Physiological adaptations contributing to stress survival in the foodborne pathogen *Campylobacter jejuni*

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ABSTRACT

In spite of being considered fragile and fastidious, the zoonotic pathogen *Campylobacter jejuni* remains the leading cause of foodborne bacterial gastroenteritis in the developed world. Lacking many of the stress responses common to other enteric pathogens, *C. jejuni* employs the survival strategies, biofilm formation and entry into the viable but non-culturable (VBNC) state, which have not been well characterized. Recent studies have indicated that these strategies are likely related at the molecular level.

The purpose of this thesis was threefold: 1) to characterize entry into the VBNC state for planktonic and biofilm cells of *C. jejuni* with starvation at 4°C; 2) to evaluate a novel PMAqPCR method to quantify viable cells (both culturable and viable but non-culturable) in planktonic and biofilm cells of C. jejuni during starvation at 4°C; and 3) to investigate changes in gene expression of selected genes involved in biofilm formation and entry into the VBNC state. The three strains *C. jejuni* NCTC 11168 V1, *C. jejuni* NCTC V26 and *C. jejuni* 16-2R were included in all studies to compare variation based on strains.

Cells were considered VBNC when there was no growth with enrichment, but cells scored as viable based on membrane integrity. Biofilm cells which became VBNC in some cases after 10 days of stress were found to enter the VBNC state earlier than planktonic cells by 10 to 50 days. Additionally, no significant reductions occurred in viable cell counts over the course of the experiments, confirming that the loss of culturability was not due to cell death ($p<0.05$).

To date, no methods have been used to quantify viable but non-culturable biofilm cells of *C. jejuni.* The novel method PMAqPCR which has been successful for the enumeration of planktonic *C. jejuni* as well as for biofilm cells of other species was validated for quantifying *C. jejuni* biofilm cells in late log phase (20 h) and once cells had entered the VBNC state.

The genes that affect both entry into a VBNC state and the ability to form biofilm in *C. jejuni* were upregulated during biofilm formation. Gene expression prior to stress treatment was 5 to 37 fold higher in biofilm cells than in their planktonic counterparts for all three strains

($p<0.001$). For the planktonic samples, only one of the 3 strains showed significant changes in gene expression during the transition to the VBNC state. In this case, all 4 target genes were significantly upregulated 4-6 fold just prior to cells becoming VBNC ($p<0.05$).

At present food and drinking water safety in Canada continues to be assessed primarily using culture-based methodology. As validated in this thesis, the ability to quantify both culturable and viable but non-culturable *C. jejuni* cells in both planktonic and biofilm forms will allow for improved evaluation of quality control methods in both research and industries where these pathogens are a concern. Also, the understanding of the interaction between biofilm formation and entry into the VBNC state at the molecular level described herein provides information which can be used to develop appropriate interventions and reduce the incidence of campylobacteriosis.

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CHAPTER 1: Literature Review

1.1 Introduction

The first recorded isolation of *Campylobacter jejuni* was in 1886 by Theodor Escherich from the stools of an infected infant who had died of what he called 'cholera infantum' (Skirrow and Blaser, 2000). He was unable to culture the organisms and therefore could not conclude they were the causative agent. It was not until the late 1970's, when the collaboration of Dr. Dekeyser, from the National Institute of Veterinary Medicine and Dr. Butzler, from St. Pierre University Hospital enabled the culturing of this fastidious organism from human feces, using methods common in veterinary medicine (Skirrow and Blaser, 2000). Since that time, *C. jejuni* has been recognized as a significant cause of gastroenteritis in humans.

C. jejuni is a Gram-negative epsilon-proteobacterium; the causative agent of campylobacteriosis, an infection characterized by severe abdominal pain, diarrhea, chills and fever that typically lasts 8–10 days. Infections tend to be self-limiting, but can lead to more serious sequelae, such as Guillain-Barré Syndrome and Reactive Arthritis (Skirrow and Blaser, 2000).

C. jejuni is a sensitive and fastidious pathogen with specific growth requirements. As an obligate microaerobe, it requires reduced levels of oxygen for growth. It is also capnophilic, so growth is enhanced with elevated carbon dioxide levels. Since it is asaccharolytic and unable to metabolize sugars, it requires specialized media for culturing. It is thermophilic and will only grow between 30°C and 47°C and is sensitive to pH and drying (Skirrow and Blaser, 2000).

Compared to other intestinal pathogens, *C. jejuni* has a small genome, with 1,641,481 base pairs (bp), encoding 1,654 proteins and 54 stable RNA species (*Campylobacter jejuni*, 2014). *Salmonella* and *Escherichia coli* both have larger genomes. Pathogenic *E. coli* O157:H7 has 5,440,000bp encoding 5,416 proteins. *Salmonella enterica* Enteritidis has 4,686,000bp and *S. enterica* Typhimurium has 5,067,000bp (*Salmonella*, 2014).

In spite of being more sensitive to environmental stresses and having fewer proteins than other enteric pathogens, *C. jejuni* remains the leading notifiable cause of enteric food- and waterborne diseases in Canada, with 10,174 cases reported in 2012 (Notifiable Diseases On-Line, 2014). In fact, reported cases of *C. jejuni* exceed reported levels for other important foodborne pathogens in other industrialized nations as well, with 20 to 150 cases per 100, 000 individuals reported annually (Olson et al., 2008). Although, recent analysis shows that there has been a downward trend in the incidence rate in Canada since 2000, the incidence rate per 100,000 in 2012 for *C. jejuni* (29.3) still remains higher than its closest competitor, *Salmonella* (19.67) or either *Shigella* (3.08) or verotoxic *E. coli* (1.94) (Fig. 1.1) (Notifiable Diseases On-Line, 2014).

Given the reduced genome size, it is not surprising that *C. jejuni* lacks many of the stress response systems commonly found in other enteric pathogens (Park, 2002). The fact that it manages to survive both *in vivo* host defenses and transmission-related environmental stresses is likely due to its ability to form biofilm and enter into a VBNC state. Although these phenotypes are central to the survival of *C. jejuni*, neither has been well characterized.

What follows is a review of the current literature, beginning with a description of *C. jejuni* in food and water, which are the main sources of infection. The next two sections cover the main survival strategies, namely biofilm formation and the viable but non-culturable state. Although limited, the understanding of the interactions between these two survival strategies at the molecular level is discussed. The literature review ends with a discussion of the rationale for the work done and the research objectives of this thesis.

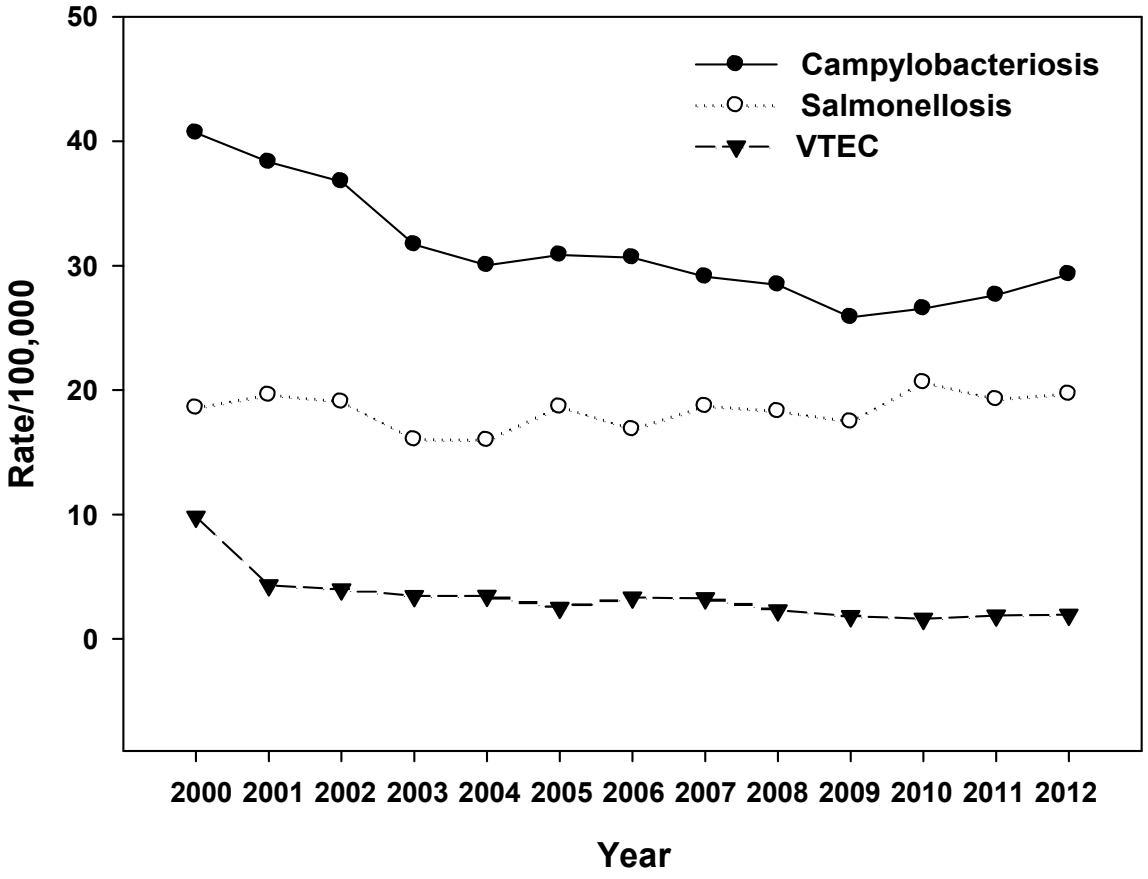

Figure 1.1. Rate per 100,000 of reported cases over time in Canada**,** both sexes (including unknown), all ages, 2000-2012 (http://dsol-smed.phac-aspc.gc.ca/dsol-smed/ndis/charts.php?c=pl).

1.2. *Campylobacter* in food and water

Poultry is the main reservoir for *C. jejuni* and the majority of human infections occur as sporadic cases associated with the consumption or handling of contaminated poultry (Alter and Scherer, 2006; Uyttendaele et al., 2006; Tam et al., 2009; Nguyen et al., 2012). When outbreaks do occur, they are usually linked to water, either due to a breakdown in treatment or as a result of cross contamination with untreated water (Pitkanen, 2013). The persistence of *C. jejuni* in poultry rearing and processing environments is a major concern and will be reviewed here, followed by a description of issues related to *C. jejuni* in drinking water.

C. jejuni likely survives the disinfection procedures commonly used in poultry production by existing within a biofilm or entering the VBNC state (Sparks, 2009; Nguyen et al., 2012). These phenotypes are known to provide protection from various stresses and may help to explain the prevalence of *Campylobacter* in the food chain.

1.2.1. Poultry rearing

C. jejuni is prevalent in poultry rearing facilities and difficult to eradicate (Nguyen et al., 2012). Newly hatched chicks are free of *Campylobacter* for 2 to 3 weeks (Newell and Fearnley, 2003), but when present in the environment, *C. jejuni* will readily colonize the cecum of chickens, residing there asymptomatically (Mead et al., 1995). Since chickens are coprophagic, *C. jejuni* spreads very quickly within flocks (Newell and Fearnley, 2003).

Attempts to determine the sources of *Campylobacter* on poultry farms have included assessment of both vertical and horizontal transmission. Vertical transmission from parent to progeny appears to play an insignificant role as various studies have shown that progeny of *Campylobacter*-positive parents were culture-negative and the recovery of identical isolates from parent and progeny flocks is rare (Newell et al., 2011; Hermans et al., 2012). Horizontal transmission appears to be the normal route and various sources of transmission have been considered, including feed, litter, water, incomplete disinfection between flocks when prior flocks

were contaminated, vermin, insects, proximity of livestock, human contact and contact with equipment (Newell and Fearnley, 2003).

Feed and litter have been found to be *Campylobacter*-free (Pearson et al., 1993), but they can be contaminated by exposure to colonized flocks (Pearson et al., 1993; Bull et al., 2006; Kassem et al., 2010). Although *Campylobacter* can be isolated from bovine faecal matter, transmission from livestock is rare (Bull et al., 2006; Johnsen et al., 2006; McDowell et al., 2008) and the greatest risk is from other poultry on the same site. Wild mammals, pets, birds and insects harbour *C. jejuni*, but their role in transmission to poultry is unclear due to limited and contradictory results (Shreeve et al., 2000; Hermans et al., 2012). *Campylobacter* is widespread in the environment around poultry houses (Hansen et al., 2007; Ridley et al., 2011) and the strains which persist in the external environment are considered a relevant source of flock colonization (Newell et al., 2011; Hermans et al., 2012).

Campylobacter can survive in water for weeks to months (Blaser et al., 1980; Lazaro et al., 1999) and isolates collected from standing water in puddles and ditches were genotypically identical to those from broilers (Hiett et al., 2002; Rivoal et al., 2005; Bull et al., 2006; Johnsen et al., 2006; Hansson et al., 2007; Messens et al., 2009). In most studies the isolates were detected concurrently, preventing determination of source, but in one study, an isolate found in ditch water prior to colonization was identical to those isolated later from broilers, indicating that the direction of transmission was from water to birds (Messens et al., 2009).

Human traffic is also an important vehicle for the transport of *Campylobacter* into the poultry house. *C. jejuni* is isolated from clothes, hands and boots of farm staff, catchers and transporters (Herman et al., 2003; Ramabu et al., 2004; Ellerbroek et al., 2010) and the strains are often identical to those of colonized poultry (Johnsen et al., 2006; Ridley et al., 2011). Depopulation/thinning is often done by independent catching crews which can result in the transport of *C. jejuni* between farms (Allen et al., 2008). Also, transport crates used during

thinning or during transport to the abattoir have been found to harbour *C. jejuni* even after cleaning (Ellerbroek et al., 2010; Ridley et al., 2011).

The importance of the contribution of the poultry water system to recurring contamination of flocks has traditionally been overlooked. This is likely related to the difficulty of detecting *C. jejuni* in water, either due to insufficient sampling volume or because cells enter a VBNC state (Zimmer et al., 2003; Sparks, 2009).

Survival of *C. jejuni* in water is enhanced by the presence of biofilms and the ability of certain *C. jejuni* strains to enter the VBNC state (Buswell et al., 1998; Trachoo et al., 2002; Lehtola et al., 2006; Hanning et al., 2008; Sparks, 2009; Hermans et al., 2012; Kudirkiene et al., 2012). In fact, the low nutrient conditions within the water lines are consistent with those known to induce the VBNC state and extend survival experimentally (Buswell et al., 1998; Cappelier et al., 1999; Lazaro et al., 1999).

It is not surprising that numerous attempts to isolate *C. jejuni* within the poultry water system using culturing have been unsuccessful (Kapperud et al., 1993; Gregory et al., 1997; Hiett et al., 2002; Ring et al., 2005; Hansson et al., 2007; Hansson et al., 2010). Detection of VBNC cells requires molecular methods often involving specialized staining and microscopy, and studies employing such methods are limited (Cappelier et al., 1999; Gangaiah et al., 2009).

In one study, the use of molecular methods and microscopy made it possible to locate non-culturable *C. jejuni* throughout the poultry water system: from the soil-water interface at the bottom of the borehole to the pipework within the chicken sheds, as well as within biofilms which were found to be prevalent and thick within the water supply system (Pearson et al., 1993). In this same study, improved hygiene, including drinking water chlorination and regular cleaning and disinfection of the water system led to a dramatic reduction from 81% to 7% of birds being colonized and a 1,000 to 10,000-fold reduction in *Campylobacters* recovered from carcasses post-slaughter (Pearson et al., 1993).

Other studies have also found that chlorination of poultry drinking water (Arsenault et al., 2007) or use of chlorinated municipal water (Guerin et al., 2007) were successful at reducing the incidence of flock colonization, but in one study, chlorination of flock drinking water had no effect on reducing colonization (Stern et al., 2002), and in some cases the treatments used to disinfect the water lines, acidification and chlorination, were found to increase the risk of colonization (Refregier-Petton et al., 2001; Stern et al., 2002; Jansen et al., 2014). This may be explained by the fact that exposure to weak organic acids like those used in water line disinfection actually induce the VBNC state in *C. jejuni* (Chaveerach et al., 2003; Smigic et al., 2009).

Hygiene scores have correlated directly with flock positivity (Johnsen et al., 2006) and poultry house cleaning has been found to be efficient at inactivating *Campylobacter*. In houses practising good hygiene, carryover from one flock to a subsequent flock in the same house was found to be rare (Gibbens et al., 2001; Colles et al., 2008; McDowell et al., 2008). However one farm with good hygiene barriers delivered more than 60% *Campylobacter*-positive slaughter batches between 2001 and 2003, but when ultraviolet light irradiation of the water was introduced, the *Campylobacter* incidence decreased to less than 10% positive slaughter batches per year (Hansson et al., 2010) confirming that *C. jejuni* in the poultry water system is a significant source of contamination (Herman et al., 2003). Studies have identified that isolates from the water supply and drinking nipples were identical to those colonizing the poultry (Bull et al., 2006; Messens et al., 2009; Cokal et al., 2011), but the sequence of contamination was not clear and the water may have been contaminated by the colonized poultry.

Early work using fluorescence *in situ* hybridization (FISH) with *C. jejuni*-specific rRNA and peptide nucleic acid (PNA) probes detected *C. jejuni* in biofilms within simulated water systems up to 20 days after cells had become non-culturable (Buswell et al., 1998; Lehtola et al., 2006). Similar methods detected non-culturable *C. jejuni* in samples taken from the inner surface of a poultry water line (Cokal et al., 2011). Although viability was not assessed in these studies,

the authors suggested that the extended presence of *C. jejuni* might be due to cells entering the VBNC state. This is a particular concern since VBNC *C. jejuni* remain able to colonize chicks (Stern et al., 1994).

The presence of *C. jejuni* in the flock drinking water lines and reservoirs, especially in the VBNC state and/or in biofilms presents a major challenge for sanitation. Both cells within biofilms and VBNC cells are more resistant to cleaning and disinfection (Alter and Scherer, 2006; Nguyen et al., 2012) and can act as a reservoir for the contamination of future flocks.

Although various sources of transmission in poultry houses have been investigated, and have been found to play a role in contamination of new flocks, efficient control measures appear to be lacking. In order to accurately assess the significance of the various sources and determine the efficacy of *Campylobacter* reduction practices, non-culture based methods for the detection and enumeration of all viable *Campylobacters* are required. These limitations are addressed in this thesis, where the development and optimization of methods to detect viable *Campylobacter* are investigated.

1.2.2. Poultry meat processing

C. jejuni enters slaughtering and poultry processing facilities during the processing of contaminated flocks. Numerous interventions are in place to reduce the load of *C. jejuni* in poultry meat processing environments. These include scalding, chilling, irradiation and the use of antibacterial agents such as organic acids and chlorine (Sparks, 2009). However, these interventions are not completely effective and *C. jejuni* is often detected on retail poultry meat (Nguyen et al., 2012). In Canada, an average of surveys done between 1998 and 2007 found that 48.5 % of retail poultry was contaminated. Regional variation exists with 36% and 40% in Quebec (Nadeau et al., 2002) 62% in Alberta (Bohaychuk et al., 2006) and 46%, 47% and 59.6% in Ontario (Galanis, 2007; Cook et al., 2009; Deckert et al., 2010). Elsewhere rates vary from 60% in Japan (Suzuki and Yamamoto, 2009), to 79% in the United States (Nannapaneni et al., 2005) and 90% in the United Kingdom (Moran et al., 2009).

During slaughter, there are several processes which can lead to contamination. Meat may be contaminated during evisceration, through cross-contamination in the scalding water or as a result of contact with equipment (Johnsen et al., 2006; Peyrat et al., 2008). In some cases, if flocks were *Campylobacter*-free upon arrival at the abattoir, the broiler carcasses were also uncontaminated (Ellerbroek et al., 2010), but negative flocks have also been contaminated at the slaughterhouse (Herman et al., 2003), and sometimes the processing water was found to be contaminated prior to slaughtering (Ellerbroek et al., 2010). Delivery of *Campylobacter*-positive birds led to contamination throughout the processing line and resulted in all carcasses being contaminated (Herman et al., 2003; Ellerbroek et al., 2010).

Due to the high numbers of *C. jejuni* present in ceca of colonized birds (up to 10^9 CFU/g faeces), it is likely that contamination occurs during evisceration (Newell et al., 2011). Genotyping studies found that isolates from the ceca of broilers are very similar to those found on corresponding carcasses (Normand et al., 2008). However, in some cases, isolates from food contact surfaces have been more prevalent on the broiler meat than the strains isolated from the ceca (Kudirkiene et al., 2012) and this may be related to the strain's ability to form biofilm. Biofilms can form on food contact surfaces (Trachoo and Frank, 2002; Cools et al., 2005; Young et al., 2007; Fravalo et al., 2009; Gunther and Chen, 2009; Sulaeman et al., 2010) as well as directly on meat (Chantarapanont et al., 2004; Jang et al., 2007) and pre-existing biofilms may enhance attachment (Hanning et al., 2008; Sanders et al., 2008). *C. jejuni* within biofilms are resistant to treatment with various chemical sanitizers, including chlorine, quaternary ammonia, peracetic acid and peroctanoic acid (Yang et al., 2001; Trachoo et al., 2002; Chantarapanont et al., 2004; Northcutt et al., 2005) and can survive overnight on food contact surfaces even after cleaning (Peyrat et al., 2008). In a study where ten strains of *C. jejuni* were isolated from food contact surfaces in a broiler meat production chain, the two strains which were isolated after disinfection were found to be significantly better at forming biofilm than the other strains (Kudirkiene et al., 2012).

Our understanding of the epidemiology of *Campylobacter* with respect to poultry is growing, but at present there are no known interventions available to prevent or reduce *Campylobacter* colonization in broilers (Hermans et al., 2011) nor the contamination that occurs during processing (Hermans et al., 2012). One goal of this thesis is to improve the understanding of survival mechanisms in *C. jejuni*. Understanding the relationship between biofilm formation and entry into a VBNC state may provide targets for intervention strategies, which have been overlooked in the past.

1.2.3. *Campylobacter* in drinking water for human consumption

Another relevant source of human infection is contaminated drinking water. A review of outbreak data linked to drinking water in Canada over a 30 year period found that of 288 waterborne enteric outbreaks, 99 were from public water systems, 138 were semi-public and 51 were private water systems. In the survey, *Campylobacter* (24 outbreaks) was second only to *Giardia* (51 outbreaks) in the list of causative agents (Schuster et al., 2005). Drinking water outbreaks can occur as a result of treatment failure, or if treated water becomes contaminated by untreated water, as a result of cross-connection with wastewater infrastructure or due to heavy rainfall overwhelming the disinfection procedures (Pitkanen, 2013).

Campylobacter spp. are often isolated from environmental water, likely due to wastewater effluents, farm animals and birds (Pitkanen, 2013). Most reports of *C. jejuni* in surface water have been qualitative (Pitkanen, 2013), but values ranging from 10^5 CFU/L in stream water in Georgia (USA) (Vereen et al., 2007) to 10^7 CFU/L in surface water in Florida (Hellein et al., 2011) have been reported. Also relevant to the levels in surface water are the *C. jejuni* counts in sewage effluents, where numbers can be as high as 10^5 CFU/L (Rechenburg and Kistemann, 2009).

Campylobacter spp. have typically shown improved survival in water at lower temperatures due to their ability to enter a VBNC state (Rollins and Colwell, 1986; Buswell et al., 1998; Lazaro et al., 1999). This adds to the difficulty in detecting these pathogens in water by

standard culturing methods, even with enrichment methods which aid in recovery of injured cells, but do not improve the culturability of VBNC cells (Whitesides and Oliver, 1997; Pitkanen, 2013). However, new PCR-based methods such as those being investigated in this thesis have shown promise for the detection of VBNC *C. jejuni* in freshwater microcosms (Bae and Wuertz, 2012). Improved detection of *C. jejuni* in surface waters which act as sources for drinking water will lead to improved decision making in the event of technical failures or overload of the water treatment system due to heavy rainfall.

The fact that *C. jejuni* can exist within biofilms and enter a VBNC state is relevant to both poultry and drinking water as sources of campylobacteriosis. Developing a better understanding of these survival phenotypes can help to inform and assess effective strategies to reduce the prevalence of *C. jejuni* in the food chain.

1.3. Biofilms

Biofilms are ubiquitous in nature, provide bacteria with protection, allow them to remain in favourable habitats and facilitate gene transfer. Most often biofilms harbour various species of bacteria, a situation where one species may alter the micro-environment, allowing others to flourish.

Biofilm formation by *C. jejuni* is a concern in poultry houses, during poultry slaughter and processing, on poultry meat, in the avian intestinal tract where they reside asymptomatically, and in the human intestinal tract where they cause disease (Sparks, 2009; Newell et al., 2011). Biofilms commonly found within water distribution systems are also a concern, as they can act as a reservoir for pathogens (Schuster et al., 2005; Pitkanen, 2013; Whiley et al., 2013).

This section begins with a description of the five stages of biofilm formation on abiotic surfaces (Fig. 1.2). For each stage there is a description of what is known for the model organism *Pseudomonas aeruginosa* and other organisms where relevant information is available, followed by what is known for *C. jejuni*. This is followed by a description of *C. jejuni* attachment and biofilm formation on cultured cell lines and *ex vivo* intestinal epithelial tissue. Conditions and

genes relevant to biofilm formation are addressed. Strain variation is reviewed and the section concludes with a discussion of the role of biofilm in extending survival by providing protection from stresses.

1.3.1. Biofilm formation on abiotic surfaces

Biofilms are microbial communities, encased in a self-produced protective polymeric matrix where cells possess distinct phenotypic differences in metabolism, cell physiology and stress tolerance, when compared to their planktonic counterparts. *C. jejuni* biofilms can form as aggregates, where bacteria attach to one another and secrete extracellular polymeric substances (EPS), as pellicles which form at an air-liquid interface, but are most commonly studied in a surface-attached mode (Joshua et al., 2006; Kalmokoff et al., 2006; Moe et al., 2010; Nguyen et al., 2010; Reuter et al., 2010).

Biofilms form in response to various environmental signals and can develop via multiple signalling pathways (O'Toole and Kolter, 1998) and different species of bacteria initiate attachment and form biofilm in response to different environmental cues (Petrova and Sauer, 2012). Biofilm formation occurs in 5 stages, (i) planktonic, (ii) attachment, (iii) microcolony formation, (iv) macrocolony formation and (v) dispersion (Sauer et al., 2002; Stoodley et al., 2002; Monds and O'Toole, 2009; Petrova and Sauer, 2012; Cappitelli et al., 2014), Fig. 1.2. Our understanding of these stages in biofilm development is based on work done with the model organism, *P. aeruginosa* (Sauer et al., 2002). Recent studies have provided insight into biofilm formation in other species, indicating a great deal of variation at all stages, not only between species, but even between strains of the same species (Petrova and Sauer, 2012; Cappitelli et al., 2014; Martinez and Vadyvaloo, 2014).

*S*tudies investigating biofilm formation in *C. jejuni* have identified many similarities to the original *Pseudomonas* model (Joshua et al., 2006; Kalmokoff et al., 2006; Moe et al., 2010) as well as certain differences (Kalmokoff et al., 2006; Reuter et al., 2010), but the understanding of biofilm formation in this relevant pathogen lags behind and more work is needed to assess how *C.*

jejuni differs from the commonly studied model bacteria so that effective biofilm reduction strategies can be put in place.

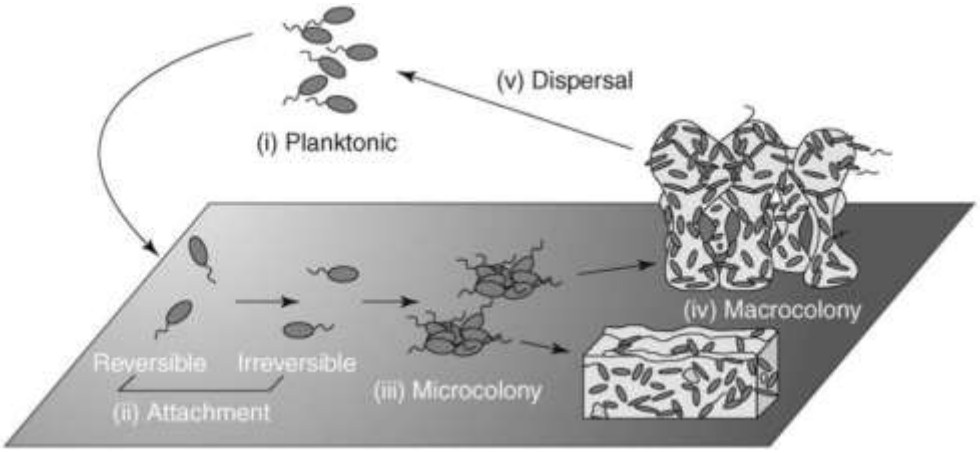

Figure 1.2. Stages in biofilm formation: (i) planktonic, (ii) reversible and irreversible attachment, (iii) microcolony formation, (iv) macrocolony formation, (v) dispersion (with permission from Monds and O'Toole, 2009).

1.3.2. Stages in biofilm formation

Stage 1 - Initial reversible attachment: Initial attachment is facilitated by flagellar motility. Motility allows cells to approach the surface, overcoming the repulsive negative electrostatic charges and increasing the probability of physical contact with cell surface receptors such as fimbriae, polysaccharides, pili and flagella, thereby increasing the chance of attachment (Bos et al., 1999). Flagella and type IV pili are important to attachment for *Pseudomonas* spp. and *V. cholera* while fimbriae like type I pili, curli and conjugative pili are important for biofilm formation in *E. coli* (O'Toole and Kolter, 1998; Watnick and Kolter, 1999). Non-motile *P. aeruginosa* cells showed a significant decrease in attachment compared to cells with flagellar motility (Sauer et al., 2002). Use of confocal scanning microscopy revealed that Psl, an exopolysaccharide important for all stages of biofilm formation in *P. aeruginosa* PAO1, occurs in a helical pattern on the bacterial cell surface at the biofilm initiation stage (Ma et al., 2009).

During this initial reversible attachment stage *P. aeruginosa* is loosely attached via a single pole and can easily detach (Sauer et al., 2002). Cells can be spinning, vibrating or moving across the surface and some cells exhibit a pilus-mediated twitching movement (Sauer et al., 2002; Toutain et al., 2007). At this stage cells are still able to leave the surface (O'Toole and Kolter, 1998) and can be removed by rinsing (Kumar and Anand, 1998).

A possible mechanism for signalling attachment and initiating polysaccharide production has been elucidated in mutation studies with *Vibrio parahaemolyticus,* where the rotation of the flagella was found to be important to attachment (McCarter and Silverman, 1990). Petrova and Sauer (2012) suggest that in this case, the flagella acts as a 'mechanosensor' for adhesion and the initial attachment which restricts flagellar rotation, triggers the twitching motility and the expression of polysaccharide biosynthesis genes leading to the production of EPS.

