BACTERIOPHAGE TREATMENT OF *CAMPYLOBACTER* BIOFILMS: FORMATION OF THE CARRIER STATE LIFE CYCLE

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ABSTRACT

Campylobacter jejuni is a gram-negative thermotolerant microaerobic pathogen that causes human gastroenteritis worldwide. The production of extracellular polymeric substances to create a biofilm is a mechanism by which *C. jejuni* can protect itself from unfavourable environments, and is a contributory factor to the survival and transmission of the organism to farms animals and into the human food chain. Bacteriophages are natural predators of bacteria that have the potential for use as targeted biocontrol agents with the advantage that they can penetrate and affect bacteria embedded in biofilms. The effects of virulent *Campylobacter*-specific bacteriophages CP8 and CP30 on *C. jejuni* biofilms formed on glass by strains NCTC 11168, PT14 and HPC5 at 37 °C under microaerobic conditions were investigated. Independent bacteriophage treatment led to 1 to 3 Log_{10} CFU/cm^2 reductions in the viable count 24 h postinfection compared with control levels. In contrast, bacteriophage applied under these conditions effected a reduction of less than 1 Log_{10} CFU/ml in planktonic cells. Resistance to bacteriophage in bacteria surviving bacteriphage treatment of *C. jejuni* NCTC 11168 biofilms was >80%, whereas bacteriophage resistance was not found in similarly recovered *C. jejuni* PT14 cells. Concomitant dispersal of the biofilm matrix by bacteriophage was demonstrated by crystal violet staining and transmission electron microscopy (TEM).

The resistant survivors of bacteriophage treatment of biofilms formed by HPC5 and PT14 remained closely associated with the phage but not NCTC 11168. Analysis of the DNA contents of these isolates by PFGE and Southern transfer confirmed the presence of phage genomic DNA (approximately 140 kb) leading to the conclusion that these strains represent examples of the carrier state life cycle (CSLC) reported for other bacterial species. TEMs of CSLC cultures demonstrated the association of bacteriophage particles with *Campylobacter* cells that were devoid of flagella. Physiological studies of the CSLC strains showed the bacteria were non-motile but able to grow at a similar rate to parental cultures until reaching the phage proliferation threshold (7 Log_{10} CFU/ml) when growth rate declined and the phage titre increased. Of further note the CSLC strains had a greater capacity to survive atmospheric oxygen under nutrient limited conditions. CSLC phages exhibited differences in host binding, efficiency of plating and host range. Transcriptome analyses of CSLC strains harvested from microaerobic cultures at early exponential phase prior to phage proliferation were performed using DNA microarrays to demonstrate changes in host gene expression as compared with parental cultures. Notably genes involved in metabolism and the modification of macromolecules were up-regulated and specific flagella biosynthesis functions down-regulated in the CSLC strains.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
µl	microlitre
A_{600}	Absorbance 600 nm
AI	Autoinducer
AHL	Homoserine-lactones
ATCC	American Type Culture Collection
BA	Blood Agar
bp	base pair
CCDA	Campylobacter blood-free selective agar
cDNA	complementary deoxyribonucleic acid
CDT	Cytolethal Distending Toxin
CFU	Colony forming unit
CJIE	*Campylobacter jejuni* insertion element
cm^2	centimetre square
CPS	Capsular polysaccharide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CsCl	Caesium Chloride
CSLC	Carrier state life cycle
DGC	Diguanylate cyclase
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNAvfc
EDTA	Ethylene Diamine Tetraacetic Acid
EF	Elongation factor
EMBL	European Molecular Biology Laboratory

EOP	Efficiency of Plating
EPS	Exopolysaccharide
EU	European Union
FDA	Food and Drug Administration
FOI	Frequency of incorporation
GMP	Guanosine monophosphate
GBS	Guillain-Barré Syndrome
GC	Guanine Cytosine
GFP	Green Fluorescence Protein
h	Hour
HCl	hydrochloric acid
IF	Initial factor
Kb	Kilo base
LOS	Lipooligosaccharide
$MgSO_4$	Magnesium sulphate
MH	Mueller-Hinton
min	Minus
ml	Millilitre
MOI	Multiplicity of Infection
MRD	Maximal Recovery Diluents
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCTC	National Collection of Type Cultures
nm	Nanometre
NZCYM	New Zealand Casamino Yeast Medium
OD	Optical Density
PBS	Phosphate Buffer Saline
PDE	Phosphodiesterase
PE	Phosphatidylethanolamine

PFGE	Pulsed Field Gel Electrophoresis
PFU	Plaque forming unit
PL	Pseudolysogen
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
RO	Reverse Osmosis
SAM	S-adenosyl-l-methionine
SD	Standard deviation
SDS	Sodium Dodecyl sulphate
SM	Salt Magnesium
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
TAE	Tris-Acetated EDTA
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TMAO	Trimethylamine-*N*-oxide
V	Voltage
VBNC	Viable but nonculturable
WHO	World Health Organisation
% w/v	Percentage weight per volume
YFP	Yellow Fluorescence Protein

CHAPTER 1

GENERAL INTRODUCTION

1.1 *Campylobacter*

1.1.1 Characteristics of *Campylobacter*

Campylobacter spp. are Gram-negative, microaerophilic bacteria that are members of the family *Campylobacteraceae*. The genus of *Campylobacter* is composed of 17 species (Korczak *et al*., 2006). There are several morphological forms of *Campylobacter* spp. including rod-shaped forms, spiral S-shaped, and curved cells in fresh cultures but in old cultures a coccoid form becomes the predominant, which is associated with a loss in viability (Moran and Upton, 1987; Penner, 1988). The cell dimensions of *Campylobacter* spp. are in the range of 0.2 to 0.8 μm wide and 0.5 to 5 μm long (Vandamme, 2000). Motility is a distinctive phenotypic characteristic, which is observed to be a corkscrew-like motion of the spiral rods using a polar unsheathed flagellum at one or both ends of the cells (Vandamme, 2000). Their guanine-cytosine (G+C) content is low at approximately 28-30 mol% (Penner, 1988). The organisms grow under microaerobic conditions, in which the atmosphere contains 3-5% oxygen and 2-10% carbon dioxide for optimal growth (Vandamme, 2000) and within a narrow temperature range of 30 to 47 °C (Skirrow and Benjamin, 1979). However, some species and strains have been documented to grow under aerobic or anaerobic conditions (Vandamme, 2000).

Campylobacter exhibit typical biochemical characteristics as regards the reduction of fumarate to succinate; negative methyl red reaction and acetoin and indole production. The additional biochemical characteristics of most species are: reduction of nitrate, absence of hippurate hydrolysis, and the presence of oxidase activity (Vandamme, 2000). The major respiratory substrates are menaquinones with a chemoorganotrophic type of metabolism (Vandamme, 2000). The main source of energy is derived from amino acids and keto acids such as glutamine, asparagine, glutathione or tricarboxylic acid cycle intermediates, not from carbohydrates due to the lack of phosphofrutokinase essential for glycolysis (Dasti *et al*., 2010). The pathways characteristic of anaerobic metabolism have been suggested as an alternative respiratory method by which *C. jejuni* can use fumarate, nitrate, nitrite, trimethylamine-*N*-oxide (TMAO), and dimethyl sulfoxide (DMSO) instead of oxygen as a terminal acceptor (Sellars *et al*., 2002; Weingarten *et al*., 2008). *Campylobacter* have an extended glycome in the form of cell surface lipooligosaccharide (LOS), capsular polysaccharide (CPS), glycolipids and glycoproteins (Karlyshev *et al*., 2005). The presence of glycoprotein in a bacterial species is unusual, where campylobacters notably exhibit both O- and N-linked protein glycosylation with the former being restricted to the flagellins that polymerise to form a major component of the flagellum (Linton *et al*., 2001).

The *Campylobacter* species are fragile in that they are unable to grow in the presence of oxygen outside the animal host, and are generally sensitive to environmental stresses such as drying, exposure to disinfectants and acidic conditions (challenged at pH 4.5) (Murphy *et al.*, 2006). However, *Campylobacter* spp. have developed survival mechanisms to overcome these stresses. The mechanisms of adaptation to environmental stresses are believed to include: a viable but non-culturable (VBNC) state, genome plasticity, quorum sensing, the formation of biofilms and stationary phase survival mechanisms (Murphy *et al.*, 2006). The VBNC state of *Campylobacter* spp. is controversial being associated with starved or aged cultures likely under oxidative stress. These result in a decline in viability with the associated morphological change from spiral to coccoid forms but that these cells can be recovered through the colonisation of chickens, which represent favourable conditions. The recovery in chickens notwithstanding, there are significant differences in the aerotolerance between strains and the culture conditions under which they can recovered, placing the concept of a VBNC state as a uniform mechanism of survival in dispute (Klančnik *et al*., 2009; Jackson *et al*., 2009; Federighi *et al*., 1998). *C. jejuni* unlike other foodborne pathogens, lacks the key regulators SoxRS and OxyR responsible for the coordinated defence against oxidative stress in *E. coli* and *S.* Typhimurium. However, it has been proposed that alternative regulators, termed Fur and PerR mediate at least part of the response to oxidative stress in *Campylobacter* by repressing the key enzymes alkyl hydroperoxide reductase (AhpC) and catalase (KatA) (Baillon *et al*., 1999; van Vliet *et al*., 2001). Campylobacters also encode other antioxidant enzymes, such as thiolperoxidase (Tpx) and bacterioferritin co-migratory protein (Bcp) that act together to protect against oxidative stress (Atack *et al*., 2008). According to Duong and Konkel (2009), campylobacters have seven hyper-variable plasticity regions (PR) identified as PR1-PR7 based on comparative genome hybridization (CGH) analysis. The PR1 region includes genes that encode the utilisation of alternative electron acceptors during respiration that contribute to overcome in restricted oxygen environments. Regions of PR2, PR3, and PR7 contain genes that encode outer membrane and periplasmic proteins, which may contribute to phenotypic variation and adaptation to different ecological niches. The genes encoded in the PR4, PR5 and PR6 regions have functions that are involved in the biosynthesis and modification of flagella, LOS and capsular polysaccharide. The hyper-variable genome plasticity regions of *Campylobacter* spp. play a critical role in the survival of the organism in harsh environments including those that create oxidative stress. Quorum sensing is a system of stimulus and response correlated to population densities of bacteria. In Campylobacter quorum sensing has been implicated in the synthesis, secretion and detection of extracellular signalling molecules known as autoinducers through the function of LuxS (Jeon *et al*., 2005; Plummer *et al*., 2011). Autoinducer (AI2) of *C. jejuni* has been reported in several food environments such as milk, fruit juice and chicken (Cloak *et al*., 2002). However, that AI2 functions as a quorum sensing molecule in *C. jejuni* at all, has been disputed (Holmes *et al*., 2009). The formation of biofilms, which may protect campylobacters in extreme conditions by enclosing them in an extracellular polymeric substance is another significant defence mechanism that will be discussed later in this chapter. Thus, these various mechanisms may allow cells to survive in the host intestinal tract, on food, and in the environment.

Factors that may contribute to infection in humans and animals include: genetic variation of the *C. jejuni* genome involving intragenomic mechanisms leading to the presence of hypervariable sequences in particular in genes involved in the biosynthesis or modification of surface structures such as capsular polysaccharide (CPS), lipooligosaccharide (LOS) and flagellum (reviewed by Young *et al*., 2007). Natural transformation has an important role in genome plasticity and in the spread of new factors such as antibiotic resistance, due to the fact that *C. jejuni* is naturally competent (Wilson *et al*., 2003). The pathogenesis of *C. jejuni* is comprised of four main stages: adhesion to intestinal cells, colonization of the digestive tract, invasion of targeted cells, and toxin production (Konkel *et al*., 2001). *C. jejuni* is a unique pathogen that can execute glycosylation of several proteins related to colonization, adherence and invasion such as flagellum, CPS, LOS and *Campylobacte*r-invasive agents (Cia) (Dasti *et al*., 2010). The presence of LOS can contribute to post-infection disorders because it may mimic human neuronal gangliosides leading to autoimmune disorders (Gilbert *et al*., 2002; Naito *et al*., 2010). The CPS and flagella motility of *C. jejuni* plays a crucial role in serum resistance, the adherence and invasion of epithelial cells, and colonization (Jones *et al*., 2004; Karlyshev *et al*., 2002). Cytolethal distending toxin (CDT) produced by *C. jejuni* that encode by *cdtABC* and has been ascribed a role in pathogenesis by contribution to secretion of interleukin (IL)-8 in INT407 intestinal cell line (AbuOun *et al.,* 2005). In conclusion many factors lead to the ultimate pathogenicity of *C. jejuni* and its transmission from food to human infection.

1.1.2 Impact on food safety and health

Campylobacters are prevalent bacterial food borne pathogens in human and animals that are ubiquitous in the environment. *Campylobacter* spp. are a cause of bacterial gastroenteritis and food borne disease worldwide, especially in developed countries. The illness is generally self-limiting and ranges from mild to severe disease leading to one or more symptoms which include inflammation, abdominal pain, fever and bloody diarrhoea (Konkel *et al*., 2001; Butzler, 2004). In severe cases, patients are treated with erythromycin (Konkel *et al*., 2001). Other diseases that have been associated with *Campylobacter* infection are the autoimmuneneuropathies Guillain-Barré syndrome (GBS) leading to acute neuromuscular paralysis and the ocular version Miller-Fisher syndrome (Stern, 2001). Campylobacter associated cases of these diseases are thought to involve molecular mimicry of human gangliosides by *C. jejuni* lipooligosaccharide, where sero-conversion results in the production of antibodies in patients recovering from *Campylobacter* infection that cross-react with nerve cell gangliosides GM1, GM1b and GD1a (Corcoran and Moran, 2007). Campylobacteriosis accounted for 198,252 human cases reported in 2009 in the EU (http://www.efsa.europa.eu/en/press/news/zoonoses110322.htm). The cases of *Campylobacter* infection reported include a 10% increase in England and Wales in 2010 compared to those recorded in 2009 (http://www.hpa.org.uk/hpr/archives/2010/news4810.htm#campy). In the United States, *C. jejuni* is reported as a major cause of bacterial diarrhoeal illness with more cases than a combination of *Shigella* spp. and *Salmonella* spp. together (Anonymous, FDA, 2008). The WHO also reported that

Campylobacter species are a major cause of gastroenteritis throughout the world, where the reported number of cases surpassed the incidence of *Salmonella* (Anonymous, WHO, 2008). More than 90% of human campylobacter infection is reported to be caused by *C. jejuni* and *C. coli* (Moore *et al.*, 2001; Dasti *et al*., 2010).

In England and Wales, cases of *Campylobacter* enteritis have been reported to be caused by transmission from food such as poultry, private water supplies or due to the consumption of raw or inadequately pasteurized milk (Pebody *et al.*, 1997; Esteban *et al.*, 2008). The sources of *Campylobacter* spp. that contaminate food are usually from animals such as chicken and other poultry, swine, cattle and other domestic animals (Newell and Fearnley, 2003; Guan and Holley, 2003). Similarly, campylobacter contamination of meat, poultry and milk has been observed in Pakistan, Belgium, Poland and other countries around the world (Hussain *et al.*, 2007; Ghafir *et al.*, 2007; Maćkiw *et al.*, 2008). Moreover, *Campylobacter* species have been isolated from untreated water sources such as lake water, drinking water supplies and water from dairy farms, resulting in waterborne campylobacteriosis (Korhonen and Martikainen, 1991; Jones and Roworth, 1996; Moore *et al.*, 2001; and Kemp *et al.*, 2005). The survival of *C. jejuni* in natural mineral water has also been observed (Tatchou-Nyamsi-König *et al.*, 2007). Consequently, campylobacter is of real public concern in terms of human health and food safety.

1.2 Bacterial Biofilm Formation

1.2.1 Development of biofilm formation

Biofilms are naturally composed of multicultural species or monocultures attached to a surface and embedded in an extracellular polymeric matrix that include polysaccharides, protein, nucleic acid and lipids (Flemming and Wingender, 2010). This is produced to protect the microorganisms from environmental stresses such as desiccation, oxygen toxicity, starvation, and antimicrobial agents (O'Toole *et al.*, 2000; Kalmokoff *et al.*, 2006; Joshua *et al.*, 2006). Biofilms create microenvironments for a consortium of bacteria including variations in pH, nutrient concentrations and oxygen levels (Else *et al.*, 2003). There are four stages of biofilm development that have been envisaged as follows: (i) Initial attachment to surface; (ii) Microbial development or growth to form microcolonies; (iii) Maturation of biofilms; and (iv) Detachment as shown on figure1.1 (O'Toole *et al.*, 2000).

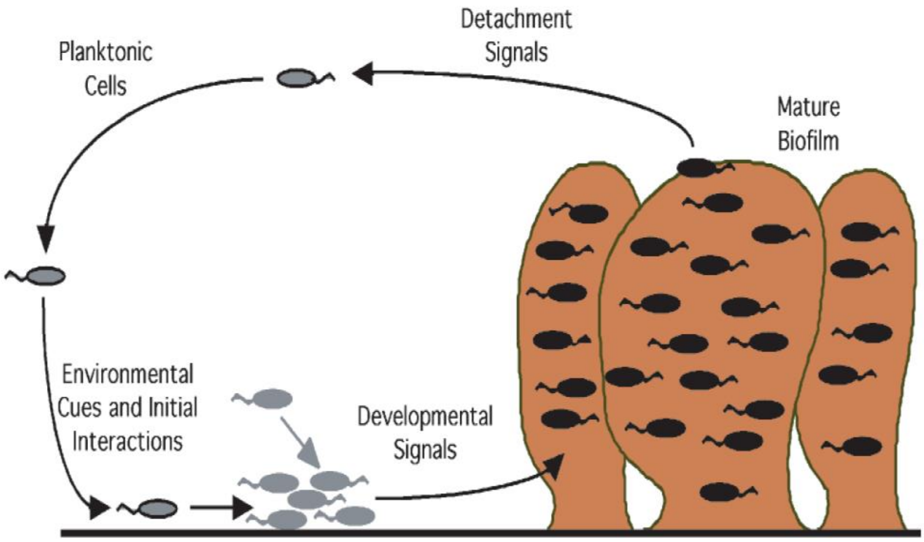


Figure 1.1-Model of biofilm development. Individual planktonic cells can form cell-to-surface and cell-to-cell contacts resulting in the formation of microcolonies. Cells in the biofilm can return to a planktonic lifestyle to complete the cycle of biofilm development.

Source: O'Toole *et al.* (2000)

In the initial stages of biofilm formation, the planktonic cells or free cells floating in liquid medium begin to attach to the surface. The Derjaguin and Landau, Verwey and Overbeek (DLVO) theory explains that the forces between charged surfaces interacting through a liquid medium are the combined effects of Van der Waals attraction and electrostatic repulsion forces, and has been used extensively to calculate the conditions for biofilm formation and microbe retention within biofilms (Russel *et al*., 1989; Hermansson, 1999). A charged surface will attract counter-ions from solution and repel co-ions to form an electrical double layer, and the interaction of these layers represented on the substrate and the approaching bacteria give rise to two energy minima.

Association of bacteria at the first minima are thought to be loosely attached or reversibly bound, whereas those that progress to overcome the repulsive forces to achieve the second minima are bound irreversibly. The magnitudes of these forces are dependent on the ionic strength of the solution. However, the attachment process of microorganisms to surfaces depends both on bacterial motility and the physiochemical properties of the bacterial cell (Kumar and Anand, 1998). Adhesion develops firstly between the bacterial cells and the substratum, which can take place in two stages: a reversible adhesion to the surface and subsequently an irreversible adhesion process (Kumar and Anand, 1998). The interaction forces involved in the reversible process between the bacterial cells and a surface include van der Waals interactions, electrostatic forces and hydrophobic interactions (Palmer *et al*., 2007). Meanwhile, the irreversible attachment of cells is a crucial stage in biofilm development. The synthesis of components for biofilm formation can proceed after the cells have attached to a surface. Bacterial cells may produce surface appendages such as flagella, fimbriae, pili and extracellular polysaccharide fibrils to support their contact with the surface (Jones and Isaaoson, 1983). The forces that are related with this mechanism are dipole-dipole interactions, hydrogen bonding, ionic, covalent bonding and hydrophobic interactions (Kumar and Anand, 1998).

