 100.0mg 7.5mg
Campylobacter Growth Selectavial (FBP)	MAST	Sodium pyruvate Sodium metabissulphite Ferrous sulphate	250mg/litre 250mg/litre 250mg/litre
Campylobacter Enrichment Selectavial (Exeter)	MAST	Trimethoprim Rifampicin Polymyxin Cefoperazone Amphotericin B	10mg/litre 5mg/litre 2500iu/litre 15mg/litre 2mg/litre
Horse Blood, Defibrinated	Oxoid	Horse blood products	
Horse Blood, lysed	Southern group laboratories	Horse blood products	
Chelex	BIORAD	Styrene divinylbenzene copolymers containing iminodiacetate ions	

APPENDIX 2 (BUFFERS)

A. Solutions used during agarose gel electrophoresis

5x TBE buffer

54 g Tris base, 465 g EDTA and 27.5 g orthoboric acid (BDH bioscience) were mixed with sterile distilled water and the volume was made up to 1000 mL. The buffer was sterilised by autoclaving at 121°C for 15 min and was then diluted 1 in 10. 0.5 × TBE was used to make agarose gel.

B. Preparation of Campylobacter growth supplement:

Using a sterile pipette, contents of the vial were dissolved with 5 mL of deionized water and added into 500 mL of prepared medium.

C. preparation of 2% Chelex:

16 gm of Chelex100Resin was transferred into a sterile100 mL borosilicate glass bottle containing 8 mL of Tris HCL and mixed. The suspension was filled up to 80 mL with dH_2O.

D. preparation of Tetracycline solution:

300 µg of Tetracycline powder was added into 20 mL Universal tube containing 10 mL of SDW and dissolved. 1 mL of this stock was added into 1 litre of mCCDA.

E. Preparation of (LB) broth:

LB broth was prepared (per 1 litre) by mixing 5 g of Yeast extract (SIGMA) with 15 g of tryptone water (SIGMA). LB agar was prepared (per 1 litre) by mixing 12 g of Bacteriological agar (SIGMA) with LB broth.

F. preparation of pseudomonas C-N selective supplement:

CN agar was prepared (per 500 mL) by adding 5 mL of glycerol into 500 mL of Pseudomonas Agar Base (Oxoid) and autoclaved at 121°C for 15 min. Both CN supplement (1 vial per 500 mL- Oxoid) and a 2 mL o the mixture (SDW and Ethanol) were added.

G. Preparation of Campylobacter Enrichment Broth (Exeter Broth)

Preparation Single Exeter Broth:

27.5 g of Nutrient Broth NO2 was dissolved in 1100 mL of distilled water and autoclaved at 121°C for 15 min, aseptically the followings were added after cooling to 50°C:

1. 11 mL of Lysed Horse Blood
2. One vial of Campylobacter Growth Selectavial (dissolved in 5 mL SDW) and one vial of Campylobacter Enrichment Selectavial (dissolved in 5 mL SDW).

Preparation Double Exeter Broth:

27.5 g of Nutrient Broth NO2 was dissolved in 550 mL of distilled water and autoclaved at 121°C for 15 min, aseptically the followings were added after cooling to 50°C:

1. 11 mL of Lysed Horse Blood
2. One vial of Campylobacter Growth Selectavial (dissolved in 5 mL SDW) and one vial of Campylobacter Enrichment Selectavial (dissolved in 5 mL SDW).

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