Stage 1 in *C. jejuni*: Motility is known to be important for initial attachment to both biotic and abiotic surfaces for *C. jejuni* as well, but whether the attachment of the flagella signals EPS production in a similar fashion to *V. parahaemolyticus,* has yet to be explored for this pathogen (Haddock et al., 2010; Moe et al., 2010). Mutations in genes related to motility in *C. jejuni* all led to reduced biofilm formation (Joshua et al., 2006; Kalmokoff et al., 2006; Asakura et al., 2007; Reeser et al., 2007). Both non-motile *C. jejuni* cells with intact flagella (Δ*motA*) and those that were aflagellate (Δ*flaA*) were equally poor at forming biofilm (Moe et al., 2010).

Stage 2 - Irreversible attachment: Irreversible attachment of *P. aeruginosa* cells to stainless steel occurred in less than one min and increased linearly over 10 min (Stanley, 1983) and high levels of attachment were observed after 30 min (Choi et al., 2013). Stage 2 is characterized by the production of exopolymers and adhesins (Petrova and Sauer, 2012; Cappitelli et al., 2014) and removal of cells from the surface at this stage requires stronger physical forces such as scrubbing or scraping (Kumar and Anand, 1998) or chemical treatment (Frank and Chmielewski, 2001). Expression of genes involved in polysaccharide production and

the reduction in surface swarming behaviour that mark the transition to irreversible attachment are associated with increased levels of cyclic dinucleotides (c-di-NMP) (Petrova and Sauer, 2012; Valle et al., 2012). This ubiquitous intracellular signalling molecule is involved in controlling the expression of flagellar biosynthesis genes and is required for biofilm formation in *P. aeruginosa* (Drenkard and Ausubel, 2002; Hickman and Harwood, 2008). Exopolysaccharides produced at this stage play an important role in adhesion (Chagnot et al., 2013), but not all polysaccharides produced contribute to adhesion (Petrova and Sauer, 2012; Martinez and Vadyvaloo, 2014). Polysaccharides Psl, Pel and alginate are relevant to attachment and biofilm formation of *P. aeruginosa*, but their roles vary by strain (Ma et al., 2009; Colvin et al., 2011; Petrova and Sauer, 2012; Wei and Ma, 2013). Disruption of immature biofilm by DNase I indicates that extracellular DNA (eDNA) also plays a role in the early stages of attachment of *P. aeruginosa* (Whitchurch et al., 2002).

Stage 2 in *C. jejuni*: *C. jejuni* was found to attach to stainless steel immediately, reaching 3.68 to 4.52 $\log_{10}$ cells/cm^2 (after rinsing in PBS) within 1 min of contact, but the strength of attachment was weak and no aggregation was observed (Nguyen et al., 2010). Only viable cells were detected on stainless steel coupons, suggesting that attachment is an active process, just as in *P. aeruginosa* (Sauer et al., 2002).

Exopolymers, adhesins and eDNA are also important in irreversible attachment of *C. jejuni*. Genes related to adhesion in *C. jejuni* such as *cadF* and *peb4* are important to biofilm initiation (Kalmokoff et al., 2006; Asakura et al., 2007). Upregulation of the adhesin *cadF* in response to aerobic stress was accompanied by increased attachment and biofilm formation in *C. jejuni* strains 81-176 and NCTC 11168 (Sulaeman et al., 2012). *C. jejuni* NCTC 11168 mutants for the adhesin, *peb4* were less able to form biofilm than the WT (Asakura et al., 2007). The role of eDNA in biofilm formation in *C. jejuni* is supported by work showing that a mutant for the gene *dps* (DNA binding protein from starved cells) which plays a role in DNA condensation and protection had 50% less biofilm than the WT (Theoret et al., 2012). Also, similar to *P.*

aeruginosa, DNase I was found to disrupt *C. jejuni* biofilm, indicating that DNA is likely an integral structural component of *C. jejuni* biofilm (Svensson, 2008).

Stage 3 - Microcolony formation: In the *P. aeruginosa* model, cells form microcolonies within 2 h, which are irreversibly attached. Cells are non-motile and the Las quorum-sensing system which becomes active at this stage positively regulates c-di-GMP production (Davies et al., 1998; Sauer et al., 2002; Petrova and Sauer, 2012). Psl, which also positively regulates c-di-GMP in *P. auruginosa* PAO1, now forms a matrix covering the entire biofilm from top to bottom (Ma et al., 2009). Cellulase treatment inhibits formation of this matrix and eliminates the helical distribution of Psl on the bacterial surface, suggesting this exopolysaccharide is critical to biofilm development for *P. aeruginosa* PAO1 (Ma et al., 2009).

Stage 3 in *C. jejuni*: Microcolonies of *C. jejuni* that were 0.5 to 2 mm in diameter formed on glass coverslips within 2 h. Real time observations with phase-contrast microscopy revealed that during initial attachment, bacteria were motile and advanced to the core of each microcolony, which may help explain the importance of quorum-sensing and motility to biofilm formation in this pathogen (Moe et al., 2010). In contrast, Kalmokoff et al (2006) reported that after 24 h, only single attached cells of *C. jejuni* 11168 (V26) could be observed on stainless steel, nitrocellulose or glass fibre filters. The fact that *C. jejuni* 81-176 cells aggregated on glass coverslips in one study (Moe et al., 2010), but no aggregation was seen for *C. jejuni* 11168 V26 (Kalmokoff et al., 2006) or for 6 other strains (Nguyen et al., 2010) on stainless steel coupons, glass fibre filters or nitrocellulose (Kalmokoff et al., 2006) may be due to strain variation, but could be related to differences in material and/or culture conditions (Kalmokoff et al., 2006). Aggregation occurred in Brucella broth, but not in Mueller Hinton broth or phosphate buffered saline (PBS) (Kalmokoff et al., 2006; Moe et al., 2010; Nguyen et al., 2010).

Quorum sensing also plays a role in biofilm formation in *C. jejuni*. Mutants for *luxS* had significantly reduced biofilm formation (Reeser et al., 2007). Acylated homoserine lactones (AHL) are used as autoinducer signalling molecules for quorum sensing in most Gram-negative

bacteria (Camilli and Bassler, 2006; Ng and Bassler, 2009). Commercially available AHLs and an AHL–like molecule encoded on the *C. jejuni* genome were found to inhibit biofilm formation of *C. jejuni*, although cells maintained some level of attachment (Moorhead and Griffiths, 2011).

Stage 4 - Macrocolony formation – Maturation: Maturation of *Pseudomonas* biofilms occurs as a result of microbial growth and recruitment of microbes from the environment. At this stage cells become immobilized within the self-produced matrix of EPS. Diffusion through the EPS is slower than cellular metabolism and the resulting chemical gradients create micro-niches allowing diverse species to coexist (Cappitelli et al., 2014).

Formation of mushroom-like macrocolonies that characterize a mature *P. aeruginosa* biofilm requires repression of twitching motility in a subpopulation of cells at the surface. This repression occurs in the presence of glucose but not when cells are grown with citrate (Klausen et al., 2003; Klausen et al., 2003). This sub-population of non-twitching cells become the 'stalk' and form a platform on which other cells can form the mushroom-like cap (Fig. 1.2. iv). Cells grown on citrate maintain twitching motility and form a flat homogeneous biofilm (Monds and O'Toole, 2009). After 20 h of biofilm growth, cell death and lysis occur in the central region of the mushroom-like structure, creating a cavity where motile (swimming) planktonic cells are observed. At this stage, Psl is observed only at the periphery and not in the centre of mushroom like structures. The even distribution of Psl in flat biofilms suggests that the reduced Psl in the centre of the microcolony contributes to cavity formation (Ma et al., 2009).

Stage 4 in *C. jejuni*: Attachment of *C. jejuni* to stainless steel, peaks at 3 h ranging from 4.5 to 5.5 ($\log_{10}$ cells/cm^2) depending on the strain and the level and strength of attachment, and counts remain constant to 5 h (Nguyen et al., 2010). On glass coverslips, continued biofilm formation can be observed from 4 to 6 h after initial contact and *C. jejuni* form net-like connections between flagella and produce excessive amounts of EPS (Moe et al., 2010). Biofilm continues to grow over 3 d if surfaces are introduced to fresh media every 24 h (Kalmokoff et al., 2006). The three-dimensional structure of *C. jejuni* biofilm has not been examined to date.

Stage 5 – Dispersion: The final stage in biofilm development involves the release of single cells or clumps of cells from the biofilm. Dispersion of cells can be an active process mediated by the cell or a passive process mediated by external forces (Cappitelli et al., 2014). Dispersion can occur: i) by erosion, with the continuous release of single cells or small cell clusters, ii) by sloughing, with the sudden detachment of large portions of biofilm , or iii) by seeding, with the rapid release of large numbers of single cells or small clusters of cells from hollow cavities (Ma et al., 2009; Kaplan, 2010; Cappitelli et al., 2014). In *P. aeruginosa*, dispersion of bacteria by seeding, from interior portions of the biofilm occurs after 9 d. At this stage, some cells in the central portion of the biofilm regain motility which allows them to leave the biofilm. However, cells closer to the surface of the mushroom-like structure remain non-motile (Sauer et al., 2002; Ma et al., 2009).

Stage 5 in *C. jejuni*: In *C. jejuni* it has been suggested that dispersion is a passive process, since biofilms shed high numbers of viable cells independent of the oxygen concentration (Reuter et al., 2010). Even though oxygen concentration does not control the release of cells from the biofilm, further investigation into other possible mechanisms for dispersion should be done to confirm that it is a passive process.

1.3.3. Biofilm and epithelial tissue

Biofilm formation is part of chronic infections like those of *P. aeruginosa* associated with cystic fibrosis patients (Valle et al., 2012). Investigations of *P. aeruginosa* attachment to epithelial cell cultures provided evidence that cell culture models are limited in their ability to mimic the conditions *in vivo*. The *P. aeruginosa* PAO1 strain commonly used in the laboratory was better at adhering to human epithelial cells in culture than to mucin, while clinical strains isolated from cystic fibrosis (CF) sputa were less able to adhere to the cells in culture, but showed enhanced attachment to human tracheobronchial mucins (Klockgether et al., 2013).

Although the acute infections caused by *C. jejuni* are believed to occur as a result of planktonic cells attaching to intestinal epithelial cells (Valle et al., 2012), *C. jejuni* does form

biofilm on intestinal epithelial tissue within 1-2 h (Haddock et al., 2010) in a similar fashion to that observed on glass (Moe et al., 2010). Formation of biofilm on absorptive surfaces of the intestine would prevent the normal absorptive transport functions of the ileal mucosa and may contribute to the symptoms of the disease (Haddock et al., 2010). Although cell culture models of infection are often used to study enteric infections at the cellular/molecular level, they are not good models for biofilm formation on tissues, as *C. jejuni* microcolonies and biofilm do not form on cell culture models (Grant et al., 2006). *Ex vivo* culture models where intestinal epithelial tissue samples are used, provide a more representative model. *C. jejuni* can form microcolonies, and in some cases, sheets of biofilm on intestinal epithelial tissue (Grant et al., 2006; Haddock et al., 2010). *C. jejuni* is chemotactic to mucin and adherence occurs preferentially to mucus on the tissue, with discrete macrocolonies as well as blankets of *C. jejuni* observed within and under mucus layers within 3-4h (Grant et al., 2006; Haddock et al., 2010).

Adhesion and invasion are dependent on motility (Grant et al., 2006; Haddock et al., 2010). SEM images indicated that bacterial flagella interact with the microvilli of the intestinal cells, as well as being involved in aggregation of the bacterial cells (Grant et al., 2006; Hu et al., 2008; Haddock et al., 2010). The morphology of the flagellum was altered in a subset of the adherent bacteria (Haddock et al., 2010), which may relate to its role in transporting *Campylobacter* invasion antigens into host cells (Grant et al., 2006). In contrast to the finding that non-motile mutants were still able to attach to stainless steel, although in lower numbers (Moe et al., 2010), non-motile mutants were unable to form microcolonies or biofilm on tissue (Haddock et al., 2010).

1.3.4. Conditions relevant to biofilm formation

Various environmental conditions such as nutrient availability, temperature, dynamic flow conditions, surface properties, osmotic pressure, and oxygen concentration play a role in biofilm formation for a variety of bacteria.

Biofilm formation is increased in low nutrient or starvation conditions. When comparing full strength arginine brilliant green glucose peptone broth (ABGP) medium and 90% (w/v) media, the low nutrient conditions led to higher levels of EPS and enhanced biofilm for *P. aeruginosa* (Myszka and Czaczyk, 2009). Also, *E. coli, Agrobacterium tumefaciens,* and *Streptococcus pneumonia* produce more biofilm in low nutrient conditions (Monds and O'Toole, 2009; Lambert et al., 2014). Consistent with findings for other bacteria, overly rich media such as Bolton broth and Brucella broth are less conducive to biofilm formation in *C. jejuni* than the less nutrient-rich Mueller Hinton broth (Reeser et al., 2007). Although most studies examine the effects of reduced carbon availability by incubating cells in sterile surface water or PBS, low concentrations of nitrogen and phosphorus have also been correlated with higher levels of biofilm formation (Monds and O'Toole, 2009). In *Pseudomonas fluorescens*, when levels of extracellular inorganic phosphate (Pi) dropped below a certain threshold, attachment to polyvinylchloride (PVC) was reduced, indicating that Pi plays a role in initial surface attachment (Monds and O'Toole, 2009).

Although no work has explored the effects of Pi starvation on *C. jejuni* biofilm formation, mutants for *phoX*, the sole alkaline phosphatase in *Campylobacter,* were enhanced for biofilm formation (Drozd et al., 2011). In *C. jejuni* PhoX becomes active upon transport to the periplasm. There it provides the cell with Pi through the hydrolysis of phosphate groups from more complex organophosphate molecules. Since Pi is typically low in the environment, PhoX is necessary for the cell to maintain adequate levels of Pi (Lamarche et al., 2008). The fact that biofilm formation of the *phoX* mutant could be returned to wild type levels by adding Pi suggests that Pi also plays a role in *C. jejuni* biofilm formation (Drozd et al., 2011).

The role of temperature in biofilm formation is more complex, varies between species and changes based on other conditions. For example, 30 *Salmonella* strains had the highest quantity of biofilm formation at 30°C after 24 h, but when measured after 48 h, there was more biofilm formed at 22°C (Stepanovic et al., 2003). For *Staphylococcus aureus* and *P. aeruginosa*,

biofilm formation was greater at 37°C than at 25°C and for *Listeria monocytogenes* biofilm formation did not differ between these two temperatures, but in this case biofilm formation was not measured beyond 24 h (Choi et al., 2013). Further work with *P. aeruginosa*, reported that optimal biofilm formation measured after 48 h, occurred at 32.5°C and was reduced at temperatures above (37.5°C) or below (27.5°C) this value (Arutchelvi et al., 2011).

C. jejuni growth occurs within the narrow temperature range of 30˚C and 47°C (Hazeleger et al., 1998; Stintzi, 2003). Biofilm formation of *C. jejuni* M129 on polystyrene is greater at 37°C than at 25°C (Reeser et al., 2007). For *C. jejuni* NCTC 11168, biofilm formation occurs equally well at both 37°C and 42°C (Kassem et al., 2012). Also, there were no significant differences in the amount of *C. jejuni* incorporated into existing mixed-culture biofilm after 24 h at temperatures of 13, 20, 37, and 40°C (Sanders et al., 2008).

Dynamic flow conditions up to a certain flow rate, lead to enhanced attachment. Attachment of *P. aeruginosa* to glass increases with increasing shear in the low shear range (3.5–5.0 mN/m^2), but attachment is reduced above this range (Raya et al., 2010). For *C. jejuni,* most studies investigate biofilms formed in static conditions (Kalmokoff et al., 2006; Moe et al., 2010; Nguyen et al., 2010) and attempts to form biofilm with shaking or in a flow cell situation, have had varied success. Similar to the effect of shear in *P. aeruginosa,* shaking at higher rpm (80-100) did not allow attachment of *C. jejuni* while slowing to 50 rpm allowed cells to attach and form biofilm (Joshua et al., 2006). *C. jejuni* monoculture biofilms could only persist at flow rates of 0.75 ml/min or lower, while mixed-culture biofilms which harboured *C. jejuni* could resist flow rates as high as 2.5ml/min (Ica et al., 2012).

Surface properties influence biofilm formation in *P. aeruginosa.* Cellular attachment and biofilm formation is better on hydrophobic surfaces (polyvinylidene fluoride) than on hydrophilic surfaces (silica) (Pasmore et al., 2001; Loo et al., 2012; Marcus et al., 2012) and increased surface roughness enhances biofilm formation (Pasmore et al., 2001). *C. jejuni* M129 had better attachment to hydrophobic plastics than hydrophilic glass or copper (Reeser et al.,

2007). In contrast, *C. jejuni* 81-176 did not form biofilm on plastic, but did on both glass and stainless steel (Gunther and Chen, 2009). No studies were found that investigated the effects of surface roughness on the ability of *C. jejuni* to form biofilm.

Osmotic stress inhibits biofilm formation and leads to dispersion of existing biofilm in the model organism *P. aeruginosa*. The inclusion of 0.5M NaCl in the growth media, inhibited biofilm formation in *P. aeruginosa* (Bazire et al., 2007) and the addition of 6 mol/L NaCL to preformed *P. aeruginosa* biofilm led to detachment of cells (van der Waal et al., 2011). Similarly, osmotic pressure created by the addition of NaCl, glucose or sucrose all led to significantly decreased biofilm formation in *C. jejuni* M129 and induced cells to transition to coccoid morphology (Reeser et al., 2007).

P. aeruginosa is capable of growth at varying oxygen levels (Skolimowski et al., 2010). *P. aeruginosa* PAO1 had less attachment in microaerobic conditions than those with higher oxygen concentrations and cells attached during high oxygen conditions tended to detach if oxygen concentrations were reduced (Skolimowski et al., 2010).

The effect of oxygen tension on biofilm formation in *C. jejuni* appears to vary greatly between strains and due to the limited data available and the variation in methods between studies it is difficult to draw any conclusions. Oxygen, which is a stress for the microaerobic *C. jejuni*, was found to significantly enhance initial attachment of the 81-176 strain (within 30 min) (Sulaeman et al., 2012). For the 11168 strain, there was no significant difference in the amount of biofilm formed in aerobic and microaerobic conditions after 24 h, and it was only after 48 h that more biofilm was observed in the aerobic conditions (Reuter et al., 2010). For the M129 strain more biofilm formation was observed in the microaerobic condition at 24 h and no data was available for 48 h (Reeser et al., 2007). More work needs to be done to better understand the impact of oxygen tension on biofilm formation of the various strains of *C. jejuni*.

1.3.5. Strain variation

The seemingly contradictory results in various studies can often be attributed to strain variation. Factors that vary among strains and influence attachment and biofilm formation include cell surface hydrophobicity, polysaccharides and the source of isolates.

1.3.5.1. Cell surface hydrophobicity

Cell surface hydrophobicity correlates positively with attachment to surfaces. Strains with higher hydrophobicity are better at surface attachment. For example, of 6 *P. aeruginosa* strains, better adhesion occurred for the 3 strains which were more hydrophobic (Roosjen et al., 2006). A significant positive correlation between cell surface hydrophobicity and the capacity of individual strains to form biofilms was found in a study of 17 strains of Salmonella (Wang et al., 2013). Attachment of *Campylobacter* to abiotic surfaces significantly correlated with cell surface hydrophobicity (Nguyen et al., 2011). Surface hydrophobicity varied considerably between 13 *C. jejuni* strains, ranging from 17.6 to 53° and cell surface hydrophobicity had a significant positive correlation with attachment to glass and stainless steel ($p<0.007$) (Nguyen et al., 2011). For some strains, the hydrophobicity was higher when cells were grown planktonically (9 of 13) and for others when growth was sessile and cells were immobilized as in a biofilm (4 of 13) (Nguyen et al., 2011). PFGE pattern assessment indicated that there were no general relationships between the genotypic and phenotypic properties of the strains in the study (Nguyen et al., 2011).

1.3.5.2. Polysaccharides

Exopolysaccharides produced during biofilm formation vary between *Pseudomonas* strains (Petrova and Sauer, 2012; Martinez and Vadyvaloo, 2014). The polysaccharide Psl produced by *P. aeruginosa* PAO1 is required for adhesion to glass and mucin-coated surfaces and connects cells to each other (Ma et al., 2009; Colvin et al., 2011; Petrova and Sauer, 2012; Wei and Ma, 2013). The polysaccharide Pel was found to play no role in the PAO1 strain, but was important for biofilm formation in the PA14 strain (Colvin et al., 2011). The exopolysaccharide alginate contributes to structural stability and water retention in biofilms and is mainly produced

in clinical isolates from patients with cystic fibrosis (CF), playing a role in the muccoid phenotype of these strains (Govan and Deretic, 1996). Although *C. jejuni* is known to produce a variety of exopolysaccharides, their role in attachment remains to be explored (Nguyen et al., 2012).

1.3.5.3. Source

Pseudomonas strains isolated from CF patients were less able to increase surface area coverage when compared to the laboratory strain PAO1 which had formed a layer of cells covering the entire surface twenty-four hours postinoculation. During this time the CF strains had shown little or no change to surface coverage and when these strains did form biofilm (day 5), each CF strain had its own distinctive and reproducible microcolony architecture (Kirov et al., 2007). No clear correlation between source and biofilm forming ability can be made for *C. jejuni* strains/isolates. Although one study reported that an evaluation of 22 strains of *C. jejuni,* indicated that clinical isolates and those originating from food processing environments had better adhesion than those from animals and animal carcasses, another study of 20 *C. jejuni* isolates from human, poultry and water, reported that significantly more biofilm formation was observed for only one of the five human isolates and for two of the 14 poultry-derived strains (Teh et al., 2010). One poultry isolate was found to be incapable of forming biofilm in the conditions used in the study and another poultry isolate gave inconsistent results. The one isolate from water was found to form moderate amounts of biofilm.

1.3.6. Commonly used *C. jejuni* strains

Since the initial identification of *C. jejuni* in 1977, the importance of identifying the origins of particular strains being used in each study is being increasingly recognized by the research community. What follows is a brief description of some of the strains more commonly used in research and discussed in this thesis (Table 1.1).

C. jejuni NCTC 11168 was first isolated from human diarrheic feces in 1977 by M. Skirrow and designated strain 5636/77 at that time (Gaynor et al., 2004). A lab-passaged version

of this strain was sequenced in 2000 (Parkhill et al., 2000). The sequenced clone was later found to colonize 1-day-old chicks and invade tissue culture cells less efficiently and to be less motile (Ahmed et al., 2002; Carrillo et al., 2004; Gaynor et al., 2004) than the original *C. jejuni* variant isolated in 1977. The lab-passaged variant also differed in morphology, displaying a straight rod shape rather than the typical helical shape of the more virulent original (Gaynor et al., 2004). More recently, additional strains of *C. jejuni* NCTC 11168 which were similar to the original clinical isolate in terms of motility, morphology and invasion were sequenced (Cooper et al., 2012; Revez et al., 2012; Thomas et al., 2014). The *C. jejuni* NCTC 11168 stains used by these authors had been obtained from ATCC, and were not subjected to lab-passaging. The genome of the non-passaged strains differed from that of the less virulent variant (Parkhill et al., 2000) by various point mutations, but the studies reported variable mutations. Four point mutations were found in all three studies: i) Gene *cj0276*, which encodes for the rod-shape determining protein MreB, and experienced an A/G change resulting in an aspartic acid switch to glycine. ii) *cj0431*, encoding for a putative periplasmic ATP/GTP binding protein, had a T/A change. This eliminated a stop codon and lead to addition of 41 amino acids. iii) Locus *cj0455c* also had an elimination of a stop codon through an A/G change. This resulted in an addition of 61 amino acids to the hypothetical protein encoded by *cj0455c*. iv) For *cj0807*, which codes for a 7-alpha-hydrosteroid dehydrogenase, an A/G change was found resulting in a lysine switch to glutamic acid. (Cooper et al., 2012; Revez et al., 2012; Thomas et al., 2014). These four loci did not change after four in vitro transfers, nor after passage in humans, mice or pigs (Cooper et al., 2012; Thomas et al., 2014) and the same point mutations were found in eight other *C. jejuni* strains through a BLASTN search of the NCBI database (Cooper et al., 2012). Of the strains used in the present study, *C. jejuni* NCTC 11168 V1 corresponds to the original clinical isolate which other authors referred to as NCTC 11168-O (Cooper et al., 2012), NCTC 11168-BN148 (Revez et al., 2012) or NCTC 11168-GSv (Thomas et al., 2014). *C. jejuni* NCTC 11168 V26 used in this thesis refers to the lab-passaged variant studied by (Carillo et al., 2004).

The highly virulent *C. jejuni* 81-176 strain was originally isolated from a raw milk-borne case of colitis (Kilcoyne et al., 2014). This strain has 37 genes not present in the reference strains NCTC 11168 (Parkhill et al., 2000) and RM1221 (Fouts et al., 2005) some of which encode respiratory functions not found in these reference strains, thereby contributing to the improved efficiency of 81-176 to colonize the intestines of human or animal hosts. The 81-176 strain also harbours the virulence plasmid pVIR which contributes to the virulence of this strain (Bacon et al., 2000).

C. jejuni RM1221 was isolated from a chicken carcass and minimally passaged prior to being sequenced (Miller et al., 2000). In a comparison of protein content, *C. jejuni* RM1221 had the highest average protein percent identity (98.41%) with *C. jejuni* NCTC 11168 (Fouts et al., 2005). *C. jejuni* RM1221 harbours no plasmids (Fouts et al., 2005).

C. jejuni M129, a human clinical isolate was used to develop a PCR assay for *C. jejuni* by researchers in the University of Arizona Department of Veterinary Science and Microbiology (Day et al., 1997). This strain was also used in the study by Reeser et al (2007) which investigated the influence of various environmental factors including temperature, oxygen tension and nutrition on biofilm formation on a variety of abiotic surfaces.

Table 1.1. *C. jejuni* strains frequently used in biofilm studies.

Strain	**Reference**
C. jejuni NCTC 11168 V1 – original clinical isolate (NCTC 11168-BN148)	(Revez et al., 2012)
C. jejuni NCTC 11168 V26 – lab-passaged version of V1 (ATCC catalog no. 700819)	(Parkhill et al., 2000)
C. jejuni 81-176 – human clinical isolate from raw milk borne case of colitis	(Korlath et al., 1985)
C. jejuni RM1221 – minimally passaged chicken isolate	(Miller et al., 2000)
C. jejuni M129 – human clinical isolate	(Day et al., 1997)

1.3.7. Extended survival and stress protection of *C. jejuni* within biofilms

The extended survival observed for *C. jejuni* cells within biofilms occurs as a result of the protection provided from environmental stresses such as desiccation, aerobic stress, temperature stress and changes in acidity (Trachoo and Frank, 2002; Joshua et al., 2006; Kubota et al., 2008) as well as from sanitation with chlorine, quaternary ammonia, peracetic acid, peroctanoic acid or trisodium phosphate (Somers et al., 1994; Yang et al., 2001; Trachoo and Frank, 2002; Chantarapanont et al., 2004; Northcutt et al., 2005).

Factors that contribute to protection from stresses include the presence of other species in the biofilm (mixed-culture biofilm), the matrix (EPS) encasing the cells and the physiological differences in gene expression and growth rate for planktonic and biofilm cells. Although many biofilm studies are done with monoculture biofilms (Joshua et al., 2006; Kalmokoff et al., 2006), mixed-culture biofilms which are more relevant outside the lab, generally enhance biofilm formation and survival of *C. jejuni*. *C. jejuni* can maintain viability in a mono-species biofilm, but will be culturable longer in a mixed species biofilm and the presence of pre-established biofilm extends survival (Buswell et al., 1998; Trachoo et al., 2002; Sanders et al., 2008). In fact, the presence of autochthonous water microflora extended culturability to twice what was found in a mono-culture water microcosm and this extended survival may have been due to reduced oxygen tension in the existing biofilm (Buswell et al., 1998; Trachoo et al., 2002; Sanders et al., 2008). Depletion of oxygen by aerobic microorganisms will lead to reduced oxygen tension within a biofilm, providing an environment hospitable to the growth of microaerobic organisms like *C. jejuni*. Microscopy with a *Campylobacter*-specific rRNA probe indicated that the pathogen was incorporated within the biofilm matrix where the oxygen tensions would be lowest (Buswell et al., 1998).

C. jejuni within a mono-species biofilm was able to survive both aerobic and temperature stress twice as long as planktonic cells, with biofilm samples remaining culturable 24 days and 12

days respectively in ambient conditions (Joshua et al., 2006). This extended survival could be due to both the protective nature of the EPS, as well as gene expression differences between the biofilm and planktonic phenotypes.

In support of the protective nature of EPS, it was recently shown that diallyl sulphide, a compound derived from garlic, was able to destroy the EPS of a *C. jejuni* biofilm, allowing the biofilm cells to be killed by antibiotics at the same rate as the planktonic cells (Lu et al., 2012). It has also been proposed that EPS may dilute or neutralize antimicrobials and it has been shown that biofilms significantly reduce but do not completely block antibiotic penetration and that the rate of penetration varies between antibiotics (Suci et al., 1994).

Physiological differences between biofilm and planktonic cells may also play a role in the increased stress resistance of biofilm cells. Analyses of gene expression and protein profiling have shown that significant differences do exist (Kalmokoff et al., 2006; Sampathkumar et al., 2006). The genes coding for chemotactic proteins, general and oxidative stress response proteins, and proteins involved in biosynthesis, energy generation, catabolic functions and those for iron uptake and membrane transport are expressed at higher levels in biofilm cells (Kalmokoff et al., 2006; Sampathkumar et al., 2006). One global regulator known to support biofilm formation in *C. jejuni* is the carbon starvation regulator (csrA). *C. jejuni csrA* mutants produced 50 percent less biofilm than the wildtype (Fields and Thompson, 2008). Mutants for the phosphate-related genes *ppk1* and *phoX* also exhibited increased levels of biofilm of 10 and 30% respectively, but this may be due to the up regulation of *csrA* seen in these mutants (Gangaiah et al., 2009). More work is needed to explore the complex mechanistic relationships of these genes during biofilm formation.

Another physiological difference that contributes to the increased resistance to antimicrobials is the reduced growth rates of cells within a biofilm. Non-dividing bacteria will be unaffected by antimicrobials targeted at growth-specific factors. Work done with *Pseudomonas*

putida harbouring a fluorescent growth activity reporter indicated that there was reduced growth activity in the centre of microcolonies and that growth activity could be correlated with location in the biofilm (Sternberg et al., 1999).

A better understanding of the relationship between biofilm formation and entry into the VBNC state, as explored in this thesis, will inform decisions about sanitation and lead to improved practices for food safety.

1.4 The viable but non-culturable (VBNC) state

Bacteria are considered VBNC when they maintain viability, but are unable to grow and form colonies on bacteriological media commonly used for their cultivation. There has been some debate over the years as to whether this state is inducible and genetically programmed or just a prelude to cell death.