The control of bacterial motility is a key to the formation of biofilms. The control of motility and therefore the commitment to biofilm formation has been linked to intracellular cyclic-di GMP levels in bacteria (Kuchma *et al*., 2007). Intracellular cyclic-di GMP levels are increased through expression of diguanylate cyclases (DGCs) or conversely degraded by c-di-GMP phosphodiesterases (PDEs). DGC activity is provided by GGDEF domains and PDE activity is provided by either EAL or HD-GYP domains, where the domain names relate to the amino acid motifs they contain and contribute to their respective enzymatic activities. Environmental and cellular signals control the expression and, through amino-terminal sensory domains, activities of DGCs and PDEs (Hengge, 2009). For example, *Pseudomonas aeruginosa*, BifA protein has PDE activity which plays a role in polysaccharide production required for biofilm formation (Kuchma *et al*., 2007). Alternatively Bassis and Visick (2010) reported that in *Vibrio fischeri* had a gene, *binA* that encodes c-diGMP phosphodiesterase. The deletion of *binA* results in an increase in biofilm formation.

Cell to cell communication and/or signalling may play an important role in biofilm formation and development, in particular regulation of the attachment and detachment steps. Quorum sensing is a mechanism of cell-cell signalling that has been reported to be involved in biofilm development. Quorum sensing is based on the process of autoinduction for self-organisation and regulation of microbial cells (Parsek and Greenberg, 2005). This mechanism is mediated by small molecules, hormone-like diffusible molecules called autoinducers (AIs) from Gram negative bacteria, which have been characterised as homoserine-lactones (AHL) in some species (Lazar, 2011). However in Gram positive bacteria peptidic molecules (AIP) have been identified as the signalling compounds responsible for intra-specific communication (Lazar, 2011). Boronated diester molecules (AI-2) are involved in inter-specific communication among both Gram-positive and Gram-negative bacteria (Simoes *et al*., 2010).

1.2.2 The impact of biofilms on the food chain

The ability of microorganisms to attach and grow on food and food-contacted surfaces under favourable conditions is a significant factor in food safety (Kumar and Anand, 1998). Moreover, the attachment and growth of microbial cells to natural surfaces not only plays an important role in the survival of pathogenic bacteria within biofilms but it also increases the resistance of the cells within the biofilm to antimicrobial agents and sanitizers (Costerton *et al.*, 1999). Possible mechanisms of biofilm resistance to antimicrobial agents include (i) the failure of the antimicrobial agents to penetrate the biofilms in which the extracellular matrix acts as a barrier, (ii) slow growth rate and stress response: bacterial cells grow slowly under starvation conditions encountered within mature biofilms leading to physiological changes that can lead to the emergence of spatially separated sub-populations that can survive in the stationary state under nutrition limitation, and can survive the application of antimicrobial agents due to reduced susceptibility of minimal or non-growing cells in, (iii) environmental heterogeneity within biofilm: bacterial cells within biofilms may grow at different rates as a result of different rates of protein synthesis and/or respiratory activity, which in turn will lead to varied responses to antimicrobial agents that effect these metabolic processes, (iv) the induction of an insensitive biofilm phenotype: sub-populations of bacteria can be selected that are altered in their expression of multidrug efflux pumps, membrane protein composition or antimicrobial resistance genes that can independently or collectively act to block the antimicrobial agent passage into the bacterial cells (Mah and O'Toole, 2001).

Arnold and Silvers (2000) reported the attachment and biofilm formation by mixed populations of bacteria from rinsed broiler carcasses on several types of surface during poultry processing. Reeser *et al*. (2007) reported the formation of biofilms by *Campylobacter jejuni* on a variety of abiotic surfaces commonly used in the provision of water for poultry flocks, including acrylonitrile butadiene styrene and polyvinyl chloride plastics. These biofilms may well harbour *Campylobacter* in the poultry processing environment and constitute a reservoir of contamination. However, Dykes *et al.* (2003) compared the survival and cellular protein expression patterns of planktonic and biofilm *Campylobacter jejuni* cultures formed on glass beads using a growth medium of heart infusion broth with 1% yeast extract. The biofilm associated bacteria were reported to decline in viable count upon storage in phosphate buffered saline faster that the planktonic cells over a 14 day period. Resolution of the cellular protein by 2D gel electrophoresis showed differences between planktonic and biofilm cells with seven unique and 12 up-regulated protein spots based on normalised pixel densities. The reduced survival ability of the biofilm bacteria coupled with the tentative identification of stress related proteins amongst those upregulated upon the 2D-gels, led Dykes *et al*. to conclude that biofilm associated campylobacters may be more sensitive inimical stresses encountered in food production.

1.3 *Campylobacter* biofilms

1.3.1 Biofilm formation by *Campylobacter*

Campylobacter strains that were able to form biofilms, or associate with biofilms formed by other organisms, have been reported by several authors. Buswell *et al.* (1998) reported that the survival and persistence of *Campylobacter* spp. was extended when attached on water conduits and in aquatic biofilms. Tang (2000) demonstrated that *C. jejuni* containing biofilms were formed when grown on surfaces pre-colonized with *Pseudomonas putida*. According to Trachoo *et al.* (2002) *C. jejuni* was attached on biofilms formed by Gram-positive chicken house isolates including named P1, Y1, W1 and a *Pseudomonas* sp. on polyvinyl chloride (PVC) coupons. *Campylobacter* cells were detected by immunofluorescence in biofilms present in the water lines of a commercial broiler house (Zimmer *et al*., 2003). Sanders *et al.* (2007) also reported that *C. jejuni* attached to other Gram-positive bacterial biofilms that were collected from the whole carcass rinses (WCR) that also included bacteria of the genera *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Bacillus,* *Enterococcus*, and *Micrococcus* on stainless steel. Furthermore, Sanders *et al.* (2008) reported that *C. jejuni* 1221 green fluorescent protein (gfp) was able to attach on the gram-positive bacterial biofilms collected from WCR and subsequently establishment biofilms on stainless steel under nutrition limitation and at various temperatures (13, 20, 37 and 42 ºC). Hanning *et al*. (2008) reported that *C. jejuni* was able to attach on the mixed bacterial poultry biofilms of *Pseudomonas* sp., *Staphylococcus* sp., *E. coli*, *Bacillus* sp. and

Flavobacterium sp. as a secondary colonizer. Later Teh *et al*. (2010) reported that *C. jejuni* strains found in New Zealand could form biofilms but were associated with mixed-population biofilms of *Enterococcus faecalis* and *Staphylococcus simulans*.

The ability of *Campylobacter* spp. to form monospecies biofilms has been demonstrated. Dykes *et al.* (1993) reported that a *C. jejuni* strain was able to attach to glass beads during incubation at 37 °C for 48 h under microaerobic conditions to form biofilms. Joshua *et al.* (2006) showed that axenic *C. jejuni* could attach and form biofilms on glass surfaces and recognised three types of forming structures that included: the attached structures on the glass surface, unattached aggregates (flocs) and a pellicle structure at the liquid-gas interface. Fratamico *et al.* (2007) reported that 14 *Campylobacter* strains were able to form biofilms on glass, stainless steel and polystyrene plastic, for example, *C. jejuni* strain 81-176 produced a visible biofilm on multiple surfaces. This strain was also reported to produce a heterogeneous biofilm containing similar quantities of spiral and coccoid forms (Gunther IV and Chen, 2009). Duffy and Dykes (2009) reported that the viability of *C. jejuni* cultures on a stainless steel surface were reduced to zero during storage in distilled water at 4 ºC over 20 days. However, the authors noted that 3.5 Log_{10} cells/cm^2 remained attached throughout the period based on observations using confocal scanning laser microscopy. The application of discriminative live/dead strains suggested the biofilm were viable but that these cells were not culturable (VBNC). Similarly Ica *et al*. (2012) investigated the survival of *C. jejuni* in mono- and mixed-culture active biofilms using confocal scanning laser microscopy in conjunction with live/dead stains. These authors found that cells from monoculture biofilms were alive according to live/dead staining but that these cells were not culturable. In contrast, in mixed-culture biofilms, *C. jejuni* remained culturable. The variation in the abilities of laboratories to culture stressed campylobacters notwithstanding these findings highlight that biofilms are a mechanism for persistence of *C. jejuni* within harsh environments.

1.3.2 The molecular mechanisms of *Campylobacter* biofilm formation

Genetic and molecular approaches have been used to study *Campylobacter* biofilms since the complete genome sequence of *C. jejuni* NCTC 11168 was documented by Parkhill *et al*. (2000). There are many reports investigating the genes involved in biofilm formation. The genes that control motility may be responsible for changing the planktonic mode of growth to the sessile mode of growth. Naoaki (2002) reported that an aflagellate (*fla*-) mutant constructed from *C. jejuni* strain 81-176 could not form biofilms. Komokoff *et al.* (2006) reported that the flagellar motility complex including flagellins (FlaA, FlaB), the filament cap (FliD), the basal body (FlgG, FlgG2) and chemotactic protein (CheA) play a crucial role in attachment of *C. jejuni* 11168 to solid surfaces during biofilm formation. Moreover, the aflagellate mutant *maf5* in *C. jejuni* did not form a pellicle or attach to a glass surface (Joshua *et al.*, 2006). According to Reeser *et al.* (2007) *C. jejuni flaAB* mutants were significantly decreased in their capability to form biofilms compared with the wild type strain. Thus, the motility may have a critical role in an initial attachment of *C. jejuni* to a surface.

The outer membrane of *Campylobacter* contains lipo-oligosaccharide (LOS), capsular polysaccharide (CPS) and N-linked polysaccharides participating in stress survival, transmission and virulence in *C. jejuni* (McLennan *et al.*, 2008). It is notable that cell surface structures may play a role in biofilm formation. Extracellular polysaccharides are component of biofilms that formed to protect microbial cells from environmental stress (Sutherland, 2001). In *C. jejuni*, capsular polysaccharides were encoded by the *kps* operon (Karlyshev *et al*., 2005). However, *kpsM* mutants deficient in capsular polysaccharide production, were able to attach to the surfaces (Naoaki, 2002). Similarly, Joshua *et al.* (2006) also reported that mutation of *kpsM* in *C. jejuni* did not affect biofilm formation. However, Naoaki (2002) also reported that the *kpsM* mutant had a shorter survival period than wild type. Thus, capsular polysaccharide may play a necessary role in protecting from environmental stresses. Strains carrying mutations of *pglH* and *neuB1* that encode a glycosyltransferase for *N*-linked protein glycosylation biosynthesis and a sialic acid synthase for lipo-oligosaccharide biosynthesis respectively were able to form unattached aggregates or flocs (Joshua *et al*., 2006). In *C. jejuni*, O-linked flagellin glycosylation was encoded by five genes including Cj1321 to Cj1326, of which Cj1325 and Cj1326 are a single gene (Howard *et al*., 2009). A *C. jejuni* 11168H strain carrying a deletion in Cj1324, resulting in the absence of two legionaminic glycan modifications that alter the surface charge of flagella, was less capable of biofilm formation than the wild type (Howard *et al*., 2009). Based on a hyper-reactive phenotype to the UV fluorescent dye calcofluor white (CFW) that binds polysaccharides. Naito *et al*. (2010) identified two mutations in *C. jejuni* 81-176 that increased biofilm formation. Surprising to the authors the mutations were located in *waaF* and *lgtF* that encode *S*-heptosyltransferase and a two-domain glycosyltransferase respectively, which would result in the complete loss of the LOS outer core region. However, the same group had previously reported that a *kps*M knock-out mutant defective in capsular polysaccharide export was also CFW hyper-reactive and hyper-biofilm forming (McLennan *et al.*, 2008). It is evident that the cell envelope of C. *jejuni* is highly dynamic, in which multiple compensatory changes can enhance or cause steric or charge clashes of carbohydrate moieties to hinder biofilm formation and other phases of the bacterial life cycle. Asakura *et al*. (2007) reported that deletion of the *peb4* gene encoding major antigenic peptide in *C. jejuni* NCTC 11168 lead to an alteration in the membrane protein content that caused a reduction in biofilm formation compared to wild type. Previously, *C. jejuni* within biofilms had been shown to express greater quantities of the *peb4* product compared to planktonic cells, which was also implicated in the binding of mammalian cells (Kalmokoff *et al*., 2006). Nguyen *et al*. (2011) additionally reported that the abilities of *C. jejuni* and *C. coli* to form biofilms on abiotic surfaces were depended on the surface hydrophobicity and surface charge of campylobacters.

Quorum sensing or cell-to-cell signalling is a cell-population density-dependent signalling system by which bacteria can regulate gene expression (Miller and Bassler, 2001). The quorum sensing molecule responsible for cell-to-cell signalling was first identified in *Vibrio harveyi and V. fischeri* (Nealson *et al*., 1970). There are two types of density-dependent signal systems that are comprised of a sensor and a quorum sensing molecule (Cloak *et al*., 2002). Signalling system 1 is composed of sensor 1 and autoinducer-1 (AI-1) and is strongly species-specific, involving homoserine lactones (Cao and Meighen, 1989). Signal system 2 is composed of sensor 2 and autoinducer-2 (AI-2) that is poorly species specific. Cloak *et al*. (2002) revealed that *C. jejuni* and *C. coli* could produce autoinducer-2 (AI-2). The *luxS* gene is required for AI-2 production in signalling system 2 (Cloak *et al*., 2002). Moreover, quorum sensing has been reported to involve attachment, biofilm development and detachment from biofilms (O'Toole *et al*., 2000; Elvers and Park, 2002; Chang, 2006). Jeon *et al*. (2003) also suggested that the motility and surface properties of *C. jejuni* was regulated by quorum sensing. According to Reeser *et al*. (2007), a *C. jejuni luxS* mutant, deficient in the production of AI-2 resulted in a reduction in the formation of biofilm when compared with wild type.

McLennan *et al.* (2008) reported that a Δ*spoT* mutant defective in the stringent response (SR) showed an increase in exopolysaccharide production with extended growth at 42 °C under anaerobic conditions. The reagent calcofluor white was used to demonstrate β1-3 and/or β1-4 linkages of polysaccharides, as components of the surface polysaccharide of *C. jejuni*. Based on the reaction with calcofluor white they suggested that the *C. jejuni* Δ*spoT* mutant was the first SR mutant identified as up-regulating biofilms. In addition, the *CsrA* (Carbon starvation regulator) gene was responsible for regulation of stationary phase metabolism and virulence determinants including host cell invasion, quorum sensing, biofilm formation, iron acquisition, type III secretion systems and outer membrane protein expression (Fields and Thompson, 2008). A *Campylobacter jejuni CsrA* mutant was able to form thin biofilms when compared with the wild type strain (Fields and Thompson, 2008). However, the reduction of biofilm formation by the *C. jejuni CsrA* mutant is consistent with the observation that there is a reduction in motility. The mutant of Δ*cprS* (Campylobacter planktonic growth regulation) that encodes the CprS kinase sensor dramatically increased biofilm formation of *C. jejuni* (Svensson *et al*., 2009). These findings suggested that several different genes are involved in the regulation of biofilm formation by *C. jejuni*, importantly those involved in the surface structure of membrane, flagella and possibly those related to quorum sensing.

1.3.3 The effect of environmental factors on biofilm formation by *Campylobacter*

Attachment and biofilm formation is one of the mechanisms by which microbial cells enhance survival and cope with environmental stresses. Factors affecting the attachment and biofilm formation of microbial cells to a surface have been studied. Firstly, microorganisms form biofilms when nutrients become limited (Poulsen, 1999). Reeser *et al.* (2007) demonstrated that biofilm formation by *C. jejuni* was inhibited by growth in nutrient rich media. Sanders *et al.* (2008) also demonstrated that nutrient limitation affected *C. jejuni* attached to stainless steel and in biofilms. These data suggest that the starvation may trigger the formation of *Campylobacter* biofilms. However, Hunt *et al.* (2004) suggested that the onset of starvation could induce detachment from biofilms due to the accumulation of metabolic products and a lack of nutrients for bacterial growth. In addition, high osmolarity in the overlaying media containing high concentrations of sugar and salt has been shown to affect biofilm formation by *C. jejuni*, resulting in reduction of biofilms (Reeser *et al.*, 2007). Temperature has been shown not only to affect the growth of microorganisms, but also to effect the development of attachment and biofilm formation on surfaces. Dykes *et al*. (2003) demonstrated that the numbers of culturable cells of *C. jejuni* grown as biofilms during storage in phosphate buffer saline (PBS), at 4 and 10 °C, was constant throughout 3 days; whereas, the viability of cells in biofilms incubated at 25 and 37 °C was dramatically decreased. Sanders *et al*. (2008) studied the attachment of *C. jejuni* 1221 marked with gfp to biofilms formed on the stainless steel by bacteria recovered from whole carcass rinses of processed broiler chickens. These authors found no significant difference in the attachment of *C. jejuni* at four temperatures examined (13, 20, 37 and 42 ºC); however, viable *C. jejuni* 1221 gfp were only recoverable at 13 and 20 ºC. Moen *et al*. (2005) reported that the amount of polysaccharide and oligosaccharide increased under non-growth survival conditions, in particular at 25 °C under anaerobic conditions resulting in the expression of a few genes that were stress related components of cell membranes. Reeser *et al*. (2007) suggested that temperature had a significant effect on biofilm formation when comparing incubation at 25 and 37 °C. It was shown that *C. jejuni* M129 was able to form a biofilm at 37 °C to a much greater extent than at 25 °C. Oxidative stress resistance of *Campylobacter* strains may involve the production of biofilms. Reeser *et al*. (2007) suggested that *C. jejuni* exposure to atmospheric oxygen led to a reduction in biofilm when compared with the presence of carbon dioxide (10%). However, Asakura *et al*. (2007) reported that *C. jejuni* NCTC 11168 was better able to form biofilms under aerobic conditions than microaerobic conditions over 24 and 48 h but not at 72 h using quantitative crystal violet staining. Reuter *et al*. (2010) suggested that biofilm formation of *C. jejuni* increased rapidly under aerobic conditions compared to microaerobic conditions and could assist in the survival of *C. jejuni* when exposed to atmospheric levels of oxygen, although the quantity of biofilm formed based on crystal violet staining was not significantly different after longer incubation times. Interestingly, Ica *et al.* (2012) reported that the quantity of oxygen in the mono-culture *C. jejuni* biofilms was not measureable, and Sulaeman *et al.* (2012) also reported the over-expression of proteins involved in biofilm initiation under oxygen-enriched conditions. In conclusion reports in the literature suggest that biofilm formation by *C. jejuni* is dependent on environmental factors including temperature, oxidative stress, nutritional limitation and the physiological phase of growth.

1.4 Bacteriophages

1.4.1 Characteristics of bacteriophage

Bacteriophages were independently discovered by Frederick W. Twort and Felix d'Hérelle in 1915 and 1917 (Stent, 1963). Bacterial viruses can kill or infect specific bacteria and are parasites to bacteria. Bacteriophages are ubiquitous and can be found in a variety of environments such as sea water, soil, food, sewage and waste water. The number of bacteriophage on earth is estimated to be in the range of 10^{30} to 10^{32}. Additionally, bacteriophages play a substantial role in regulation of the microbial balance in the ecosystems where the roles of bacteriophages have been explored (Kutter and Sulakvelidze, 2005). Bacteriophages are constantly diffusing, decaying, or exploring bacteria to adsorb and infect that has profound impact on bacterial communities, and on ecosystem nutrient flow (Abedon, 2008). Individual phage particles contain a nucleic acid genome (DNA or RNA) enclosed in a protein or lipoprotein coat or capsid (Guttman *et al*., 2005). They have no machinery for producing energy and no ribosomes for making proteins. High levels of specificity to a particular bacterial host is due to the fact that bacteriophage can attach to specific receptors on the surface of the target bacteria including lipopolysaccharides, teichoic acids and flagella (Guttman *et al.*, 2005). The majority of the identified phages are tailed phage, of which 60% are in the family *Siphoviridae,* 25% in *Myoviridae* and 15% in *Podoviridae* (Guttman *et al.,* 2005). Tailless phages are often differentiated by shape compared to the tailed phages, where morphologies such as rods, spherical, lemon shapes or pleiomorphic shapes have been observed (Guttman *et al.*, 2005). Besides morphology, bacteriophages have been classified by having either double or single stranded DNA or RNA (Figure 1.2).