Nystrom (2003) has asserted that current knowledge does not "support the notion that non-culturability is an inducible, genetically programmed capacity of cells to ensure survival under adverse environmental conditions, as stated by the VBNC hypothesis." However, he does concede that "…bacteria can exist in a reversible, non-culturable mode" (Nystrom, 2003). Sachidanandham and Gin (2009) provide support for the VBNC state, describing it as "an orderly and spontaneous adaptation to circumvent adverse conditions."

The pioneering study in Rita Colwell's lab over 30 years ago, provided evidence that both *E. coli* and *Vibrio cholerae* were able to exist in this non-culturable form (Xu et al., 1982). In 1986, researchers from the same lab reported that *C. jejuni* was also capable of becoming VBNC (Rollins and Colwell, 1986). Since that time numerous bacteria, both pathogenic and non-pathogenic, have been found capable of entering the VBNC state (Table 1.2). Bacteria enter the VBNC state in response to certain unfavourable conditions or stresses and can remain dormant for extended periods. Although numerous cases exist showing that bacteria can resuscitate from the VBNC state, there is still little knowledge about the factors that lead to resuscitation. This

lack of understanding is a serious concern for any industry (water quality monitoring, food industry, medicine) where detection of pathogens is based on culturability.

This section includes an overview of conditions which are known to induce the VBNC state, the characteristics common to cells in this state, the ability of cells to resuscitate and methods to detect and quantify both culturable and VBNC cells.

1.4.1. Induction of VBNC

There are numerous stresses that induce a VBNC state. These include low nutrient stress (starvation), temperature stress, osmotic stress, acid stress, aerobic stress, heavy metals, white light and antibiotics. Many of the methods used to induce VBNC in the laboratory mimic conditions that bacteria would encounter during the cycle of infection, either in the environment or within the host. The most effective methods to induce a VBNC state in *C. jejuni* are 1) starvation at 4°C, which requires days to weeks before cells become non-culturable or 2) incubation in formic acid (pH=4) where cells become VBNC within a few hours. A survey of commonly used methods is provided in Table 1.3.

1.4.1.1. Low nutrient stress (starvation)

C. jejuni is unable to metabolize sugars and instead uses amino acids and small organic acids (i.e. pyruvate, serine, aspartate) commonly found in the gut, as a source of energy and carbon (Kelly, 2001). When these become unavailable, as in nutrient poor conditions (starvation), *C. jejuni* mounts a stringent response, which aids in long term survival (see Section 1.5, Gaynor et al., 2005). Authors using low nutrient stress to induce the VBNC state have incubated cells in aged, sterile stream water (Rollins and Colwell, 1986), filter-sterilized surface water (Cappelier et al., 1999), surface water (Tholozan et al., 1999), sterile tap water (Buswell et al., 1998), artificial seawater (Baffone et al., 2006), distilled water (Duffy and Dykes, 2009), bottled water (Guillou et al., 2008), and PBS (Hazeleger et al., 1998; Lazaro et al., 1999). The range of time for cells to become VBNC varied, but in one study, cells remained culturable up to 4 months and viable up to

7 months when incubated in phosphate buffered saline at 4°C (Rollins and Colwell, 1986; Lazaro et al., 1999).

1.4.1.2. Temperature stress

C. jejuni has a narrow range of temperatures which permit growth. Growth is only observed between 30˚C and 47°C, with sharp declines at both ends (Hazeleger et al., 1998; Stintzi et al., 2005). However, although *C. jejuni* has been shown to be quickly destroyed by heating (60˚C for 1 min), this pathogen was able to survive 3 cycles of freeze thaw in either -20˚C or -70˚C (Alter and Scherer, 2006).

C. jejuni has 17 identified heat shock proteins (Stintzi, 2003) and no known cold shock proteins (Hazeleger et al., 1998), but responds quickly to changes in temperature by altering gene expression (Stintzi, 2003). In a DNA microarray analysis, the greatest proportion of gene expression changes occurred within 10 minutes of the temperature change from 37˚C to 42˚C (Stintzi, 2003).

Induction of the VBNC state in *C. jejuni* has been most successful when cells were incubated at 4°C (Table 1.3). Loss of viability occurred sooner at higher temperatures (10˚C, 24˚C, 37˚C) (Rollins and Colwell, 1986; Buswell et al., 1998). For the temperatures used in various studies, both culturability (4 months) and viability (7 months) were maintained longest at 4°C (Rollins and Colwell, 1986; Lazaro et al., 1999).

1.4.1.3. Acid stress

In order to cause gastroenteritis, bacterial cells need to survive passage through the stomach. *C. jejuni* has a very low infectious dose (500 cells) implying that it must be able to rapidly adapt to the acidic conditions in the stomach and GI tract. Optimum pH for growth of *C. jejuni* is between 6.5 and 7.5 (Chaveerach et al., 2003). Both *C. jejuni* NCTC 11168 and ATCC 81-176 possess an acid tolerance response (ATR) stimulated by acid and /or oxygen stress, which requires protein synthesis to be activated (Reid et al., 2008; Ma et al., 2009). Exposure to pH 5.5 led to the up regulation of 26 genes and the down regulation of 68 others (Reid et al., 2008) and

cells adapted to low pH for 100 min were able to survive in pH of 4.5 (Ma et al., 2009). In the literature, low pH stress is one of the more common methods used to quickly induce a VBNC state in *C. jejuni* (Table 1.3). With the use of formic acid at pH 4, 10 strains of *C. jejuni* became VBNC within 2 hours (Chaveerach et al., 2003). Other organic acids (acetic and propionic) and hydrochloric acid were unable to induce the VBNC state in *C. jejuni*, suggesting a link between formate metabolism and VBNC formation (Kassem et al., 2013).

1.4.1.4. Osmotic stress

Cells respond to high osmotic environments by producing compatible solutes or increasing uptake of K+ and solutes (Jackson et al., 2009). One K+ transport system has been identified in *C. jejuni*, but it has not yet been determined if this system is induced by osmotic stress (Park, 2002; Jackson et al., 2009). *E. coli, Klebsiella pneumonia* and *Enterobacter* sp. all enter the VBNC state when exposed to osmotic stress (Sachidanandham and Gin, 2009). Generally *C. jejuni* is very sensitive to salinity and will not grow at concentrations above 2% NaCl (w/v), but Doyle and Roman (1982) showed that culturability in 4.5% NaCl was extended by reducing temperature. Although osmotic stress is used with other organisms, no studies were found that used osmotic stress to induce a VBNC state in *C. jejuni*.

1.4.1.5. Other factors

Although there is no work to date examining the ability of heavy metals, white light, or antibiotics to induce a VBNC state in *C. jejuni*, work with other species indicates that these factors contribute to the VBNC state. Navarrete et al (2014) have recently shown that increased zinc concentrations hasten entry into a VBNC state by one to three days for the plant pathogen *Xyella fastidiosa.* Zinc also caused the plant pathogen to form stronger biofilms with increased EPS. White light (sunlight) can induce a VBNC state in *E. coli* and *S. enterica* (Idil et al., 2011) as well as in *Helicobacter pylori* inoculated onto spinach (Buck and Oliver, 2010). The antibiotic Vancomycin induced the VBNC state in *S. aureus* growing in biofilms, which remained viable for 150 days after cells lost culturability (Pasquaroli et al., 2013).

1.4.2. Characteristics of cells in the VBNC state

Entry into a VBNC state is a response to stressful conditions and so most of the changes observed in the cells are attempts to mitigate the damage done by the stress and minimize maintenance requirements.

1.4.2.1. Changes in morphology and size

During exponential growth, *C. jejuni* exhibits a spiral or comma-shaped morphology. It is often the case that cells become coccoid during entry into a VBNC state. Coccoid cells have been observed in stressed cultures of *H. pylori* (Rudnicka et al., 2014), *V. parahaemolyticus* (Su et al., 2013), *V. cholerae* (Senoh et al., 2010) and *E. coli* (Signoretto et al., 2005) among others. Coccoid cells have also been reported for stressed cultures of *C. jejuni* (Lazaro et al., 1999; Tholozan et al., 1999).

Although initially it was believed that entry into the VBNC state was equivalent with the cells becoming coccoid, there is evidence suggesting that the transition to a coccoid morphology is independent of loss of culturability (Cook and Bolster, 2007). Lazaro et al (Lazaro et al., 1999) found that only 1/3 of a population of VBNC *C. jejuni* (strain C-1) cells were coccoid. Tholozan et al (Tholozan et al., 1999) reported that of three *C. jejuni* strains, one became coccoid (shorter and thicker) during stress, while for the other two, most cells remained spiral-shaped (and were longer) for 15 days after becoming VBNC. At the same time, all three strains had similar increases from 1.73 ml/mg of protein for the culturable form to 10.96 ml/mg of protein in the VBNC cells (Tholozan et al., 1999). By inhibiting protein synthesis with chloramphenicol and damaging DNA with irradiation during transition to the VBNC state in *C. jejuni*, Hazeleger et al (1996) showed that transition to the coccoid state was a passive process. Rollins and Colwell (1986) observed that cell size and shape vary significantly within a VBNC population, but acknowledge that *C. jejuni* exhibits condensation of the cytosol during the transition to the VBNC state. This is similar to what has been observed for *E. coli*, which exhibit size reduction or "dwarfing" in the VBNC state (Signoretto et al., 2005).

1.4.2.2. Respiration rates are maintained at a reduced level

Respiration indicates substrate transport and an active electron transport chain and confirms cell viability. *C. jejuni* entered the VBNC state after 15 days of starvation at 4°C and was still actively respiring after 30 days (Cappelier et al., 1999), while induction in PBS at 4°C allowed cells to remain culturable for 48 days and maintain respiratory activity for up to 7 months (Lazaro et al., 1999). In both cases, respiratory activity was reduced as cells entered the VBNC state (Cappelier et al., 1999; Lazaro et al., 1999).

1.4.2.3. ATP and membrane potential are maintained at a reduced level

Culturable *C. jejuni* continue to generate ATP during storage at 4°C (Hazeleger et al., 1998). This indicates that the electron transport chain is active and generating the required proton motive force to produce ATP. ATP levels decreased during starvation at 4°C and fell below detectable levels after 15 days (Tholozan et al., 1999). The membrane potential of *C. jejuni* cells in late log phase was 66mV and dropped to 35mV after 15 days of treatment (Tholozan et al., 1999).

1.4.2.4. Continued gene expression

Continued gene expression, as well as being an indicator of viability, can also provide evidence of the production of toxins or virulence factors. Chaisowwong et al (2012) found that expression of the virulence genes *flaA, flaB, cdtA, cdtB, cdtC, cadF* and *ciaB* was maintained in VBNC *C. jejuni,* leading these authors to suggest that VBNC *C. jejuni* may still be capable of causing disease. Even though expression of these genes was reduced, with the relative expression ratios compared to stationary phase ranging from 0.14 to 0.61 times, the authors suggest that cells in the VBNC state maintain an attenuated virulence (Chaisowwong et al., 2012). Patrone et al (2013) also reported continued expression of the adhesin CadF in VBNC *C. jejuni* and with 27 to 40% reduced efficiency of adherence to Caco-2 cells.

1.4.2.5. Changes to protein profile

Protein synthesis is known to occur in *C. jejuni* at temperatures as low as 4°C (Hazeleger et al., 1998) and protein content was maintained for 196 days in cells held in PBS at 4°C, but not at 20°C (Lazaro et al., 1999). Two dimensional gel electrophoresis results indicated that there were several proteins up or down regulated during the transition to the VBNC state, but the proteins were not identified (Lazaro et al., 1999).

1.4.2.6. Changes to membrane fatty acids:

Although changes in the membrane fatty acid composition during transition to the VBNC state have been reported for *Vibrio vulnificus* (Day and Oliver, 2004), *E. coli* (Muela et al., 2008), *Enterococcus faecalis* (Heim et al., 2002) and *V. parahaemolyticus* (Lai et al., 2009), no changes have been observed for *C. jejuni* (Hazeleger et al., 1995).

1.4.2.7. Changes to peptidoglycan and formation of "blebs"

Analysis of peptidoglycan in VBNC *E.coli* has shown 3 fold increases in DAP-DAP cross linking, shorter glycan strand length, and increases in muropeptides with covalently bound lipoprotein (Signoretto et al., 2002). This may explain the greater mechanical resistance in the VBNC state of *E. coli*, *Klebsiella* and *Enterococcus* cells which were able to withstand 3 cycles of freeze/thaw in liquid nitrogen (Sachidanandham and Gin, 2009). No work has been done to explore the changes in peptidoglycan in *C. jejuni,* but a similar mechanism may exist as *C. jejuni* was able to survive 3 cycles of freeze thaw in either -20˚C or -70˚C and cold-adapted stationary phase *C. jejuni* were more resistant to high hydrostatic pressure (Sagarzazu et al., 2010).

Another adaptation noticed in VBNC *C. jejuni* is the formation of 'blebs' (Lazaro et al., 1999). These extrusions in the outer membrane may be a way of improving substrate uptake in response to the reduced surface area of coccoid cells. Adjusting cell volume to surface area ratio will minimize cell maintenance requirements, while at the same time recycling membrane components in response to starvation.

1.4.2.8. Virulence and ability to cause infection

There is some evidence to support the premise that cells in a VBNC state are still able to initiate infection. VBNC *C. jejuni* has been shown to cause systemic disease (Klancnik et al., 2009) and death (Jones et al., 1991) in mice. It was recently shown that VBNC *C. jejuni* continue to express virulence genes (Chaisowwong et al., 2012), are able invade Caco-2 cells (Klancnik et al., 2009; Chaisowwong et al., 2012) and can survive within these cells for up to 4 days (Klancnik et al., 2009).

Although this evidence suggests that *C. jejuni* retains virulence in the VBNC state, in one study, VBNC *C. jejuni* had no effect on stimulating an immune response as measured by interleukin 8 (IL-8) production in the cell lines used and the decline in culturability was found to be linearly correlated to a decline in adherence and invasion (Verhoeff-Bakkenes et al., 2008; Verhoeff-Bakkenes et al., 2009). This study was done using cell cultures which are not a good model for *C. jejuni* behaviour *in vivo* (Grant et al., 2006; Haddock et al., 2010). Further work with *ex vivo* epithelial tissue where mucus is present, may provide a more accurate representation of what would occur *in vivo* and could be beneficial to future investigations of the virulence potential of VBNC *C. jejuni*.

1.4.3. Resuscitation of VBNC

The idea that cells can become dormant is only relevant if they can resuscitate and resume growth. This has been a contentious issue since the conception of the VBNC state and although it is gaining traction in the food safety community, it continues to be viewed with skepticism. The frequently cited paper by Whitesides and Oliver (1997) used a dilution series in combination with timing to prove that growth of new cultures from previously non-growing cells could not be the result of growth of a few culturable cells, but was only possible if there were resuscitation occurring.

Other methods which show that there was genuine resuscitation and not just recovery of injured cells include the addition of antibiotics to prevent the growth of injured cells, molecular

typing to prove the identity of recovered cells (Cappelier et al., 1999), and extensive work with flow cytometry to show resuscitation of VBNC *E. coli, K. pneumonia* and *Enterobacter* (Sachidanandham and Gin, 2009). Methods which have been explored for their ability to resuscitate VBNC bacteria include reversal of stress, the presence of resuscitation promoting factors and contact with higher organisms. Due to the fact that very little work has been done to explore resuscitation in *C. jejuni*, this section examines resuscitation in other species and includes what is known for *C. jejuni*.

1.4.3.1. Reversal of stress:

Starvation is known to induce the VBNC state in many bacteria and it might follow that a simple reversal of the stress by the addition of nutrients would reinitiate growth. Instead, many authors have found that providing starved cells with rich media has been inhibitory to resuscitation (Whitesides and Oliver, 1997; Lindback et al., 2010; Nicolo et al., 2011). Richards (2011) found that VBNC *H. pylori* induced by oxygen stress were easier to resuscitate by removing the oxygen stress than starved cells were by adding nutrients. They suggest that starved cells experienced "nutrient shock" where rapid transport of nutrients into cells led to more oxidative stress than cells were able to manage and prevented rather than induced resuscitation. The fact that diluted media was more effective than undiluted for resuscitation of *Yersinia pestis* in the VBNC state supports this (Pawlowski et al., 2011). Resuscitation in rich media was successful for *E.coli* and *Shigella flexneri* after acid-induced (grapefruit juice) VBNC entry. In this case VBNC induction was not related to starvation, so resuscitation was likely due to relief from acid stress rather than to the addition of rich media (Nicolo et al., 2011).

Attempts to induce resuscitation by providing a variety of reactive oxygen species (ROS) scavengers led authors to conclude that the resuscitative action of certain ROS scavengers was likely not related to their antioxidant properties (Morishige et al., 2013; Ducret et al., 2014). Of the scavengers tested, only pyruvate, glutamate and alpha-ketoglutarate led to resuscitation (Mizunoe et al., 1999; Morishige et al., 2013; Ducret et al., 2014). In fact, when hydrogen

peroxide (H_2O_2) was used to induce the VBNC state in *S. enterica*, the H_2O_2 treated cells did not retain H_2O_2 (Morishige et al., 2013).

Morishige et al (2013), provided evidence that pyruvate was triggering the synthesis of macromolecules in VBNC *Salmonella*. With the addition of pyruvate, DNA synthesis which had decreased to 1.8% of control during the H_2O_2 stress, rapidly increased in the first 15 minutes and continued a more gradual increase for 60 minutes. This same pattern was observed for proteins but at a slower rate. The presence of pyruvate allowed the use of significantly more radio-labeled precursors during the resuscitation process. These authors tested pyruvate analogues and the results suggested that the 'alpha-keto' residue was needed for the resuscitation effect, but that this structure alone was inadequate(Morishige et al., 2013).

Other authors have successfully resuscitated *E.coli* (Mizunoe et al., 1999; Pinto et al., 2011), *V. parahaemolyticus* (Griffitt et al., 2011) and *Legionella* (Ducret et al., 2014) with the addition of amino acids or peptone. Glutamate is one of the most abundant amino acids and pyruvate is easily converted to the amino acid alanine. These results suggest that amino acids may play a role in resuscitation by initiating the synthesis of macromolecules.

For some cells a simple change back to optimal temperatures was able to resuscitate the VBNC cells. This was the case for *V. vulnificus* which resuscitated upon temperature upshift from 4 to 25°C (Wong et al., 2004).

1.4.3.2. Rpfs: Resuscitation promoting factors

Many bacterial cells secrete auto-inducers in late log phase and these extracellular bacterial proteins have been used to resuscitate various bacteria. Bacteria resuscitated by spent culture supernatant (cell free supernatant) with secreted auto-inducers include *S. aureus* (Pascoe et al., 2014), *E. coli* (Pinto et al., 2011), *S. typhimurium*, EHEC, *Citrobacter freundii,* and *Enterobacter agglomerans* (Reissbrodt et al., 2002). Moorhead and Griffiths (2011) identified an auto-inducer homologue cjA, in *C. jejuni*. Addition of cjA or homoserine lactases allowed earlier entry into the VBNC state in times of stress and also inhibited biofilm formation. More work

needs to be done to better understand the mechanisms behind these responses and the role of Rpfs in resuscitation in *C. jejuni*.

Bacteria will also secrete auto-inducers in response to contact with the hormones epinephrine and norepinephrine which are produced in response to severe tissue injury (Nicolo and Guglielmono, 2012). Enteric pathogens ingested in the VBNC state may encounter tissue damage in the intestinal tract and be exposed to the associated hormones which could lead to their resuscitation.

1.4.3.3. Contact with higher organisms

C. jejuni has been resuscitated in embryonated eggs (Cappelier et al., 1999; Talibart et al., 2000; Chaveerach et al., 2003; Guillou et al., 2008), mice (Cappelier et al., 1999; Baffone et al., 2006; Klancnik et al., 2009) and human volunteers (Black et al., 1992), as well as in their natural host, chicks (Stern et al., 1994; Cappelier et al., 1999). Chaisowwong et al (2012) found that co-culture with human epithelial cells was able to resuscitate cells in some cases, but results were inconsistent. No attempts to resuscitate with *ex vivo* tissue have been made, but studies done with culturable *C. jejuni* have shown that cells behave differently on tissue in the presence of mucus than on *in vitro* cell cultures which lack mucus (Grant et al., 2006; Haddock et al., 2010).

1.4.4. Methods to detect and quantify VBNC cells:

Accurate quantification of *C. jejuni* in the environment and in clinical and food samples is central to protecting human health. An outbreak in Japan with over 1500 cases of salmonellosis from contaminated squid, led to research showing that the causative agent, *S. enterica* serovar Oranienburg was able to enter a VBNC state in response to salt stress (Asakura et al., 2002). This was also the case in the more recent German outbreak, where the less common EHEC strain (104:H4) caused over 3000 cases of hemorrhagic diarrhea with 45 deaths. After this outbreak, the strain isolated from salad sprouts, was shown to enter a VBNC state within 3 days when left in local tap water, with 75% of cells found to be viable after 10 days (Aurass et al., 2011).

Development of detection assays based on viability and policy changes to replace old standard culturability methods with new "viability-based" methods is beginning to be implemented. Molecular detection methods have been approved for norovirus and STEC in Europe (ISO/TS 15216-1:2013; ISO/TS 13136). These are the first two ISO standard reference methods based on RT-PCR and their approval may pave the way for the use of molecular methods for other pathogenic bacteria. In Canada, molecular detection methods for food are currently under development (Carrillo, 2011) and a PCR-based method for *C. jejuni* has recently been AOAC approved for screening of ready to eat turkey and chicken carcass rinses (AOAC, 2014).

This review of methods begins with a description of enumeration methods based on culturability, focusing on detection of *C. jejuni* in food in Canada. This is followed by a description of methods used to assess viability in non-culturable cells.

1.4.4.1. Detection and enumeration of culturable cells

In order to culture specific bacteria, the correct culture conditions and media must be used. *C. jejuni* is a good example of a bacterium that remained undetected for many years due to its specific growth requirements. Although it was originally isolated in 1886 by Theodor Escherich from the stools of an infected infant, the lack of knowledge about its growth requirements at the time meant that it could not be cultured and was not confirmed as the cause of disease (Kist, 1986). Despite the fact that culture methodology has advanced, there are still many limitations (Melero et al., 2011; Trevors, 2011; Fittipaldi et al., 2012).

In Canada, regulations regarding food safety are established by Health Canada and enforced by the Canadian Food Inspection Agency (CFIA). The approved laboratory procedures for isolating bacteria of the genus *Campylobacter* in foods as described in the Compendium of Analytical Methods, Volume 3, MFLP-46 were last updated in March, 2002 (Laboratory Procedures for the Microbiological Analysis of Foods. Vol 3. The Compendium of Analytical Methods, 2014). The procedure consists of four stages, selective enrichment, colony formation

on selective agars, purification and identification. Specific sample preparation is described for fresh raw meat, pork and poultry samples, milk, shellfish, frozen foods and swab samples. All samples are incubated in Park and Sanders enrichment broth in microaerobic conditions for 3-4 hours at 37°C, then transferred to 42°C for 24 and 48 h. After the period of enrichment, samples are streaked onto *Campylobacter* blood-free selective agar (CCDA) and Preston agar and incubated at 37°C for up to 72 h in microaerobic conditions. Suspect colonies are those which are smooth, convex, translucent and colourless to cream coloured are selected for purification by streak plating followed by microscopy and biochemical tests. In wet mount preparations of each colony, young *Campylobacter* cells will appear S-shaped and have a cork-screw like motility. Cells older than 72 h may appear coccoid. Biochemical tests are used to confirm identification. *C. jejuni* is catalase and oxidase positive, negative for resistance to nalidixic acid and positive for nitrate reduction.

1.4.4.2. Detection and enumeration of 'viable' cells

Cellular viability requires i) the presence of functional and intact nucleic acids, ii) minimum cellular energy to allow basic functioning of cellular processes and iii) an intact and polarized cytoplasmic membrane (Hammes et al., 2011). In a review of microbial injury and recovery in food, Wu (2008) points out that membrane damage is the most frequent type of damage and the most likely to lead to cell death. Membrane integrity is recognized as one of the most significant indicators of viability and use of the compromised cytoplasmic membrane as an indicator of cell death is a valuable research tool (Nebe-von-Caron et al., 2000; Trevors, 2012)

Numerous assays have been developed to assess the viability of cells that are not culturable. Indicators of viability include: continued gene expression as measured by reverse transcriptase PCR (Buck and Oliver, 2010; Chaisowwong et al., 2012; Patrone et al., 2013), protection of DNA from Dnase I digestion (Pawlowski et al., 2011), continued cell growth in the presence of cell division inhibitors (Kogure et al., 1979), ATP generation (Lindback et al., 2010), metabolic activity indicated by reduction of tetrazolium salts (Cappelier et al., 1999; Lazaro et al.,

1999; Cook and Bolster, 2007; Perez et al., 2010), uptake of labeled amino acids (Pawlowski et al., 2011) and the presence of an intact membrane (Nogva et al., 2003; Rudi et al., 2005; Duffy and Dykes, 2009; Chen and Chang, 2010; He and Chen, 2010; Chaisowwong et al., 2012).

The advent of reverse transcription made it possible to use PCR based molecular methods to assess viability. Continued gene expression, as measured by reverse transcription PCR is a good indicator of viable cells. Although there is a wide range of values for mRNA half-life cited in the literature, there is general agreement that mRNAs are not very stable and their presence indicates a functioning cell. The half-life of mRNA in *E. coli* ranges from seconds to 20 minutes (Sheridan et al., 1999; Conway and Schoolnik, 2003). According to Smith and Oliver (2006) the upper limit in VBNC cells is 60 minutes, but Lindback (2010) states that the mRNA half-life in *L. monocytogenes* increases from 2-4 minutes in culturable cells to 6-10 hours for cells in the VBNC state. Given that cells can remain in a VBNC state for up to 7 months (Lazaro et al., 1999) the presence of mRNA will at least indicate that cells were functioning in the last 10 hours. Reverse transcription PCR was successful for detecting non-culturable *H. pylori* within a biofilm formed in drinking water from a surface water reservoir (Linke et al., 2010).

One of the first attempts to assess the presence of viable but non-culturable cells was the 'Kogure method' (Kogure et al., 1979). In this substrate responsive assay, nalidixic acid was used to prevent cell division and the presence of elongated cells indicated viability (Rollins and Colwell, 1986; Griffitt et al., 2011).

Given that metabolic activity is a strong indicator of viability, numerous authors have used tetrazolium salt reduction to measure cellular respiration (Cappelier et al., 1997; Lazaro et al., 1999; Cook and Bolster, 2007; Gangaiah et al., 2009). Perez et al (2010) developed a colorimetric assay with tetrazolium salt reduction and used it to assess VBNC cells in biofilms. The biofilms produced by diluted municipal treated wastewater, were grown in 24 well plates and OD values provided indications of viability. Ducret et al (2014) used the ChemChrome V6 procedure where a fluorogenic ester is converted to free fluorescein by cytoplasmic esterases.

This shows metabolically active cells and when used in conjunction with NIS-Element (Nikon) software it allows enumeration and manual differentiation between individually labeled cells, cells in aggregation, and/or auto-fluorescent particles which can interfere with automated analysis. Another strong indicator of viability, ATP generation, was measured with the Luciferase assay by Lindback et al (2010) showing that the ATP concentration in VBNC *L. monocytogenes* cells remained constant for 12 months. Pawlowski et al (2011) assessed viability in VBNC *Y. pestis* 25 days after cells became non-culturable, by measuring uptake of labeled amino acids, protection of DNA from DNase I digestion and cellular membrane integrity.

The use of fluorescent staining to indicate membrane integrity has become one of the most commonly used methods to assess cell viability. Although membrane integrity alone does not prove viability, the absence of an intact membrane is a good indication of a non-viable cell (Wu, 2008; Hammes et al., 2011; Trevors, 2012). Numerous authors have employed *Bac*Light™ Live/Dead stain to determine viability in their samples (Nogva et al., 2003; Rudi et al., 2005; Duffy and Dykes, 2009; Chen and Chang, 2010; He and Chen, 2010; Chaisowwong et al., 2012). Often it is used with epifluorescent microscopy, but it can also be applied to flow cytometry. Cell counts can be done manually but image analysis software is often employed and has been shown to be more accurate (Seo et al., 2010).

1.4.4.3. Using EMAqPCR and PMAqPCR for detection of viable planktonic cells

Reverse transcription PCR confirms viability and provides information about the expression of particular genes, but can't be used to quantify the number of viable cells in a sample. A newly developed method addresses this knowledge gap by incorporating intercalating agents with quantitative PCR (Nogva et al., 2003)

This method includes pre-treatment of samples with ethidium-monoazide (EMA) or propidium monoazide (PMA) which enters dead cells and cells with membrane damage, where it intercalates with the DNA. Any remaining EMA or PMA is deactivated by light exposure prior to

DNA extraction and PCR. This means that only DNA from intact cells will be amplified, making it possible to use qPCR to quantify the number of viable (intact) cells in the sample.

EMA concentrations of 10-100 µg/ml were evaluated with *E.coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* and although the authors found that 100 µg/ml was most efficient, they cautioned that the PCR signal for viable controls treated with EMA was lower than for viable cells with no EMA added, indicating that EMA may have entered some of the viable cells (Nogva et al., 2003).

Further investigation of EMAqPCR indicated that although transport pumps actively exported EMA from metabolically active cells, in some species, small amounts of EMA remained and led to substantial loss of detection of DNA in intact cells (Nocker and Camper, 2006). *Streptococcus sobrinus, Micrococcus luteus, S. aureus, L. monocytogenes* and *Mycobacterium avium* were less efficient at removing EMA from viable cells than*, E. coli* O157:H7*, Serratia marcescens, S. typhimirium* or *Pseudomonas syringae* (Nocker et al., 2006). In this same study, in an attempt to address the issue of reduced DNA detection from viable cells when using EMA, a similar compound propidium monoazide (PMA) was investigated and compared to EMA. PMA is identical to the commonly used stain propidium iodide (PI), except for presence of an azide group that allows cross-linking with DNA. PI is membrane impermeant and generally excluded from viable cells. PMA has a similar reduced ability to penetrate live cells as EMA, due to its higher charge. PMA at 50 µM was found to be effective for all the species in the study (Nocker et al., 2006).

Other authors also had success with PMAqPCR. PMA at 50 µM was effective for *Listeria monocytogenes* (Pan and Breidt, 2007), *S. aureus* and *S. epidermis* (Kobayashi et al., 2010) and 50-100 µg/ml of PMA when used with for 10^7CFU/ml *of Enterobacter sakazakii* completely inhibited amplification from dead/damaged cells, but caused no significant inhibition of the amplification from viable cells (Cawthorn and Witthuhn, 2008). Josefsen et al (2010) found that 10µg/ml was sufficient for use with *C. jejuni* in chicken carcass rinse.

There are conflicting reports for the effectiveness of EMAqPCR for the accurate quantification of viable cells. In one study, EMA at 100 µg/ml used with *C. jejuni*, 1) gave a good quantitative prediction of the fraction of viable cells in a sample, 2) was not influenced by background microflora and 3) could be used on spiked chicken breast and leg (Rudi et al., 2005). EMAqPCR at 20 µg/ml was successful for the enumeration of viable *C. jejuni* in the mixed cultures with *E.coli* and *P. fluorescens,* as well as being applicable to all growth phases, but when assessing *C. jejuni* with EMA at concentrations above 20 µg/ml, DNA from some of the viable cells was lost (He and Chen, 2010). Lower concentrations of EMA were also found to be effective for *V. vulnificus* (3.0 µg/ml) (Wang and Levin, 2006), *Salmonella* in chicken and eggs (10µg/ml) (Wang and Mustapha, 2010) and Legionella in water (2.5µg/ml) (Mansi et al., 2014).

Other studies found EMAqPCR to be inadequate. Flekna and Hein (2007) found that when assessing EMA concentrations between 1-100 µg/ml with *C. jejuni* and *L. monocytogenes*, using enough to prevent amplification of dead DNA was toxic to viable cells. EMA also inhibited amplification of viable cells in *E. sakazakii* (Cawthorn and Witthuhn, 2008). There appear to be no studies citing problems with the use of PMA.

1.4.4.4. Using EMAqPCR and PMAqPCR for detection of viable cells in a biofilm:

Although most work done with EMA and PMAqPCR has been with planktonic cells, there are some examples of these assays being applied to biofilms. No work to date has investigated the use of these techniques with *C. jejuni* biofilms. EMA was found to be successful for *E.coli* O157:H7 biofilm cells at a concentration of 100 µg/ml (Marouani-Gadri et al., 2010) and for *Legionella* in biofilms at 2.3 µg/ml. Even at low concentrations EMA was found to be toxic for *L. monocytogenes* biofilm cells (Pan and Breidt, 2007) and *Streptococcus mutans* and *S. sobrinus* from oral biofilms (Yasunaga et al., 2013), but in the latter 2 cases PMA was successful at concentrations of 50µM and 25µg/ml respectively. This preliminary work demonstrates the potential of this new technique, but further optimization may be required.

Biofilms are being accepted as the normal mode of growth for microorganisms (Davies, 2003). Given that *C. jejuni* finds protection and extended survival within a biofilm, it is critical to develop techniques to determine the potential for cells in a biofilm to enter the VBNC state. One of the objectives of this thesis was to assess PMAqPCR as a technique that can be applied to *C. jejuni* biofilm cells.

Table 1.2. Pathogens known to enter a VBNC state. (with permission from Dr. Jim Oliver)

Acetobacter aceti
Acinetobacter calcoaceticus
Aeromonas hydrophila
A. salmonicida
Agrobacterium tumefaciens
Alcaligenes eutrophus
Aquaspirllum fasciculus
Arcobacter butzleri
Azotobacter vinelandii
Bacillus megaterium
Bifidobacterium animalis
B. lactis
B. longum
Burkoldaria cepacia
B. pseudomallei
Campylobacter coli
C. jejuni C. larii
Chlamydia spp.
Citrobacter freundii
Cytophaga allerginae
Edwardsiella tarda
Enterobacter aerogenes
E. agglomerans
E. cloacae
E. sakazakii
Enterococcus faecalis
E. facium
E. hirae
Erwinia amylovora
Escherichia coli
(including O157:H7*)*
Francisella noatunensis
F. tularensis
Glaciibacter superstes
Gordonia jinhuaensis
Halococcus dombrowski
Helicobacter pylori
Klebsiella aerogenes
K. pneumoni K.
planticola
Lactobacillus lactis
L. lindnerii
L. paracollinoides
L. plantarum
Legionella pneumophila
Listeria monocytogenes
Listonella pelagia
Methylocystis parvus
Microbacterium sp.
Micrococcus flavus
M. luteus
M. varians
Mycobacterium avium
M. smegmatis
M. tuberculosis
Oenococcus oeni
Pasteurella piscida
Pseudomonas aeruginosa
P. fluorescens
P. putida
P. syringae
Ralstonia solanaceae
Rhizobium leguminosarum
Rhodococcus rhodochrous
Salmonella enteritidis
S. typhi
S. typhimurium
Serratia marcescens
Shigella dysenteriae
S. flexneri
S. sonnei
Sinorhizobium meliloti
Staphylococcus aureus
S. epidermidis
Stappia sp.
Tenacibaculum sp.
Vibrio anguillarum
V. campbellii
V. cholerae
V. fischeri
V. harveyi
V. mimicus
V. natriegens
V. parahaemolyticus
V. proteolytica
V. shiloi
V. tasmaniensi
V. vulnificus (Bt 1 & 2)
Xanthomonas axonopodis
X. campestris
Yersinia pestis

Table 1.3. Summary of VBNC induction methods and results.

Author Year	Strain	VBNC induction method	Remain culturable on (X) with enrichment (Y) until (Z)	VBNC = Remain viable using (X) method	Summary
Aurass 2011	*E.coli* O104:H4	9% saline 9% saline with Cu^{2+} Tap water (tap 1) Tap water (tap 2) 23°C and 4°C 15-fold higher Cu^{2+} in tap 1 than tap 2	X= Nutrient Agar Y = no enrichment Z = Day 5 (copper, 4°C) Z = Day 3 (copper, 23°C) Z = Day 3 (tap 1, 23°C) All others remained culturable to Day 40	*Bac*Light™ Live/Dead staining (*Bac*Light)	Bacteria linked to 2011 outbreak in Germany: A fraction of *E. coli* O104:H4 outbreak strain cells remained viable in microcosms for more than 30 days in the VBNC state. Recovery of copper induced VBNC cells by washing in cold EDTA and plating on rich media
Baffone 2006	*C. jejuni* ATCC 33291 nine clinical isolates	Sterile artificial sea water (ASW) at 4°C	X = Columbia agar base Y = Nutrient Broth No. 2 with: 1. Exeter broth – with supplements (0.02% ferrous sulphate, sodium pyruvate and sodium metabisulphate) and antimicrobial agents 2. Wriable broth – aerotolerant as above with different antimicrobials 3. Preston broth - Karmali agar Z = 12 to 35 days, enrichment prolonged culturability 3-7 days	CTC-DAPI (5-cyano-2,3-ditolyl tetrazolium chloride and 4,6-diamidino-2-phenylindole staining) Resuscitate in mouse model	Testing various enrichment methods to extend culturability
Buswell 1998	*C. jejuni* CH1 and 9752	a) Sterilized ground water from a borehole b) Two-stage continuous-mixed-culture aquatic biofilm model	a) Columbia blood agar (CBA) b) CBA with Skirrow selective supplement Y = no enrichment a) Z = 230 h at 4C b) Z = 700 h at 4C	Immuno-fluorescent antibody staining, and *Campylobacter*-specific RNA probe	Viability maintained to end of experiment (42 days) in 4°C

Cappelier 1999a	*C. jejuni* 79, 85, and Bf,	Sterile surface water	X = CBA Y = Park and Sanders without antibiotics - plate on Karmali and Columbia agars Z = 17 days	CTC-DAPI, Injected diluted cells into yolk sac (25 VBNC cells/ml) Adherence to HeLa cells varied by strain	Recovery in embryonated eggs of VBNC *C. jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation
Cappelier 1999b	*C. jejuni* Bf	Sterile surface water	X = Columbia blood agar Y = Park and Sanders without antibiotics - 48hr incubation at 37°C then spread plate on Karmali agar as well as Columbia agar Z = 15 days	CTC-DAPI Better resuscitation in mice than chicks	Recovery of VBNC *C. jejuni* cells in two animal models Good summary of early resuscitation attempts 4°C cells become VBNC 25°C cells die
Chaisowwong 2012	*C. jejuni* CG8486	Bolton broth at 4°C	X= modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) Y = Bolton and/or Preston broth with supplement SR 0232 (Oxoid) Z = 38 days	*Bac*Light RTqPCR	Virulence genes still expressed in VBNC *C. jejuni* Also, adherence and invasion did occur and in some cases cells were resuscitated by co-culture with epithelial cell lines
Chaveerach 2003	*C. jejuni* and *C. coli 10* strains isolated from chickens main 3: C350, C4602, C144	Incubation in Mueller Hinton broth with formic acid (pH=4) 37°C for 2hr	X = charcoal, cefoperazone deoxycholate agar (CCDA) without antibiotics Y = buffered peptone water Z = 1 to 2 h	CTC-DAPI Recovery in embryonated eggs	Survival and resuscitation of 10 strains of *C. jejuni* and *C. coli* under acid conditions
Chen and Chang 2010	*Legionella pneumophila*	Water from cooling towers and hot water systems	X = buffered charcoal yeast extract agar - according to ISO 1998 Z = not done	*Bac*Light	EMA and PMAqPCR Viable counts higher than culturable counts
Cook and Bolster 2007	1. *C. jejuni* ATCC 49943 2. *E.coli*	Ground water at 4°C	1. X = *Campylobacter* selective agar with Preston selective supplement (Oxoid SR0204) Z = 42 to 85 days (by source); 2. X = Luria agar Z = up to 470 days	CTC - DAPI 1. viable for 100 days 2. viable for 140 days	Comparison of the survival of *C. jejuni* and *E. coli* in ground water

Ducret 2014	*Legionella pneumophila*	HOCl treatment	X = buffered charcoal yeast extract Y = no enrichment	ChemChrome V6 Kit: Quantitative microscopic analysis-	Resuscitation on agar with ROS scavengers (pyruvate, glutamate) which help injured cells to recover after a stress. (see Morishige)
Duffy and Dykes 2009	*C. jejuni* from beef cattle feces B1, B2, retail chicken meat C1, C2, and ATCC 33560	Sterile deionized water at 3.5°C for 30 days	X = charcoal cefazolin sodium deoxycholate agar - no antibiotics Y = no enrichment Z = 10 to 20 days	*Bac*Light	VBNC cells still attach to stainless steel
Gangaiah 2009	*C. jejuni* 81-176	Acid stress, pH=4	X = MHA Y = no enrichment Z = 1h After 3 hours % viable : 42.4 % of WT 2.8 % of *ppk1* mutant	CTC-DAPI	ppk1 mutant: - deficient in poly-P accumulation - decreased ability to form VBNC cells under acid stress. - biofilm increased
Griffitt 2011	*Vibrio parahaemolyticus*	artificial sea water at 4°C	X = T1N3 agar plates (10-g Tryptone, 30-g NaCl, 7-g agar per 1000-mL diH2O, pH 7.2) and CHROMagar Vibrio Y = no enrichment Z = 50 days	RING-FISH probe	Resuscitation in alkaline peptone water
Guillou 2008	*C. jejuni* NCTC 11168 and Bof	Bottled water 4°C	X = Columbia blood agar (non-selective) and Karmali (selective)agar Y = Preston broth Z = 20 to 48 days	Recovery in embryonated eggs	Recovery of VBNC *C. jejuni* from bottled water
Hazeleger 1998	*C. jejuni* strains 104 and ATCC 33560	Cold stress – but not to full VBNC state	NA	Chemotaxis and aerotaxis observed at all temperatures	Oxygen consumption, catalase activity, ATP generation, and protein synthesis observed at temperatures as low as 4°C

Klancnik 2009	*C. jejuni* K49/4	Ringer solution with chloramphenicol	X = Karmali agar Y = no enrichment Z = 3 days for log phase cells Z = 23 days for stationary phase cells	*Bac*Light	Starved cells had: - lower metabolic activity - induce heat stress resistance - survive 4 day in Caco-2 cells - cause disease in mouse
Lai 2009	*Vibrio parahaemolyticus*	Morita mineral salt-0.5% NaCl at 4 °C for six weeks	Investigation of protein profile	*Bac*Light	13 up-regulated proteins associated with transcription, translation, ATP synthase, gluconeogenesis-related metabolism, antioxidants
Lazaro 1999	*C. jejuni* C-1 and same strain adapted to mouse intestine: $C\text{-}1_{RR}$	Phosphate buffered saline at 4°C and 20°C	X = CBA Y = no enrichment At 4°C: Z = 50 days C-1 Z = 100 days $C\text{-}1_{RR}$ At 20°C: Z = 7 days for C-1 Z = 7 days for $C\text{-}1_{RR}$	CTC-DAPI Cellular integrity, intact DNA, respiring cells – all detected up to 7 months at 4°C	Spiral to coccal shape does not directly correspond to loss of culturability, Percent of cells respiring after 30 days starvation was higher than % spiral or % culturable Starved at 4°C formed blebs Overall many proteins still at 4°C after 196 days but very few at 20°C, Less intact DNA after 20 days at 20°C than 116 days at 4°C
Mizunoe 1999	Late-exponential-phase cells of Escherichia coli O157:H- strain E32511/HSC	sterile distilled water at 4 °C.	X = Luria-Bertani Y = no enrichment Z = 21 days	*Bac*Light Resuscitation on agar with pyruvate and alpha-ketoglutaric acid	VBNC samples inoculated onto agar medium amended with catalase or nonenzyme peroxide-degrading compounds such as sodium pyruvate or a-ketoglutaric acid, plate counts increased to 10^4–10^5 CFU/ml within 48 h.

Moorhead 2011	*C. jejuni* 81-176 *C. jejuni* cj11	Sterile distilled water at 4°C and 10°C	X = Nutrient broth2 agar Y = no enrichment Z = Experimental conditions were maintained until the viable count was undetectable over three consecutive days. Time for a 5 $\log_{10}$ reduction was calculated	CTC-DAPI	Short chained HSLs and the novel compound cjA prolonged the delay to a VBNC state as well as inhibiting biofilm formation
Morishige 2013	*Salmonella*	0.1 to 1.0 mM H_2O_2 for 60 minutes	X = Luria-Bertani agar Y = no enrichment Z = VBNC based on log reduction	*Bacstain* CTC Rapid Staining Kit	Addition of pyruvate initiated resuscitation by triggering synthesis of DNA and protein
Nicolo 2011	*E. coli* *L. monocytogenes* *S. enterica* *S. flexneri*	Grapefruit juice	X = tryptone soya broth (*L. monocytogenes*) X = LB (other 3) Y = no enrichment Z = 24 h (except *S. flexneri* – still culturable at 48 h)	*Bac*Light	Entry in to VBNC and survival in spiked grapefruit juice All except *L. monocytogenes* were viable at 24 h
Pascoe 2014	*S. aureus*	50 ml of sterile distilled water and held at 4uC for up to a month	X = MPN - inoculate 5 replicates of 10-fold serial dilutions in TSB medium Y = no enrichment Z = 30 days (<10CFU based on MPN)	Resuscitation with spent media	Dormant cells of Staphylococcus aureus are resuscitated by spent culture supernatant
Pasquaroli 2013	*S. aureus* 10850 biofilms	Antibiotics (vancomycin or quinupristin/ dalfopristin) with nutrient depletion 37C	X = TSA Y = no enrichment Z = 10 - 30 days (antibiotic) = 40 days without antibiotic = 60 days with rich media Viable cells remained constant for 150 days beyond loss of culturability when VBNC from antibiotics, shorter time with starvation	*Bac*Light, RTqPCR, resuscitation in rich media with pyruvate	Antibiotics induce VBNC cells to form within biofilms

Patrone 2013	*C. jejuni* ATCC 33291 and a human clinical isolate *C. jejuni* 241	Freshwater 4°C	X = CBA with Preston *Campylobacter* Selective Supplement Y = no enrichment Z = 48 (241), Z = 46 (33291)	CTC-DAPI Note: addition of pyruvic acid prior to CTC RTq PCR 10^6 cells viable at day 60 by CTC	*CadF* expression in VBNC cells still expressed at VBNC entry, In the VBNC state, *C. jejuni* 241 and *C. jejuni* ATCC 33291 showed 26.9 and 40% reductions in efficiency of adherence to Caco-2 cells
Pawlowski 2011	*Yersinia pestis*	Autoclaved tap water, river water at 4°C	X = TSA Y = TSA with pyruvate Z = 21 days (autoclave tap water) Others all growing at day 28	*Bac*light Viable cells at 46 days	DNase I protection in VBNC *Y. pestis,* Radiolabeled amino acid uptake in VBNC cells
Pinto 2011	*E. coli*	Deionized water with varying degrees of salinity at 4°C	X = TSA Y = TSA with pyruvate Z = 29 weeks	Kogure method: use nalidixic acid and look for cell elongation(Kogure et al., 1979)	Resuscitation of VBNC *E. coli* with addition of amino acids or CFS
Rollins and Colwell 1986	*C. jejuni* HC clinical isolate, minimally passaged prior to storage in liquid nitrogen	1)brucella broth agar biphasic system 2) 50ml of brain heart infusion broth with yeast 3) filter sterilized, aged stream water (pH 7.1)	X = Spread plate counts on 5% sheep blood agar Y = ND *In stream water:* Z = $>10^4$ CFU for more than 4 months at 4°C Z = 28 days at 25°C Z = 10 days at 37°	Kogure method, Metabolic assay - evolution of labeled CO_2 from cells $>10^6$ viable cells/ml in VBNC samples	Initial study investigating the VBNC state in *C. jejuni,* Loss of culturability occurs sooner in shaken systems, Significant numbers of culturable cells in the biphasic cultures as late as 1 year postinoculation
Sachidanandham 2009	*Enterobacter* sp. mcp11b *Klebsiella pneumonia* mcp11d *E. coli*	Osmotic stress	not done	*Bac*light	Flow cytometry to assess cell viability and resuscitation by removal of stress

Senoh 2010	i) *Vibrio cholerae* O139 VC-280 ii) *V. cholerae* O1 N16961 *V. cholerae* O139 iii) VC- 280/pG13, GFP labeled version of O139 VC-280	Artificial sea water 4°C	X = nutrient agar supplemented with 1% NaCl Y = Alkaline peptone water Z = 48 days without enrichment Z = 70 days with enrichment	*Bac*Light Resuscitation by co-culture with epithelial cells	Conversion of viable but nonculturable *Vibrio cholerae* to the culturable state by co-culture with eukaryotic cells
Signoretto 2005	*E. coli* KN126	Sterilized lake water at 4°C	X = 10 ml of water filtered on membrane - placed face-up onto LB agar - colony appearance two days later Y = no enrichment Z = 14 days	Kogure method	Description of modification of the peptidoglycan in VBNC *E. coli*
Smigic 2009	*C. jejuni* 603 and 608	Exposure to lactic acid (pH=4)	X = Columbia base agar with horse blood Y = Bolton Broth Z = cells remained culturable during the 12 minutes of testing	Not done	Resuscitation after lactic acid treatment Assessing cell pH levels using Fluorescence Ratio Imaging Microscopy
Stern 1994	*C. jejuni* 6 poultry isolates	Phosphate buffered saline at 4°C	X = Brucella-FBP agar Y = selective enrichment (Stem and Line 1992) Z = 4-7 weeks without enrichment Z = 8 weeks with enrichment	Colonization of poultry	Colonization of chicks by non-culturable *Campylobacter* spp.
Talibart 2000	85 strains of *Campylobacters,* *C. jejuni* ATCC 33560	Sterile water at 4°C	X = Columbia blood agar Y = 48 h in Preston medium with blood Z = 8 to beyond 60 days	Resuscitation in embryonated chicken eggs	Resuscitation of VBNC *C. jejuni* after 30 days, 51% of the VBNC samples were recovered by injection in 9-day chicken eggs
Tholozan 1999	*C. jejuni* Bf, 79, and 85	Sterile surface water at 4°C for up to 30 days	X = Columbia Blood agar Y = Preston media Z = 14 to 16 days	CTC-DAPI	Physiological characterization of VBNC *C. jejuni*

Whitesides 1997	*Vibrio vulnificus* C7184 opaque	ASW at 5°C	X = heart infusion agar Y = no enrichment Z = 4 to 6 days	Kogure method	Resuscitation of *Vibrio vulnificus* from the VBNC state – frequently cited paper – proof on resuscitation not just regrowth – based on CFU increasing too rapidly for growth to be occurring
Wong 2004	*Vibrio parahaemolyticus* ST550, a serotype O4:K13 and KP+ clinical strain - 20 clinical and 4 environmental strains	Morita mineral salt solution at 4°C	X = TSA-3% NaCl Y = TSA-3% NaCl with catalase Z = 35 to 49 days Catalase did not enhance culturability	*Bac*Light	Resuscitation of viable but non-culturable *Vibrio parahaemolyticus* in a minimum salt medium by temperature upshift

1.5. Selection rationale and description of target genes

1.5.1. Selection of target genes

Although little work has been done to explore the interactions between the VBNC and biofilm survival strategies, there are studies which suggest that the molecular mechanisms of these phenotypes may be related. Gene mutation studies have revealed that genes involved in the transition to a VBNC state also play a role in biofilm formation (Candon et al., 2007; Gangaiah et al., 2009; Drozd et al., 2011). Four genes have been found to influence both biofilm formation and stress survival, the carbon starvation regulator (*csrA*), the stringent response regulator (*spoT*), polyphosphate kinase1 (*ppk1*), and the alkaline phosphatase (*phoX*).

Mutants for *ppk1, phoX* and *spoT* all exhibit enhanced biofilm formation, and low nutrient survival defect indicated by reduced survival in media with no carbon or phosphate (Gaynor et al., 2005; Gangaiah et al., 2009; Drozd et al., 2011) (Table 1.4). The ability to enter the VBNC state has only been investigated for the *ppk1* mutant. After 1 hour of acid stress both the *ppk1* mutant and WT cells were non-culturable and while 96% of WT cells remained viable, only 36% of the mutant did (Gangaiah et al., 2009). Expression of *csrA* in Δ*ppk1* and Δ*phoX* was increased 5 and 2.6 fold respectively (Gangaiah et al., 2009; Drozd et al., 2011). *CsrA* expression was not measured in the *spoT* mutant. CsrA is known to positively regulate biofilm in *C. jejuni*, but no work has been done to determine its role during low nutrient stress (Fields and Thompson, 2008).

The next section provides a more thorough description of each gene beginning with a general description followed by what is known in *C. jejuni*. This is concluded with a summary of likely interactions and potential roles for each gene in the VBNC process in *C. jejuni*.

1.5.2. Description of target genes

1.5.2.1. The carbon starvation regulator (CsrA)

Global regulatory networks such as the Carbon Starvation Regulator (CsrA) allow bacteria to coordinate the expression of large sets of genes in response to changing environmental and physiological conditions. CsrA is a small dimeric RNA binding protein that regulates the translation of various target transcripts. CsrA operates by binding to mRNA transcripts thereby either activating or repressing translation (Timmermans and Van Melderen, 2010). In *E.coli* it regulates stationary phase metabolism, activating exponential phase processes such as motility and glycolysis, and repressing various stationary phase functions, like gluconeogenesis and biofilm formation (Edwards et al., 2011). CsrA also regulates various virulence factors: host cell invasion, quorum sensing, iron acquisition, type III secretion systems, outer membrane protein (OMP) expression, and oxidative stress resistance. CsrA is widespread in eubacteria, but has some variation in function between species. Control of CsrA has been studied extensively in *E. coli* (see (Timmermans and Van Melderen, 2010; Edwards et al., 2011). CsrA expression is under the positive control of σ^{38} which is upregulated at the onset of stationary phase or during exposure to stress (Timmermans and Van Melderen, 2010). Further control occurs as CsrA is sequestered by the non-coding RNAs, CsrB and CsrC (CsrB/C) which are upregulated by the presence of ppGpp (Edwards et al., 2011).

1.5.2.2. CsrA in C. jejuni

Two studies directly examine the role of CsrA in *C. jejuni*, the initial investigation by Fields and Thompson in 2008 and their follow up work in 2012 (Fields and Thompson, 2008, 2012). The initial mutation study revealed roles for motility, biofilm formation, adherence to epithelial cells and oxidative stress defense for CsrA in *C. jejuni*. In order to further explore the molecular mechanisms of CsrA in *C. jejuni*, Fields and Thompson (2012) investigated the ability of CsrA from *C. jejuni* to rescue the phenotypes of an *E.coli csrA* mutant. The role of CsrA in *C. jejuni* is consistent with *E. coli* for activating motility, and providing protection from oxidative

stress, but *C. jejuni* is incapable of glycolysis and CsrA positively regulates biofilm formation rather than repressing it. A phylogenetic comparison of CsrA in *C. jejuni* and *E. coli* found that there were amino acid differences in both of the RNA binding domains known to be functional in *E. coli*. In spite of these differences, the *C. jejuni* CsrA was able to restore motility and reduce the excess biofilm formation back to WT levels in the *E.coli* mutant. More work is needed to better understand the roles of CsrA in *C. jejuni*.

1.5.2.3. The stringent response

The stringent response (SR) is a global stress response mediated by the alarmone, guanasine tetraphosphate (ppGpp). Much of the early work characterizing the SR was done in the Gram negative, gamma-proteobacterium *E.coli*, where the SR is mediated by a ppGpp synthetase, Rel A, and a bifunctional synthetase/hydrolase, SpoT. The SR is typically activated by amino acid starvation. The reduced number of aminoacylated tRNAs cause the ribosome to stall, which is believed to trigger ribosomal bound RelA to catalyze the synthesis of pppGpp. This is hydrolyzed to ppGpp which then binds to the β sub-unit of RNA polymerase (RNAP) altering its promoter affinity. Without ppGpp, RNAP in association with σ^{70} and *dksA* will initiate transcription with AT rich promoters such as the *rrn* promoters. However, when ppGpp binds to RNAP there is preferential transcription of GC rich promoters (Dalebroux et al., 2010). This repression of *rrn* promoters leads to reduced production of ribosomes, which in turn results in reduced protein synthesis. This response, often associated with stationary phase in bacteria, is induced by low nutrient conditions, and alters gene expression to favour survival over growth (Goelzer and Fromion, 2011). In gamma-proteobacteria the SR can also be initiated by low levels of carbon, iron, fatty acids, and phosphorus (Dalebroux et al., 2010).

1.5.2.4. The stringent response is mediated by spoT in C. jejuni

Alpha and epsilon-proteobacteria and Gram positive bacteria have a single bifunctional ppGpp synthetase/hydrolase. In *C. jejuni* this enzyme, encoded by *spoT*, catalyzes both the

synthesis and hydrolysis of (p)ppGpp . Although the mechanism for synthesizing (p)ppGpp in *C. jejuni* has not been studied, it has been suggested that spoT is bound to the ribosome and catalyzes the synthesis of (p)ppGpp in response to amino acid starvation in a manner similar to that described above for *E.coli* (Gaynor et al., 2005). Wild-type(WT) *C. jejuni* accumulated large amounts of (p)ppGpp in response to nutrient downshift from MHB to MOPS-MGS which contains no carbon or phosphate, but had no increase in ppGpp levels in nutrient-rich conditions (Gaynor et al., 2005; Wells and Gaynor, 2006). Mutants for *spoT* were (p)ppGpp^0 (ppGpp was absent in the mutants) and were defective for stationary phase/low nutrient stress survival , with an 8-12 fold decrease in viability compared to the WT after 24h (Gaynor et al., 2005). These mutants also had reduced levels of poly-P at stationary phase when compared to the WT where poly-P accumulated during the transition from log to stationary phase (Candon et al., 2007). *SpoT* mutants show reduced survival of aerobic stress, along with reduced adherence, invasion and intracellular survival, which indicates that ppGpp is important to virulence (Dalebroux et al., 2010). The *spoT* mutant had increased expression of certain stress response genes (*groELS, dnaK, htrA, clpB*) and phosphate uptake genes, but had no effect on tolerance of osmotic stress, serum sensitivity or colonization (McLennan et al., 2008). Unlike the mutants in *E. coli,* the *C. jejuni spoT* mutant had increased biofilm formation.

1.5.2.5. Polyphosphate kinase is required for stationary phase/nutrient stress survival

Polyphosphate kinase (*ppk1*) catalyzes the synthesis of inorganic polyphosphate (poly-P), a linear polymer of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds. Poly-P acts as a reservoir for energy and phosphate. Poly-P is widely distributed among bacterial species, playing a role in host colonization and pathogenicity, adaptation to environmental changes and survival (Ogawa et al., 2000; Kim et al., 2002; Jahid et al., 2006). More specifically, poly-P impacts various cellular processes including ATP production, entry of DNA through membrane channels, capsule composition, motility, biofilm formation and survival during stationary phase or nutrient stress (Gangaiah et al., 2009).

1.5.2.6. Polyphosphate is required for transition to a VBNC state in C. jejuni

The roles of poly-P differ between bacteria. In *C. jejuni*, poly-P plays a role in low nutrient survival, natural transformation, osmotolerance, resistance to antimicrobials, intracellular survival, colonization, and biofilm formation, but does not affect motility or oxidative stress resistance (Candon et al., 2007; Gangaiah et al., 2009). Mutants for *ppk1* had significantly reduced levels of poly-P and were less able to enter the VBNC state, indicating that poly-P is required for maintaining viability during stress (Gangaiah et al., 2009). Although in many bacteria mutants for *ppk1* have a reduced biofilm phenotype (Rashid et al., 2000; Chen et al., 2002; Shi et al., 2004), the *C. jejuni ppk1* mutant displayed an increase in biofilm formation (Candon et al., 2007).

1.5.2.7. Alkaline phosphatases provide cells with inorganic phosphate

Cellular phosphate levels provide cells with information about the nutritional state of the environment. Orthophosphate (Pi) which is typically low in the environment is required for Ppk1 mediated formation of poly-P which plays a role in basic metabolism and stress response (Rao et al., 2009). Low phosphate levels stimulate the stringent response, flagella growth, quorum sensing and production of virulence factors (Brown and Kornberg, 2004; Yuan et al., 2005; Lamarche et al., 2008). Alkaline phosphatases (PhoX) provide cells with inorganic phosphate (Pi) through hydrolysis of phosphate groups from more complex organophosphate molecules (Rajashekara et al., 2009). The *phoX* genes in *V. cholerae,* which are genetically similar to those in *C. jejuni*, play a role in biofilm formation, aerobic and heat stress tolerance, stringent response and flagella function (Lamarche et al., 2008; Pratt et al., 2009; Silby et al., 2009).

1.5.2.8. PhoX is the sole alkaline phosphatase in C. jejuni

C. jejuni senses environmental phosphate levels via the two component system phosS/phosR (phosphate sensor/phosphate response regulator) which activates expression of *phoX* in response to phosphate starvation (Wosten et al., 2006). PhoX is then translocated to the periplasm by the Twin Arginine Translocation system (TAT) where it becomes active (Drozd et

al., 2011). A TAT mutant had basic stress response defects, likely due to the inability to translocate PhoX, leading to reduced Pi levels, poly-P production and poly-P mediated stress responses (Drozd et al., 2011). Mutation studies reveal that the PhoX mediated acquisition of Pi is required for poly-P production (Drozd et al., 2011). The *phoX* mutants had lower levels of poly-P and showed nutrient stress defects which could be rescued by the addition of Pi or glutamine (note that glutamine synthesis requires Pi). Reduced expression of *ppk1* in the *phoX* mutant may indicate that this gene is down regulated in response to low Pi, allowing cells to use the available Pi rather than storing it in the form of poly-P. These mutants were also defective for invasion and colonization. Although many bacteria exhibit reduced biofilm in their *phoX* mutants, the opposite was true for *C. jejuni.* In this case, the *phoX* mutant had enhanced biofilm formation, which is more common in plant and soil pathogens. The increased biofilm phenotype reverted to WT levels with the addition of Pi. The increased biofilm formation may be related to the fact that *CsrA* was found to be upregulated in the *phoX* mutant. As was mentioned earlier CsrA positively regulates biofilm in *C. jejuni*. The enhanced biofilm may also be relevant to the increased resistance to antimicrobials of Δ*phoX.*

1.5.3. Interactions between the genes

Although the relationship between these genes is not completely understood, there are some interactions which may provide insight into the molecular pathways that are involved in the VBNC and biofilm survival strategies of *C. jejuni* (Fig. 1.3).