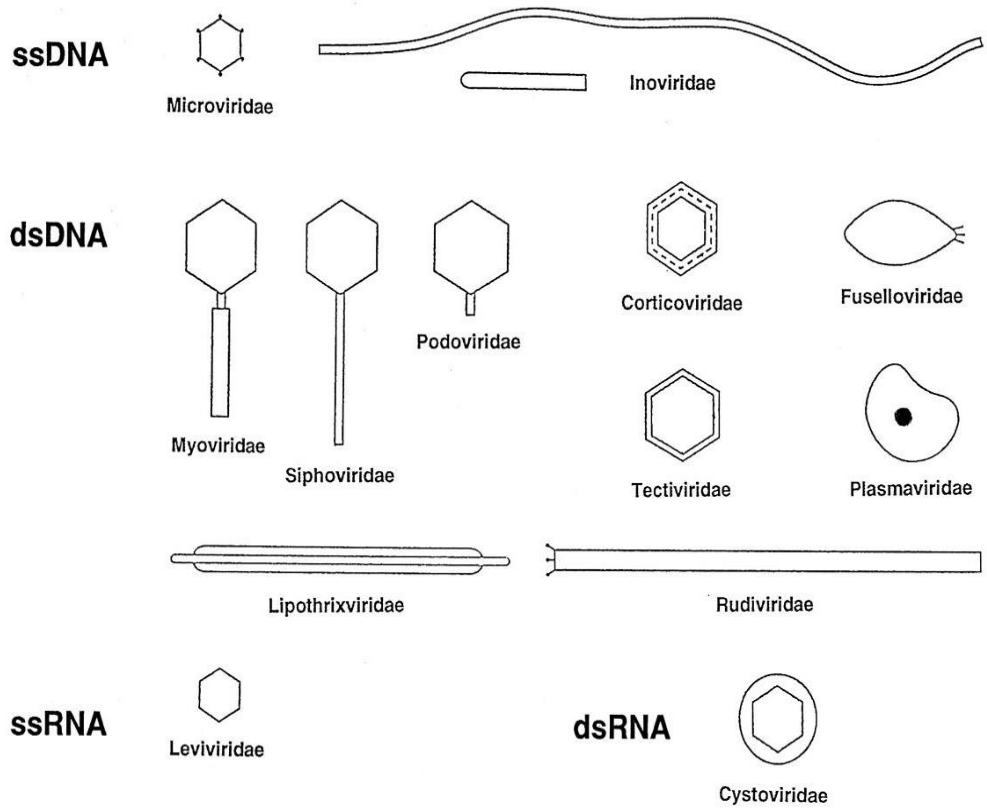


Figure 1.2-Basic morphologies of different families of virions of prokaryotes. Source: Ackermann (2006)

1.4.2 Bacteriophage life cycles

According to Abedon (2006) the general phage life cycle basically involves adsorption, infection and phage release, followed by phage decay (Figure 1.3). Bacteriophages are divided into two classes based on lifecycle: virulent phage that have a lytic cycle, and temperate phage that potentially have a lysogenic phase in their life cycle. Virulent bacteriophage infect and insert their genome in to a bacteria host cell, where upon it takes over the bacterial energy and synthesis mechanisms to produce progeny, leading to host lysis and progeny phage release, for instance, the bacteriophage T4 life cycle (Figure 1.4).

Whereas, when temperate phage infect bacteria the phage genome can become integrated into the bacterial host genome and remain dormant until stimulated by environmental factors to enter the lytic cycle and produce progeny (Guttman *et al.*, 2005), for example, the lysogenic life cycle of λ phage (Figure 1.5). Additionally, pseudolysogeny or the carrier state has been reported as an alternative life cycle of both virulent and temperate phages (Miller and Ripp, 2002).

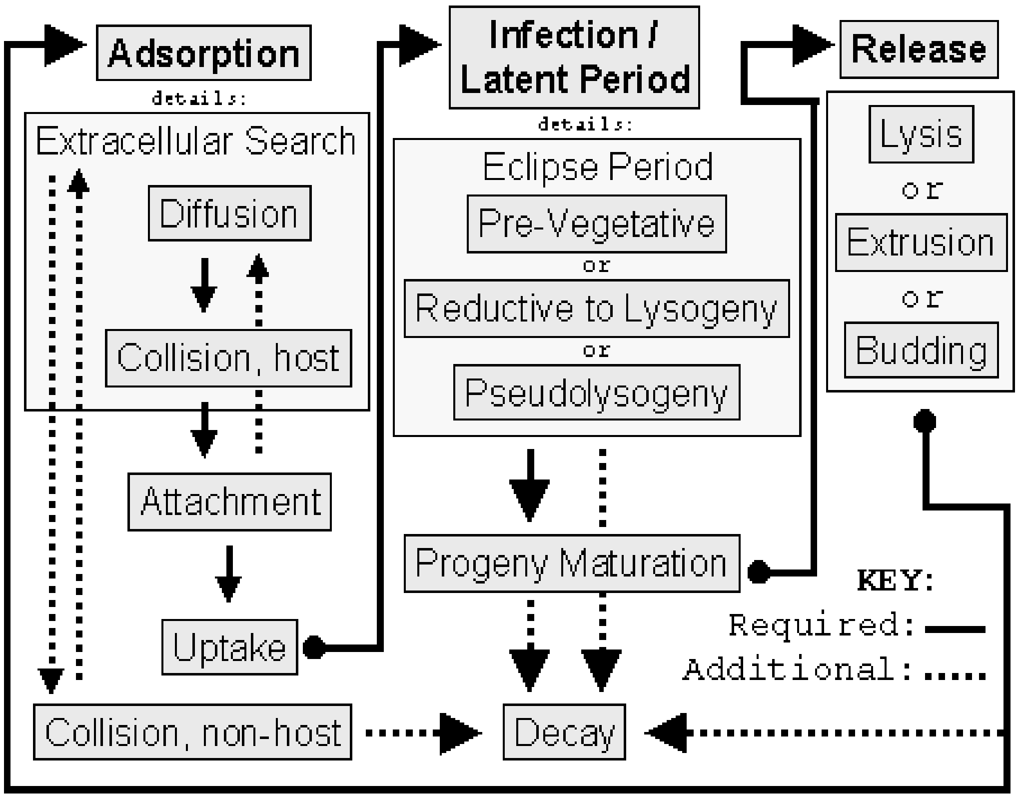


Figure 1.3- General phage life cycle

Source: Abedon (2006).

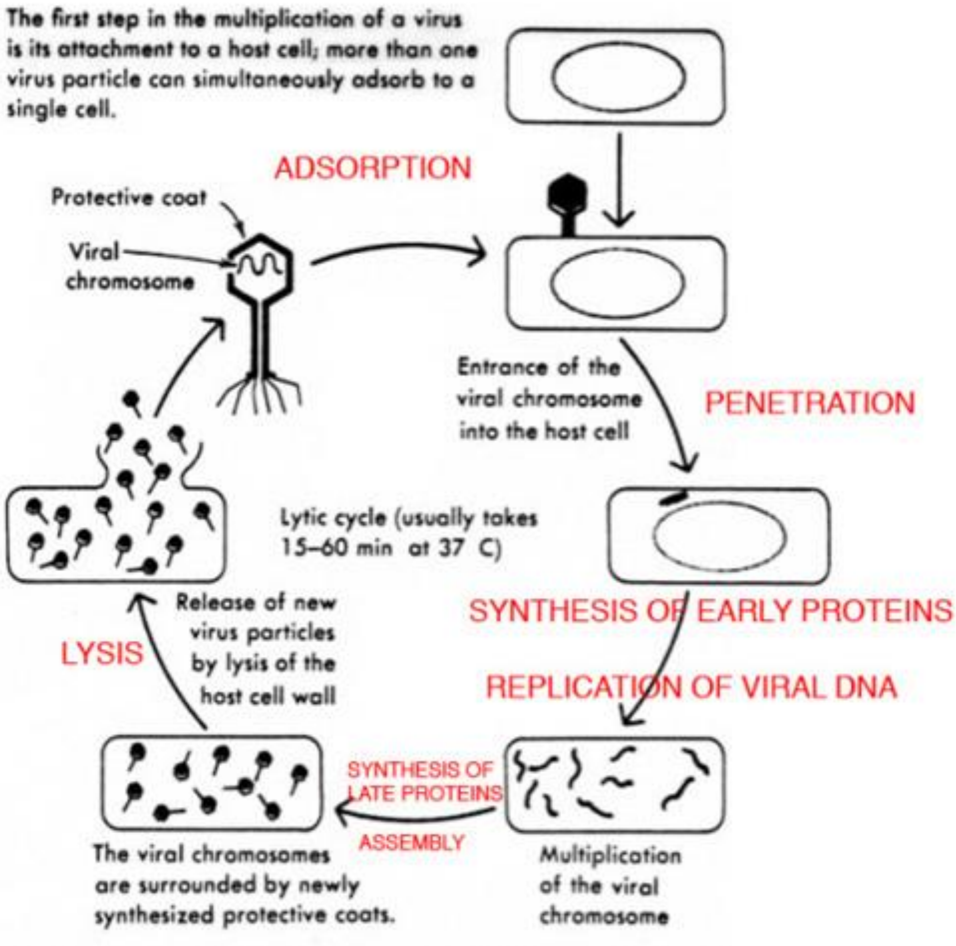


Figure 1.4-Bacteriophage T4 life cycle.

Source: http://bacteriophageucd.files.wordpress.com/2011/04/t4-lifecycle.jpg

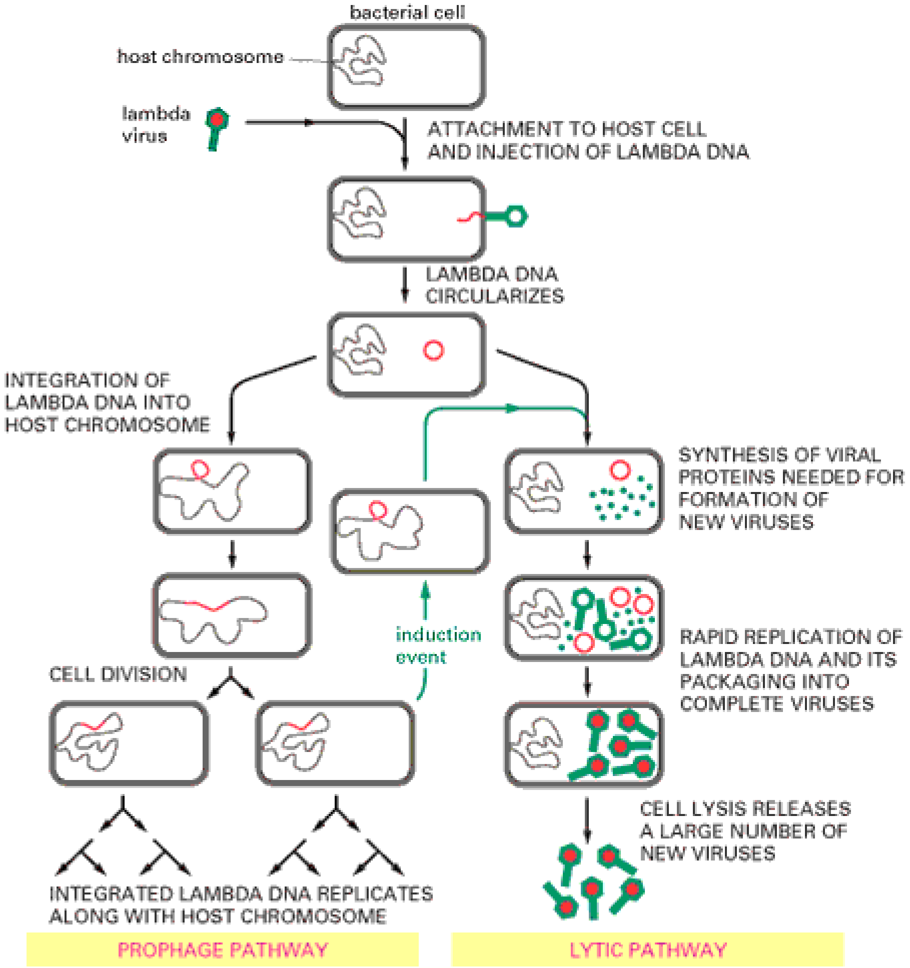


Figure 1.5-Lysogenic life cycle of λ phage.

Source: http://bacteriophageucd.files.wordpress.com/2011/04/lambda-life-jpg.gif

1.5 Pseudolysogeny

The definition of pseudolysogeny is an unstable state where the viral DNA is maintained or carried in a bacterial culture, in contrast to true lysogeny where the bacteriophage genome is integrated in to or replicates with the bacterial genome (Grange, 1975; Ripp and Miller, 1997, 1998). Pseudolysogenic bacteria can form plaques spontaneously when cultured on suitable media (Grange, 1975). Pseudolysogens have been documented for more than 30 years but research in to the phenomenon has been limited. The formation of pseudolysogens by *Mycobacterium* had been reported in 1970s, where the characteristics of pseudolysogenic *Mycobacterium diernhoferi* ATCC 19341 were observed, and that cured variants showed no cultural, biochemical or serological variation compared to the original culture (Grange, 1975). Pseudolysogenic *M. chelonei* with phage Φ630 was reported by Jones and Greenberg (1977), who showed differences in host modification and restriction with a mycobacteriophage isolated from this bacteria. The resultant progeny phage propagating on an alternative host exhibited a marked reduction in efficiency of plating compared to the original host. Furthermore, the establishment of a pseudolysogenic state in *Azotobacter vinelandii* strain O, was identified as resulting in the phenotypic conversion of the host, including loss of polysaccharide coat, flagella, motility and the acquisition of a yellow pigmented appearance (Thompson *et al*., 1980a). Subculture of these strains led to the loss of phage A21 production but retention of their phenotypic differences (Thompson *et al*., 1980b). Similarly, Ripp and Miller (1997) reported that a phage genome had stabilised in *Pseudomonas aeroginosa* but was no longer able to replicate along with host genome replication and cell division. The same investigators showed that pseudolysogeny played an important role in the long-term survival of bacteriophage in a natural freshwater environment under nutrient limitation. Additionally, establishment of pseudolysogeny in *P. aeroginosa* infected with either phage F116 or UT1 was increased when the bacterial cells were starved leading to decreased phage numbers and followed by an increase in the number of bacteria containing phage DNA (Ripp and Miller, 1998). Burst sizes of phages decreased when bacteria were starved resulting in higher occurrence of pseudolysogens, which was probably due to the fact that the starved cells did not supply sufficient energy for phage to initiate either a lysogenic or lytic life cycle (Miller and Ripp, 2002). A pseudolysogenic-like interaction between Φ HSIC virus and *Listonella pelagia,* showing a high rate of spontaneous induction under stimulated growth and low titres under unsuitable growth conditions was reported by Williamson *et al*. (2001) and Williamson and Paul (2006). Los *et al*. (2003) reported formation of T4 pseudolysogens of *Escherichia coli* in slowly growing cells in carbon-limited chemostats. Phage (ΦAsp2) of an *Actinoplanes* strain SN223 was able to form a putative pseudolysogenic state based as the presence of phage DNA and the observation of phage particles present in bacterial cells by electron microscopy (Jarling *et al*., 2004). Pseudolysogeny was also reported in sporulating *Bacillus subtilis* where the phage genome survived storage in endospores (Sonenshein, 2005). *Vibrio harveyi* infected with Siphoviriae-like phage (VHS1) exhibited pseudolysogens where the VHS1 DNA was carried on non-phage producing progeny but no longer in daughter cells (Khemayan *et al*., 2006). A characteristic of pseudolysogens is that they are able to produce viable phage particles along with a population of bacterial cells but do not produce phage as a result of induction with a mutagenic agent such as mitomycin C and that they lack integration of their nucleic acid (Williamson *et al*., 2001). In conclusion the literature suggests that many bacteria are able to form pseudolysogens particularly where conditions for bacterial growth are limited, such as nutrition limitation or on sporulation. Pseudolysogeny is often associated with conversion phenotypes that distinguish them from the original bacteria. These phenotypes can be beneficial for the survival of the host bacteria under stress conditions. Bacteriophage associated with the surviving bacteria remain with their host offering shelter but retain the ability to undergo lysis at a later time. Pseudolysogens therefore play an important role in the maintenance and evolution of bacteriophages in natural environments.

1.6 *Campylobacter* Bacteriophages

1.6.1 Characteristics of *Campylobacter* bacteriophages

The majority of phages that have been isolated with specificity to *Campylobacter* strains are virulent few being reported as temperate (Sails *et al*., 1998). *Campylobacter* bacteriophages are typically found in poultry and pig products, and sewage effluent (Frost *et al*., 1999; Atterbury *et al*., 2003b; El-Shibiny *et al*., 2005; Hansen *et al.,* 2007; Tsuei *et al*., 2007). In the 1970s, temperate bacteriophage isolates were reported from *Vibrio fetus*, a species that is currently known as *Campylobacter fetus* var. *intestinailis* (Firehammer and Boder, 1968; Bryner *et al*., 1973; Hallett *et al*., 1977). *Campylobacter* phages that have been extensively characterised belong to either the *Myoviridae* or

Siphoviridae families (Atterbury *et al*., 2003b; Loc Carillo *et al.*, 2005; Timms *et al*., 2010). The *Myoviridae* family is classified into three categories of bacteriophages specific to *Campylobacter* based on genomic size: Class I-a genome size of approximately 320 kb, head 140 nm; Class II-a genome size of approximately 194 kb, head 99 nm and refractory to digest with *Hha*I; Class III-a genome size of approximately 140 kb, head 100 nm and digestible by *Hha*I (Table 1.1) (Sails *et al*., 1998; El-Shibiny *et al*., 2009; Timms *et al*., 2010). Electron microscopy showed these phages have icosahedral heads and long contractile tails with average head sizes in the range of 92-100 nm and overall lengths of 190-200 nm (Sails *et al*., 1998; Loc Carrillo *et al*., 2005; 2007; Figure 1.6). The group III phage of *Campylobacter* (virulent phages) were typically isolated from chicken, (El-Shibiny *et al.*, 2005; Loc Carrillo *et al*., 2005, 2007).

Bacteriophage can alter or provoke the succession of *Campylobacter* types colonising broiler chickens during production where environmentally exposed birds can be exposed to multiple *Campylobacter* types over the rearing cycle (El-Shibiny *et al*., 2005; El-Shibiny *et al*., 2007). The selection of the dominant type may be influenced by the presence of bacteriophages as observed in longitudinal studies of broiler houses demonstrating strain and bacteriophage carry over from one flock to another (Connerton *et al*., 2004). This was further demonstrated experimentally by Scott *et al*. (2007b) in a broiler chicken colonisation model, who showed that in the absence of bacteriophage, a bacteriophage-sensitive strain out competed a bacteriophage insensitive strain to become the dominant strain. However, when bacteriophage were administered to birds co-infected with both strains, the situation was reversed, with the insensitive strain becoming dominant and the sensitive strain being reduced to a minority population. Individually the strains were equally able to colonise birds but it was clear that the strain insensitive to the bacteriophages was associated with a competitive fitness disadvantage in the absence of bacteriophages but not in their presence. These findings have implications regarding the types of strains isolated from different sources as the presence of bacteriophages may bias the isolation rates of different strains colonising the same intestinal environment. Overall the role of bacteriophage on *Campylobacter* population dynamics depends on the proportion of insensitive and susceptible bacteria and the fitness of these bacteria in the environmental niche in which they are challenged (Cairns *et al*., 2009).

Table 1.1-*Campylobacter* bacteriophage (*Myoviridae* family) classification and sources

Groups	Sources	Phages	References
I	NCTC	Φ4 (NCTC 12676)	Sails *et al*. (1998)
		Φ12 (NCTC 12677)	Coward *et al*. (2006)
II	Chicken	CP220	El-Shibiny *et al*. (2009)
		CPt10	Timms *et al*. (2010)
	NCTC	Φ10 (NCTC 12683)	Sails *et al*. (1998)
		Φ14 (NCTC 12675)	Coward *et al*. (2006)
		Φ8 (NCTC 12681)	
		Φ3 (NCTC 12682)	
		Φ15 (NCTC 12684)	
III	Chicken	CP1-8, CP34	Loc Carrillo *et al*. (2005,
		CP30, CP81	2007)
	NCTC	Φ1 (NCTC 12673)	Scott *et al*. (2007a); Hammerl
		Φ9 (NCTC 12669)	*et al*. (2011)
		Φ16 (NCTC 12670)	Sails *et al*. (1998)
		Φ7 (NCTC 12671)	Coward *et al*. (2006)
		Φ13 (NCTC 12672)	
		Φ2 (NCTC 12674)	
		Φ5 (NCTC 12678)	
		Φ11 (NCTC 12679)	
		Φ6 (NCTC 12680)	

Φ1-Φ16 are used in phage typing for discrimination of *C. jejuni* and *C. coli*

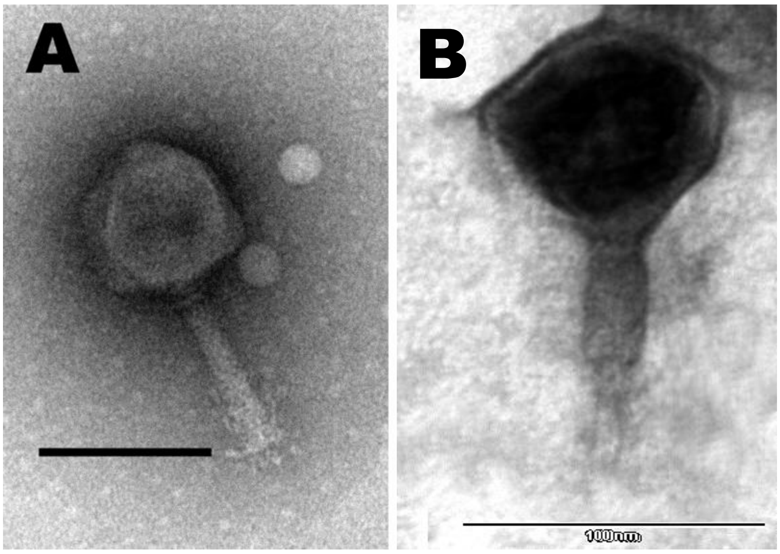


Figure 1.6-Electron micrographs of bacteriophages CP8 (A), Bars, 0.1 µm. (Loc Carrillo *et al*., 2005) and CP30 (B).

1.6.2 Influence of bacteriophage on genomic diversity of *Campylobacter*

Campylobacter prophage have been reported to have an effect on the genomic diversity of *Campylobacter*. Fouts *et al*. (2005) reported that there were three insertion sequences of phage DNA into the genome of *C. jejuni* RM1221 including *Campylobacter jejuni* insertion element 1 (CJIE1) containing a Mu-like phage, CJIE2 encoding phage-related endonuclease, methylases or repressors and CJIE4-phage structural proteins. Parker *et al*. (2006) showed through the use of a PCR screen and DNA microarray hybridisation that several *C. jejuni* isolates carry inserted sequences with one or two being similar to CJIE of *C. jejuni* RM1221. Bacteriophages affect Pulsed Field Gel Electrophoresis (PFGE) patterns of *C. jejuni* because of insertions or deletions of bacteriophage genes (Barton *et al*., 2007). Genomic rearrangements were identified in phage-treated isolates from chickens, resulting from recombination by Mu-like prophage DNA sequences in the *Campylobacter* genomic DNA (Scott *et al*., 2007b). Furthermore, the genomic DNA of *C. jejuni* HPC5 was found to carry on insertion of Mu-like phage DNA (CJIE1) similar to these seen in *C. jejuni* RM1221 (John *et al*., 2011). The prophages might have a role in the biology and virulence of bacteria. Clark and Ng (2008) provided further evidence for recombination of prophages in the DNA of several *C. jejuni* isolates by southern blotting using probes designed from three putative prophages of *C. jejuni* RM1221. Scott *et al*. (2007a) reported that phage resistance/insensitivity could be acquired by horizontal gene transfer between the *C. jejuni* phage sensitive and phage resistant strains that lead to the creation of a new phenotype; however, the phage resistant strains were less capable of colonising broiler chickens.

1.6.3 Phage genome sequences

Whole genome sequences of *Campylobacter* phage CP220, CPt10 NCTC 12673 and CP81 have been lodged in the database (Genebank no: FN667788.1, FN667789.1, GU296433.1, and EMBL: FR823450, respectively). These genomic sequences represent group II and group III *Campylobacter* specific bacteriophages (Table 1.2). Interestingly, the CP220 and CPt10 genomes contained many copies of radical *S*-adenyl-methionine genes that may play a role in expanding bacterial metabolism during infection through enhanced biosynthesis of metal containing oxidoreductases (Timms *et al*., 2010; Connerton *et al*., 2011). Additionally, phage CP81 (group III) showed a relationship with group II CP220 and Cpt10 resulting in the presence of a number of similar proteins relating to capsid structure, DNA replication and recombination; however these virion-related modules are scattered throughout the phage genome and are not conserved as they are in other T4 related bacteriophage (Hammerl *et al*., 2011). Notably the CP81 phage genome does not contain the metabolic enzymes present in CP220/CPt10 that include 12 proteins with *S*-adenosylmethionine domains, transposases and membrane proteins (Hammerl *et al*., 2011). Interestingly, the vB_Ccom-IBB_35 phage (Genebank no: HM246720- HM246724) contains genes that encode carbohydrase enzymes that have the potential to degrade bacterial capsular polysaccharides (CPS) (Carvalho *et al*., 2012). The genome sequence of the group 2 bacteriophage CP21 (Genebank no: HE815464) is composed of large modules separated by long DNA repeat regions which may trigger recombination and modular shuffling. However, the genome sequences of CP21 and vB_Ccom-IBB_35 bacteriophage are incomplete , with the former composed of five contigs (Hammerl *et al*. 2012).

Table 1.2- The sequenced whole genome of *Campylobacter* phages

Phages	Group	Length (bp)	DNA	%GC	References
CP220	II	177534	circular	27.4	Timms *et al*. (2010)
CPt10	II	175720	circular	27.3	Timms *et al*. (2010)
CP21	II	182833	unknown	27	Hammerl *et al*. (2012)
vB_Ccom-IBB_35	II	172065	unknown	27	Carvalho *et al*. (2012)
NCTC 12673	III	135041	circular	26	Szymanski *et al*. (2011)
CP81	III	132454	unknown	26.1	Hammerl *et al*. (2011)

1.6.4 Application of *Campylobacter* bacteriophage

Phage can be exploited to detect bacterial pathogens or to reduce bacterial pathogens upon infection; as an antimicrobial agent in both medical and agricultural settings; as biotherapeutic agents and for biosanitation for food safety purposes (Kutter and Sulakvelidze, 2005; Hagens and Loessner, 2007; Mahony *et al*., 2011). Phage therapy has been applied in food animals such as cattle, sheep, pigs and poultry to reduce the microbial loads of food borne pathogens (Johnson *et al*., 2008). *Campylobacter* bacteriophages have been applied to both phage typing and phage therapy. *Campylobacter* phage typing was developed for discrimination of *C. jejuni* and *C. coli* (Grajewski *et al*., 1985; Salama *et al*., 1990; Khakhria and Lior, 1992). There are 16 phages used in the UK phage typing scheme to characterise *C. jejuni* and *C. coli* (Sails *et al*., 1998; Frost *et al*., 1999). The 16 phages were classified into three groups based on structure criteria and belong to *Myoviridae* family of phages. The phages were generally isolated from poultry faeces, poultry manure and sewage effluent (Sails *et al*., 1998). Khakhria and Lior (1992) reported the application of *Campylobacter* phage typing using 19 typing phages to differentiate *C. jejuni* and *C. coli* isolated from human and non-human established 46 different phage types. Frost *et al*. (1999) reported the utilisation of phage typing in combination with serotyping to discriminate both species into subtypes. Phage typing may therefore be of epidemiological use in conjunction with serotyping (Frost *et al*., 1999). Coward *et al*. (2006) reported the 16 typing bacteriophages (Φ1-16 or PT1-PT16) could be assigned to four groups based on their reaction with spontaneous resistant mutants arising from the *Campylobacter* typing strains using a spot lysis method.

Bacteriophage therapy has been shown to be potentially useful to reduce *Campylobacter* in broiler chicken flocks (Wagenaar *et al*., 2005; Loc Carillo *et al*., 2005; El-Shibiny *et al*., 2005). For instance, application of a group II *Campylobacter* bacteriophage (CP220) was able to reduce numbers of *C. jejuni* and *C. coli* colonizing broiler chickens (El-shiny *et al*., 2009). *Campylobacter* phages have been used for biological control on food products, for instance, phages infecting *C. jejuni* could inactivate their host strains on raw and cooked beef (Bigwood *et al.*, 2008). Goode *et al*. (2003) showed that *Campylobacter* bacteriophage (NCTC 12673 or Φ1 on phage type strain) treatment, on *C. jejuni* C222, on chicken skin at a multiplicity of infection (MOI) of 1, reduced the bacteria less than 1 Log_{10} unit and at a higher dose the phage led to reduction in the number of bacterial cells to lower than the control. Atterbury *et al*. (2003a) reported that the application of bacteriophage on chicken skins was able to reduce the recovery number of *Campylobacter* approximately 1 Log_{10} CFU at 4 °C. The optimal conditions for phage treatment of food products were dependent on the interaction of both *Campylobacter* phage and concentration of host (Bigwood *et al*., 2009). The same investigators suggested that the application of phage on food products was dependent on host concentration and phage concentration. Hwang *et al*. (2009) showed that *in vitro*, the phage CPS2, when had been isolated from chickens in Korea, was able to reduce the growth of *C. jejuni* in liquid culture. Carvalho *et al*. (2010) reported a reduction of *C. coli* and *C. jejuni* numbers in chicken faeces of an approximately 2 Log_{10} CFU/g following treatment with a phage cocktail. These findings suggest the feasibility of exploiting bacteriophage to control *Campylobacter* in the food chain and therefore a reduction in transmission to humans.

1.6.5 Acquisition of phage resistance

The acquisition of phage resistance is a problem that may affect any bacteriophage therapy application. Loc Carrillo *et al*. (2005) reported that 97% of isolates after phage treatment of chicken experimentally infected with campylobacter remained phage sensitive, and that those that were phage resistant were unable to colonise chickens efficiently. Furthermore the resistant types were found to revert to bacteriophage sensitivity upon passage through chickens. Similarly, Carvalho *et al*. (2010) reported that 86% of bacteria isolated from chickens colonised with resistant strains without phage treatment were susceptible to phage, while 54% of the resistant strains isolated from phage treatment on chicken reverted to susceptibility to phage sensitivity. Scott *et al*. (2007b) reported evidence that phage resistance in *C. jejuni* strain HPC5 was associated with genomic rearrangements. Bacteriophage resistance mechanisms may therefore impact on the use of phage therapy. Receptor modification to prevent phage entry to the host cells is one mechanism of phage resistance. Variation in the surface membrane structure of *C. jejuni* may therefore play a crucial role in the characteristics of phage-host range as well as the ability to colonise chickens. For instance, *C. jejuni* with defects in motility or capsular polysaccharide were reported to exhibit phage resistance (Coward *et al*., 2006). Restriction-modification systems are another mechanism by which bacterial cells prevent foreign DNA from replicating, in particular phage DNA leading to phage resistance. The diversity of the DNA restriction and modification systems encoded by *C. jejuni* strains had been recently studied. For example, *C. jejuni* 81-176 encodes both type I and type II DNA restriction and modification systems, in contrast, the sequenced strains, NCTC 11168 and RM1221 have only the type II restriction and modification system (Hofreuter *et al*., 2006). However, phage DNA may avoid restriction enzymes, leading to the process of the lytic life cycle (reviewed by Labrie *et al*., 2010). Many bacteria and Achaea contain clustered regularly interspaced short palindromic repeats (CRISPRs) that are responsible for resistance to invasive genetic elements (Haurwitz *et al*., 2010). The CRISPR/Cas system mediates phage resistance by acting in at least two general ways: the adaptation stage and the interference stage (Deveau *et al*., 2010). Additionally, other mechanisms of phage resistance are by blocking the entry of phage DNA into host cells and through abortive infection mechanisms (Sturino and Klaenhammer, 2006).

1.7 Bacteriophage dispersal of biofilm

The persistence of foodborne pathogens on contact surfaces will negatively affect the quality and safety of food products. The presence of biofilms forms harbourage for pathogens where several outbreaks of foodborne disease have been attributed at least in part to the presence of biofilms (Jahid and Ha, 2012). Pathogenic microorganisms can be found in mono- and mixed-biofilms which have proved difficult to remove, increasing pathogen survival and enabling dissemination during processing (Simuões *et al*., 2010). The application of disinfectants and other antimicrobial agents to treat bacteria within biofilms are often of limited success with compounds such as chlorine, commercial chlorinated sanitizer, sodium hyprochlorite, quanternary ammonium compounds having to be applied at increasing concentrations (Simuões *et al*., 2010). These stringent conditions are necessary for high risk food production facilities but increase the risk of antimicrobial resistance emerging, and making the control of pathogens within biofilms difficult (Mah and O'Toole, 2001). The necessity to identify alternative methods to control pathogens has led to the discovery that bacteriophages are a potentially useful biological agent to control biofilm formation and the control of pathogenic bacteria within biofilms. Hibma *et al.* (1997) suggested that phage could inhibit the L-form of *L. monocytogenes* biofilms on stainless steel and also reduce viable cell numbers. Hughes *et al.* (1998) also reported that bacteriophage isolated from sewage could be used to treat biofilms produced by two *Enterobacter agglomerans* strains and an exopolysaccharide (EPS)-producing pseudomonad, resulting in production of very large plaques in the biofilm. The clearing of the biofilm by bacteriophage has been linked with the production of a polysaccharide depolymerase that can degrade the exopolysaccharide. For instance, phage φ15, a T7-like bacteriophage; has a virion-associated expolysaccharide depolymerase that was able to degrade single-species *Pseudomonas putida* biofilms of strains PpG1 and RD5PR2 (Cornelissen *et al*., 2011). The same authors also confirmed that the φ15 tail spike protein (Gp17) showed similarity to pectin lyases with the potential to bind and degrade EPS. Sillankorva *et al.* (2004) reported that bacteriophage Φ S1, at a multiplicity of infection (MOI) of 0.5, infected both planktonic cells and biofilms of *Pseudomonas fluorescens* resulting in reduction in culturability by approximately 85% in both cases. However, when *Escherichia coli* O157:H7 attached to stainless steel was treated with bacteriophage, there was a reduction in viability of the attached cells but not in the number of cells in the biofilm (Sharma *et al.*, 2005). Additional examples of bacteriophage trials with biofilms are shown in Table 1.3. Generally research has indicated that bacteriophage may be useful in the reduction of attached cells and cells in biofilms on food surfaces and food-contacted surfaces.

An alternative approach was suggested by Whitchurch *et al*. (2002), who attempted to use DNase I to inhibit *Pseudomonas* biofilm formation due to the requirement for extracellular DNA to be present for the establishment of the biofilm. The developments of other novel agents that inhibit biofilm formation including inhibitors of inter- and intracellular signals have been reviewed by

Kaplan (2010). Quorum sensing inhibitory compounds have been suggested as agents to prevent biofilm formation, such as AHL antagonist inactivation of LuxR homologs, inhibition synthesis of AHL and degradation of AHL (Hentzer *et al*., 2002). The use of an engineered BdcA gene in the initial colonizer cells could be able to reduce c-di-GMP levels, resulting in an increase in motility and reduction in adhesion leading to dispersion of the initial colonising cells (Ma *et al*., 2011).

Table 1.3-Examples of bacteriophage trials with biofilms

Bacteria	Phages	Main Outcome	References
Escherichia coli	K29	Ability of phage to penetrate the polysaccharide capsule of *E. coli*.	Bayer *et al*. (1979)
E. coli	T4	*E. coli* in biofilms was successfully lysed with phage.	Dolittle *et al*. (1995)
E. coli	Modified T7	Engineered phage reduced biofilm cell counts by over 99%.	Lu and Collins (2007)
E. coli	OP7061, OP10081	Exposure of *E. coli* to phage resulted in the phage-tolerant subpopulation exhibiting stimulation of biofilm formation.	Lacqua *et al*. (2006)
E. coli	T4	The phage reduced bacterial biofilms by approximately 90%	Carson *et al*. (2010)
Staphylococcus aureus	K	Reduction in biofilm mass varied depending on strain tested.	Cerca *et al*. (2007)
Listeria monocytogenes	H387,H378A, 2671	Synergistic effect found with a quaternary ammonium compound and phage.	Roy *et al*. (1993)
Enterobacter cloace	11229, ΦEnt,Φ1.15, Blackburn, Philipstown	Three phages successfully removed the single species biofilms. The phage Φ1.15 enzyme was capable of depolymerising the extracellular polysaccharide.	Tait *et al*. (2002)
Pseudomonas aeruginosa	F116	Phage caused 1-2 Log_{10}reduction in biofilm viable count dependent on the ratio of phage per cell.	Hanlon *et al*. (2001)
P. aeruginosa	Phage cocktail	The phage cocktail reduced the biofilm cell density by 99.9%	Fu *et al*. (2010)
P. fluorescens	φIBB-PF7A	Biomass removed by 63-91% depending on the biofilm age and biofilm forming condition	Sillankova *et al*. (2008)
Proteus mirabilis,	Coli-proteus,	The phage reduced bacterial biofilms by approximately 90%	Carson *et al*. (2010)

Modified and adapted from the review by O'Flaherty *et al*. (2009)

1.8 Objectives of this study

Campylobacter species are of great concern as a cause of food borne disease and are harmful to human health. Knowledge of the mechanisms of prolonged environmental survival of these bacteria and host cell virulence still remains unclear. The survival and persistence of *Campylobacter* spp. in the natural environment and on food surfaces may involve biofilm formation. The formation of *Campylobacter* biofilms may help these organisms to survive in harsh environments and in the persistence and colonization of animal digestive tracts leading to contamination of food, during processing. Thus, it is necessary to understand biofilm formation by *C. jejuni* and to better understand how biofilms respond to environmental factors in order to help eliminate them from food and food-contacted surfaces. Bacteriophages are an alternative treatment as bio-control agents to reduce or eliminate the biofilms from food and food contacted-surfaces. The effect of bacteriophage exposure on attachment of cells and on pre-formed biofilms by *Campylobacter* has not previously been studied.

The specific objectives of this study are:

1) To investigate the dispersal of *Campylobacter* biofilms by bacteriophage under microaerobic conditions.
2) To evaluate the formation of the carrier state life cycle (CSLC) in *Campylobacter* biofilms after bacteriophage treatment and the

physiology of the CSLC cultures and parental bacteria under microaerobic, low oxygen (<1% O_2), and atmospheric conditions.

3) To demonstrate the characteristics of the CSLC phages compared to parental phages.
4) To determine the transcriptomics of CSLC cultures and parent strains at early exponential phase of growth under microaerobic conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth, storage media and chemical solutions

2.1.1 Blood agar (BA)

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

Typical Formula	g/litre
Proteose peptone	15.0
Liver digest	2.5
Yeast extract	5.0
Sodium chloride	5.0
Agar	12.0
pH 7.4 ± 0.2 @ 25°C	

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

Campylobacter blood-free selective agar (Oxoid), supplemented with 2% (w/v) bacteriological agar No. 1 (Oxoid) was prepared according to manufacturer's instructions. A 1 Litre of CCDA contained 55 g of *Campylobacter* blood-free selective agar and 20 g of agar No. 1. Typical formula of CCDA contains substances as shown below.

Typical Formula	g/litre
Nutrient Broth No.2	25.0
Bacteriological charcoal	4.0
Casein hydrolysate	3.0
Sodium desoxycholate	1.0
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.0
pH 7.4 ± 0.2 @ 25°C	

2.1.3 New Zealand casamino yeast medium (NZCYM) basal agar

Difco™ NZCYM broth (Becton, Dickinson and Company, Sparks, MD, USA), supplemented with bacteriological agar No. 1 (Oxoid) to a final concentration of 1.5% w/v, was prepared according to manufacturer's instructions. A 1 Litre of NZCYM basal agar contained 22 g of NZCYM broth and 15 g of agar No.1. Typical formula of NZCYM broth contains substances as shown below.

Typical Formula	g/litre
Pancreatic Digest of Casein	10.0
Casamino acids	1.0
Yeast extract	5.0
Sodium chloride	5.0
Magnesium sulfate (anhydrous)	0.98
pH 7.0 ± 0.2 @ 25°C	

2.1.4 NZCYM overlay agar

Difco™ NZCYM broth, supplemented with bacteriological agar No. 1 to a final concentration of 0.6% (w/v) was prepared according to manufacturer's instructions. Following sterilisation, 6 ml aliquots were aseptically dispensed to sterile glass universals and stored at 4°C for a maximum of 8 weeks. Immediately before use the agar was melted in a microwave and cooled to 50°C. A 1 Litre of NZCYM overlay agar contained 17.6 g of NZCYM broth and 6 g of agar No.1.