In WT *C. jejuni,* poly-P (synthesized by *ppk1*) is accumulated during the transition from exponential to stationary phase. A *C. jejuni spoT* mutant, unable to produce ppGpp, had significantly reduced levels of poly-P at stationary phase (Candon et al., 2007). In *E. coli* the roles of *spoT* and *ppk1* are related. ppGpp blocks exopolyphosphatase (Ppx), from hydrolyzing poly-P, thereby maintaining high levels of this reservoir of energy and phosphate during stress (Candon et al., 2007). Recent research in *C. jejuni* has shown that *ppx* mutants, accumulate more poly-P, but have less ppGpp than the WT (Malde et al., 2014). Taken together these results suggest that

ppGpp may block Ppx mediated hydrolysis of poly-P in *C. jejuni* as well, but more work needs to be done to confirm this.

Gene expression was not explored in all the mutants, but the *ppk1* mutant had increased expression of both *spoT* and *csrA* (Gangaiah et al., 2009). In the *ppk1* mutant, unable to synthesize poly-P, the increased expression of *spoT* and the subsequent accumulation of ppGpp would serve to maintain existing stores of poly-P. The increased expression of *csrA* would enhance biofilm growth and provide protection from stress.

The *spoT, ppk1* and *phoX* mutants all had increased biofilm, while the *csrA* mutant was deficient in biofilm formation (Gaynor et al., 2005; Fields and Thompson, 2008; Gangaiah et al., 2009; Drozd et al., 2011). In *E.coli*, ppGpp and CsrA form a negative feedback loop where ppGpp represses the activity of CsrA and CsrA represses synthesis of ppGpp. If a similar mechanism existed in *C. jejuni*, it could explain the increased biofilm in the *spoT* mutant. It would be interesting to investigate the expression of *spoT* in the *csrA* mutant.

In summary, the *C. jejuni spoT, ppk1 and phoX* mutants, which all had reductions in poly-P levels, also exhibited increased biofilm formation. In the case where the addition of Pi allowed for production of poly-P, biofilm returned to WT levels (i.e. Δ*phoX*). However, addition of Pi to Δ*ppk1* which was still unable to synthesize poly-P led to further increases in biofilm.

This suggests that *C. jejuni* cells which are unable to enter the VBNC state due to limiting levels of poly-P, may up regulate biofilm formation as an alternative strategy for survival. This premise is supported by the fact that both Δ*ppk1* and Δ*phoX* had up regulation of *csrA*, which is known to positively regulate biofilm in *C. jejuni*. Conversely, adequate levels of poly-P may act as a negative feedback for *csrA* (as ppGpp has been shown to do in *E. coli*) inhibiting excessive biofilm formation during times of abundance.

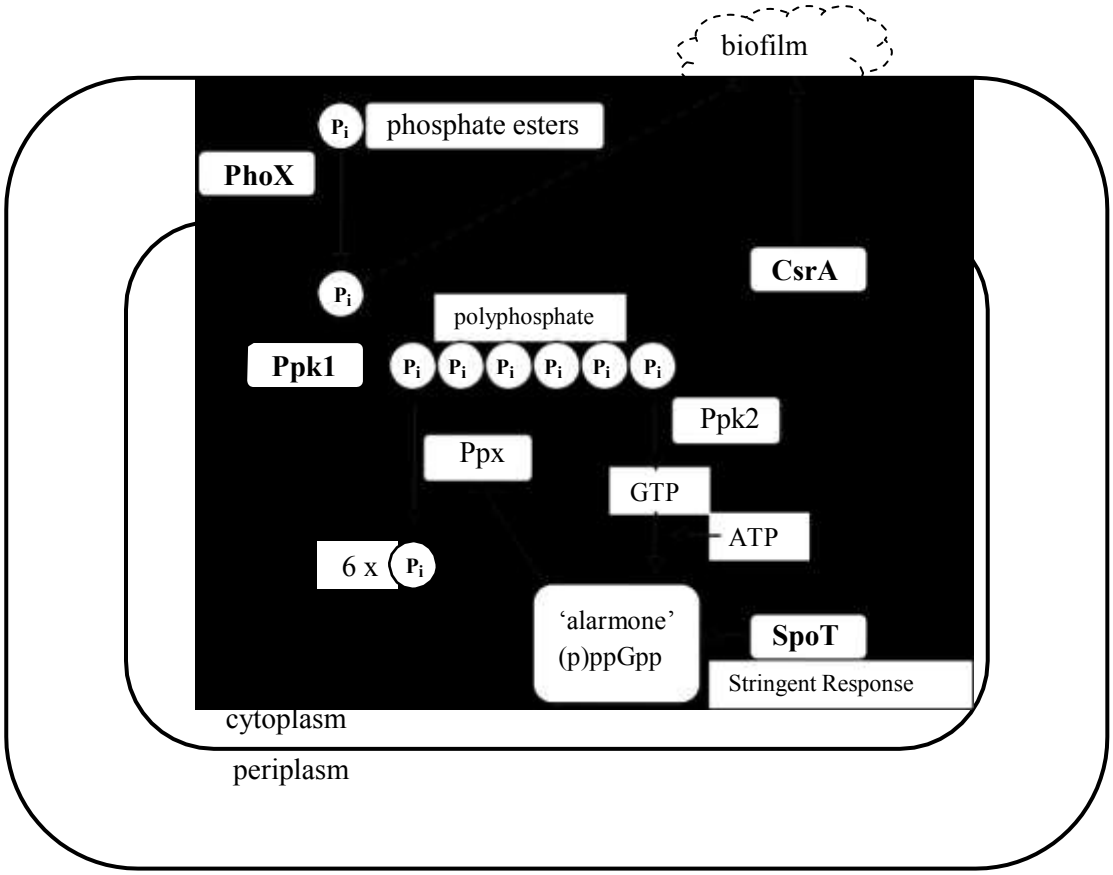

Figure 1.3. Possible interactions of target gene products in *C. jejuni.* (adapted from (Drozd et al., 2011). Alkaline phosphatase (PhoX) hydrolyzes organophosphate esters in the periplasm, providing inorganic phosphate (Pi) which is used for polyphosphate (poly-P) synthesis by polyphosphate kinase 1 (Ppk1). Polyphosphate kinase 2 (Ppk2) uses poly-P to generate GTP. Ppx is an exopolyphosphatase that hydrolyzes poly-P to Pi, but is blocked by ppGpp. SpoT catalyzes the synthesis of (p) ppGpp from GTP and ATP which mediates the stringent response. Dashed lines indicate possible interactions.

Table 1.4. Target genes and their mutants in *C. jejuni*.

***spoT*: global effector** • activates Stringent Response (SR) • expressed in response to: - starvation - contact with epithelial cells - contact with tissue • catalyzes synthesis of alarmone – (p)ppGpp • ppGpp binding to RNAP activates transcription of GT-rich promoters: - increased amino acid biosynthesis - increased expression of stress response proteins *References*: (Gaynor et al., 2005; Dalebroux et al., 2010)	***ΔspoT:*** • (p)ppGpp^0 • defect in stationary phase and low nutrient survival (8-12 fold decrease in CFU) • reduced poly-P at stationary phase • increased biofilm • increased expression of phosphate uptake genes • increased expression of stress response genes • reduced expression of respiratory and metabolic genes • reduced survival of aerobic stress • reduced adherence, invasion and intracellular survival *References*: (Gaynor et al., 2005; Dalebroux et al., 2010)
***csrA:* global effector** • regulating transcription of mRNA • roles in biofilm formation, motility, adherence to epithelial cells, oxidative stress defense *References*: (Fields and Thompson, 2008, 2012)	***ΔcsrA:*** • reduced biofilm formation • reduced motility • reduced adherence to epithelial cells *References*: (Fields and Thompson, 2008, 2012)
***phoX*: sole alkaline phosphatase** • acquisition of P_i *Reference*: (Rajashekara et al., 2009)	***ΔphoX:*** • reduced levels of poly-P • low nutrient survival defect - (rescued with addition of P_i) • reduced expression of *spoT, ppk1* • increased expression of *csrA* • increased biofilm - (returns to WT level with the addition of P_i) *Reference*: (Drozd et al., 2011)
***ppk1* – polyphosphate kinase** • catalyzes synthesis of poly-P • expressed in response to starvation • poly-P reservoir for energy and phosphate *Reference*: (Gangaiah et al., 2009)	***Δppk1:*** • reduced ability to go VBNC • low nutrient survival defect • increased biofilm • increased expression of csrA • increased expression of *spoT* (ppGpp prevents hydrolysis of poly-P by blocking *ppx*) • increased Pho regulon genes • reduced osmotolerance, natural transformation, intracellular survival and resistance to antimicrobials *Reference*: (Gangaiah et al., 2009)

1.6. Rationale and Objectives

Biofilms are a substantial problem in the food industry. Bacteria can evade sanitation within biofilms and because they are so difficult to remove, biofilms on food contact surfaces can become a source of recurring contamination. The work done herein addresses the need to determine if biofilm growth is conducive to entry into a VBNC state.

Although it has been confirmed that planktonic *C. jejuni* transition to the VBNC state in response to stress, this is the first study to examine the ability of *C. jejuni* biofilm cells to respond to stress in this manner. Based on the fact that planktonic *C. jejuni* cells were able to transition to the VBNC state, it was postulated that *C. jejuni* biofilm cells would also enter into the VBNC state in conditions of stress. The work done to assess the ability of *C. jejuni* biofilm cells to become VBNC is described in Chapter 2.

The results of the work described in Chapter 2 indicated the need for improved methods capable of detecting both planktonic and biofilm VBNC cells, which would be feasible for use in routine monitoring. The finding that the novel method PMAqPCR had been successful for quantifying planktonic *C. jejuni* as well as biofilm cells of other species, led to the hypothesis that it would also be successful for *C. jejuni* biofilm cells. The investigation of this premise is described in Chapter 3.

As described in Chapter 2 and Chapter 3, biofilm cells consistently entered the VBNC state earlier than their planktonic counterparts, which led to the hypothesis that the expression of genes known to play a role in both biofilm formation and entry into the VBNC state would differ between biofilm and planktonic cells and that these differences would help to elucidate the molecular mechanisms involved in these two related survival strategies.

The objectives of this work were:

1. To determine if three strains of *C. jejuni* (V1, V26, 16-2R) were able to enter a VBNC state and to characterize this entry for both biofilm and planktonic samples,
2. To evaluate PMAqPCR as a method for measuring viable cells in both biofilm and planktonic samples of *C. jejuni* and
3. To determine if the selected genes were differentially expressed in biofilm vs planktonic cells prior to, during, and after entry into the VBNC state.

The work of this thesis expands our understanding of how *C. jejuni* biofilm and planktonic cells transition to the VBNC state, how the novel molecular method, PMAqPCR can be used to quantify VBNC *C. jejuni* cells in both biofilm and planktonic states and how the increased expression of certain genes in *C. jejuni* biofilm cells may predispose them to becoming VBNC. The work described here represents a significant advancement in our understanding of the survival strategies used by *C. jejuni* and validates a much needed novel detection methodology that could be used in routine monitoring. The main goal of this thesis was to add to the limited understanding of how *C. jejuni* survives in the environment and remains the leading cause of foodborne disease.

CHAPTER 2: *Campylobacter jejuni* biofilm cells become viable but non-culturable (VBNC) in low nutrient conditions at 4°C more quickly than their planktonic counterparts

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Abstract

Campylobacter jejuni remains the leading cause of foodborne disease in the developed world. In order to assess the ability of biofilm cells to enter and survive in a viable but non-culturable state, biofilm and planktonic cells of three strains of *Campylobacter jejuni* were incubated at 4°C in phosphate buffered saline. Culturability was monitored by standard drop plating on Mueller Hinton agar and viability was measured using the LIVE/DEAD® *Bac*Light ™ assay which assesses membrane integrity. Both biofilm and planktonic cells became non-culturable prior to becoming non-viable. Biofilm cells became non-culturable as early as 10 days for one strain, while planktonic cells became non-culturable after 30 to 40 days of treatment. Planktonic cells were still viable after 60 days of stress treatment. Biofilm cells showed significantly reduced viability by day 50 for the two clinical isolates and by day 60 for the poultry isolate. Of the media assessed for their ability to extend the culturability of the VBNC cells, *Campylobacter* Agar Base with the addition of *Campylobacter* Growth Supplement was most successful at prolonging culturability, but even with enrichment in Bolton broth, cells still remained viable and potentially infectious, longer than they were culturable.

2.1. Introduction

Campylobacter jejuni is currently considered the main cause of bacterial gastroenteritis in the developed world (Suzuki and Yamamoto, 2009; Whiley et al., 2013). Most cases are associated with the consumption or handling of contaminated poultry and although poultry is considered the main reservoir, infections have also been linked to raw milk, untreated water, pets and farm animals (Whiley et al., 2013).

C. jejuni is a fastidious pathogen that can only grow at 30 – 45˚C in a microaerobic atmosphere, conditions found in the avian host. The pathogen also lacks many stress-response mechanisms commonly found in other Gram negative bacteria. Despite this sensitivity to stresses found outside the host, *C. jejuni* is prevalent in poultry houses and slaughter facilities (Ellerbroek et al., 2010; Cokal et al., 2011). Various hypotheses have been put forth to explain this conundrum, including the suggestion that *C. jejuni* survives in the environment by forming biofilms and by entering a viable-but non culturable (VBNC) state (Murphy et al., 2006; Pitkanen, 2013).

C. jejuni can form mono-culture biofilms or establish in pre-existing biofilms of strong biofilm producers, such as *Pseudomonas* spp., *Flavobacterium* spp., *Corynebacterium* spp., *Staphylococcus* spp, or *Enterococcus* spp. (Trachoo et al., 2002; Teh et al., 2010; Ica et al., 2012). Such biofilms can develop in food processing environments, in drinking water systems, and also in water systems of poultry houses (Trachoo et al., 2002; Sparks, 2009; Wingender and Flemming, 2011; Pitkanen, 2013). *C. jejuni* cells in biofilms are very resistant to environmental stresses (Stoodley et al., 2002) and many disinfectants (Alter and Scherer, 2006) and they can survive aerobic and low-temperature stress twice as long as planktonic cells (Joshua et al., 2006). *C. jejuni* cells can detach from biofilms. In food production environments, this leads to contamination of product; in water distribution systems, detached biofilm clusters may cause infection of humans or colonization of poultry (Trachoo and Frank, 2002; Schuster et al., 2005; Lehtola et al., 2007; Wingender and Flemming, 2011). Thus, *C. jejuni* in biofilms, pose a

significant public health risk and are also considered an important contributor to the persistence and spread of *C. jejuni* in poultry houses and slaughter facilities.

C. jejuni can become VBNC in response to various stressors, such as starvation, low temperature, and low pH (Cappelier and Federighi, 1998; Chaveerach et al., 2003; Gangaiah et al., 2009; Trevors, 2011). VBNC *C. jejuni* are more resistant to disinfection than actively growing cells (Davies, 2003), they can survive in the VBNC state for up to 7 months (Lazaro et al., 1999), and they will not be detected by culture-based methods, even when an enrichment step is used to resuscitate injured cells (Baffone et al., 2006). Recent work shows that VBNC *C. jejuni* continue to express virulence genes and adhere to epithelial cells (Chaisowwong et al., 2012) substantiating the concern that these cells may remain infectious. Existence of VBNC *C. jejuni* was demonstrated for both planktonic cells and biofilm-associated cells (Buswell et al., 1998; Trachoo et al., 2002; Ica et al., 2012). Planktonic *C. jejuni* in the VBNC state can attach to surfaces, initiating biofilm formation (Duffy and Dykes, 2009).

Despite the public health concern of VBNC *C. jejuni*, there is limited quantitative information regarding the time course of VBNC development and the percentage of viable cells found in non-culturable populations of *C. jejuni* (Trevors, 2011). The objective of this study was to quantitatively assess and compare the development of VBNC *C. jejuni* in a planktonic and biofilm state using the LIVE/DEAD® *Bac*Light™ assay and culturing in media commonly used to detect and enumerate *C. jejuni.*

2.2. Materials and Methods

2.2.1. Bacterial strains and culture conditions

Campylobacter jejuni NCTC 11168 V1 was purchased from the ATCC and is representative of the original clinical isolate from a case of human enteritis in 1977 (Ahmed et al., 2002). *C. jejuni* NCTC 11168 V26 (Carrillo et al., 2004), the laboratory passaged version of V1, was kindly donated by Dr. Brenda Allan from the Vaccine and Infectious Disease Organization (VIDO) in Saskatoon. *C. jejuni* 16-2R, a poultry isolate, was kindly donated by Dr. Joseph Odumeru,

Laboratory Services Division, University of Guelph. All three strains were maintained at -80°C in an ultra freezer (Thermo Electron). Cells from stock cultures were resuscitated on Mueller Hinton agar (MHA) by incubating at 42°C under microaerobic conditions (5% 02, 10% C02 and 85% N2) for 24 h. Cells were then transferred onto fresh MHA and incubated at 37°C under microaerobic conditions for 24 h prior to preparation of inocula.

2.2.2. Preparation of biofilm and planktonic cells

For each experiment, inocula were prepared fresh from frozen stock in order to avoid the transcriptional variation inherent in these bacteria which may undergo phenotypic changes as a result of subculturing (Carrillo et al., 2004). Bacteria were grown as a lawn on MHA for 24 h at 37°C and then all cells were transferred from the subculture to 5 ml sterile phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) using a polyester tipped sterile swab (Fisherbrand). Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK), the resulting suspension was standardized to an OD_{600} of 0.3± 0.015, which was equivalent to approximately 10^8 CFU/ml as confirmed by plate counting.

For development of biofilm cells, 0.1 g of glass fibre filters (pore size 0.7 µm,Whatman GF/F) were placed in 250 ml glass bottles (Pyrex) which were then autoclaved at 121°C for 20 min. Glass fibre filters provide extensive surface area for cell attachment and are amenable to the removal of cells (Kalmokoff et al., 2006). Bottles were cooled overnight and 20 ml of sterile Mueller Hinton broth (MHB) were added to each bottle. Each bottle was inoculated with 1.0 ml of standardized inoculum. Bottles were incubated microaerobically (5% O_2, 10% CO_2, 85% N) at 37°C with gentle agitation (25 rpm) in an incubator shaker (New Brunswick Scientific, *Innova*™ 4430) for 24 h. Growth of planktonic cells followed the same procedure without the addition of glass fibre filters.

2.2.3. Harvesting and enumeration of biofilm and planktonic cells by plate counting

After 24 h of growth, bottles were removed from the incubator and placed on ice. For bottles with glass fibre filters, the broth containing planktonic cells was aseptically removed and discarded.

The glass fibre filters were washed 3 times with 25 ml of cold PBS to remove any remaining planktonic cells. Biofilm cells were removed as described by Trachoo and Frank (2002). Filters were aseptically transferred to sterile 100 ml glass bottles containing 5 g of glass beads (SEPHEX, 450-600nm) and 10 ml of PBS and vortexed vigorously for 2 min using a Fisher Scientific Vortex Mixer (Cat# 02215365, 50/60 Hz Phase) set at maximum speed (10). The supernatant was then filtered through sterile stomacher bags to remove excess glass fibre and the removed biofilm cells were collected in sterile 15 ml tubes. Both the removed biofilm cells and planktonic cells (grown separately) were enumerated by drop plating on MHA, incubated microaerobically at 42°C. Colonies were counted at 24 and 48 h and CFU/ml determined for each sample.

2.2.4. Induction of cells into the viable but non-culturable state

C. jejuni cells grown and harvested as described above were resuspended in 10 ml of PBS to give concentrations 10^8 CFU/ml for planktonic cells and 10^7 CFU/ml for biofilm cells. Samples were then incubated at 4°C for up to 60 days in air without shaking. At specific time intervals (day 0, 10, 20, 30, 40, 50 and 60), 500 µl of each sample was stained with *Bac*Light™ Live/Dead stain, imaged and biovolume values were obtained as described below. At the same time culturable counts for each sample were determined by drop plating on MHA as described above.

2.2.5. Extended culturability on alternative media

Once samples became non-culturable on Mueller Hinton agar, recovery by plating on supplemented agar and enrichment in Bolton broth was investigated. Supplemented agar was prepared using *Campylobacter* Agar Base (Oxoid CM0689) with the addition of *Campylobacter* Growth Supplement (Oxoid SR0232) which contains sodium pyruvate, sodium metabisulphite and ferrous sulphate. No selective supplements or antibiotics were added in order to prevent growth inhibition of injured cells. Plates were incubated microaerobically at 42°C for 48 h prior to counting. For enrichment, 1 ml of cell suspension was added to 5 ml of sterile Bolton Broth (Oxoid CM0983) and incubated microaerobically at 37°C with gentle agitation for 24 h. A 100 µl

aliquot of this suspension was then plated on supplemented agar prepared as stated above and incubated at 42°C for 48 h prior to counting.

2.2.6. Estimation of biovolumes using LIVE/DEAD® *Bac*Light™ stain in conjunction with confocal scanning laser microscopy and PHLIP analysis

C. jejuni biofilm and planktonic cells grown as described above were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Invitrogen). The fluorescent dyes propidium iodide (PI) (20 mM in DMSO) and SYTO 9 (3.34 mM in DMSO) were mixed in a 2:1 ratio (PI:SYTO 9). Samples (500 µl) were incubated with 1.5 µl of dye mixture at 24°C in the dark for 15 min. Samples were then immediately filtered onto 0.2 µm black membrane filters (Isopore™ membrane filters GTBP02500, Millipore) using a millipore vacuum filtration unit. The filters were placed on glass slides with one drop of mounting oil (Millipore) followed by sealing of the coverslips. Slides were immediately taken for viewing and image capture using sequential scanning with the Laser Scanning Microscope Fluoview, version 4.3 FV300 (Olympus FV300 CSLM) and a 60X PlanApo NA 1.4 oil immersion lens. A HeNe Green (1 mW, 543nm) laser was used to excite the propidium iodide (ex/em 535/617nm) and an argon (10 mW, force air cooled, blue 488nm) laser was used to excite the SYTO 9 (ex/em 480/500nm). Images were analyzed for total biovolume with the biofilm image analysis program, PHLIP (Phobia Lasers Image Processing Software - The New Laser Scanning Microscope Image Processing Package) (Mueller et al., 2004). Biovolumes for each condition and strain are averages of 3 trials with 3-5 random fields of view for each sample. Cell morphology and cell motility were qualitatively assessed during image capture.

2.2.7. Statistical analysis

For each strain and condition, *t*-tests were used to determine statistically significant difference with an alpha value of 0.05. Error bars represent standard deviation of the mean for three independent replicates.

2.3. Results and Discussion

2.3.1. Time to become non-culturable varies with media supplementation and enrichment.

Biofilms cells lost culturability more quickly than their planktonic counterparts on all media tested. These differences ranged from 20 to 25 days and were significant on both MHA and supplemented agar. All samples became non-culturable on MHA first, then on the supplemented agar and finally with enrichment in Bolton broth (Fig. 2.1).

Biofilm cells became non-culturable within 10-20 days of starvation and low temperature stress, while planktonic cells remained culturable for 30-40 days (Fig. 2.2). The use of supplemented agar when compared to MHA was able to extend culturability by 20 – 30 days (Fig. 2.1). Enrichment in Bolton broth further extended culturability by 10 -15 days, but only for planktonic cells (Fig. 2.1). These differences are not due to innate differences between biofilm and planktonic cells, as there were no significant differences between plate counts on MHA and supplemented agar when samples were enumerated before exposure to starvation and low temperature stress ($p<0.05$) (data not shown).

However, supplementing agar with sodium pyruvate and sodium metabisulphite is known to aid in the recovery of injured and stressed cells by quenching toxic compounds such as reactive oxygen species (Corry et al., 1995). This suggests that the plate count differences observed after stress were due to recovery of injured cells on the supplemented agar. Also, pyruvate provides an immediate source of energy since it is rapidly and efficiently metabolized via the pyruvate:flavodoxin oxidoreductase and the citric acid cycle (Guccione et al., 2008). Enrichment in Bolton broth goes further in aiding the recovery of sub-lethally injured cells and encouraging growth which may be because it contains sodium carbonate, a source of carbon dioxide. On agar, cells are stationary and can only access nutrients from the immediate vicinity. In broth, not only are cells able to move freely and access more nutrients, but exposure to the toxic by-products of growth is reduced, as these will diffuse through the liquid (Corry et al., 1995).

Results from other studies are comparable to those obtained here. In a study with identical stress conditions, planktonic cells remained culturable for 48 days on *Campylobacter* Agar Base (Oxoid CM0689) without enrichment (Lazaro et al., 1999). Similar stress conditions followed by plating on Karmali Agar (CM0935) was less effective at prolonging culturability, as samples remained culturable for only 23 days (Klancnik et al., 2009). On Columbia Agar Base (Oxoid CM0331) samples remained culturable 12-35 days when induced in artificial sea water (ASW) (Baffone et al., 2006) and 15-18 days when induced in surface water (Cappelier et al., 1999). Similar to the present study, the addition of an enrichment step prior to plating extended culturability by 12 days (Cappelier et al., 1999), but only by 3-7 days when the enrichment included selective supplements containing antibiotics (Baffone et al., 2006).

The importance of both media composition and enrichment are recognized in sample testing for both food and water samples (Pitkanen, 2013). Although there are slight variations in the enrichment media and selective agents recommended by various regulatory bodies worldwide (Table 2.1), most enrichment broths contain sodium pyruvate, sodium metabisulphate, sodium carbonate, or blood, components known to aid in the recovery of injured cells (Corry et al., 1995; Pinto et al., 2011). However, even the use of supplemented media and enrichment may not be successful in recovering stressed *Campylobacter*s from water and environmental samples (Sparks, 2009; van Frankenhuyzen et al., 2011) or from food samples (Baffone et al., 2006) due to low cell numbers and the presence of VBNC cells which are not resuscitated with enrichment(Pinto et al., 2011; Richards et al., 2011).

2.3.2. Cell viability was maintained for 60 days.

Samples were stained with two intercalating fluorescent stains; Syto9 which enters all cells producing green fluorescence, and propidium iodide which can only enter dead or damaged cells and fluoresces red (*Bac*Light™). The red fluorescence quenches the green, making it possible to obtain values for both the total biovolume and the biovolume of cells considered viable based on membrane integrity. In this study, cells were considered VBNC once there was

no evidence of growth on the supplemented media after 24h enrichment in Bolton broth, but viability was detected using the *Bac*Light™ assay (Baffone et al., 2006; Josefsen et al., 2010).

For all three strains, biofilm cells were VBNC by day 40, while planktonic cells remained culturable with enrichment to day 60 (Figure 2.2). The biovolumes of viable planktonic cells showed no significant decline over 60 days of treatment ($p<0.05$), with differences between total biovolume and viable biovolume ranging from 0.20 to 0.62 $\log_{10} \mu m^3$/ml. In contrast, differences were larger for the biofilm samples, ranging from 1.51 to 2.71 $\log_{10} \mu m^3$/ml. These differences were significant for strains V26 and 16-2R ($p<0.05$), and this was not due to an overall decline in *C. jejuni* biomass, as the total biovolume remained constant in all samples during the 60 days of exposure to starvation and low temperature stress ($p<0.05$).

The fact that biofilm cells transitioned to the VBNC state earlier than planktonic cells may be related to gene expression differences between biofilm and planktonic cells (Davies, 2003). *C. jejuni* mutants for polyphosphate kinase (Δ*ppk1*) produced more biofilm than the wild type (WT), but were less able to enter the VBNC state (Gangaiah et al., 2009). These mutants also showed increased expression of the carbon starvation regulator gene (*csrA*), which is involved in biofilm formation. More work is needed to understand the role of gene expression in the transition to the VBNC state.

C. jejuni V1, the original clinical isolate, was more successful at maintaining the VBNC state in both, biofilm and planktonic forms, than V26, the lab-passaged strain. *C. jejuni* V1 also transitioned more quickly to the coccoid state than the other strains and motility of coccoid cells was observed up to the end of the experiment (data not shown). Planktonic cells of the poultry isolate, *C. jejuni* 16-2R, maintained viability in a similar fashion to V1, but biofilm cells of this strain were less successful, showing a greater reduction by day 60 than V26 (2.71 and 2.65 $\log_{10}$ μm^3/ml respectively).

Strain variation is common in *C. jejuni*. Certain strains are better at biofilm formation (Kudirkiene et al., 2012) and not all strains are able to enter the VBNC state (Tholozan et al.,

1999). One study has shown that of 22 strains tested, clinical isolates and those from food processing facilities had better adhesion to surfaces than isolates from animals or carcasses (Sulaeman et al., 2010) supporting the idea that biofilm formation is linked to virulence. *C. jejuni* V1, the original clinical isolate is known to be more virulent than the lab-passaged strain V26 (Carrillo et al., 2004). The fact that this strain was also the most successful at maintaining the VBNC state may indicate that there is also a link between the ability to transition to the VBNC state and virulence.

2.4. Conclusions

This study provides evidence that *C. jejuni* biofilm cells are able to enter the VBNC state and appear to do so more quickly than their planktonic counterparts. The strain variation observed in this study demonstrates the need for further work to explore the relationship between the ability to enter the VBNC state and virulence. Entry into a VBNC state during times of stress may impede the detection of potentially infectious *C. jejuni*, thereby contributing to the prevalence of campylobacteriosis. A better understanding of the VBNC state along with the development and use of non-culture based detection methods may lead to improved food safety and reduced incidence of this disease.

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Table 2.1. Standard microbiological methods for detecting *C. jejuni* in food and water.

Country	Enrichment
Canada (HC) MFLP-46 2002	Park and Sanders enrichment broth (Sigma-Aldrich 17189)
USA (FDA) BAM 2001	Bolton broth (Oxoid AM7526) with antibiotics (Oxoid NDX131)
ISO 10272 2009 (original - 2006)	1A – Stressed *Campylobacters* in low background: Bolton broth (OxoidCM0983) 1B – *Campylobacters* in high background: Preston broth (Oxoid SR0232)

HC – Health Canada, FDA – Food and Drug Administration, ISO – International Organization for Standardization.