2.1.5 Mueller-Hinton (MH) Broth and Agar

Mueller-Hinton broth (Oxoid) was prepared according to manufacturer's instructions. Following sterilisation, MH broth was stored at room temperature in the dark for a maximum of 8 weeks. Addition of 1.5% (w/v) of bacteriological agar No.1 (Oxoid) was prepared to obtain the MH agar. A 1 Litre of MH broth contained 21 g of MH broth and 15 g of agar for MH agar No. 1. Typical formula of MH broth contains substances as shown below.

Typical Formula	g/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar (MH Agar)	15
pH 7.3 ± 0.1 @ 25°C	

2.1.6 *Campylobacter* motility agar

Mueller-Hinton (MH) broth (Oxoid), supplemented with bacteriological agar No. 1 to a final concentration of 0.4% (w/v) was prepared according to manufacturer's instructions. A 1 Litre of motility agar contained 21 g of MH broth and 4 g of agar No. 1.

2.1.7 Bacterial storage medium

Nutrient broth (NB) No. 2 (Oxoid) was dispensed according to manufacturer's instructions and dissolved in RO water supplemented with 20% (v/v) glycerol (Fisher Scientific, Loughborough, UK). This was dispensed in 30 ml aliquots to sterile glass universals and, following sterilisation, stored at room temperature for a maximum of 8 weeks. A 1 Litre of bacterial storage medium contained 23 g of nutrient broth and 200 ml of glycerol. Typical formula of NB No.2 contains substances as shown below.

Typical Formula	g/litre
`Lab-Lemco' powder	10.0
Peptone	10.0
Sodium chloride	5.0
pH 7.5 ± 0.2@ 25°C	

2.1.8 Maximal Recovery Diluents (MRD)

Maximum recovery diluent (Oxoid) was prepared according to manufacturer's instructions. Following sterilisation, MRD was stored at room temperature for a maximum of 8 weeks. A 1 Litre of MRD contained 9 g of MRD. Typical formula of MRD contains the substances as shown below.

Typical Formula	g/litre
Peptone	1.0
Sodium chloride	8.5
pH 7.0 ± 0.2 @ 25°C	

2.1.9 Phosphate Buffer Saline (PBS)

Phosphate buffered saline (Oxoid) was prepared according to manufacturer's instructions. Following sterilisation, PBS was stored at room temperature for a maximum of 8 weeks. The PBS contains the chemical agents as shown below.

Formula	g/litre
Sodium chloride	8.0
Potassium chloride	0.2
Disodium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
pH 7.3	

2.1.10 10 mM Tris-HCl, 1 mM disodium ethylene diamine tetraacetic acid (EDTA), pH 8 (TE buffer)

TE buffer was prepared by addition 1.214 g/l of 2-Amino-2-(hydroxymethyl)-1,3-propanediol Tris (hydroxymethyl) aminomethane (Trisma® base, Sigma-Aldrich, St Louis, MO, USA), 0.3722 g/l of disodium ethylene diamine tetraacetic acid (EDTA, Sigma-Aldrich), and adjusting the pH to 8.0 by adding HCl (Acros Organics, New Jerseys, USA). Following sterilisation, TE buffer was stored at room temperature for a maximum of 8 weeks.

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

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

2.1.12 Salt Magnesium buffer (SM buffer)

SM buffer was prepared by dissolving sodium chloride (Fisher Scientific) to a final concentration of 5.8 g/l, magnesium sulphate heptahydrate (Sigma-Aldrich) to a final concentration of 2 g/l, Trisma® base (Sigma-Aldrich) to a final concentration of 6 g/l, and gelatin (Sigma-Aldrich) to a final concentration of 0.01% w/v in RO water. The pH was adjusted to 7.5 using HCl (Acros Organics). This was dispensed in 30 ml aliquots to sterile glass universals and, following sterilisation, stored at room temperature for a maximum of 8 weeks.

2.1.13 Magnesium sulphate

Magnesium sulphate (1 M) was prepared by dissolving of magnesium sulphate heptahydrate (Sigma-Aldrich) to final concentration 246.7 g/l. To obtain 10 mM $MgSO_4$ was performed by dilution with sterile RO water.

2.2 *Campylobacter*

2.2.1 Bacterial strains and growth conditions

Campylobacter strains (Table 2.1) were stored in Nutrient Broth no.2 (Oxoid) supplement with 20 % glycerol (2.1.6) at -80 °C. For routine growth they were subcultured on Blood Agar base no. 2 (Oxoid) supplemented with 5% defibrinated horse blood (TCS) as described in 2.1.1 and incubated at 42 °C in a gas jars (Oxoid) under microaerobic conditions achieved by gas replacement (85% N_2, 5% O_2 and 10% H_2) for approximately 18-20 h.

2.2.2 Enumeration of *Campylobacter*

Campylobacter enumeration was carried out using a modification of the technique described by Miles and Misra (1938). *Campylobacter* suspensions were enumerated by performing ten-fold serial dilutions using MRD using 900 µl of MRD (2.1.8) and 100 µl of suspension. Multiple 10 microlitre spots of each dilution were dispensed onto *Campylobacter* Blood-Free Selective Agar (CCDA; Oxoid) containing addition of agar to 2% (w/v) (2.1.2) to prevent swarming and allowed to dry for 20 min. The use of the multiple spot methodology was adopted to ensure dispersal of the *Campylobacter* colonies and reduce swarming that is evident when using spread plates to obtain reliable colony counts. The plates were then incubated under microaerobic conditions at 42 ^{o}C for 48 h and the colonies counted.

Table 2.1-*Campylobacter* Strains used in this thesis

Strains	**Sources**	**Reference**
NCTC 11168	Human NCTC	Parkhill *et al*., 2000
HPC5	Chicken (caeca)	Loc Carrillo *et al*., 2005
PT14	HPA Collindale UK	Frost *et al.*, 1999
HPC5CP8PL	*Campylobacter* biofilms	In this thesis
HPC5CP30PL	*Campylobacter* biofilms	In this thesis
PT14CP8PL	*Campylobacter* biofilms	In this thesis
PT14CP30PL	*Campylobacter* biofilms	In this thesis
11168GFP	Laboratory	Karlyshev and Wren, 2005
11168YFP	Laboratory	Karlyshev and Wren, 2005
PT14GFP	Laboratory	Laboratory Stock
PT14YFP	Laboratory	Laboratory Stock
PT14YFPCP8PL	Planktonic phage-treated	In this thesis
PT14YFPCP30PL	Planktonic phage-treated	In this thesis

2.3 Bacteriophage

2.3.1 Bacteriophage propagation

The propagating *Campylobacter* strains were sub-cultured onto blood agar plates over night (2.2.1). One hundred microlitres of bacteriophage suspension

10^9 PFU/ml (Table 2.2) was mixed with *Campylobacter* cells (10^9-10^{10} CFU/ml) that were harvested into 2 ml of sterile 10 mM $MgSO_4$ (2.1.13) with the aid of sterile swab. The propagating strain suspension was incubated for 40 min at 42 ^oC in order to allow bacteriophages to adsorb. Five hundred microliters of the suspension was then aliquoted to 4 ml of molten Difco™ NZCYM supplemented with 0.6 %(w/v) agar bacteriological No.1 (Oxoid) overlay agar (2.1.4) and then applied to the surface of NZCYM basal plates (2.1.3) (Sambrook *et al*., 1989). The overlay agar plates were left to set at room temperature for 20 min and then incubated under microaerobic conditions at 42 ^oC for 48 h. Five mililitres of MRD (2.1.8) was added to the surface of each plate and the bacteriophage allowed to elute onto the buffer by shaking gently overnight at 4 ^oC on a gyratory shaker at 60 cycles / min. The final harvested bacteriophage suspension was filtered through 0.2 µm Minisart® filters (Satorious Stedim Biotech, Surrey, UK) and the phage stock titres 10^9 PFU/ml and transferred to a thick wall universal bottle and stored at 4 ^oC in the dark. These phage stocks retained their titres for at least two years.

Table 2.2-Bacteriophages used in this thesis and their propagating strains

Bacteriophages	Propagating strains	Sources
CP8	NCTC 11168 or PT14 or HPC5	Dr. C. Loc Carrillo
CP30	NCTC 11168 or PT14 or HPC5	Dr. P. Connerton
CP8PL	PT14CP8PL or HPC5CP8PL	This thesis
CP30PL	PT14CP30PL or HPC5CP30PL	This thesis
CP220	PT14 or HPC5	Dr. P. Connerton

2.3.2 The carrier state life cycle (CSLC) phage propagation

For propagation, the CSLC strains (Table 2.1) were sub-cultured on blood agar at 42 ^{o}C under microaerobic conditions for 18-20 h. The growth from the whole plate (10^9-10^{10} CFU/ml) was resuspended into 5 ml of sterile 10 mM $MgSO_4$ (2.1.13) and an aliquot of 0.5 ml of this suspension was added into 4 ml of molten NZCYM (2.1.4) and then carried on the same as described in 2.3.1.

2.3.3 Bacterial lawn preparation

Cultures of *C. jejuni* (2.2.1) were resuspended in 10 ml of 10 mM $MgSO_4$ (2.1.13). Five hundred microlitres of bacterial suspensions (10^9 CFU/ml) were added to 4 ml of molten NZCYM containing 0.6% (w/v) agar as overlay agar (2.1.4) and then dispensed on the basal NZCYM plates (2.1.3). The overlay agar plates were left to set at room temperature for 20 min. The lawns were used for the phage titration. Ten microlitre spot assays were used to locate the phage on the plates and minimise the adsorption times for phage suspension into the agar plate to prevent spread. This procedure enable the comparison of phage titres between wild type host *Campylobacter* strains and those carrying bacteriophage that give rise to background plaque formation.

2.3.4 Bacteriophage titration

To enumerate bacteriophage suspension, ten-fold serial dilutions were performed in 900 of MRD (2.1.8) using 100 µl of suspension and subsequently 10 µl aliquots dispensed, in triplicate, onto bacterial lawns (2.3.3), a volume that would readily adsorb before placing in a microaerobic environment. The plates were incubated at 42 °C under microaerobic conditions for 18-20 h. The plaques were counted and used to calculate the plaque forming unit (PFU)/ml.

2.4 Biofilm formation and bacteriophage treatment

2.4.1 Initial culture for biofilm formation

Campylobacter jejuni cultures (2.2.1) were resuspended in Mueller-Hinton (MH) broth (2.1.7) at OD_{600nm} 0.3-0.4, using a sterile swab and then incubated at 42 °C under microaerobic conditions to use as inocula. The microaerobic atmospheric was achieved using either 2 x 3.5 L Oxoid CampyGen™ gas packs placed in sealed incubation boxes or using gas jars employing gas replacement (85% N_2, 5% O_2 and 10% H_2) .

2.4.2 Attachment and biofilm formation

The *Campylobacter* cultures (2.2.1) were diluted to contain approximately 10^5 CFU/ml with fresh MH broth. Approximate *Campylobacter* count was estimated using the empirical equation:

$$\textit{Campylobacter}\ \text{count in CFU/ml} = (OD_{600} * 2 \times 10^9) - 6 \times 10^6$$

The equation was derived from a standard *Campylobacter* dilution series (Scott, 2006). A 10 ml aliquot of each culture was dispensed into a Petri dish containing six glass cover slips (18x18 mm, Scientific Laboratory Supplies Ltd, Nottingham, UK) on which the biofilms were to be formed. The Petri dishes were then incubated at 37 ^{o}C under microaerobic conditions for 1, 2, 5 and 7 days and followed the enumeration of *Campylobacter* as described in 2.4.5.

2.4.3 Effect of temperature on attachment and biofilm formation

The attachment and biofilm formation by *C. jejuni* NCTC 11168 was performed by method described in 2.4.1 and 2.4.2 following incubation at 37 or 42 ^{o}C for 1, 2, 5 and 7 days. The viability of attached and planktonic cells was determined by method described in 2.4.5.

2.4.4 Bacteriophages treatment of biofilms

Biofilms of *C. jejuni* NCTC11168, PT14 and HPC5 were allowed to form under microaerobic conditions over 5 days at 37 ^{o}C. Glass cover slips covered with these *Campylobacter* biofilms were moved to a clean Petri dish for phage treatment and 1 ml of the residual unattached cells (approximately 90%) collected in a tube for independent treatment with phage. Bacteriophage stock suspensions (2.3.1) were diluted in fresh MH broth to final concentration of 10^6

or 10^9 PFU/ml, from which 100 µl of bacteriophage suspension was placed onto each coverslip surface and added to the corresponding planktonic phase in a microcentrifuge tube. The coverslips and tubes were incubated at 37 °C, under microaerobic conditions for 0, 2, 4, 7, and 24 h in a petri dish containing a buffer reservoir to maintain a water saturated environment. Control samples were mock-treated with fresh sterile MH broth instead of phage suspension. Three slides of each treatment at each sampling time were removed and processed for the determination of the viable bacterial count (2.4.5) and bacteriophage titration (2.4.6). The remaining glass slides were left to incubate undisturbed until the next sampling time point. All experiments were performed using triplicate slides with a minimum of three independent biological replicates. The data were presented as mean counts (± standard deviation) and their significance determined using T-test ($P<0.05$).

2.4.5 Enumeration of viable bacterial cells

After gently washing with 2 ml of MRD, bacterial cells attached to the glass surfaces were resuspended in 2 ml of MRD (2.1.8) using a repeated pipetting action up and down 20 times to detach bound cells. The campylobacters were enumerated as described in 2.2.2. Planktonic cells were serially diluted and enumerated in the same way. The limit of detection for the enumeration of bacterial populationsfrom biofilm and planktonic cultures was 1 log CFU/cm^2 and 2 log CFU/ml, respectively. Recovery (efficiency of detachment) of the cells from the glass slides was monitored by repetition of the steps with subsequent enumeration to establish no significant changes in the viable count.

2.4.6 Enumeration of bacteriophage

Bacterial lawns of *C. jejuni* PT14 or HPC5 or 11168 were prepared as described in 2.3.2. Serial dilutions of the recoverable attached phage populations in MRD were retained over night at 4 ºC before titering of the bacteriophage as described in 2.3.3. After washing with 2 ml of MRD, the unattached bacteriophage on biofilms were eradicated. The recoverable attached phage numbers of plaque forming units (PFU) per cm^2 of surface area of glass cover slip were calculated by The recoverable attached phage numbers of plaque forming units (PFU) per cm^2 of surface area of glass cover slip were calculated by dividing the plaque forming units determined per coverslip with surface area (cm^2) of the glass coverslip. The limit of detection for the enumeration of bacteriophage in biofilms and planktonic phases was 1 log PFU/cm^2 and 2 PFU/ml, respectively.

2.4.7 Crystal violet staining for quantity of biofilms

Crystal violet is a vital dye which binds to carbohydrate, protein and nucleic acid and is therefore useful for staining and quantifying biofilm material (Kim and Wei 2007). *Campylobacter* biofilms attached to glass cover slips were washed twice with 2 ml of MRD (2.1.8) at ambient temperature prior to staining. One hundred microlitres of 0.1% (w/v) crystal violet (Sigma-Aldrich) was added to the surface of each glass coverslip and allowed to stain for 1 h. The biofilms were then washed three times with sterile RO water. The bound crystal violet was extracted from biofilms by dissolving in 2 ml of 95% ethanol. The absorbance of the extract was measured at 570 nm by Pharmacia Biotech Ultrospec®2000 UV/Visible Spectrophotometer. Efficiency of the crystal violet stained on biofilm was monitored by repetition of the method to prove no significant different.

2.4.8 Determination of the frequency of phage resistance

The 30-100 colonies produced by viable cells recovered from biofilms post-treatment with either bacteriophage CP8 or CP30 were sub-cultured on blood agar plates (2.2.1). Bacterial lawns were prepared from these sub-cultures to test for bacteriophage sensitivity (2.3.2). Phage stocks were applied to the bacterial lawn as 10 µl aliquots at the routine test dilution (10^5 PFU/ml; Frost *et al.*, 1999). Bacteriophage stocks included bacteriophage CP8 and CP30 propagated on different host strains. Phage resistance was identified on the basis of the inability of the phage stock to produce confluent lysis or generate any plaques on the bacterial lawn. The frequency of phage resistance was calculated as the percentage of resistant isolates per total number of isolates tested.

2.4.9 Motility (swarming) determination

The motility of *Campylobacter* isolates was assessed by centre-inoculating 0.4% (w/v) Muller-Hinton agar plates (2.1.6) and subsequently incubating them under microaerobic conditions for 48 h according to Goossens *et al*. (1989). The diameter (cm) of the growth was measured as the degree of swarming.

2.5 DNA preparation and analysis

2.5.1 *Campylobacter jejuni* genomic DNA extraction

Campylobacter jejuni genomic DNA was extracted and purified by using GenElute™ Bacterial Genomic DNA kit following manufacturer's instructions (Sigma-Aldrich). Briefly, the *Campylobacter* cultures (2.2.1) were resuspended in 1.5 ml of PBS (2.1.9) and centrifuged at 13,000 x *g* for 1 min. Pellets were harvested and resuspended in 180 µl of lysis solution T (EDTA disodium [0.5 M solution], 1-S-Octyl-beta-D-thioglucopyranoside [9-8%], NaCl, Tris-HCl [pH 8.0, 1.0 M stock solution]). The 20 µl of RNase A (20 mg/ml) was mixed and incubated at room temperature for 2 min to get RNA free DNA. Cell lysis was carried out by the addition of 20 µl of 20 mg/ml of Proteinase K solution, incubation for 30 min at 55 ^{o}C followed by the addition of 200 µl of lysis solution C (guanidine hydrochloride, Tris-HCl [pH 7.0, 1.0 M sock solution], EDTA disodium [0.5 M solution], Tween® 20, p-tertiary-Octylphenoxy polyethyl alcohol, hydrochloric acid, water), incubated for 10 min at 55 ^{o}C to obtain the lysate. The mixture of the entire lysate suspension and 200 µl of 100% ethanol were transferred into prepared binding column using a wide bore pipette tip, then centrifuged at 6,500 x *g* for 1 min. The washing step was performed firstly by addition of 500 µl of washing solution 1 (ethanol), centrifugation at 6,500 x *g* for 1 min and subsequently by addition of 500 µl of wash concentration solution (withheld as a trade secret of the manufacturer), centrifugation at 13,000 x *g* for 3 min to dry the column. The DNA was eluted by addition of 200 µl of the elution solution (10 mM TrisHCl, 0.5 mM EDTA, pH 9.0) directly onto the centre of the column, incubation at room temperature for 5 min and then centrifugation at 6,500 x *g* for 1 min. The DNA solution was kept at 4 °C or -20 °C.

2.5.2 Bacteriophage DNA extraction

Two hundred microlitres of phage suspension (approximately 10^9 PFU/ml) was mixed with 200 µl of detergent solution containing 20 mM Tris-HCl (pH 8), 200 mM EDTA (pH 8), 2% (w/v) N-lauroyl sarcosine (Sigma-Aldrich) and 0.4% (w/v) sodium dodecyl sulphate (SDS, Sigma-Aldrich). Twenty microlitres of 10 mg/ml of proteinase K (Sigma-Aldrich) was then added and incubated at 55 °C 2 h to digest capsid proteins. Phenol solution (Fisher Scientific) was added (0.5 ml), mixed by inverting, and centrifuged at 13,000 x *g* for 1 min. The top phase was collected and transferred to a new tube. Chloroform (Sigma-Aldrich) was added (0.5 ml) in the new tube and the tube was mixed by inverting, and then centrifuged at 13,000 x *g* for 1 min. This step was repeated once and then a 1: 10 volume of 7.5 M ammonium acetate (Sigma-Aldrich) was added. Two volumes of 100% ice cold ethanol was added and mixed gently by inverting. The tube was then kept at -20 °C overnight. The sample was centrifuged at 13,000 x g for 10 min. The pellet was collected and washed with 70% ethanol and then centrifuged at 13,000 g for 10 min. the pellet was resuspended in 50 µl of TE buffer (2.1.10) and kept at 4 °C until use. The DNA content was measured by using a Nanodrop ND-1000 spectrophotometer (Fisher Thermo Scientific) and by agarose gel electrophoresis (2.5.4).