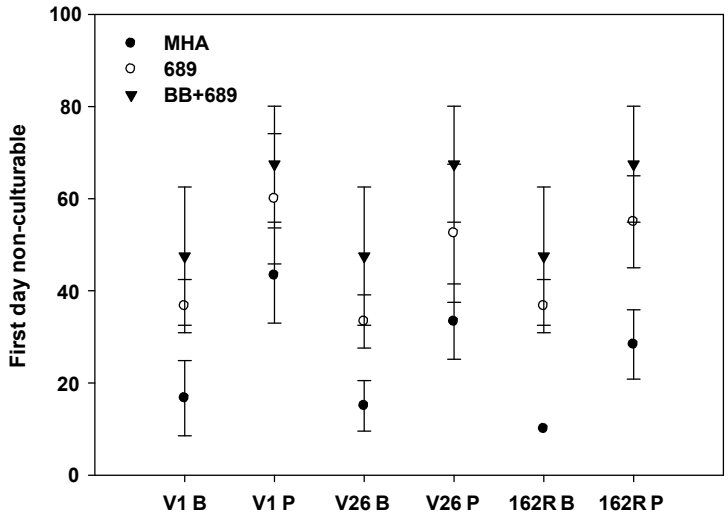

Figure 2.1. Assessment of extended culturability on alternative media. Symbols represent the average for the first day with no growth on each media. MHA – Mueller Hinton agar, 689 – supplemented *Campylobacter* agar, BB+689 – 24 hour enrichment in Bolton broth followed by drop plating on supplemented agar, V1 – *C. jejuni* NCTC11168 strain V1, V26 – *C. jejuni* NCTC11168 strain V26, 162R – *C. jejuni* poultry isolate 16-2R, B – biofilm, P – planktonic. Error bars represent standard deviation from the mean for three independent replicates.

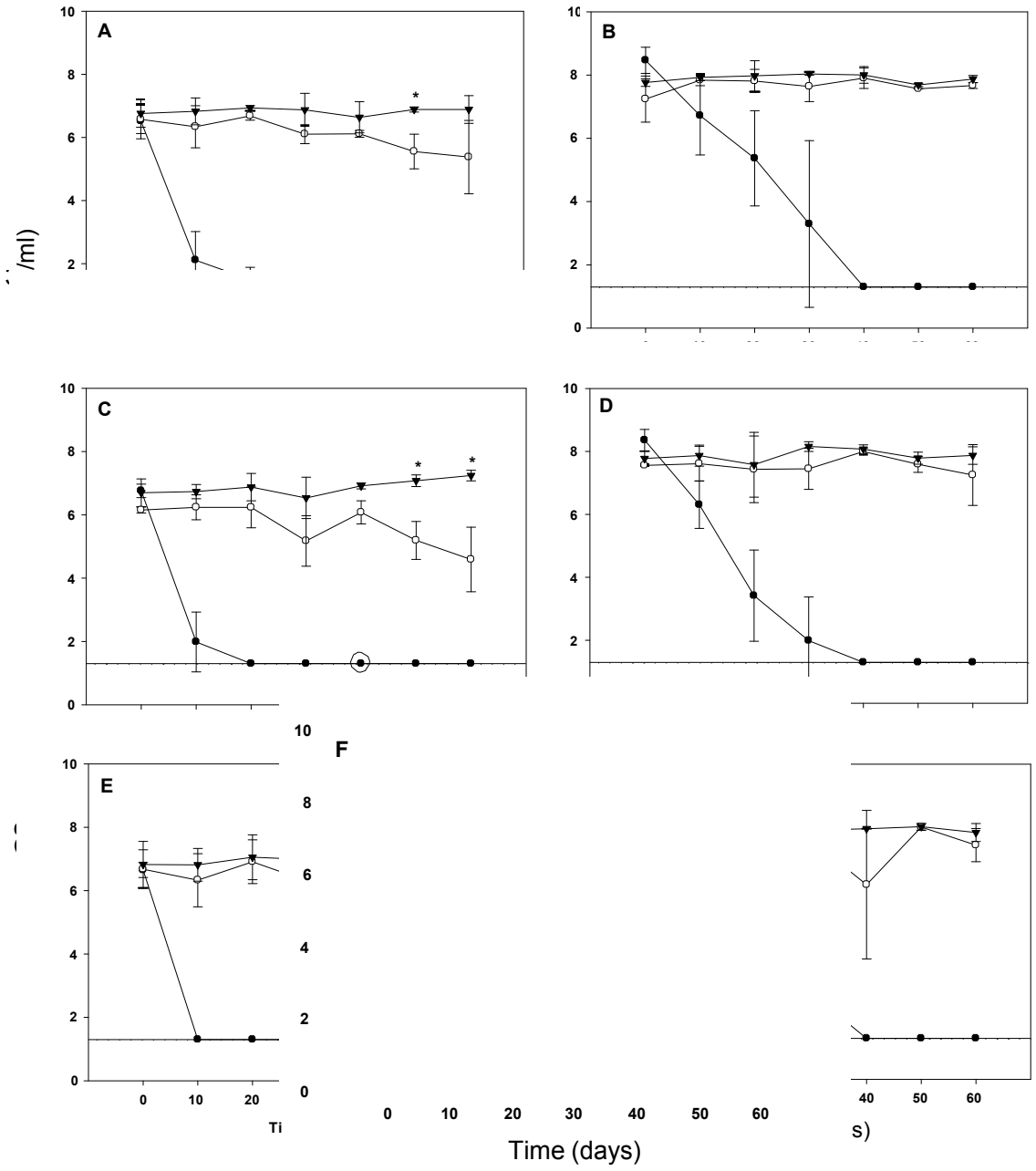

Figure 2.2. Comparison of plate counts and total and viable biovolume for *C. jejuni* V1 biofilm (A) and planktonic cells (B), *C. jejuni* V26 biofilm (C) and planktonic cells (D) and *C. jejuni* 16-2R biofilm (E) and planktonic cells (F) kept in phosphate buffered saline (PBS) at 4°C for 70 days. (●) plate counts on Mueller Hinton Agar, (▼) total biovolume, (o) viable biovolume. Dashed line indicates detection limit of 1.3 $\log_{10}$CFU/ml. Circled plate count values indicate first day non-culturable with enrichment in Bolton broth. Samples where viable biovolume was significantly less than total biovolume are indicated by * ($p<0.05$). Error bars represent standard deviation from the mean for three independent replicates.

Transition to Chapter 3

The results of the initial investigation presented in Chapter 2, indicated that *C. jejuni* biofilm cells were able to enter the VBNC state and could remain viable for extended periods. Given the impact biofilms have for food safety, it was clear that methods for quantifying the VBNC *C. jejuni* biofilms cells was needed. A review of available methods capable of quantifying VBNC cells indicated that no such methods had been validated for *C. jejuni* biofilm cells. The novel method PMAqPCR had been successful at quantifying *C. jejuni* VBNC planktonic cells. The work presented in Chapter 3 describes the ability of this method to accurately enumerate *C. jejuni* biofilm cells, both in log phase as well as after extended periods in the VBNC state.

CHAPTER 3: Evaluation of propidium monoazide and quantitative PCR to quantify viable *Campylobacter jejuni* biofilm and planktonic cells in late log phase and in a viable but non-culturable state.

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Key words: *Campylobacter*, biofilm, viable but non-culturable, PMAqPCR

Abstract

In spite of being considered fragile and fastidious, *Campylobacter jejuni* remains the leading cause of bacterial gastroenteritis in the developed world. *C. jejuni* survives stresses by forming biofilm or entering a viable but non-culturable (VBNC) state. In order to investigate the number of viable cells in samples exposed to starvation and low temperature stresses, a novel method, propidium monoazide qPCR (PMAqPCR) was compared to *Bac*Light biovolume analysis and standard plate counting for the enumeration of *C. jejuni* biofilm and planktonic cells in late log phase (20hr). There were no significant differences between viable cell counts obtained from PMAqPCR, with those from plate counts, or from *Bac*Light biovolume analyses for each sample, thus confirming that this method provides results consistent with those from accepted enumeration methods ($p>0.05$). To induce a VBNC state, *C. jejuni* biofilm and planktonic cells were incubated in phosphate buffered saline at 4°C for up to 60 days. Even with enrichment, biofilms cells lost culturability by day 10, while their planktonic counterparts remained culturable to day 60. The non-culturable biofilm cells remained viable in high numbers to day 60 and viable cell counts from the PMAqPCR (6.15 $\log_{10}$ cell count equivalents/ml) were not significantly different from those obtained using the *Bac*Light assay (6.98 $\log_{10}$ cells/ml) ($p>0.05$), confirming that the novel method is also reliable for cells exposed to stress for extended periods. PMAqPCR shows promise for use in settings where *C. jejuni* exists in biofilms or in the VBNC state (medical, drinking water, food industry). The adoption of PMAqPCR in routine monitoring, in conjunction with improved biofilm cell collection methods, will allow for more accurate enumeration of viable and potentially virulent cells leading to improved sanitation and reduced incidence of infection.

3.1. Introduction

Detection of viable *C. jejuni* is complicated by the fact that cells can enter a viable but non-culturable (VBNC) state and hence will not grow on media commonly used for their enumeration. Most cases of campylobacteriosis occur as sporadic infections associated with the consumption or handling of contaminated poultry (Hermans et al., 2011) with 10,174 cases reported in Canada in 2012 (Notifiable Diseases On-Line, 2014). Outbreaks when they do occur are usually associated with contaminated water and the number of exposed persons ranged from 20 to 20, 000 between 1978 and 2010 (Pitkanen, 2013).

C. jejuni is a zoonotic pathogen requiring a microaerobic environment and a temperature range of 30 - 45°C for growth. Given the fastidious nature of this pathogen, it should not survive the harsh conditions found in poultry rearing environments, in poultry processing facilities nor those found in surface water. *C. jejuni* is however regularly detected in these locations (Jones, 2001; Savill et al., 2001; Diergaardt et al., 2004; Vereen et al., 2007; Jokinen et al., 2010; Hellein et al., 2011; Hokajarvi et al., 2013). The fact that acid stress in poultry processing, and starvation in water have been shown to induce a VBNC state in *C. jejuni* may help to explain the pathogen's persistence in these areas.

VBNC *C. jejuni* have reduced metabolic rates, do not grow and divide, but can remain viable for up to 7 months (Lazaro et al., 1999). The ability of pathogenic bacteria to enter this state is of particular concern to the food industry, where many of the processes meant to achieve bactericidal effects may instead lead bacteria to become VBNC (Josefsen et al., 2010; Nascutiu, 2010). For example, certain strains of *C. jejuni* enter the VBNC state when exposed to refrigeration temperatures in either nutrient-poor (Cappelier et al., 1999; Baffone et al., 2006) or nutrient-rich conditions (Chaisowwong et al., 2012) or with exposure to acidic conditions *(10).* VBNC *C. jejuni* cells are capable of resuscitation by mouse passage, or with inoculation into yolk sacs of embryonated eggs (Cappelier et al., 1999; Talibart et al., 2000; Baffone et al., 2006). They also continue to express virulence genes and retain their ability to invade human intestinal

epithelial cells, substantiating the concern that such cells may be able to switch to the infectious stage once in the host organism (Chaisowwong et al., 2012).

Biofilms also play a role in *C. jejuni* environmental persistence (Buswell et al., 1998; Gunther and Chen, 2009; Petrova and Sauer, 2012; Pitkanen, 2013; Cappitelli et al., 2014). Biofilms consisting of surface-attached bacteria in a self-produced matrix of extracellular polymeric substance (EPS) differ from their planktonic counterparts with respect to gene expression, cellular physiology and resistance to stresses (O'Toole and Kolter, 1998; Joshua et al., 2006). In particular, bacteria in biofilms pose significant food safety risks. The ability of *C. jejuni* to form biofilms both on food, such as poultry (Jang et al., 2007; Kudirkiene et al., 2012) and fresh produce (Lu et al., 2011), as well as on various abiotic surfaces within food processing environments (Kalmokoff et al., 2006; Nguyen et al., 2012), provide this pathogen with protection from cleaning and disinfection procedures (Costerton et al., 1999; Yang et al., 2001; Trachoo et al., 2002; Chantarapanont et al., 2004; Northcutt et al., 2005; Joshua et al., 2006; Kudirkiene et al., 2012; Nguyen et al., 2012). The difficulty of removing bacteria attached to food contact surfaces in biofilms is a major problem in the food industry (Nguyen et al., 2012).

The existence of VBNC cells within biofilms has also been reported. Early studies using non-culture based methods were able to detect non-culturable *C. jejuni* cells within biofilms, but were unable to quantify them (Buswell et al., 1998; Trachoo et al., 2002; Lehtola et al., 2006). The advent of ethidium monoazide (EMA) qPCR in 2003 (Nogva et al., 2003) and PMAqPCR in 2006 (Nocker et al., 2006) made it possible to quantify viable cells, including those which are VBNC. Both stains cannot permeate membranes of intact, healthy cells, but will intercalate with DNA from dead or damaged cells, ensuring that only DNA from intact viable cells is amplified and used for quantification. EMAqPCR and PMAqPCR have been used to quantify viable *C. jejuni* cells in mixed cell populations (Rudi et al., 2005) and in chicken carcass wash (Josefsen et al., 2010).

In spite of the fact that bacteria often survive on food contact surfaces by existing within a biofilm, very few studies have examined the efficacy of either EMA- or PMA-qPCR for biofilm samples. In one study, EMAqPCR was able to accurately quantify viable *legionellae* in biofilm samples from cooling towers and hot water systems (Chen and Chang, 2010) and in another, PMAqPCR was found to be more appropriate than EMAqPCR for estimating viable *L. monocytogenes* in biofilms (Pan and Breidt, 2007). This is the first study to evaluate PMAqPCR with *C. jejuni* biofilm cells. In this study PMA was chosen since it was less able to enter viable cells than EMA (Pan and Breidt, 2007) and because viable cell counts obtained using PMAqPCR were found to correlate well with plate counts for samples of mixed viable/nonviable *C. jejuni* (R^2=0.98) (Seinige et al., 2014).

Another method to quantify viable cells, the *Bac*Light assay, provides an accurate and sensitive analysis of stressed samples and is a valuable research tool, but the need for epifluorescence microscopy or flow cytometry as well as the difficulties with analyzing mixed-culture biofilms precludes the acceptance of this assay in routine food safety testing. PMAqPCR only requires PCR technology which is increasingly being used for testing of food and environmental samples (Laboratory Procedures for the Microbiological Analysis of Foods. Vol 3. The Compendium of Analytical Methods, 2014) and PCR methods are appropriate for use with mixed cultures making them a better choice for samples which may harbour numerous microbial species (Josefsen et al., 2010). Both EMAqPCR and PMAqPCR have had results consistent with those from *Bac*Light analysis for planktonic *C. jejuni* (Rudi et al., 2005; He and Chen, 2010; Josefsen et al., 2010).

Methods capable of quantifying VBNC *C. jejuni* within biofilms would be advantageous for both research and routine monitoring in food processing and poultry production, allowing for accurate evaluation of intervention strategies aimed at reducing levels of *C. jejuni* in areas of concern, such as on food contact surfaces where biofilms persist.

In this study, we investigated the ability of PMAqPCR to provide results consistent with those from standard fluorescence-based viability detection methods (*Bac*Light) and plate counting for both biofilm and planktonic cells prior to stress. The ability of PMAqPCR to accurately detect and quantify cells in a VBNC state was also evaluated.

3.2. Materials and Methods

3.2.1. Bacterial strains and culture conditions.

Campylobacter jejuni NCTC 11168 V1 was purchased from the ATCC and is representative of the original clinical isolate from a case of human enteritis in 1977 (Ahmed et al., 2002). *C. jejuni* NCTC 11168 V26 (Carrillo et al., 2004), the laboratory passaged version of V1, was kindly donated by Dr. Brenda Allan from the Vaccine and Infectious Disease Organization (VIDO) in Saskatoon. *C. jejuni* 16-2R, a poultry isolate, was provided by Dr. Joseph Odumeru, Laboratory Services Division, University of Guelph. All three strains were maintained at -80°C in an ultra-freezer (Thermo Electron). Cells from stock cultures were resuscitated on Mueller Hinton agar (MHA) by incubating at 42°C under microaerobic conditions (5% 0_2, 10% $C0_2$ and 85% N_2) for 24 h. Resuscitated cells were then transferred onto fresh MHA and incubated at 37°C under microaerobic conditions for 24 h prior to preparation of inocula.

3.2.2. Preparation of biofilm and planktonic cells.

For each experiment, inocula were prepared fresh from frozen stock in order to avoid the transcriptional variation inherent in these bacteria which may undergo phenotypic changes as a result of subculturing (Carrillo et al., 2004). Bacteria were grown as a lawn on MHA for 24 h at 37°C after which cells were transferred from the subculture to 5 ml sterile phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) using a polyester tipped sterile swab (Fisherbrand). Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK), the resulting suspension was standardized to an OD_{600} of 0.3± 0.015, which was equivalent to approximately 10^8 CFU/ml as confirmed by plate counting. For development of biofilm cells, 0.1 g of glass fibre filters (Whatman GF/F, 2.1cm diameter, 0.7

pore size) were placed in 250 ml glass bottles (Pyrex) which were then autoclaved at 121°C for 20 min. Glass fibre filters provide extensive surface area for cell attachment and are amenable to the removal of cells (Kalmokoff et al., 2006). Bottles were cooled overnight and 20 ml of sterile Mueller Hinton broth (MHB) were added to each bottle. Each bottle was inoculated with 1.0 ml of standardized inoculum. Bottles were incubated microaerobically (5% O_2, 10% CO_2, 85% N) at 37°C with gentle agitation (25 rpm) in an incubator shaker (New Brunswick Scientific, *Innova* 4430) for 24 h. Growth of planktonic cells followed the same procedure without the addition of glass fibre filters.

3.2.3. Harvesting and enumeration of biofilm and planktonic cells by standard plate counting.

After 24 h of growth, bottles were removed from the incubator. For bottles with glass fibre filters, the broth containing planktonic cells was aseptically removed and discarded. The glass fibre filters were washed 3 times with 25 ml of cold PBS to remove any remaining planktonic cells. Filters were aseptically transferred to sterile 100 ml glass bottles containing 5 g of glass beads (SEPHEX, 425-600μm) and 10 ml of PBS and vortexed vigorously for 2 minutes using a Fisher Scientific Analog Vortex Mixer set at the maximum speed of 10. The supernatant was then filtered through sterile mesh-lined stomacher bags (Fisherbrand) to remove excess glass fibre and the resuspended biofilm cells were collected in sterile 50 ml tubes. Both the removed biofilm cells and planktonic cells (grown separately) were enumerated by drop plating on MHA, incubated microaerobically at 42°C. Colonies were counted at 24 and 48 h and CFU/ml determined for each sample.

3.2.4. Induction of cells into the viable but non-culturable state.

C. jejuni cells, grown and harvested as described above, were resuspended in 30 ml of PBS in sterile 50ml centrifuge tubes (Falcon) to give concentrations of approximately 10^7 CFU/ml. Initial culturable cell counts were confirmed by drop-plating on MHA as described above as well as on supplemented *Campylobacter* Agar base (Oxoid CM0689) for comparison. Samples were then

incubated at 4°C for up to 60 days in air without shaking. Samples were tested at 10 day intervals as described in Table 3.1.

3.2.5. Assessment of culturability.

Samples were drop plated on both Mueller Hinton Agar (MHA) and *Campylobacter* Agar Base (Oxoid CM0689) supplemented with *Campylobacter* Growth Supplement (Oxoid SR0232) which contains sodium pyruvate, sodium metabisulphite and ferrous sulphate. No selective supplements or antibiotics were added in order to prevent inhibition of injured cells. Plates were incubated microaerobically at 42°C for 48 h prior to counting. A 500µl aliquot of the cell suspension was added to 800µl of sterile Bolton Broth and incubated microaerobically at 37°C with gentle agitation for 24 h. A 100 µl aliquot of the broth was then plated on CM0689 medium prepared as stated above and incubated at 42°C for 48 h prior to counting.

3.2.6. Quantification of total and viable cells using LIVE/DEAD® *Bac*Light™ stain in conjunction with confocal scanning laser microscopy and PHLIP analysis.

C. jejuni biofilm and planktonic cells grown as described above were stained with the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Invitrogen). The fluorescent dyes propidium iodide (PI) (20 mM in DMSO) and SYTO 9 (3.34 mM in DMSO) were mixed in a 2:1 ratio (PI:SYTO 9). Samples (500 µl) were incubated with 1.5 µl of dye mixture at 37°C in the dark for 15 minutes. Samples were then immediately filtered onto 0.2 µm black membrane filters (Isopore membrane filters GTBP02500, Millipore) using a Millipore vacuum filtration unit. The filters were placed on glass slides with one drop of mounting oil (Millipore) and sealed with coverslips. Slides were immediately taken for viewing and image capture using sequential scanning with the Laser Scanning Microscope Fluoview, version 4.3 FV300 (Olympus FV300 CSLM) and a 60X PlanApo NA 1.4 oil immersion lens. A HeNe Green (1 mW, 543nm) laser was used to excite propidium iodide (ex/em 535/617nm) and an argon (10 mW, force air cooled, blue 488nm) laser was used to excite SYTO 9 (ex/em 480/500nm). For each sample, two to four random fields of view were analyzed for both total and green biovolume with the biofilm image

analysis program, PHLIP (Phobia Lasers Image Processing Software - The New Laser Scanning Microscope Image Processing Package) (Mueller et al., 2004). To calculate converted cell counts derived from biovolume analysis, direct counting of both green and total cells in images captured as described above was performed. Ratio of biovolume per cell was calculated for 85 fields of view . A conversion factor based on these ratios was then used to convert biovolume values from PHLIP analysis to either total converted counts (TCC) or green converted counts (GCC).

3.2.7. Assessment of viable counts by PMAqPCR.

PMA dissolved in water (Biotium Inc., Hayward, CA) was added to 500µl of a 1:10 dilution of each cell suspension to a final concentration of 50µM as suggested by the manufacturer. These were incubated in Eppendorf tubes in the dark for 5 minutes at room temperature. Following incubation, the tubes were placed on ice and exposed to a 650-W halogen light source (ColorTran Industries Inc., model LQBM-10F/TV, Burbank, California) at a distance of 20 cm for 1 minute as suggested by the manufacturer. The tubes were swirled by hand continuously during light exposure and turned over after 30s of illumination to ensure complete cross-linking of the available DNA and the conversion of free PMA to hydroxylamino propidium. For each sample, one-millilitre volumes of cell suspension were centrifuged at 10,000 X g for 5 min at 4°C and DNA extraction was performed on the pellets using the Wizard® SV Genomic DNA Purification System A2361 (Promega) as specified by the manufacturer. Due to the low yield of DNA in the VBNC samples, DNA was eluted into 200µl of Nuclease Free Water instead of the 500µl suggested by the manufacturer. A target locked nucleic acid *Campylobacter* probe 5'[6FAM]CA[+T] CC[+T]CCA CGC G[+T]T GC[BHQ1]3' (Sigma Aldrich) and forward primer OT1559 (CTG CTT AAC ACA AGT TGA GTA GG;43) and reverse primer 18-1(TTC CTT AGG TAC CGT CAG AA; designed to amplify a 287-bp sequence of the 16S rRNA gene of *C. jejuni* were chosen based on Josefsen et al (Josefsen et al., 2010). Each qPCR was performed in a 20µl volume containing 5µl of template DNA, 0.3µM of forward and reverse primers, 0.2µM of target locked nucleic acid *Campylobacter* probe (Sigma Aldrich) and 10µl of SsoFast Probes

Supermix buffer (BioRad). All qPCR were performed on a Bio-Rad Real-time thermal cycler CFX96 with a cycle profile as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 58°C for 60 s, and 72°C for 30 s. Fluorescence measurements were obtained online and analyzed with the BioRad CFX Manager software (version 2.0). In every qPCR analysis, the *C. jejuni* standard for absolute quantification and non-template controls were included in duplicate. The DNA standard for quantification of qPCR products was constructed as follows. A 287-bp DNA fragment of the 16S rRNA gene was amplified from the strain *C. jejuni* NCTC 11168 V1 with the forward and reverse primers mentioned above. The PCR product was purified using The Wizard® SV Gel and PCR Clean-Up System (Promega) and subsequently cloned into a pGEM®-T Easy Vector Systems (Promega). The concentration of the resulting plasmid was determined using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE) at an absorbance of 260nm. Tenfold serial dilutions of the plasmid, resulting in a range of 10^9 to 10^2 gene copies/µl were used as templates to generate a standard curve in each qPCR assay. The copy numbers of the DNA standards were calculated using the formula: number of copies = (amount*6.022x10^{23})/(length * 1x10^9 * 650) (http://cels.uri.edu/gsc/cndna.html). As in Josefsen et al (Josefsen et al., 2010), values generated by the qPCR were referred to as '*Campylobacter* cell equivalents' (CCE). For each sample two PCR reactions was performed. In order to obtain total cell counts, PCR was performed on gDNA that had not been pre-treated with PMA and these values were referred to as 'Total *Campylobacter* cell equivalents' (TCCE). Values obtained from samples pre-treated with PMA providing counts of only viable cells were referred to as 'Viable *Campylobacter* cell equivalents' (VCCE). *C. jejuni* is reported to have three copies of the 16S rRNA gene (Hansson et al., 2008) which is the target of the primers used in the qPCR. Since plasmids were constructed to contain only one copy of the gene insert, values generated by the BioRad CFX Manager software indicated the number of gene copies per sample. These values were then divided by three to estimate the number of *C. jejuni* cells present.

3.2.8. Statistical analysis.

Analysis of variance (ANOVA) and Student T-tests were done on log-transformed data to assess differences in total cell counts derived from *Bac*Light total biovolume analysis with total counts from qPCR. These tests were also used to assess differences in viable cell counts derived from i) plate counting, ii) *Bac*Light green biovolume analysis, and iii) PMAqPCR on day 0 as well as comparing day 0 and day 60 values. All statistical analyses were performed using IBM SPSS version 20 with a significance level of 5% ($p<0.05$). Error bars represent standard deviation of the mean. Biovolumes for each condition and strain are averages of three independent trials with 2 to 4 random fields of view for each sample. PMAqPCR values are averages of the same three independent trials with two technical replicates per sample.

3.3. Results

3.3.1. Confirming the presence of VBNC cells by culturing.

Drop plating on supplemented agar indicated that all samples had initial concentrations of 7.41 (+/- 0.15) $\log_{10}$ CFU/ml with a range from 7.15 to 7.54 $\log_{10}$ CFU/ml (Fig. 3.1 and Fig. 3.2). *C. jejuni* biofilm cells became non-culturable on both Mueller Hinton agar (MHA) and supplemented agar by day 10 (Fig. 3.1). Even with enrichment in Bolton Broth and subsequent plating on supplemented agar, none of the biofilm samples were culturable at day 10. This was much earlier than their planktonic counterparts which remained culturable on supplemented agar until day 50 for the V1 strain and day 60 for the other 2 strains. Planktonic samples of all three strains were non-culturable with enrichment at day 60 (Fig. 3.1). Cells were considered VBNC when they were no longer culturable even with enrichment, but remained viable in the *Bac*Light assay.

On day 10 and 60 respectively, when biofilm and planktonic cells were nonculturable after enrichment, viable biovolume assessed with the *Bac*Light assay was 6.22 to 6.92 ($\log_{10}$ GCC/ml) for biofilm cells and 5.91 to 6.65 ($\log_{10}$ GCC/ml) for planktonic samples and these values were not significantly different from corresponding day 1 values ($p>0.05$) (Fig 3.1).

Although there was a slight reduction in viable biovolume over the 60 days (0.25 - 0.59 $\log_{10}$ GCC/ml), the total biovolume and viable biovolume were not significantly different for all samples and time points tested ($p>0.05$), Fig. 3.1. Thus, for all 3 strains biofilm cells were VBNC on day 10 and planktonic cells were VBNC on day 60.

3.3.2. Assessing methods for the quantification of viable and culturable cells.

Three methods of enumerating viable cells were assessed. The standard microbiological method of plate counting, *Bac*Light staining in conjunction with confocal microscopy and the novel method,

PMAqPCR. In order to determine if PMAqPCR provided accurate estimates of the number of viable cells, tests were performed on day 0, on both *C. jejuni* biofilm cells and planktonic cells in late log phase (20hr). Day 0 plate counts (PC), viable cell counts obtained from PMAqPCR (VCCE) and converted cell counts for cells staining green in *Bac*Light images (GCC) were not significantly different ($p<0.05$), although the green converted cell counts (GCC) obtained from the *Bac*Light method on day 0 were on average 0.62 $\log_{10}$ lower than plate count values and 1 $\log_{10}$ lower than the viable cell counts from PMAqPCR (Fig. 3.2).

Methods were also assessed once samples had become VBNC. Again there were no significant differences between values obtained from PMAqPCR and from the *Bac*Light assay for any of the samples at either day 10, when the biofilm cells were VBNC or at day 60 when the planktonic cells were also VBNC ($p<0.05$) (Fig. 3.3). On day 10 the viable cell counts from the PMAqPCR (VCCE) were on average 1 $\log_{10}$ higher than GCC from *Bac*Light analysis. In contrast, at day 60 VCCE tended to be 0.5 $\log_{10}$ lower than GCC values. None of these differences was statistically significant ($p<0.05$).

3.3.4. Assessment of changes in cell counts over the 60 days of treatment.

The PMAqPCR indicated reductions of 1.42 to 1.70 ($\log_{10}$ VCCE/ml) in viable biofilm cell counts over the 60 days of treatment with significant reductions for the V26 and 16-2R samples ($p<0.05$), Fig 3.4B. Reductions in viable planktonic cells by this method ranged from 1.41 to

2.40 (log_{10} VCCE/ml), but this was only significant for the 16-2R sample ($p<0.05$), Fig. 3.4B. Cell counts from the *Bac*Light assay also showed reductions over the 60 days, for both biofilm and planktonic samples, but none of these were significant ($p<0.05$) (Fig. 3.1).

Total cell counts did not change significantly in any sample over the 60 days by either method ($p<0.05$). Reductions in total cell counts obtained by qPCR with no PMA treatment (TCCE), ranged from 1.01 log_{10} CCE/ml to 1.37 log_{10} CCE/ml (Fig. 3.4A), while reductions in total cell counts obtained using the *Bac*Light assay (TCC) ranged from 0.03 to 0.36 log_{10} TCC/ml (Fig. 3.1).

3.4. Discussion

Although PMAqPCR has been successful for enumerating both culturable and viable but non-culturable *C. jejuni* in the planktonic state (Josefsen et al., 2010), this is the first study to report the ability of this novel method to accurately quantify both culturable and VBNC *C. jejuni* biofilm cells.

Biofilms are a major concern within the food industry. Food safety depends on effective cleaning and disinfection of food contact surfaces. Numerous authors have shown that biofilms are difficult to remove from surfaces and provide *C. jejuni* with protection from stresses (Trachoo and Frank, 2002; Joshua et al., 2006; Kudirkiene et al., 2012). Therefore methods are required to assess the efficacy of cleaning and disinfection, particularly with respect to viable cells within biofilms. In one study, where EMAqPCR was used to assess cleaning and disinfection of food-contact surfaces in a beef processing plant, a comparison of CFU, VBNC and total cell counts for the bacterial microflora present, including *Acinetobacter, Aeromonas, Arthrobacter, Microbacterium, Pseudomonas, Psychrobacter* and *Staphylococcus* sp. among others, reported high numbers of VBNC bacterial cells on the food contact surfaces, with viable cell counts up to 2.5 log_{10} greater than culturable cell counts (Khamisse et al., 2012). The presence of such high numbers of VBNC cells on food contact surfaces even after aggressive cleaning procedures

confirms the need to quantify these potentially virulent bacteria and provides support for the use of molecular detection methods such as PMAqPCR in routine monitoring.

Routine monitoring of cells within biofilms could include placing coupons in production environments and removing them for testing at specific intervals or scraping surfaces with more force than the standard swabbing that is commonly used to collect biofilm cells for enumeration (Khamisse et al., 2012). Swabbing has been shown to be greatly variable with as much as 97% or as little as 2% of the CFUs being collected depending on the microbial species, the age of the biofilm and the type of surface (Midelet and Carpentier, 2002; Assere et al., 2008). In this context, the ability to accurately quantify viable cells within the biofilm is important. Our assessment of PMAqPCR, as confirmed by comparison to the *Bac*Light assay and when possible, plate counting, validated the ability of this method to accurately enumerate viable *C. jejuni* biofilm and planktonic cells in late log phase (20h) as well as once the cells had entered the VBNC state.

PMAqPCR could be used to routinely evaluate intervention strategies in food processing situations as well as being valuable in research settings. The protocol described here could easily be adapted using universal 16SrRNA primers to include counts of total viable cells of all microbial species alongside selected pathogens such as *C. jejuni*. In cases like the one described by Khamisse et al (Khamisse et al., 2012) where cleaning and disinfection were ineffective, it is relevant to know the extent of the biofilm remaining on the food contact surface, especially for pathogens such as *C. jejuni* which are able to find protection within existing biofilms (Trachoo and Frank, 2002).

In this study, *C. jejuni* biofilm cells transitioned to the VBNC state 50 days earlier than their planktonic counterparts and remained viable in high numbers to the end of the experiment. Biofilm cells have a unique physiology with different gene-expression than planktonic cells and have been described as a separate phenotype (Costerton et al., 1999; Davies, 2003). Biofilm cells have slower growth rates than planktonic cells, likely a result of reduced access to nutrients for

cells deeper in the biofilm (Davies, 2003). Since biofilm cells already exist in a state of low nutrition (Sauer et al., 2002), their altered gene expression may support earlier transition to the VBNC state when exposed to the low nutrient and low temperature stresses of storage in PBS at 4°C as used in this study. These gene expression differences which may explain the earlier transition of biofilm cells to the VBNC state warrant further study.

There is concern that VBNC *C. jejuni* remain capable of causing disease (Baffone et al., 2006; Nascutiu, 2010) and recent evidence that VBNC *C. jejuni* continue to express virulence genes and maintain the ability to invade epithelial cells, supports this concern (Chaisowwong et al., 2012). Retail poultry can be contaminated with up to 4.12 (log_{10} CFU/ml of rinsewater) (Bashor et al., 2004; Purnell et al., 2014) and these values do not include VBNC cells. In the present study, there were no reductions in biofilm cell counts over the first 10 days when the cells were becoming non-culturable and after 60 days the average log reduction was only 1.61 log_{10} cells/ml with viable cells counts remaining as high as 10^6 cells/ml. The infectious dose of *C. jejuni* is only 500 cells and the ability of these cells to remain viable at refrigeration temperatures for such an extended period is a huge concern for food safety.

The results presented in this study highlight the need for culture-independent detection methods like PMAqPCR for *C. jejuni,* particularly in the food industry where food contact surfaces can harbour *C. jejuni* within biofilms. Present day food safety detection methods are still primarily based on culturing, but PCR protocols are currently in place for VTEC/EHEC and *L. monocytogenes* in Canada (Laboratory Procedures for the Microbiological Analysis of Foods. Vol 3. The Compendium of Analytical Methods, 2014) and a PCR-based method for *C. jejuni* has recently been AOAC approved for screening of ready to eat turkey and chicken carcass rinses (AOAC, 2014).

In conclusion, PMAqPCR appears to be a sensitive and efficient method for quantifying viable *C. jejuni* in both biofilm and planktonic samples. The ability to detect and quantify not

only culturable, but all viable *Campylobacters* will lead to improved food safety and reduced incidence of disease.

Acknowledgments

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). We thank Aletta Schurter for her assistance with confocal image analysis and Misung Yim for technical support.

Table 3.1 Test schedule summary.

	Plate counts on:		Enrichment in Bolton broth and plate counts on 689	*Bac*Light assay (TCC[c], GCC[d])	PMAqPCR (TCCE[e], VCCE[f])
	MHA[a]	689[b]			
Day 0	√	√	√	√	√
Day 10	√	√	√	Biofilm cells only	Biofilm cells only
Day 20, 30, 40, and 50	√	√	√		
Day 60	√	√	√	√	√

[a] Mueller Hinton agar. [b] Supplemented *Campylobacter* agar base. [c] Total converted cell counts from *Bac*Light biovolume analysis. [d] Green converted cell counts from *Bac*Light biovolume analysis. [e] Total *Campylobacter* cell equivalents derived from PMAqPCR. [f] Viable *Campylobacter* cell equivalents derived from PMAqPCR. √ - indicates that all samples were tested.

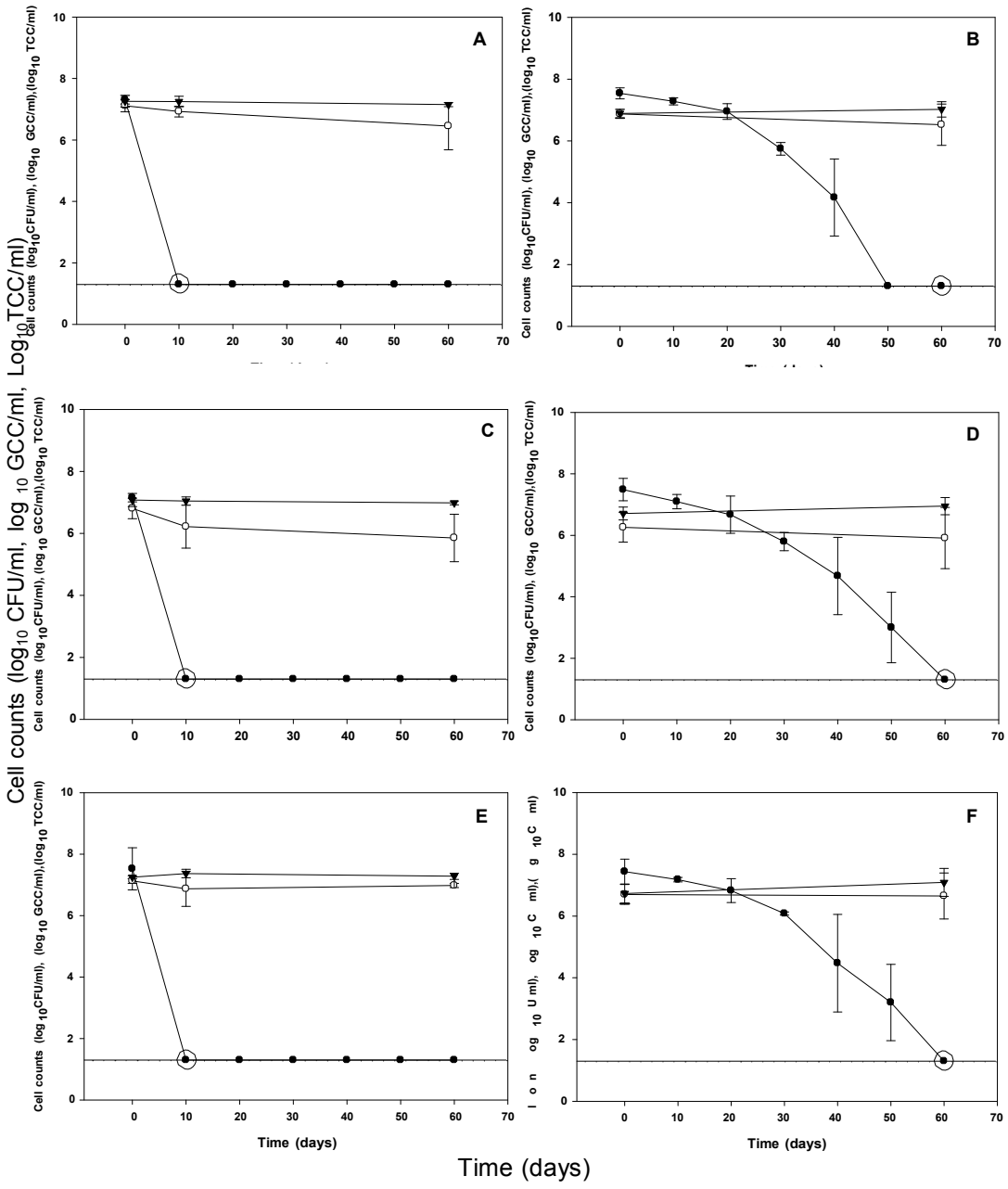

Figure 3.1. Comparison of plate counts on supplemented agar (●), total biovolume (▼) and viable biovolume (O) for *C. jejuni* V1 biofilm (A) and planktonic cells (B), *C. jejuni* V26 biofilm (C) and planktonic cells (D) and *C. jejuni* 16-2R biofilm (E) and planktonic cells (F) kept in phosphate buffered saline (PBS) at 4°C for 60 days. Dashed line indicates detection limit of 1.3 $\log_{10}$ CFU/ml. Circled plate count values indicate first day non-culturable with enrichment in Bolton broth. Error bars represent standard deviation from the mean of three independent replicate trials.

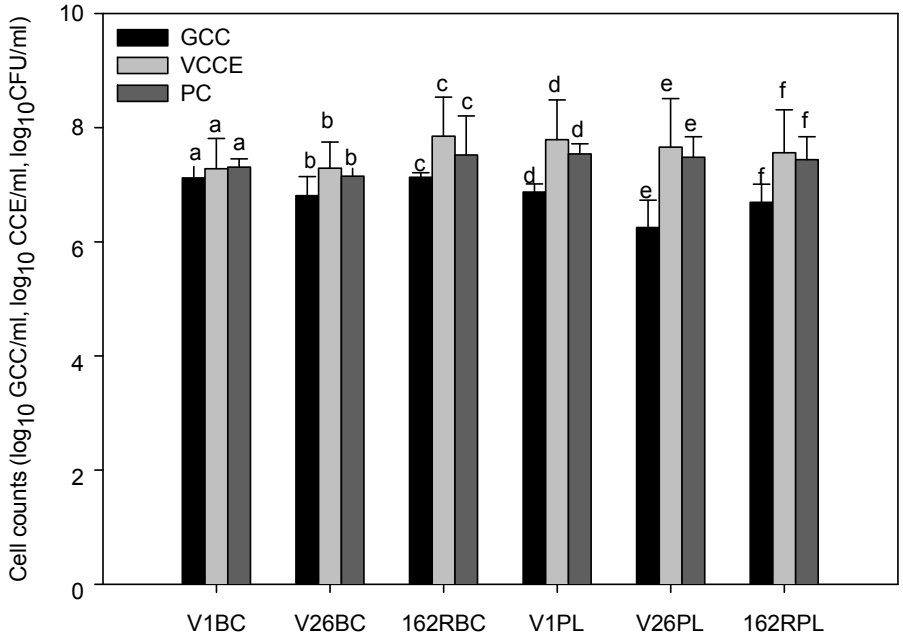

Figure 3.2. Comparison of values for viable cell counts obtained from *Bac*Light biovolume analysis (GCC), PMAqPCR (VCCE) and plate counting (PC) prior to stress. BC – biofilm cells, PL – planktonic cells, V1 - *C. jejuni* NCTC 11168 variant 1, V26 - *C. jejuni* NCTC 11168 variant 26, 162R - *C. jejuni* poultry isolate 16-2R. Small letters indicate significant differences between counts for each sample ($p<0.05$). Error bars represent standard deviation from the mean of three independent replicate trials.

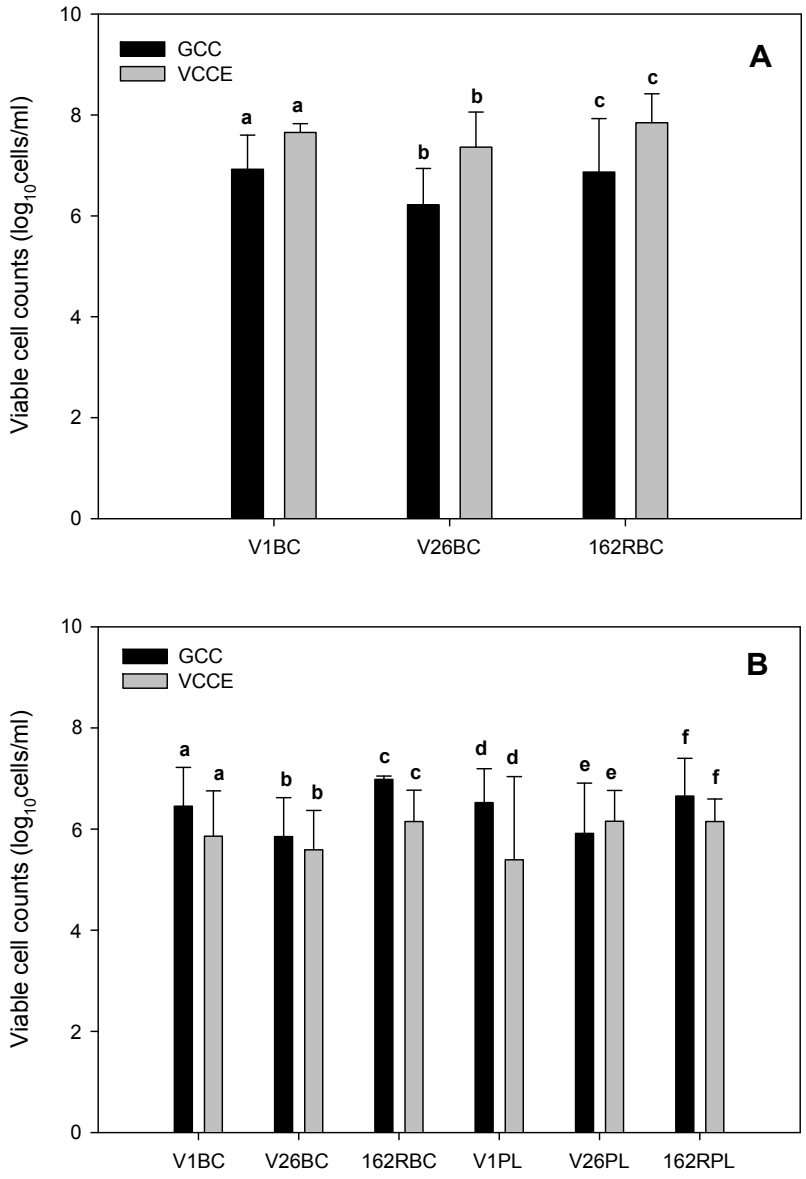

Figure 3.3. Comparison of viable cell counts from the *Bac*Light assay and the PMAqPCR in biofilm samples at day 10 when they became VBNC (A) and planktonic samples at day 60 when they became VBNC (B). GCC – green converted counts from *Bac*Light assay, VCCE – viable cell counts from PMAqPCR, BC – biofilm cells, PL – planktonic cells, V1 – *C. jejuni* NCTC 11186 variant 1, V26 – *C. jeju*ni NCTC 11168 variant 26, 162R –*C. jejuni* poultry isolate 16-2R. Small letters indicate significant differences between methods for each sample ($p<0.05$). Error bars represent standard deviation from the mean of three independent replicate trials.

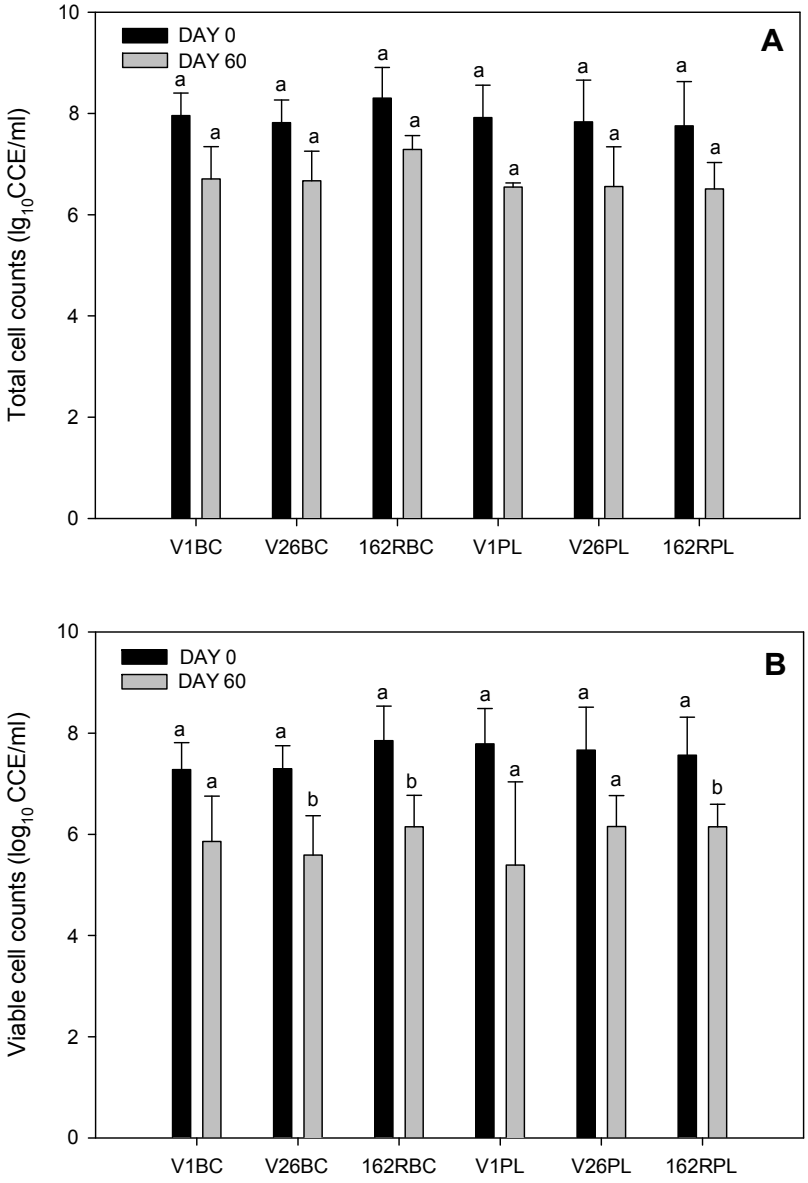

Figure 3.4. Changes in *C. jejuni* total cell counts (A) obtained from qPCR and viable cell counts (B) obtained from PMAqPCR over 60 days. BC – biofilm cells, PL – planktonic cells, V1 - *C. jejuni* NCTC 11168 variant 1, V26 - *C. jejuni* NCTC 11168 variant 26, 162R - *C. jejuni* poultry isolate 16-2R. Small letters indicate significant differences between days for each strain and sample type ($p<0.05$). Error bars represent standard deviation from the mean of three independent replicate trials.

Transition to Chapter 4

The initial work in this thesis revealed that biofilm cells enter the VBNC state earlier than their planktonic counterparts. This variation made it possible to investigate differences in gene expression which might influence the earlier transition and shed light on the molecular mechanisms involved. Genes were selected based on their relevance to biofilm formation and survivalof nutrient stress or ability to enter a VBNC state.

CHAPTER 4: Gene expression of stress-related genes in biofilm and planktonic *Campylobacter jejuni* in late log phase and during transition to the viable but non-culturable state

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Abstract

As the leading cause of bacterial gastroenteritis in the developed world, *Campylobacter jejuni* may be evading detection by entering a viable but non-culturable (VBNC) state. In order to better understand the molecular mechanisms associated with the transition to the VBNC state, gene expression of selected genes was compared in *C. jejuni* biofilm and planktonic cells prior to any applied stress (in late log phase), during transition to the VBNC state and once samples had become fully VBNC. Target genes, *spoT, ppk1, phoX* and *csrA* were selected based on their involvement in both biofilm and VBNC formation in *C. jejuni*. The VBNC state was induced by incubating samples in phosphate buffered saline at 4°C. Biofilm samples entered the VBNC state an average of 10 days earlier than planktonic samples. Gene expression prior to stress was 5 to 37 fold higher in biofilm cells than their planktonic counterparts for all three strains ($p<0.001$). Although the differences were not statistically significant, the planktonic sample that exhibited increased gene expression during transition to the VBNC state, displayed improved survival, with higher numbers of viable cells at day 60. These results suggest that upregulation of the target genes during biofilm formation contributes to the earlier transition of biofilm cells to the VBNC state.

4.1. Introduction

In spite of its fastidious nature, *Campylobacter jejuni* (*C. jejuni*) remains the leading cause of bacterial gastroenteritis in the developed world (Suzuki and Yamamoto, 2009). Campylobacteriosis, characterized by fever, severe abdominal cramping and diarrhea is most often self-limiting, but can lead to more serious sequelae such as reactive arthritis, inflammatory bowel disease and the acute neuromuscular paralysis, Guillain-Barré syndrome. *Campylobacter*s reside asymptomatically in many domestic animals and birds. Poultry is the main reservoir and source of sporadic infections with up to 57% of retail poultry in Canada being contaminated (Suzuki and Yamamoto, 2009). Outbreaks are less common but when they occur it is most often in relation to contaminated water (Pitkanen, 2013).

Despite the prevalence of this enteric pathogen, little is known about the mechanisms *C. jejuni* uses to adapt to and survive stresses. *C. jejuni* lacks the stress response systems commonly found in other enteric pathogens, but is able to resist stresses by existing within biofilms or by entering a viable but non-culturable state (Rollins and Colwell, 1986; Trachoo and Frank, 2002). Although there is little research to date on the relationship between these phenotypes, recent mutation studies have identified genes that affect both entry into a VBNC state and the ability to form biofilm in *C. jejuni,* suggesting that these two systems may be related at the molecular level (Gangaiah et al., 2009; Drozd et al., 2011). Four genes in particular, polyphosphate kinase1 (*ppk1*), an alkaline phosphatase (*phoX*), the stringent response regulator (*spoT*) and the carbon starvation regulator (*csrA*), have been reported to influence both biofilm formation and stress survival in *C. jejuni* (Gaynor et al., 2005; Candon et al., 2007; Fields and Thompson, 2008; Gangaiah et al., 2009; Drozd et al., 2011).

Polyphosphate kinase 1 (*ppk1*) catalyzes the synthesis of inorganic polyphosphate (poly-P). Poly-P consists of a long chain of phosphate residues linked by high energy phosphoanhydride bonds and acts as a reservoir for energy and phosphate. In *C. jejuni* , poly-P accumulates during the transition from exponential to stationary phase and plays a role in both

low nutrient survival and biofilm formation as well as natural transformation, osmotolerance, resistance to antimicrobials, intracellular survival and colonization, (Candon et al., 2007; Gangaiah et al., 2009). Mutants for *ppk1* have significantly reduced levels of poly-P and are less able to enter the VBNC state, indicating that poly-P is required for maintaining viability by allowing cells to enter the VBNC state during stress (Gangaiah et al., 2009).

Although the ability to transition to the VBNC state was not tested for mutants of the other three genes, their relationships to polyphosphate and biofilm formation as outlined below suggest roles for each gene in the interaction between these two survival phenotypes.

The sole alkaline phosphatase (*phoX*) in *C. jejuni*, provides the cell with inorganic phosphate (Pi) by hydrolysis of phosphate groups from organophosphate molecules. Pi which is typically low in the environment is required for the *ppk1* mediated formation of poly-P and *phoX* mutants are less able to accumulate poly-P (Drozd et al., 2011).

C. jejuni mounts a stringent response mediated by a single bifunctional guanasine pentaphosphate (pppGpp) synthetase/hydrolase encoded by *spoT* (Gaynor et al., 2005). The stringent response is a global stress response typically activated by amino acid starvation, altering gene expression to favour survival over growth. *C. jejuni* accumulates large amounts of (p)ppGpp in response to carbon and phosphate starvation, but has no increase in ppGpp levels in nutrient-rich conditions (Gaynor et al., 2005; Wells and Gaynor, 2006). A *C. jejuni spoT* mutant, unable to produce ppGpp, had significantly reduced levels of poly-P at stationary phase (Candon et al., 2007).

The global post-transcriptional regulator, *csrA* (carbon starvation regulator), a small regulatory protein that activates or represses the translation of mRNA into protein, plays a role in motility, biofilm formation, adherence to epithelial cells and oxidative stress defense in *C. jejuni* (Fields and Thompson, 2008; Timmermans and Van Melderen, 2010). Mutants for *csrA* are deficient in biofilm formation (Fields and Thompson, 2008).

The *spoT, ppk1 and phoX* mutants, which all had reductions in poly-P levels, also exhibited increased biofilm formation. In the *phoX* mutant, where the addition of Pi allowed for production of poly-P, biofilm returned to WT levels. However, addition of Pi to Δ*ppk1* which was still unable to synthesize poly-P, led to further increases in biofilm. This suggests that cells which are unable to enter the VBNC state due to limiting levels of poly-P, may up regulate biofilm formation as an alternative strategy for survival. This premise is supported by the fact that both Δ*ppk1* and Δ*phoX* had up regulation of *csrA*, which is known to positively regulate biofilm in *C. jejuni*.

The objective of this study was to determine if gene expression of *ppk1, phoX, spoT,* and *csrA* differed between biofilm and planktonic cells prior to any stress treatment, and to track changes in their expression early in the transition to the VBNC state and then once cells had become completely non-culturable in order to elucidate possible molecular mechanisms involved in the transition to the VBNC state and clarify the relationship between the biofilm and VBNC phenotypes at the molecular level.

4.2. Materials and methods

4.2.1. Bacterial strains and culture conditions.

Campylobacter jejuni NCTC 11168 V1 was purchased from the ATCC and is representative of the original clinical isolate from a case of human enteritis in 1977 (Ahmed et al., 2002). *C. jejuni* NCTC 11168 V26 (Carrillo et al., 2004), the laboratory passaged version of V1, was kindly donated by Dr. Brenda Allan from the Vaccine and Infectious Disease Organization (VIDO) in Saskatoon. *C. jejuni* 16-2R, a poultry isolate, was provided by Dr. Joseph Odumeru, Laboratory Services Division, University of Guelph. All three strains were maintained at -80°C in an ultra-freezer (Thermo Electron). Cells from stock cultures were resuscitated on Mueller Hinton agar (MHA) by incubating at 42°C under microaerobic conditions (5% 0_2, 10% $C0_2$ and 85% N_2) for

24 h. Resuscitated cells were then transferred onto fresh MHA and incubated at 37°C under microaerobic conditions for 24 h prior to preparation of inocula.

4.2.3. Preparation of biofilm and planktonic cells.

For each experiment, inocula were prepared fresh from frozen stock in order to avoid the transcriptional variation inherent in these bacteria which may undergo phenotypic changes as a result of subculturing (Carrillo et al., 2004). Bacteria were grown as a lawn on MHA for 24 h at 37°C after which cells were transferred from the subculture to 5 ml sterile phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) using a polyester tipped sterile swab (Fisherbrand). Using a NovaSpec light spectrophotometer (Biochrom LTD, Cambridge, UK), the resulting suspension was standardized to an OD_{600} of 0.3± 0.015, which was equivalent to approximately 10^8 CFU/ml as confirmed by plate counting. For development of biofilm cells, 0.1 g of glass fibre filters (Whatman GF/F) were placed in 250 ml glass bottles (Pyrex) which were then autoclaved at 121°C for 20 min. Glass fibre filters provide extensive surface area for cell attachment and are amenable to the removal of cells (Kalmokoff et al., 2006). Bottles were cooled overnight and 20 ml of sterile Mueller Hinton broth (MHB) were added to each bottle. Each bottle was inoculated with 1.0 ml of standardized inoculum. Bottles were incubated microaerobically (5% O_2, 10% CO_2, 85% N) at 37°C with gentle agitation (25 rpm) in an incubator shaker (New Brunswick Scientific, *Innova*™ 4430) for 20 h. Growth of planktonic cells followed the same procedure without the addition of glass fibre filters.

4.2.4. Harvesting and enumeration of biofilm and planktonic cells by standard plate counting.

After 20 h of growth, bottles were removed from the incubator. For bottles with glass fibre filters, the broth containing planktonic cells was aseptically removed and discarded. The glass fibre filters were washed 3 times with 25 ml of cold PBS to remove any remaining planktonic cells. Filters were aseptically transferred to sterile 100 ml glass bottles containing 5 g of glass beads (SEPHEX, 450-600nm) and 10 ml of PBS and vortexed vigorously for 2 minutes using a

Fisherbrand vortex set at maximum speed. The supernatant was then filtered through sterile mesh-lined bags to remove excess glass fibre and the resuspended biofilm cells were collected in sterile 50 ml tubes. Both the removed biofilm cells and planktonic cells (grown separately) were enumerated by drop plating on MHA, incubated microaerobically at 42°C. Colonies were counted at 24 and 48 h and CFU/ml determined for each sample.

4.2.5. Induction of cells into the viable but non-culturable state.

C. jejuni cells, grown and harvested as described above, were resuspended in 30 ml of phosphate buffered saline (PBS) in sterile 50ml centrifuge tubes (Falcon) to give concentrations of approximately 10^7 CFU/ml. Initial culturable cell counts were confirmed by drop-plating on MHA as described above as well as on supplemented *Campylobacter* Agar base (Oxoid CM0689) for comparison. Samples were then incubated at 4°C for up to 60 days in air without shaking. Samples were tested for culturability with enrichment at 10 day intervals.

4.2.6. Culture-based enumeration.

Samples were drop plated on *Campylobacter* Agar Base (Oxoid CM0689) supplemented with *Campylobacter* Growth Supplement (Oxoid SR0232) which contains sodium pyruvate, sodium metabisulphite and ferrous sulphate. No selective supplements or antibiotics were added in order to prevent inhibition of injured cells. Plates were incubated microaerobically at 42°C for 48 h prior to counting. For enrichment, a 500µl aliquot of the cell suspension was added to 800µl of sterile Bolton Broth and incubated microaerobically at 37°C with gentle agitation for 24 h. A 100 µl aliquot of the broth was then plated on supplemented *Campylobacter* Agar base prepared as stated above and incubated at 42°C for 48 h prior to counting.

4.2.7. Estimation of biovolumes using LIVE/DEAD® *Bac*Light™ stain in conjunction with confocal scanning laser microscopy and PHLIP analysis.