2.5.3 Agarose Gel Electrophoresis

DNA was routinely analysed using agarose gel electrophoresis. A solution containing 0.8% or 1.0% (w/v) agarose (Melford Laboratories Ltd., Ipswich, UK) in TAE buffer (2.1.11) was melted in a microwave and allowed to cool to 70 ºC. Ethidium bromide was added to a final concentration of 0.4 µg/ml and the solution poured into casting trays and allowed to set for 30 min at room temperature. DNA samples were mixed with 0.2 volumes of 6 x blue/orange loading dye (Promega UK, Southampton, UK) and loaded to wells in the gel. Standard DNA for comparison was the 1 kb ladder and 100 bp ladder markers (Promega). The samples were electrophoresised for 1 – 2 h at 80 V in 1x TAE buffer (2.1.11) before visualisation using a Molecular Imager Gel Doc system (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

2.5.4 Pulsed Field Gel Electrophoresis

Macro restriction profiling of *Campylobacter* genomes was performed according to the protocol of Ribot *et al.* (2001). PFGE was used to determine *Campylobacter* and CSLC bacteriophage-associated genomic DNA fragments from 20 – 1000 kb in size. *Campylobacter* genomic DNA plugs were made by collecting the growth from a blood agar plate into 1.5 ml of PBS (2.1.9) using a sterile swab. The suspensions (400 µl) were then mixed with 400 µl of molten 1% (w/v) ultra-pure PFGE agarose (Bio-Rad) in 1X TE buffer (2.1.10) and dispensed to plug moulds (Bio-Rad) to set. The genomic DNA plugs were incubated in freshly prepared cell lysis buffer (50mM Tris-HCl [pH 8.0], 50mM EDTA, 1% (w/v) N-lauroyl sarcosine, 0.1 mg/ml proteinase K) at 55°C without shaking for 20 min. The plugs were then washed by incubating at 55 °C in RO water for 20 min, followed by three incubations in TE buffer at 55 °C without shaking for 15 min. DNA plugs were stored at 4°C in TE buffer until use.

Campylobacter genomic DNA plugs were digested with *Sma*I to produce fragments of 20 – 1000 kb size. The plugs were cut into 2 mm slices using a sterile scalpel and separately placed into 100 µl of SuRE/cut buffer A (33 mM tris-acetate adjusted with glacial acetic acid to pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol) for 15 min at room temperature. This was replaced with 100 µl of fresh SuRE/cut buffer A and *Sma*I (Roche) was added to a final concentration of 0.4 U/µl. This was incubated for 2 h at 30 °C without shaking, when the buffer/ *Sma*I mixture was removed and replaced by 100 µl of TE buffer (2.1.10).

The digested DNA plugs were inserted into the PFGE 1% (w/v) of ultra-pure agarose (BioRad) gel in TAE (2.1.11). Electrophoresis was performed for 18 h at 14°C in a CHEF-DRII PFGE apparatus (Bio-Rad) in 1X TAE buffer with an initial switch time of 6.8 s and a final switch time of 38.4 s (gradient of 6 V/cm and an included angle of 120°). Following electrophoresis the gel was immersed in a 50 µg/ml ethidium bromide solution for 30 min followed by two washes in RO water for 30 min each and visualized under UV illumination.

2.5.5 Southern blotting of pulsed field gel electrophoresis (PFGE)

Southern blotting of PFGE was used to identify the bacteriophage genomes associated with the CSLC strains. The PFGE gel was transferred to 200 ml of 0.25 M HCl for 10 min at room temperature and washed with RO water. The gel was soaked twice in 200 ml of denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min and washed with RO water. Subsequently, the gel was soaked twice in 200 ml of neutralization solution (0.5 M Tris-HCl, pH 7.5 and 3 M NaCl) for 15 min and washed with RO water. The southern blot was performed as described by Sambrook *et al*. (1989) using Hybound-N+ nylon membrane (GE healthcare, Buckingham, UK). After transfer the DNA was cross-linked on the membrane using UV light for 2 min. The membrane was kept in TE buffer (2.1.9) at 4 ^{o}C.

2.5.6 Hybridization

Hybridization was performed by using Digoxigenin (DIG) high prime DNA labeling and detection starter kit (Roche Applied Science, Mannheim, Germany) as described in manufacturer's instruction.

The preparation of DIG probes was carried out as following: Template DNAs (0.5-1 µg) were denatured by heating in a boiling water bath in a 1.5 ml microfuge tube for 10 min and quickly chilling on ice. Four microliter of DIG-High prime (vial 1) was mixed with the denatured DNA and incubated overnight at 37 ^{o}C. Reaction was stopped by heating at 65 ^{o}C for 10 min. The denatured DIG-labeled DNA probe was added into 7 ml of pre-heated DIG Easy Hyb buffer at 42 ^{o}C as a probe mixture and carried on hybridization.

Pre-hybridization the blot was performed by adding 20 ml of pre-heated DIG Easy Hyb at 42 ^{o}C for overnight with gentle agitation. The pre-hybridization solution was poured off and then added the probe mixture to the blot. Hybridization was performed overnight with gentle agitation at 42 ^{o}C. The membrane was washed subsequently twice in 100 ml of 2X SSC, 0.1 % SDS at 25 ^{o}C for 5 min under constant shaking. The blot was washed twice in pre-heat 100 ml of 0.5X SSC, 0.1% SDS at 65 ^{o}C for 15 min under constant agitation at 65 ^{o}C. After that the blot was washed briefly with washing buffer [0.1 M Maleic acid, 0.15 M NaCl, pH 7.5 and 0.3% (v/v) Tween 20] for 2-5 min at 25 ^{o}C under constant agitation. The blocking buffer [1: 10 blocking solution (bottle 6) and Maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5)] was added to block the blot for 60 min at 25 °C under constant agitation. The diluted 1:5000 Anti-Digoxigenin-Alkaline phosphatase conjugate (vial 4) with blocking buffer was added on the blot and incubated under constant agitation for 60 min at 25 °C. The membrane was washed twice with washing buffer for 15 min under the same conditions. Subsequently, the membrane was equilibrated with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 2-5 min at room temperature. The mixture of 200 µl of NBT/BCIP stock solution (vial 5) and 10 ml of detection buffer was added to the blot and then incubated in the dark until the band visible (2-3 hours). The reaction was stopped by transferring to TE buffer.

2.6 Transduction of chloramphenicol resistance gene via phages and the carrier state life cycle (CSLC) phages

Campylobacter jejuni 11168 GFP, 11168YFP, PT14GFP and PT14YFP strains that contained a plasmid which expressed green fluorescence protein (GFP) or yellow fluorescence protein (YFP) and *cam*R (chloramphenicol resistance gene) were used for phage propagation. The CSLC phages were propagated from *C. jejuni* PT14YFPCP8PL and PT14YFPCP30PL obtaining from phage treatment on *C. jejuni* PT14YFP. The phages and CSLC phages (10^9-10^{10} PFU/ml) were added to suspensions of *C. jejuni* 11168 and PT14 (10^7 CFU/ml) at 42 °C under microaerobic conditions for 1 h. The viable *Campylobacter* counts were performed by using Muller-Hinton plates (2.1.7) supplemented with 5 % horse blood (TCS) and 10 µg/ml chloramphenicol (2.2.1). The resistant colonies were enumerated and the transduction rate calculated by division of transductant cells by the bacteriophage density.

2.7 Characteristics of CSLC strains and phages

2.7.1 Growth curve of *Campylobacter jejuni* and CSLC strains

Campylobacter jejuni and CSLC isolates were sub-cultured as previously described (2.2.1). The cultures were inoculated into 50 ml of sterile MH broth to a concentration of approximately 10^5 CFU/ml as described in 2.4.2 and subsequently determined by viable count to obtain the initial *Campylobacter* counts. Samples were incubated in a shaking incubator selecting an orbital rotating speed of 100 rpm (IS-971R, Fishers Scientific, UK) at 42 °C using either microaerobic conditions, or low oxygen (<1% O_2) conditions generated by AnaeroGen™ gas packs (Oxoid) or atmospheric oxygen (aerobic conditions). A 100 µl aliquot was removed every 2 h for 24 h for microaerobic and low oxygen (<1% O_2) conditions or 1 h for aerobic conditions. The samples were immediately ten-fold serially diluted using MRD. The number of viable *Campylobacter* and bacteriophages were enumerated by following the method as described above (2.2.2 and 2.3.4). All experiments were performed in triplicate at least twice.

2.7.2 Survival of *Campylobacter jejuni* and CSLC strains

Campylobacter jejuni and CSLC isolates were sub-cultured as previous described (2.2.1). The cultures were inoculated into 50 ml of sterile MRD to final concentration approximately 10^5 CFU/ml as described in 2.4.2 and subsequently determined by viable count to obtain the initial *Campylobacter* counts. The cultures were incubated in a shaking incubator at 100 rpm (Fishers Scientific) at 42 °C under either under microaerobic conditions, or low oxygen (<1% O_2) conditions achieved using AnaeroGen™ (Oxoid) or atmospheric conditions (aerobic). The number of viable *Campylobacters* and bacteriophages were enumerated by following the method described in 2.2.2 and 2.3.4. Percentage of survival of *Campylobacter* and bacteriophage were calculated. All experiments were performed in triplicate.

2.7.3 Effect of temperature on survival of *C. jejuni*

Campylobacter jejuni parent strains and CSLC isolates were sub-cultured as previously described (2.2.1). The cultures were inoculated into 50 ml of sterile MRD to final concentration of approximately 10^7 CFU/ml as described in 2.4.2 and subsequently determined by viable count to obtain the initial *Campylobacter* counts. The cultures were then incubated in an orbital shaking incubator set at 100 rpm at 42 °C under microaerobic conditions for 2 h. The cultures were then aliquoted (1 ml) into microcentrifuge tube and then incubated under ambient atmospheric conditions either at 4 °C for 1, 3, 5, 7 and 9 days, or for 0.5, 1, 1.5 and 2 h using a water bath set at 50 °C . The survival of bacterial cells and bacteriophages were determined as described in 2.2.2 and 2.3.3. Percentage of survival of *Campylobacter* and bacteriophage were calculated relative to numbers of bacteria and bacteriophages at the initial time. All experiments were performed in triplicate.

2.7.4 Comparison of the ability of parent phages and CSLC phages to form plaques on different hosts

Lawns were prepared as described in 2.3.2. The routine phage concentrations (approximately 10^5 PFU/ml) of that were propagated from distinct strains were applied on the lawns. The ability of phages and CSLC phages to form plaque on the different hosts was evaluated.

2.7.5 Efficiency of plating (EOP) of parent phages and CSLC phages

The efficiency of plating (EOP) of phages and CSLC phages was determined by calculating from the phage titre when applied to the lawns of *C. jejuni* HPC5 and 11168 and comparing them to the type strain, *C. jejuni* PT14. The lawns were prepared using the method described in 2.3.2. The phages and CSLC phages were serial-diluted and onto each lawn. Phage numbers were counted and the EOP calculated. All experiments were performed in triplicate.

2.7.6 Host binding assay of bacteriophages

Campylobacter jejuni NCTC 11168, PT14 and HPC5 were sub-cultured as previously described (2.2.1). The hosts were prepared in 10 ml of MH broth to give final counts approximately 10^9-10^{10} CFU/ml**.** Phages and CSLC phages were employed to a final phage titre of 10^5 PFU/ml**.** The mixtures were incubated in a shaking incubator operating at 100 rpm (Fishers Scientific) at 42 °C under microaerobic conditions, interval sampling every 30 min until 90 min. Samples were centrifuged at 13,000 x g for 5 min and the supernatants collected for examining the phage number by following the method described above (2.3.3). The percentages of phage binding were calculated. All experiments were performed in triplicate twice. The data were presented as the means and SD of the percentage of bound phage determined from triplicate determinations of the residuals of the input titres less the free phage post centrifugation, and their significant determined using unpaired t-tests, ANOVA (P<0.05) and further multiple comparison of the means with Tukey's 95% confidence intervals.

2.8 Transmission Electron Microscope (TEM)

Biofilms attached to glass coverslips (2.4.2) were fixed and the capsular material stained using 1% (w/v) alcian blue 8GX (Sigma-Aldrich) (Karvlyshev et al., 2001) and 3% (v/v) glutaraldehyde prepared in 0.075 M sodium cacodylate (Sigma-Aldrich) buffer, pH 7.4. They were post –fixed in 1% (v/v) osmium tetroxide in the same buffer following this, a thin layer of 1% (w/v) molten agar (Oxoid) that had been cooled to 45 °C was applied to cover the fixed biofilms and allowed to solidify. The agar-encased biofilms were then removed from the glass cover slips and cut into 2-3 mm pieces to allow processing using standard procedures, by dehydration in ethanol, infiltration and embedding in resin (TAAB Laboratories, Aldermaston, UK). Ultra thin sections were stained with uranyl acetate (Sigma-Aldrich) and lead citrate (Sigma-Aldrich) before observation using Tecnai™ Biotwin transmission electron microscope (FEI Company) operated at an accelerating voltage of 100 kV.

The CSLC strains were examined by using negative staining technique. Bacterial cultures were applied to a copper grid and then stained with uranyl acetate. The images were observed by TEM operated at an accelerating voltage of 100 kV.

2.9 RNA isolation and purification

Campylobacter jejuni and CSLC isolates (Table 2.1) were sub-cultured as previously described in 2.2.1. Three independent experiments of each culture were inoculated into 150 ml of sterile MH broth to a final density approximately 10^5 CFU/ml as described in 2.4.2 and then incubated in a shaking incubator at 100 rpm (Fishers Scientific) at 42 °C under microaerobic conditions for 9 h. Seven independent cultures of *C. jejuni* HPC5 were grown in 150 ml of sterile Nutrient broth (Oxoid) using the same conditions, from which independent RNA extractions were prepared and then pooled to act as a control for normalization of the hybridization levels to oligonucleotide spots in the microarray experiments. After centrifugation at 12,000 x *g* for 15 min, at 4 °C, in a Beckman JS-21 centrifuge using a JA14 rotor, the pellets were pooled. Total RNA were isolated by using TRIzol® Max™ Bacterial RNA isolation kit (Invitrogen, Paisley, UK) following manufacturer's instructions. The pre-heated Max Bacterial Enhancement Reagent at 95 °C was added by pipetting up and down to inhibit RNase activity and incubated at 95 °C for 4 min. TRizol® reagent was added to the lysate and incubated at room temperature for 5 min. Phase separation was proceeded by adding cold chloroform (Sigma-Aldrich) and mixed by shaking the tube vigorously by vortex for 15 sec. The mixture was incubated at room temperature and centrifuged at 12,000 g for 15 min at 4 °C. The mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless aqueous phase containing RNA. The aqueous phase (400 µl) was transferred into a fresh tube for proceeding to precipitating RNA by adding 0.5 ml cold isopropanol (Fluka) and mixing by inverting the tube, incubated at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was removed carefully without disturbing the RNA pellet. The pellet was resuspended in 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 min at 4 °C. The RNA pellet was resuspended in 50 µl RNase free water by pipetting up and down after air-dry. The RNA was treated by DNase I (QIAGEN) to digest away contaminated DNA and purified by using the RNeasy mini kit (QIAGEN) following manufacturer's instructions. Briefly, the RNA was cleaned up by buffer RLT (containing quinidine thiocynate) and subsequently with 96-100% of ethanol by using RNeasy mini spin column and centrifuge at 8000 x g for 15 sec. each step. The RNA was then cleaned twice by buffer RPE (containing ethanol) using the mini spin column and centrifuge at 8000 x g for 15 sec. and 2 min, respectively. The RNA was collected into RNase free water. RNA concentration was determined by using Nanodrop ND-1000 spectrophotometer (Fisher Scientific). The RNA was stored at -80 ^{o}C.

2.10 cDNA synthesis, labeling and hybridization

Reverse transcription and labeling with Alexa Fluor® AF555 and AF647 dyes (Molecular Probes, Invitrogen, Eugene, OR, USA) was carried out using a SuperScript™ direct cDNA labelling kit (Invitrogen) according to the manufacturer's instructions following an incubation for 3 h at 46 ºC, the hydrolysis reaction was performed by adding 15 µl of 1 M NaOH and incubating it at 70 ºC for 15 min. Neutralisation of the reaction was performed by adding the same quantity of 1 M HCl. Synthesised cDNA was purified by using QIA quick PCR purification kit (QIAGEN) and subsequently ethanol precipitation to concentrate samples. The eluted cDNA was precipitated with ethanol and was stored at -20 ºC prior to labelling. To 5 µl of the cDNA, 3 µl of 2.9 M of sodium bicarbonate (Sigma Aldrich, UK) was added and Alexa Fluor (Fisher, UK) dyes were added by dissolving in 2 µl of dimethyl sulfoxide (DMSO; Sigma Aldrich, UK). This was incubated for 1 h in the dark, and the labelled cDNA was purified by using the QIA PCR Purification kit according to the manufacturer's instructions. The synthesized cDNA was quantified by using a Nanodrop spectrophotometer. Labelling efficiency was measured by calculating the frequency of incorporation (FOI) of the dye defined as the number of labeled nucleotides incorporated per 1000 nucleotides of cDNA (nucleotides / dye ratio = pmol dNTP / pmol dye) and should be >15. The experimental samples were competitively hybridized against pooled HPC5 cDNA control, prepared as described above (2.9), labelled with AF555.

The arrays are composed of synthetic oligonucleotide 70-mers (Eurofins MWG Operon, Germany): Tm 71 °C ± 5 °C, located from 3′ end of gene >70 bp, poly (N) tract <8 bp, stem length in potential hairpin <8 bp contiguous bases common to any non-self ORF <20 bp with cross-hybridisation identity to all other genes <70%. The slides used for the microarray were γ-aminopropyltriethoxysilane (γ APS) coated, A^+ bar coded SCHOTT Nexterion® slides (Item number 1064875). The slides were printed according to a campy dense array gal file by Microgrid II GOD robot using TAS software version V2.4.03. The slides had 2500 pins with a 26 × 26 pin grid configuration. The spacing between the spots was 0.165 mm and the slides were prepared at 60%

humidity. The slides were rehydrated by incubation at room temperature in a humidity chamber for 30 min followed by 30 min incubation at 60 °C to dry. The slides were blocked in a solution containing 100 mM ethanolamine, 1 M Tris (pH 9.0), and 0.1% SDS (Sigma Aldrich) for 15 min at 50 °C, then thoroughly rinsed with water and spun dry before use. Hybridizations of microarray slides were performed at the Queens Medical Centre, University of Nottingham. The slides represent oligonucleotide microarray contains the gene repertoires (locus tags) from *C. jejuni* 11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000), *C. jejuni* RM1221 (Fouts *et al.*, 2005), *C. coli* RM2228 (Fouts *et al.*, 2005), and all new non-redundant locus tags associated with *C. jejuni* and *C. coli* genomes available in the GenBank database at the time of construction (May 2009). In addition, oligonucleotides representing all the *Campylobacter* bacteriophage genes for representative class II (CP220 Accession No: FN667788.1; Timms *et al.*, 2010) and class III (CPX Accession No: JN132397.1; Al-kandari *et al.*, unpublished) bacteriophage.

The microarray slides were printed in triplicate and each of the hybridizations were repeated to yield two technical replicates for each sample containing three independent spots for each oligonucleotide probe. The microarray slides were scanned using Axon Genepix 4200AL scanner (Molecular Devices Corporation, Sunnyvale, CA, USA). Fluorescent spot intensities were analyzed using Genespring GX11 software (Agilent Technologies UK Ltd, Wokingham, UK) and the data normalized against the AF555-labelled controls (Lowess), merged and expressed in Log_2 values. The normalized data were compared between wild type and the CSLC strains. Differentially expressed genes were identified on the basis of $P<0.05$ by ANOVA and using the Benjamini

Hochberg False Discovery Rate correction (Benjamini and Hochberg, 1995) and exhibiting a minimum of a two-fold change in transcript abundance in all three independent biological replicates. At the time of writing these microarray data have not been published are not available in the Gene Expression Omnibus (GEO) database.