C. jejuni biofilm and planktonic cells grown as described above were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Invitrogen). The fluorescent dyes propidium iodide (PI) (20 mM in DMSO) and SYTO 9 (3.34 mM in DMSO)

were mixed in a 2:1 ratio (PI:SYTO 9). Samples (500 µl) were incubated with 1.5 µl of dye mixture at 37°C in the dark for 15 minutes. Samples were then immediately filtered onto 0.2 µm black membrane filters (Isopore™ membrane filters GTBP02500, Millipore) using a millipore vacuum filtration unit. The filters were placed on glass slides with one drop of mounting oil (Millipore) and sealed with coverslips. Slides were immediately taken for viewing and image capture using sequential scanning with the Laser Scanning Microscope Fluoview, version 4.3 FV300 (Olympus FV300 CSLM) and a 60X PlanApo NA 1.4 oil immersion lens. A HeNe Green (1 mW, 543nm) laser was used to excite propidium iodide (ex/em 535/617nm) and an argon (10 mW, force air cooled, blue 488nm) laser was used to excite SYTO 9 (ex/em 480/500nm). For each sample, twelve random fields of view were analyzed for both total and green biovolume with the biofilm image analysis program, PHLIP (Phobia Lasers Image Processing Software - The New Laser Scanning Microscope Image Processing Package) (Mueller et al., 2004).

4.2.8. Calculation of converted cell counts derived from biovolume analysis.

Direct counting of both green and total cells in images captured as described above was performed on 85 fields of view. A conversion factor based on these ratios was then used to convert biovolume values from PHLIP analysis to either total converted counts (TCC) or green converted counts (GCC).

4.2.9. Reverse transcription quantitative PCR.

For each sample, 10ml volumes of cell suspension were centrifuged at 12,500 X g for 20 min at 4°C, followed by pellets being resuspended in 1 ml of PBS and centrifuged at 21.1 x g for 5 min. RNA extraction was performed on these pellets using the Aurum™ Total RNA Mini Kit (BioRad) using the spin protocol as specified by the manufacturer. RNA was immediately converted to cDNA using the iScript™ Select cDNA Syntheis Kit (BioRad) using the random primer mix as directed by the manufacturer. Samples were normalized using 25ng of total RNA in each conversion reaction based on the maximum amount of RNA recommended by the manufacturer and the amount obtained by processing the samples. Each qPCR was performed in a

20µl volume containing 2µl of template cDNA, 0.3µM of forward and reverse primers, and 10µl of SsoFast™ EvaGreen® Supermix buffer (Bio-Rad). All reactions were performed on a Bio-Rad CFX96™ Real-Time 1000 thermal cycler with a cycle profile as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 51°C for 10 s. Melt curves from 65°C to 95°C with 0.5°C increments were included in each run. Fluorescence measurements were obtained online and analyzed with the Bio-Rad CFX Manager software (version 3.1). In every qPCR analysis, the *C. jejuni* standard and non-template controls for each primer set were included in duplicate. All samples were also normalized using the 16S rRNA gene which was not significantly different in any of the samples ($p>0.05$).

4.2.10. Statistical analysis.

Student T-tests were done on log-transformed data to assess differences in cell counts derived from *Bac*Light™ biovolume analysis. Error bars represent standard deviation of the mean. Biovolumes for each condition and strain are averages of three independent trials with twelve random fields of view for each sample. Gene expression values are averages of the same three independent trials with three technical replicates per sample.

4.3. Results

4.3.1. Transition to the VBNC state

4.3.1.1. Culturability

Although there was considerable variability in loss of culturability, trends were still apparent. Biofilm cells became non-culturable before planktonic cells for all 3 strains on both supplemented agar and with enrichment (Fig. 4.1). Results for V1 (original clinical isolate) were similar to those for V26 (the lab passaged version of V1). V1 and V26 planktonic cells remained culturable 10 days longer than their biofilm counterparts, on both supplemented agar and with enrichment. For the 16-2R strain biofilm cells became non-culturable on supplemented agar on average 10 days earlier than planktonic cells, but with enrichment there was almost no difference (Fig.4.1).

4.3.1.2. Plate counts and viable cell counts

For all samples, plate counts continuously declined over the 60 days, reaching levels below the detection limit between day 40 and 60 (Fig. 4.2). For biofilm samples, plate counts declined by 1.4 to 1.9 $\log_{10}$ CFU/ml between day 0 and 20, while for planktonic samples the decline over the first 20 days was smaller, ranging from 0.7 to 1.1 $\log_{10}$ CFU/ml.

When comparing culturable cell counts with viable cell counts at day 20, it was noticed that the differences were much greater for the biofilm samples (1.4 to 1.7 $\log_{10}$cells/ml) than for the planktonic samples (0.1 to 0.8 $\log_{10}$cells/ml), but this difference was only significant for V1 biofilm cells. It is interesting to note that 86 to 91% of the planktonic cells remained culturable at day 20, while only 75 to 80% of biofilm cells were still culturable at this time. Day 0 viable cells' values were not assessed as they had been consistent with plate counts in previous trials.

4.3.1.3. Total and viable cell counts

Biovolume analysis indicated that there were no significant reductions in viable cell counts from day 20 to day 60, confirming that the loss of culturability was not due to cell death ($p>0.05$). There were also no significant changes in total cell counts over the course of the experiment (Fig 4.2), ($p>0.05$).

4.3.1.4. VBNC

The VBNC state was defined in a manner similar to Chaisowwong et al., (2012), where cells were considered VBNC once there was no evidence of growth on the supplemented media after enrichment, but cells remained viable based on *Bac*Light live/dead staining. Thus samples became non-culturable with enrichment between day 50 and 60, but maintained viability as determined by the *Bac*Light live/dead staining indicating that these cells had entered the VBNC state (Fig. 4.2).

4.3.2. Gene Expression

In order to investigate the changes in transcription of four genes known to play a role in nutrient stress response and biofilm formation, quantitative RT-PCR analysis was performed at 4

time points: prior to treatment, after 20 days of treatment, at day 50, when biofilm cells became non-culturable with enrichment (and were considered VBNC) but planktonic cells were still culturable, and at the end of the experiment at day 60. Timepoints for sampling were chosen based on earlier trials where samples transitioned to the VBNC state as early as day 20 and as late as day 60.

4.3.2.1. Gene expression prior to stress treatment

All 4 target genes were significantly upregulated in biofilm samples at day 0 ($p<0.001$) (Fig. 4.3). Gene expression prior to stress treatment was 5 to 37 fold higher in biofilm cells than in their planktonic counterparts for all three strains (Fig. 4.3). The differences between expression in biofilm and planktonic samples were largest for the lab-passaged strain V26 (22 to 37 fold) and smallest for the poultry isolate 16-2R (5 to 10 fold) (Fig. 4.3).

Of the 4 genes, *spoT* (9, 21, 36 fold) and *ppk1* (10, 18, 37 fold) had the greatest increased expression in biofilm cells as compared to planktonic cells prior to stress treatment, regardless of strain ($p<0.001$). Even for *csrA* which had the smallest differences in gene expression between biofilm and planktonic cells for all 3 strains prior to stress (5, 12, 22 fold), these differences were significant ($p<0.001$). The differences in expression of *phoX* in biofilm and planktonic cells prior to stress (7, 17, 33 fold) was intermediate (Fig. 4.3).

4.3.2.2. Changes in gene expression over the 60 days:

Between day 0 and day 20, *csrA* was significantly down regulated 3 to 6 fold, in all but the 16-2R biofilm sample (Fig. 4.4) ($p<0.05$). *SpoT* was also down-regulated at this time, in all samples except V1 biofilm cells. This was significant for V26 biofilm and planktonic samples and the 16-2R biofilm sample, with a fold range of 3-9 fold ($p<0.05$). For V1 and 16-2R planktonic samples the change was 2 ($p=0.09$) and 9 fold ($p=0.56$) respectively.

Between day 20 and day 50, gene expression was significantly upregulated 4 to 6 fold in all four genes for the V1 planktonic sample and 2 fold for *csrA* and *spoT* in the 16-2R biofilm samples ($p<0.05$). Also, for these 2 samples gene expression was down-regulated 3 to 8 fold

between day 50 and 60 which was significant for all genes in the 16-2R biofilm sample and for *ppk1* and *spoT* in the V1 planktonic sample ($p<0.05$).

For the V1 biofilm sample *csrA* and *spoT* were significantly upregulated 2 fold between day 50 and 60 ($p<0.05$) and although non-significant, *pho X* was also upregulated 2 fold and *ppk1* down-regulated 2 fold during this period.

4.4. Discussion

Gene expression of all four selected genes was significantly higher in biofilm cells than in planktonic cells prior to stress and all biofilm samples transitioned to the VBNC state earlier than the planktonic samples. This suggests that growth in a biofilm predisposes cells to enter the VBNC state by upregulating the four genes tested. This is supported by previous work for the *ppk1* gene, where mutants were less able to maintain viability by entering the VBNC state when exposed to stress (Gangaiah et al., 2009). No work has yet been published that explores the ability of mutants for the other three genes to enter a VBNC state during stress. The fact that at day 20, a larger percent of cells in the biofilm samples were VBNC than in planktonic samples also supports the idea that growth in a biofilm predisposes cells to become VBNC.

Of the 4 genes, the difference in expression between biofilm and planktonic samples prior to stress was greatest for *spoT* and *ppk1* regardless of strain ($p<0.001$). These genes are known to respond to low nutrient stress and their greatly increased expression may have been a response to low nutrient conditions during growth within the biofilm. Starvation in the presence of phosphate allowed the cells to produce polyphosphate and may explain the extended survival in the VBNC state observed in the experiments. However, since both biofilm and planktonic cells were incubated with phosphate, the presence of phosphate is not relevant to the differences in gene expression observed at day 0.

The increased expression of *spoT* would lead to increased levels of the alarmone, ppGpp thereby initiating the stringent response and the increased production of amino acids and expression of stress response proteins (Dalebroux et al., 2010). The increased expression of *ppk1*

which catalyzes the addition of Pi to poly-P would lead to reduced levels of Pi in the cells. This in turn may be sensed by the PhoS/R system and lead to upregulation of *phoX* in order to provide the cell with more Pi, which it does by catalyzing the removal of phosphate groups from organophosphate molecules (Wosten et al., 2006).

Even for *csrA,* the differences in gene expression between biofilm and planktonic cells prior to stress were significant ($p<0.001$). The fact that this gene was the least upregulated in biofilm of the 4 selected genes prior to stress was a surprise and may indicate that either only small amounts are required for biofilm formation, that it had been upregulated early in biofilm formation (samples were processed after 20h) and then down-regulated again and/or that the mechanisms involved are more complex. Little is known about the regulation of *CsrA* in *C. jejuni*. In *E. coli*, *CsrA* is controlled by the small RNAs CsrB and CsrC that have no apparent orthologs in the *C. jejuni* genome. In *E. coli, csrA* expression is under the positive control of σ^{38} which is upregulated at the onset of stationary phase or during exposure to stress (Zimmerman et al., 2009). *CsrB* and *CsrC* are highly expressed in the absence of amino acids, the same conditions used to induce the VBNC state in the present work, as well as at the onset of stationary phase. The increased concentrations of CsrB and CsrC lead to the sequestration of CsrA (Zimmerman et al., 2009). A similar form of feedback regulation may be occurring in *C. jejuni*, because although the mechanisms are not known, it was recently shown that when expressed in an *E.coli csrA* mutant, *C. jejuni csrA* was able to recover defects in motility, biofilm formation and cellular morphology (Fields and Thompson, 2012). More work needs to be done to explore the role of *csrA* in the low nutrient stress response in *C. jejuni.*

Changes in gene expression during transition to the VBNC state were observed for the 16-2R biofilm sample and the V1 planktonic sample. For both of these samples, all four genes were upregulated between day 20 and 50 (Fig. 4.4). During this time these samples were non-culturable on the supplemented agar, but would still culture with enrichment, indicating that they were transitioning to the VBNC state (Fig. 4.2). From day 50 – 60, all four genes were

downregulated and by day 60 the samples were fully VBNC suggesting that the four genes are upregulated during transition to the VBNC state and then downregulated once it is reached.

For V1 and V26 biofilm cells the values on day 60 provide insight on changes in gene expression that occur after the cells are VBNC. For the V1 biofilm cells there are slight upregulations in all genes except *ppk1* which is down regulated. The V26 biofilm sample maintained viability in spite of a lack of gene expression changes. This may be explained by the overall higher expression of genes in this sample.

In conclusion it was found that all four target genes were significantly upregulated during biofilm formation for all three strains in this study. In all cases, biofilms samples transitioned to the VBNC state earlier than their planktonic counterparts. Also, biofilm samples had higher numbers of non-culturable cells than their planktonic counterparts at day 20 which suggests that cells are already entering the VBNC state during biofilm formation which may be due to the low nutrient status within biofilms. Although not statistically significant, the planktonic sample of V1, which showed increased gene expression prior to VBNC entry, maintained a higher number of viable cells at day 60 than the planktonic sample of V26 which had no increased gene expression. The poultry isolate maintained culturability longer than the clinical isolates in both biofilm and planktonic samples.

The results of this study suggest that the upregulation of the 4 selected genes in the biofilm samples contributes to the earlier transition to the VBNC state for these cells. Although, it appears that the four target genes play a role in VBNC formation, further research is required to clarify the mechanisms and the role each gene product plays in this particular survival strategy.

Table 4.1. Primers for use in gene expression analyses.

Name	Sequence 5' to 3'	Position in sequence *C. jejuni* NCTC 11168*	Amplicon size	Reference
16S rRNA F	CTGCTTAACACAAGTTGA	1) 39434 – 39720	287bp	(Josefsen et al., 2010)
16S rRNA R	TTCTGACGGTACCTAAGGAA	2) 394315 – 394601	287bp	(Josefsen et al., 2010)
		3) 696609 – 696895	287bp	(Josefsen et al., 2010)
csrA F	TTATCGGAGAAGGTATAG	1038147 – 1038243	97bp	(Drozd et al., 2011)
csrA R	TTTCTAAGTATCATAAGGG			
spoT F	GTAACCACTCGCACAATATC	1205365 – 1205546	182bp	(Drozd et al., 2011)
spoT R	GATGTCGCAGTTTATTCTCC			
ppk1 F	TGAAGCAAGTATGGAAGGAG	1292747 – 1292976	230bp	(Drozd et al., 2011)
ppk1 R	ATATAGGAGTCATAAGTTCTAAGC			
phoX F	AGGGCCTATTGCTTGTGAATTAAC	150423 – 150510	88bp	(Wosten et al., 2006)
phoX R	ACCTTCTCCTGGATGTTGTATGC			

C. jejuni has 3 copies of the 16S rRNA gene. *Primer sets were tested on the non-sequenced strain 16-2R.

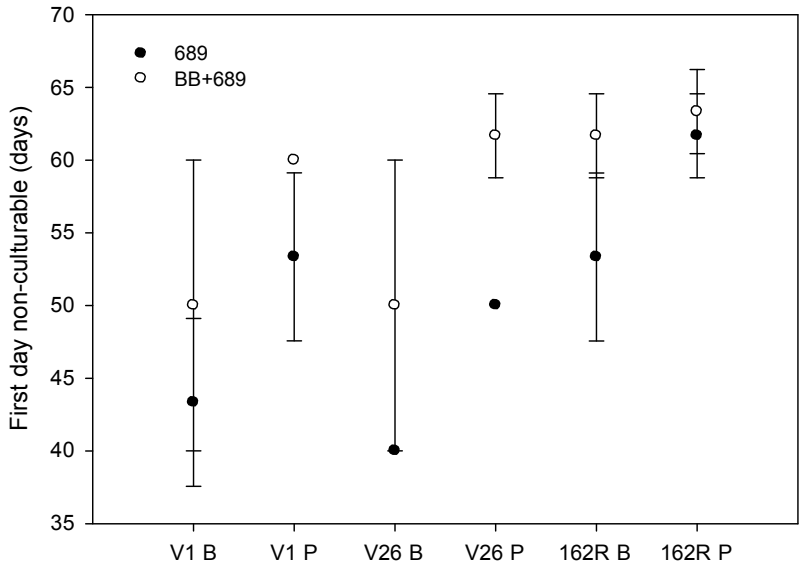

Figure 4.1. Loss of culturability for *C. jejuni* in phosphate buffered saline at 4°C. Symbols represent the average for the first day with no growth on supplemented agar (●), or with 24 h enrichment in Bolton broth followed by drop plating on supplemented agar (○). Error bars represent standard deviation of 3 biological replicates. V1 – *C. jejuni* NCTC 11168 strain V1, V26 – *C. jejuni* NCTC 11168 strain V26, 162R – *C. jejuni* poultry isolate 16-2R, B – biofilm, P – planktonic.

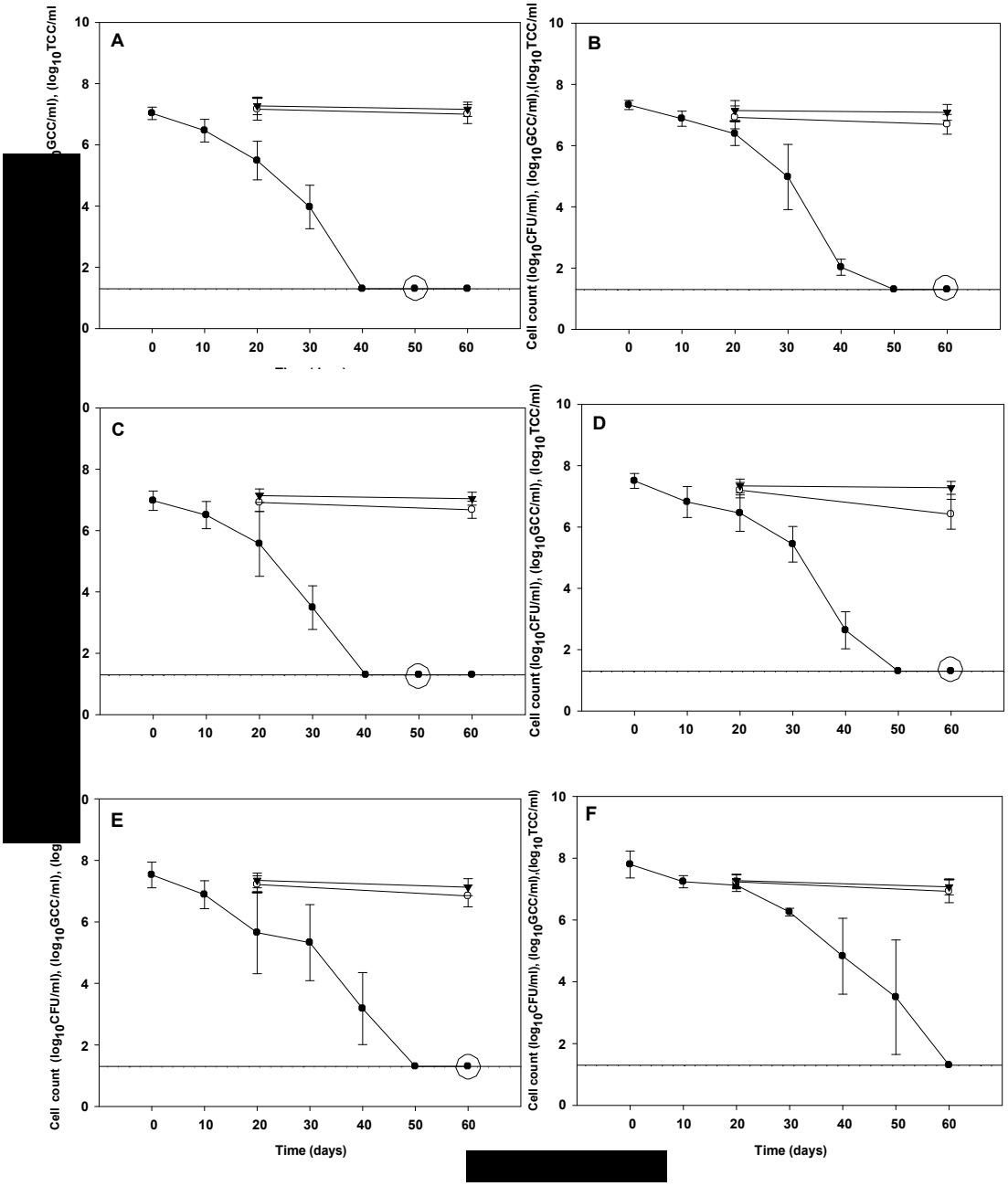

Figure 4.2. Comparison of plate counts on supplemented agar (●), total (▼) and viable (o) cell counts based on biovolume analyses for *C. jejuni* V1 biofilm (A), and planktonic cells (B), *C. jejuni* V26 biofilm (C) and planktonic cells (D) and *C. jejuni* 16-2R biofilm (E) and planktonic cells (F) kept in phosphate buffered saline (PBS) at 4°C for 60 days. Dashed line indicates plate count detection limit of 1.3 $\log_{10}$ CFU/ml. Circled plate count values indicate first day non-culturable with enrichment in Bolton broth.

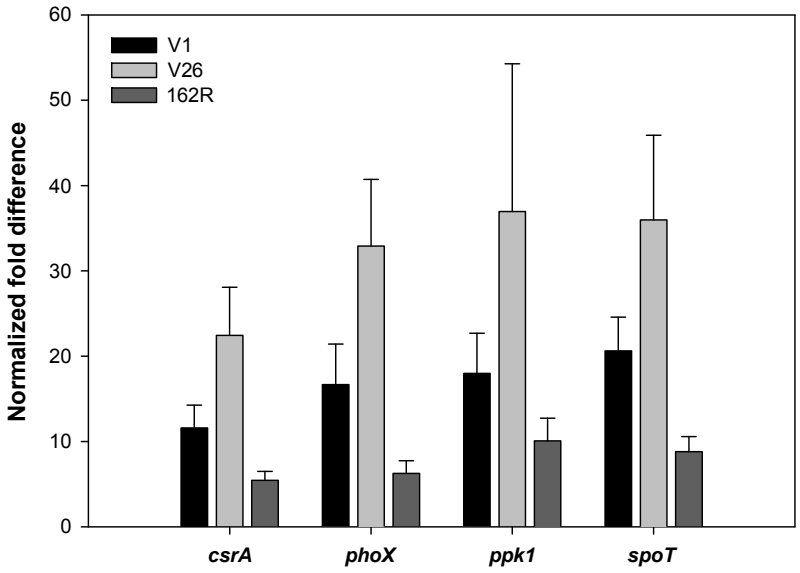

Figure 4.3. Relative differences in the expression of target genes between biofilm and planktonic samples. Gene expression was significantly higher in biofilm samples for all genes in all samples ($p<0.05$). The relative difference ($2^{-\Delta\Delta CT}$) in gene expression was calculated from the ΔΔCT by subtracting the value of the planktonic sample from the value for the biofilm sample after normalization with a 16S rRNA housekeeping gene. V1 indicates fold difference between the V1 biofilm and planktonic samples. V26 indicates fold difference between the V26 biofilm and planktonic samples. 162R indicates fold difference between the 16-2R biofilm and planktonic samples. Genes with a 2-fold or greater difference ($p<0.05$) were considered to be significantly upregulated or down-regulated. Each bar represents the mean ± SE of the relative fold difference in expression from three independent experiments with triplicate reactions for each sample. *csrA* – carbon starvation regulator gene, *phoX* – alkaline phosphatase gene, *ppk1* –polyphosphate kinase gene, *spoT* – stringent response gene, V1 – *C. jejuni* NCTC 11168 strain V1, V26 – *C. jejuni* NCTC 11168 strain V26, 162R – *C. jejuni* poultry isolate 16-2R.

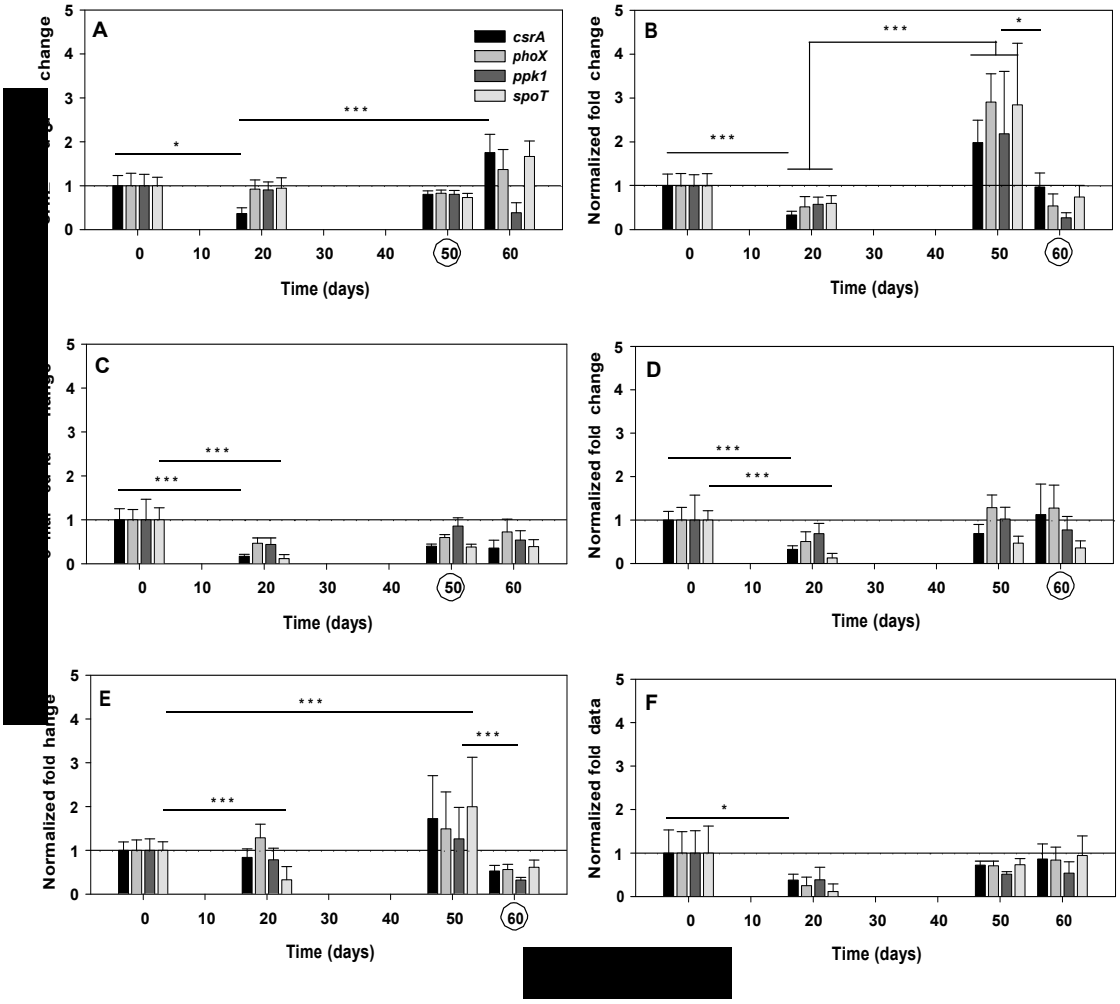

Figure 4.4. Changes in gene expression over the 60 days of treatment for *C. jejuni* V1 biofilm (A), and planktonic cells (B), *C. jejuni* V26 biofilm (C) and planktonic cells (D) and *C. jejuni* 16-2R biofilm (E) and planktonic cells (F) kept in phosphate buffered saline (PBS) at 4°C for 60 days. Dashed line indicates day 0 baseline values. Circled days indicate first day non-culturable with enrichment in Bolton broth. Significant differences in gene expression are indicated by * ($p<0.05$), *** ($p<0.001$). *csrA* – carbon starvation regulator gene, *phoX* – alkaline phosphatase gene, *ppk1* –polyphosphate kinase gene, *spoT* – stringent response gene.

CHAPTER 5: Conclusions and Future Directions

Campylobacter jejuni has been the leading cause of bacterial foodborne disease since it was first isolated in the late 1970s. It remains a conundrum how such a sensitive, fastidious pathogen continues to uphold this status. In spite of numerous studies done over the last 30 years, there has been no significant reduction in the incidence of disease.

The investigations described in this thesis were motivated by the rationale that *C. jejuni* has remained the leading cause of bacterial foodborne disease due to its ability to transition to the VBNC state, particularly within biofilms and hence evade detection. Although the first published account of VBNC *C. jejuni* in 1986 initiated discussion of this state, it has taken time to be accepted within the research community. The knowledge that biofilms provide protection for microbes has been well established, along with the fact that biofilms on food contact surfaces are resistant to removal and act as a source of recontamination.

One of the novel findings of this thesis is that *C. jejuni* biofilm cells can enter a VBNC state and remain viable in high numbers (10^6 cells/ml) for an extended period (60 days) in refrigeration temperatures (4°C). Although there was variation with respect to time to become VBNC, biofilm cells consistently transitioned earlier than planktonic cells in three separate sets of experiments (Ch2, 3 and 4). The fact that existing within a biofilm is conducive to entry into the VBNC state has major implications for food safety and substantiates the importance of being able to detect and quantify VBNC biofilm cells. The work described in Chapter 3 validated the ability of the molecular method PMAqPCR to accurately quantify *C. jejuni* VBNC biofilm cells. These results can be used to inform decision making about methods for routine monitoring on food contact surfaces where biofilms may be present. For example, PMAqPCR could be used in assessing novel sanitation methods for their efficacy in killing cells within biofilms since the values provided will indicate if any cells remain in the VBNC state.

The earlier entry of biofilm cells to the VBNC state provided an opportunity to explore differences in gene expression and begin to describe the molecular mechanisms involved (Ch 4).

All four selected genes were found to be expressed at significantly higher levels in biofilm cells prior to the application of stress, suggesting that upregulation of these genes in biofilm cells contributes to the earlier transition to the VBNC state. For planktonic samples, the changes in gene expression over the 60 days of treatment varied by strain, but increased gene expression was associated with improved survival.

Further work needs to be done to clarify the role each gene plays in biofilm and VBNC formation and gene expression experiments would benefit from improved predictability of the timing of entry into the VBNC state. Although we know that the *ppk1* mutant has a reduced ability to enter a VBNC state when exposed to stress, and the *csrA, spoT*, and *phoX* mutants lose culturability more quickly than the WT, it remains unknown if these other mutants are entering a VBNC state or simply dying (Gangaiah et al., 2009). Measuring viability using either the *Bac*Light assay or PMAqPCR, alongside loss of culturability would provide this information and give a better understanding of the function of *csrA, spoT* and *phoX* in VBNC formation.

Other future studies are needed to address the ability of VBNC *C. jejuni* to resuscitate *in vivo* and the potential of these VBNC cells to initiate disease. Improved models of infection are required which provide a more accurate representation of the human gut than the cell line methodology used at present.

The methodology developed in this thesis could also be applied to investigations of the ability of *C. jejuni* to enter the VBNC state in multispecies biofilms.

In conclusion, the work done in this thesis provides the basis for improved food safety methods which offer a more accurate assessment of the number of viable and potentially infectious *C. jejuni* cells in both planktonic and biofilm samples. Also, understanding the interaction between biofilm formation and entry into the VBNC state at the molecular level as described here can act as a starting point for the development of appropriate interventions to reduce the incidence of campylobacteriosis. Together these results will help to oust *C. jejuni* as the leading cause of foodborne disease.

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