2.11 Identification CSLC phage DNA in CSLC strains

To identify the CSLC phage DNA in the CSLC strains, DNA of the CSLC strains was prepared as described in 2.5.1. Primers were designed on a non-coding region of CP30 that distinguish the DNA from other phages and synthesized by Eurofins MWG Operon (Ebersberg, Gemany) and the sequence of the primers shown below:

Name	Sequence
CP30tp-Frd	5’-GAAATTGCATGAATCAATGCGG-3’
CP30tp-Rev	5’-GTTTGTCACTTTTTATGGAACTAC-3’

PCR reactions were performed in a total volume of 50 µl consisting of 1 µl of DNA template (100 ng/µl), 5 µl of 2.5 mM dNTPs (Promega), 5 µl of 25 mM $MgCl_2$, 1 µl of each primer (listed above), 5 µl of 10 x buffer (500 mM tris-HCl [pH 9.2], 140 mM ammonium sulphate, 17.5 mM magnesium chloride), variable volume of RO water and 1 Unit of *Taq* DNA polymerase (Thermo Scientific) in a thin-walled 0.5 ml PCR tubes (Fisher). PCR conditions were as follows: predenaturation at 96 °C for 3 min for 1 cycle and 30 cycles of denaturation at 96 °C for 0.5 min, annealing at 60 °C for 0.25 min and elongation at 72 °C for 1 min before a final elongation at 72 °C for 7 min and storage of the PCR products at 4°C for analysis.

2.12 Analysis of different DNA sequences of CP8 and CP30

DNA sequences discriminating between bacteriophage CP8 and CP30 were identified to confirm the identities of the initial phages used for treatment with those produced by the CSLC cultures. The primers were designed on a unique region in the CP8 DNA sequence that distinguishes it from other phage sequences available. The oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and the sequence of primers shown below:

Name	Sequence
CP853B	5'-TCG TTA TAC CAC GGA TAT AG-3'
CP854B	5'-TAT AGG AGG GTT GTG AAA TG-3'

Phage DNA was prepared as described in 2.5.2. PCR reaction mixtures were performed by following in total 50 µl consisting of 1 µl of DNA template (100 ng/µl), 5 µl of 2.5 mM dNTPs (Promega), 5 µl of 25 mM $MgCl_2$, 1 µl of each primer (above list), 5 µl of 10 x buffer (500 mM tris-HCl [pH 9.2], 140 mM ammonium sulphate, 17.5 mM magnesium chloride), variable volume of RO water and 1 U of *Taq* DNA polymerase (Thermo Scientific) in thin-walled 0.5 ml PCR tubes (Fisher). PCR conditions were as follows: predenaturation at 96 °C for 5 min for 1 cycle and 30 cycles of denaturation at 96 °C for 1 min, annealing at 52 °C for 0.5 min and elongation at 72 °C for 5 min before a final elongation at 72 °C for 10 min and storage of the PCR products at 4°C for analysis. Sequencing of DNA

DNA sequencing was performed on the PCR products after purification using a PCR purification kit (Qiagen). DNA sequencing was performed by MWG Biotech AG (Ebersberg, Germany) using their ValueRead service (dye terminator chemistry).

DNA sequencing results were received as chromatograms. These were analysed using Chromas v1.45 (Conor McCarthy, Griffith University, Australia - the latest version of this program can be obtained from http://www.technelysium.com.au/chromas.html) and the raw sequence data exported to the BioEdit sequence alignment tool (Hall, 1999) for further analysis. Sequences were compared to database sequences using the BLAST suite of tools (Altschul *et al.,* 1997) which was located at http://www.ncbi.nlm.nih.gov/blast/.

2.13 Analysis of the inactivation of CP8 with antiserum

The *C. jejuni*, CP8PL (CSLC phage) and CSLC cultures were treated with either rabbit pre-immune serum or serum raised against bacteriophage CP8 following dilution with PBS buffer at a final concentration 1:15. Viable *Campylobacter* counts and bacteriophage titres were determined post treatments as described above in sections 2.2.2 and 2.3.4 respectively.

CHAPTER 3

BACTERIOPHAGE-MEDIATED DISPERSAL OF *CAMPYLOBACTER JEJUNI* BIOFILMS

3.1 Introduction

Campylobacter jejuni is a Gram-negative thermotolerant microaerobic pathogen that causes human gastroenteritis worldwide. In the United States, it is estimated there are 2 to 3 million people infected each year (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/campylobacter/technical.html#incidence), whereas over 190,000 cases were reported in the European Union (www.efsa.europa.eu/de/scdocs/doc/1533.pdf). Infection usually causes self-limiting diarrhoea but can occasionally lead to serious illnesses such as Guillain-Barré syndrome and reactive arthritis (Stern, 2001). The majority of human disease is attributed to *C. jejuni* whilst infection by other members of the *Campylobacter* genus is relatively infrequent. Consumption of undercooked or contaminated meat or poultry is generally believed to be the cause of a significant proportion of the total number of infections (Wilson *et al*., 2008).

Bacteria in their natural environments frequently form biofilms that are composed of single or multiple bacterial species embedded in a surface extra-cellular polymeric matrix, which can include polysaccharides, protein, nucleic acid, phospholipid and teichoic acid (Chmielewski *et al*., 2003; Costerton *et al*., 2004; O'Toole *et al*., 2000; Sutherland, 2001). Thermophilic campylobacters may be considered as part of the commensal communities of poultry (Hendrixson and DiRita, 2004) and many other wild and farmed animals. Typically these bacteria form densely packed parcels of cells within the luminal crypts, attached to the mucus but not directly to the epithelium (Beery *et al*., 1988). In the laboratory, *Campylobacter* has been shown to be capable of forming three distinct types of biofilm (Joshua *et al*., 2006): the surface-attached type, a pellicle type in liquid culture and an unattached aggregate or floc type, probably similar to that observed in the luminal crypts *in vivo*. Biofilm formation may help the bacteria to overcome environmental stresses such as aerobic conditions, desiccation, heating, disinfectants and acidic conditions (Murphy *et al*., 2006; Reeser *et al*., 2007), and thereby increase their potential to cause disease. *Campylobacter* strains have been demonstrated to attach or associate with biofilms containing multiple organisms such as Gram-positive bacteria. For example, campylobacters comprise part of the mixed microflora of biofilms present in water lines and on many types of surface, including materials commonly used in industrial settings such as polyvinyl chloride (PVC) and stainless steel (Buswell *et al.*, 1998; Sanders *et al*., 2007; Sanders *et al*., 2008; Trachoo *et al*., 2002; Zimmer *et al*., 2003). Campylobacters have also been demonstrated to form monoculture biofilms on various surfaces under microaerobic (Dykes *et al*., 2003; Gunther IV *et al*., 2009; Joshua *et al*., 2006; Kalmokoff *et al*., 2006; Moe *et al*., 2010; Reeser *et al*., 2007) and aerobic conditions (Reuter *et al*., 2010). Under conditions of biofilm formation *Campylobacter* strains have been reported to be enhanced in their ability to resist antimicrobial agents (Joshua *et al*., 2006; Kalmokoff *et al*., 2006). Sessile cells of *C. jejuni* within biofilms exhibit a greater resistance to treatments with the anitbiotics ciprofloxacin or erythromycin compared to non-biofilm planktonic cells, possibly because these antibiotic agents do not diffuse through *C. jejuni* biofilm EPS (Lu *et al*., 2012). The formation of biofilms has therefore been suggested as a mechanism by which *Campylobacter* are able to survive harsh environments, and is probably a contributing factor in their persistence and colonization of the digestive tracts of poultry and other animal species.

Bacteriophages represent an alternative to conventional antimicrobial agents to control food-borne microorganisms. The abilities of bacteriophages to reduce food-borne bacteria as a means of bio-sanitization and phage therapy have been investigated. *Campylobacter* bacteriophages have been shown to reduce viable *Campylobacter* cells on chicken skin (Atterbury *et al*., 2003; Goode *et al*., 2003) and on raw and cooked beef (Bigwood *et al*., 2008). Bacteriophage therapy has been shown to reduce *Campylobacter* in the intestines of chickens (El-Shibiny *et al*., 2005; El-Shibiny *et al*., 2009; Loc Carrillo *et al*., 2005; Scott *et al*., 2007a; Wagenaar *et al*., 2005). Acquisition of bacteriophage resistance is commonly cited as a disadvantage of bacteriophage; however, implementation of the treatment of broiler chickens at the end of the rearing cycle should ensure maximal effect of the treatment and the removal of resistant survivors upon depopulation of the barn (El-Shibiny *et al*., 2009). Resistant *Campylobacter* isolates recovered from chickens treated with bacteriophage have been reported to be compromised in their ability to recolonize chickens

(Loc Carrillo *et al*., 2005), however alternative studies using bacteriophage cocktails have been reported to lead to resistant types capable of re-colonization (Carvalho *et al*., 2010).

The application of bacteriophage to reduce biofilms of several different bacterial species has been demonstrated (Hibma *et al*., 1997; Hughes *et al.*, 1998; Sillankorva *et al*., 2004). Moreover, engineered bacteriophage enzymes have been employed to disperse biofilms by breaking down components of the extra-cellular polymeric matrix (Lu and Collins, 2007). However, when stainless steel adherent *Escherichia coli* O157:H7 were treated with bacteriophages there was a reduction in the viability of the exposed adhered cells but not in the cells within the biofilm itself due to inability of bacteriophage to gain access through the biofilm (Sharma *et al*., 2005).

In this chapter the optimal conditions required for the formation of stable biofilms by *C. jejuni* have been investigated as a prerequisite to assess the impact of bacteriophage. The effect of bacteriophage treatment of *Campylobacter* biofilms had not previously been studied, and therefore this study was undertaken to assess the ability of *Campylobacter* specific bacteriophage to prey on bacteria *in situ* and disperse *Campylobacter* biofilms. Bacteriophage treatment of bacteria is known to involve non-linear kinetic phenomena which are independent of the concentration of bacteriophage applied on the bacteria population (Payne and Jansen, 2001). However, the bacteriophage treatment is dependent on the adsorption properties of the bacteriophage to the host. The phage infection cycle within a biofilm environment has been considered to consist of three stages: settlement on the biofilm surface, production of phage progeny inside the biofilms and emigration of phage progeny out of the current focus infection (Gallet *et al*., 2009). High adsorption rates are beneficial for phage settlement but could be detrimental to phage productivity and emigration (Gallet *et al*., 2009). How these factors influence the formation and maintenance of biofilms *in vivo* or in the environment will require further research.

3.2 Results

3.2.1 Attachment and biofilm formation

The biofilm forming ability of the type strain *C. jejuni* NCTC 11168 has been examined at 37 and 42 ºC under microaerobic conditions over 7 days. The viability of attached cells on a glass surface at these temperatures were not significantly different (P>0.05, paired t-test) (Figure 3.1A). The quantities of biofilm produced at both temperatures were assessed by staining with crystal violet and measuring the optical density of the stained material solubilised in ethanol. At 5 days post-inoculation the quantity of biofilm material recovered from the glass surface at 37 ºC was higher than that at 42 ºC (Figure 3.1B). Therefore, all subsequent experiments of biofilm formation by *C. jejuni* and bacteriophage treatment were performed at 37 ºC after 5 days incubation in Muller-Hinton broth under microaerobic conditions.

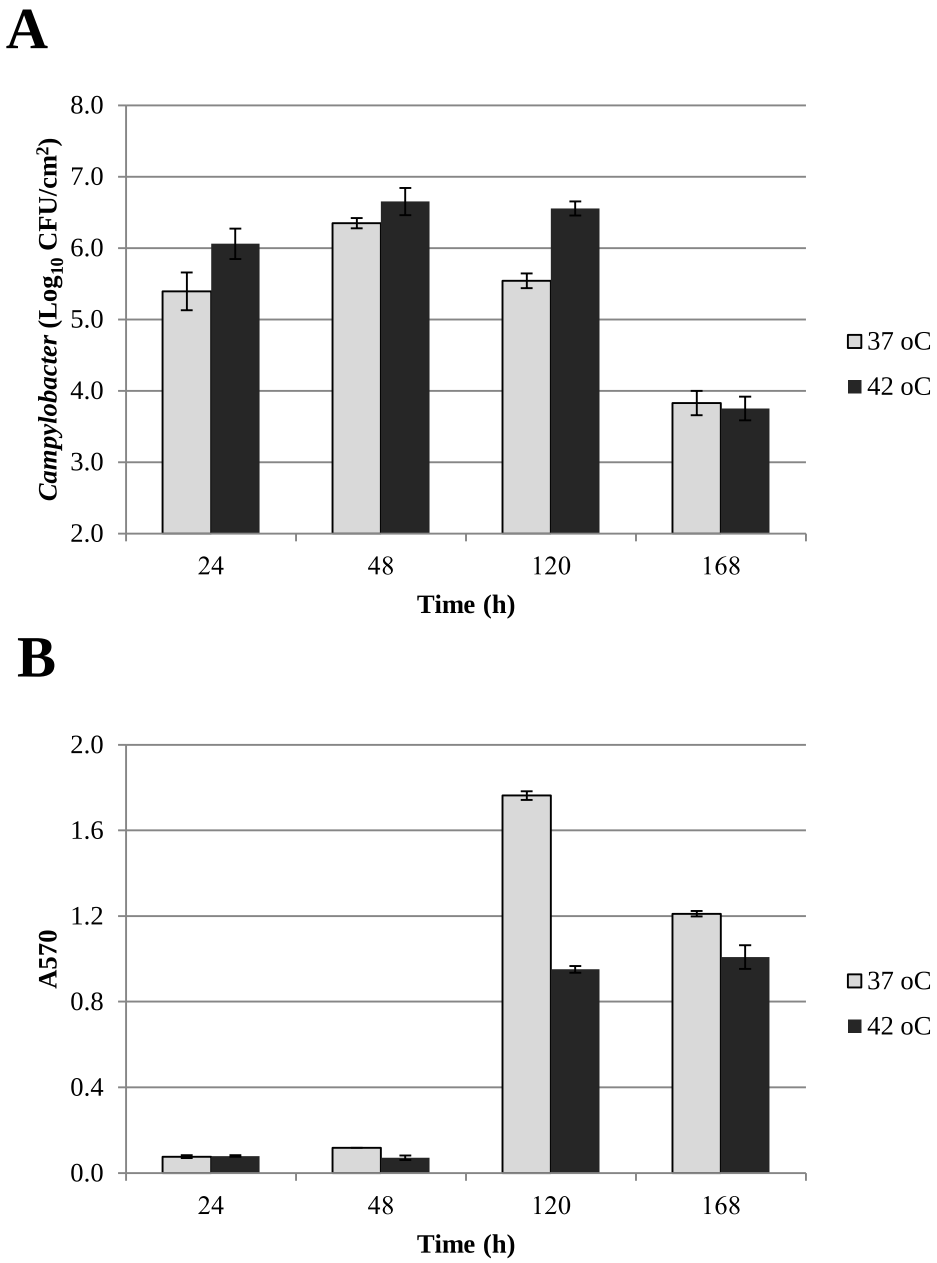


Figure 3.1-Viablity of *C.jejuni* NCTC 11168 attached on a surfaces at 37ºC and 42ºC under microaerobic conditions (A) and the quantity of biofilm produced as measured by recording the optical density at 570 nm after ethanol solubilisation of crystal violet stained surfaces (B).

The abilities to attach and form biofilms of *C. jejuni* strains NCTC11168, PT14 and HPC5 were investigated to monitor the development of *Campylobacter* biofilms at 37 ºC under microaerobic conditions (Figure 3.2). Figure 3.2A shows that *C. jejuni* strains NCTC 11168, PT14 and HPC5 were able to initiate attachment on a surface after incubation for 24 h with an increase in the viable count (Figure 3.2A). Strains NCTC 11168 and PT14 showed no significant difference in viability over 5 days but at 7 days post-incubation, the cells began to detach from the surface, resulting in a loss of viability. On the other hand, the viability of the planktonic cells remained similar throughout the experiment (Figure 3.2B). The quantity of biofilm increased under microaerobic conditions at days 5, suggesting that *C. jejuni* produces extracellular polymeric materials to form mature biofilms, however a reduction in the recoverable biofilm was observed at 7 days. Interestingly, *C. jejuni* HPC5 has the least ability to produce extracellular matrix materials when compared to the other strains. Sulaeman *et al*. (2010) reported that 46 strains of *C. jejuni* and *C. coli* displayed different adhesion capabilities ranging from no adhesion to strong adhesion. The ability of *C. jejuni* to attach on surfaces varied depending on the surface charge and composition of the support (Nguyen *et al*., 2011).

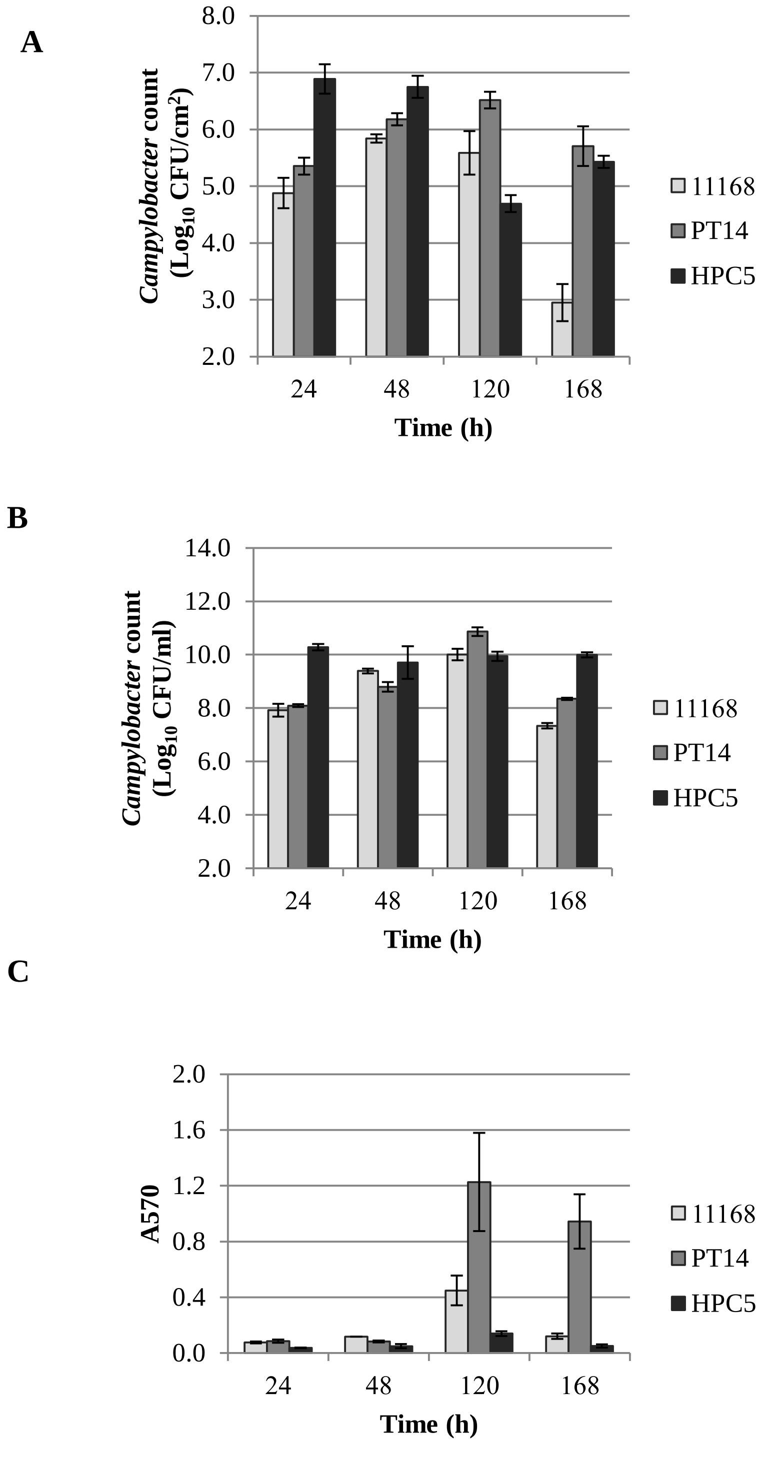


Figure 3.2-Viable count of attachment (A) and planktonic cells (B) of *Campylobacter jejuni* NCTC 11168, PT14 and HPC5 at 37 ºC under microaerobic condition and biofilm formation determined by recording the optical density at 570 nm after ethanol solubilisation of crystal violet stained surfaces (C).

3.2.2 Viability of *Campylobacter* cells within biofilms following bacteriophage treatment

Biofilms that were allowed to form on glass surfaces over 5 days (time of maximum observed attachment and biofilm formation) were treated with either bacteriophage CP8 or CP30, at a multiplicity of infection (MOI) of approximately 1 PFU per viable *Campylobacter* cell (Figure 3.3). Planktonic cultures from these biofilms were recovered and treated simultaneously at an equivalent MOI. Initial viable counts within biofilms of *C. jejuni* NCTC 11168 were observed to vary batch to batch over the range Log_{10} 4 CFU/cm^2 to Log_{10} 6 CFU/cm^2. A minimum of 5 independent experiments were therefore performed in which each experiment contained 3 replicate control and treated biofilms from the same batch of glass coverslips. The data are recorded as the means and standard deviations of replicates within these independent experiments. Viable cell counts from the *C. jejuni* NCTC 11168 biofilm were significantly reduced by 3 Log_{10} CFU/cm^2 at 2 h post-infection by either of the phages compared to mock treated biofilms (P <0.05; Figure 3.4A). In contrast with the bacteriophage treated biofilms the control (mock treated) *C. jejuni* NCTC 11168 biofilms exhibited an increase in the viable count over the period to reach approximately Log_{10} 5.5 CFU/cm^2 at 8 h. The initial fall in the bacteriophage treated biofilms was followed by a rapid recovery in the number of attached viable cells that could not have occurred by normal cell division alone within the 2 h period. The observation may be associated with bacteriophage mediated detachment of *Campylobacter* containing biofilm from the glass surface and its loss in the primary surface washes before agitation to recover the biofilm and enumeration of the bacteria. Later time points would have sufficient time for re-attachment of *Campylobacter* containing biofilm and therefore record higher viable counts. The CP8-treated culture experienced a further decline in the viable cell numbers over the next 24 h to barely detectable levels.

Bacteriophage treatments of *C. jejuni* PT14 biofilms with CP8 resulted in a reduction of 1 Log_{10} CFU/cm^2 in the viable count at 24 h compared to the mock-treated control (Figure 3.5A). Treatment of *C. jejuni* PT14 biofilms with bacteriophage CP30 by comparison produced a 2.5 Log_{10} CFU/cm^2 reduction in the viable count 4 h post-infection, before experiencing a temporary recovery at 8 h. No significant reductions beyond that recorded for the control culture were observed over the 4 h post-infection for *C. jejuni* PT14 biofilms treated with CP8 bacteriophage (Figure 3.5A).

Bacteriophage treatment of *C. jejuni* HPC5 biofilms with CP30 or CP8 resulted in reductions in the viable counts of approximately 1 and 1.5 Log_{10} CFU/cm^2 at 2 h and 7 h, respectively when compared to the mock infected control biofilms (Figure 3.6A). However, the counts recorded for the attached bacteria of experimental and control samples exhibited a reduction in viability at 24 h. The *C. jejuni* strain HPC5 notably produced less crystal violet stainable extracellular polymeric matrix material than the other *C. jejuni* strains used in this study. Over the course of the experiment bacteria embedded in this matrix could be more easily detached and afforded less protection from desiccation or atmospheric oxygen. Planktonic cells removed from the liquid phase of the *C.*

jejuni NCTC 11168, PT14 and HPC5 static cultures were treated in parallel with bacteriophages CP8 and CP30 (Figure 3.4B; 3.5B and 3.6B). The *C. jejuni* PT14 treated with CP30 showed a significant reduction of 1 Log_{10} CFU/ml (P <0.05) in the viable count at 4 h, before recovery to levels recorded for mock-treated controls. Otherwise, the *C. jejuni* HPC5 treated with CP8 and CP30 exhibited a reduction in viability of 2 Log_{10} CFU/ml at 24 h post-infection (Figure 3.6B).

Bacteriophage titres following application to the biofilms declined probably as a result of binding host cells but thereafter remained static over the experimental period (broken lines in Fig.3.4A and 3.5A). However, it is notable that bacteriophage CP8 and CP30 bind the *C. jejuni* NCTC 11168 host in the biofilm or planktonic phase to a greater degree than *C. jejuni* PT14 (Figure 3.4A to 3.5B).

Summary of bacteriophage treatment of Campylobacter biofilms

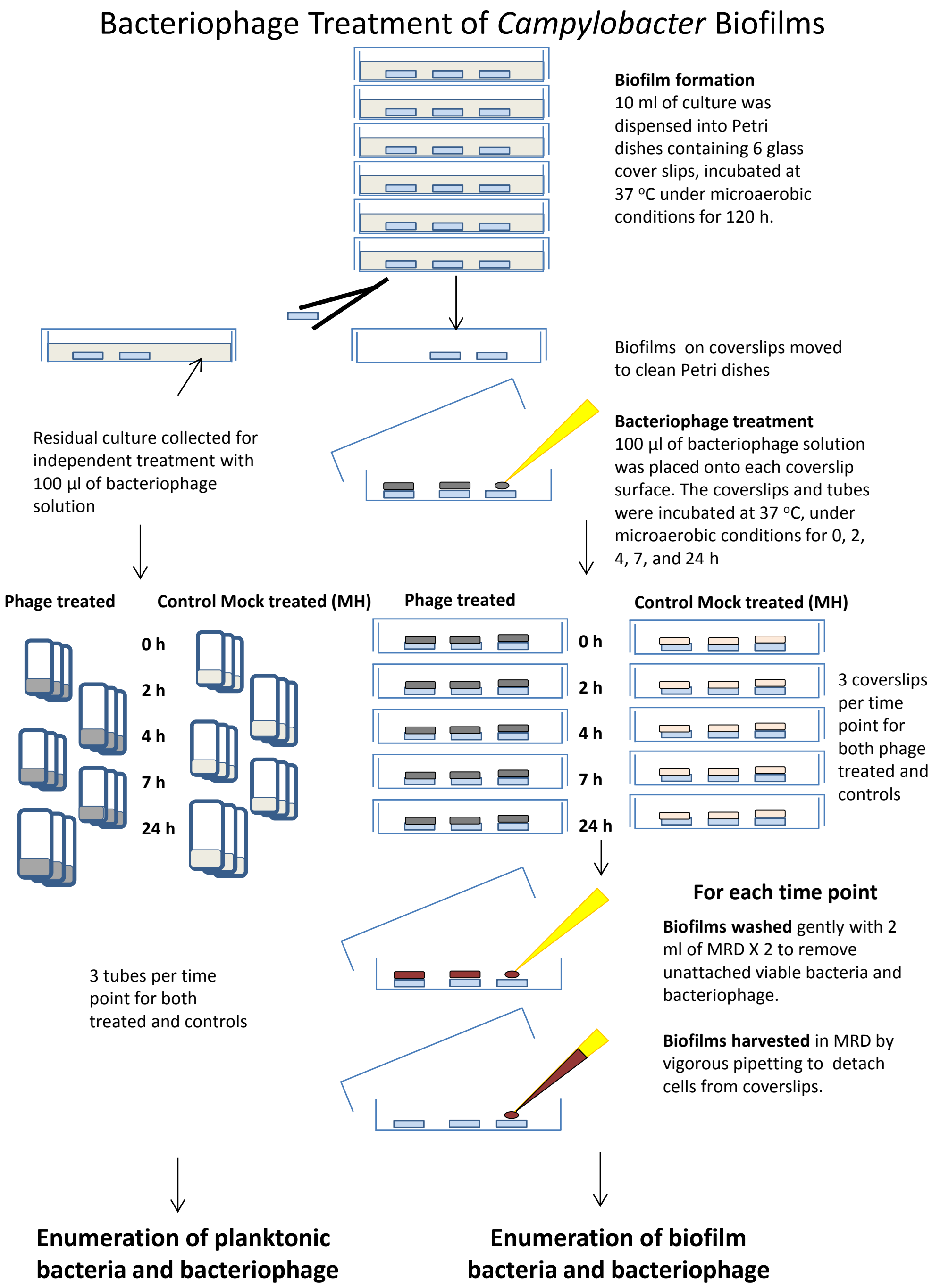


Figure 3.3-Flow diagram of the protocol adopted for the bacteriophage treatment of *Campylobacter* biofilms

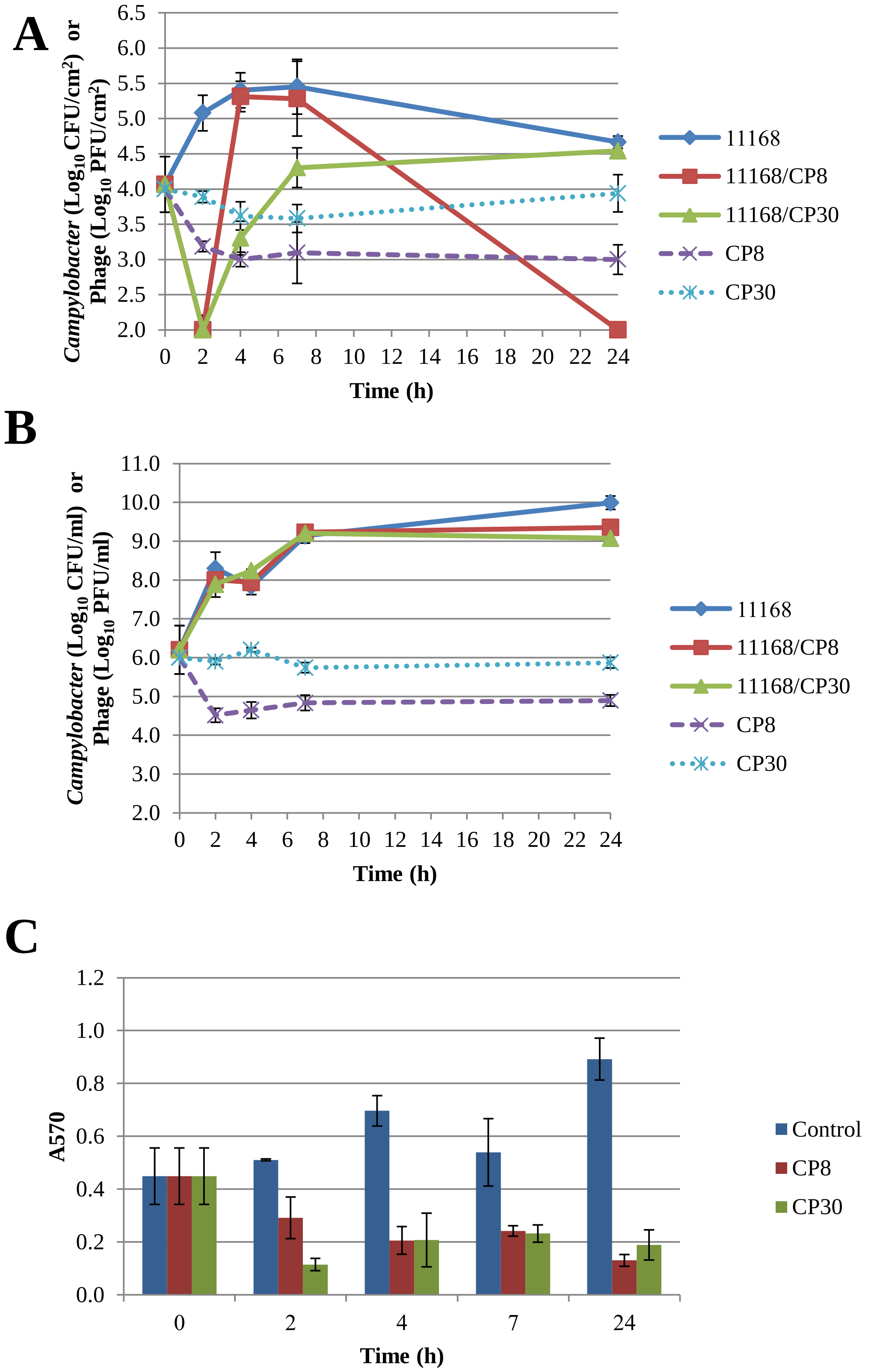


Figure 3.4-Viablity of *C. jejuni* NCTC11168 following bacteriophage treatment of biofilms (A) compared to that following treatment of planktonic cultures (B). The bar chart (C) compares the effect of bacteriophage treatment of biofilms, quantified using crystal violet staining. For the biofilm cultures the limit of detection is 1.3 Log_{10} CFU or PFU/cm^2 and for planktonic cultures 2.4 Log_{10} CFU or PFU/ml.

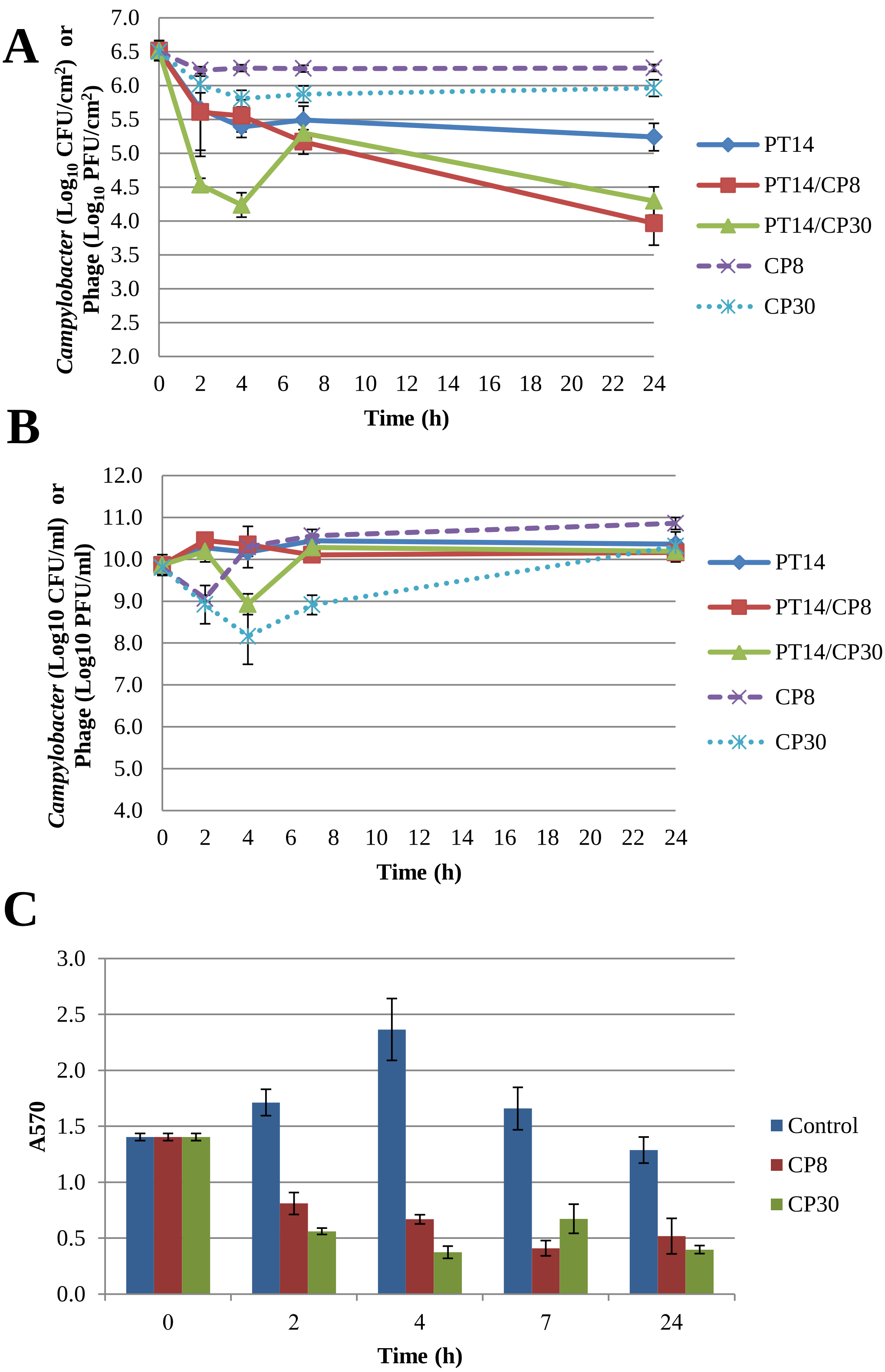


Figure 3.5-Viability of *C. jejuni* PT14 following bacteriophage treatment of biofilms (A) compared to that following treatment of planktonic cultures (B). The bar chart (C) compares the effect of bacteriophage treatment of biofilms, quantified using crystal violet staining. For the biofilm cultures the limit of detection is 1.3 Log_{10} CFU or PFU/cm^2 and for planktonic cultures 2.4 Log_{10} CFU or PFU/ml.

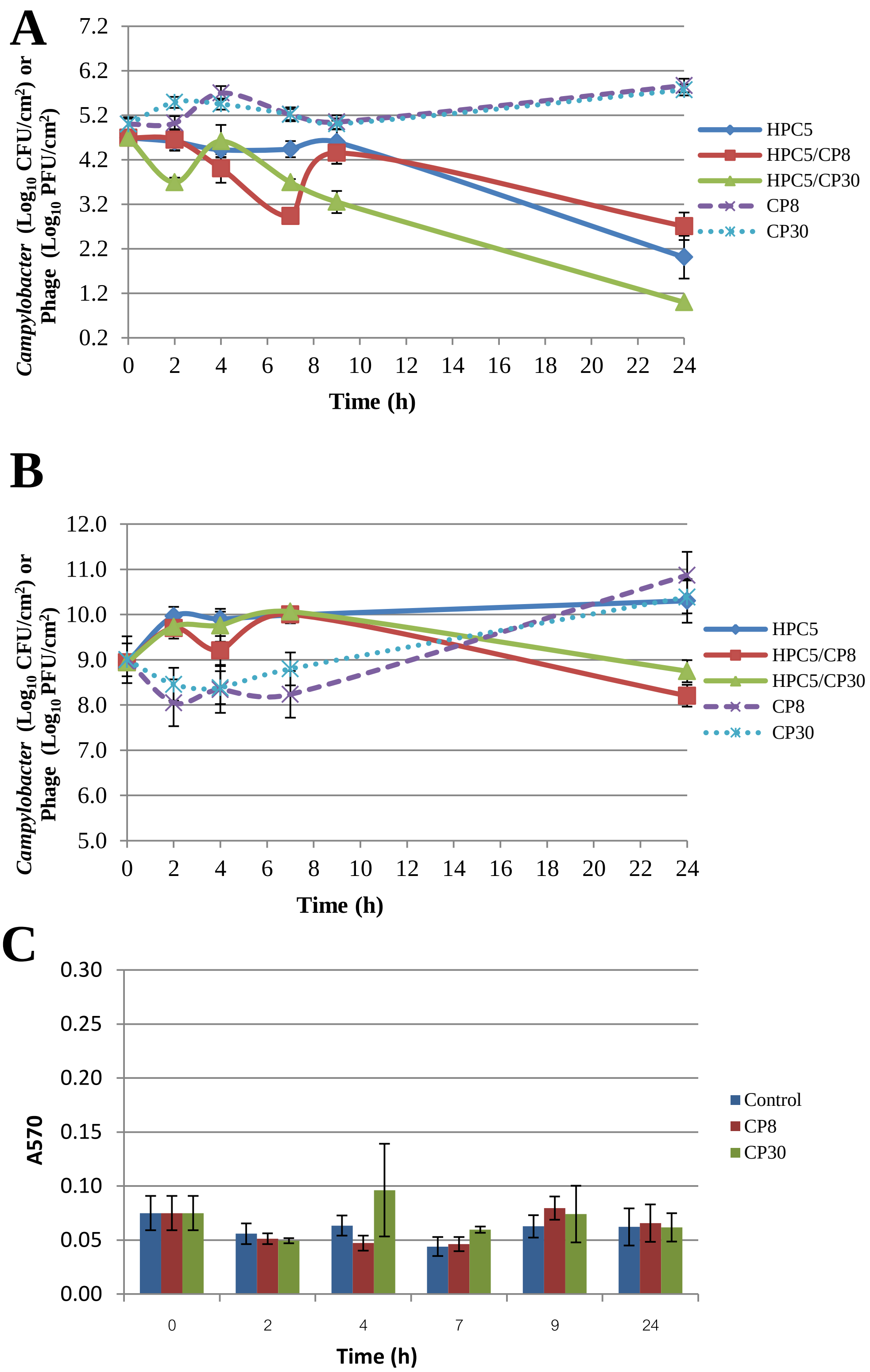


Figure 3.6-Viability of *C. jejuni* HPC5 following bacteriophage treatment of biofilms (A) compared to that following treatment of planktonic cultures (B). The bar chart (C) compares the effect of bacteriophage treatment of biofilms, quantified using crystal violet staining. For the biofilm cultures the limit of detection is 1.3 Log_{10} CFU or PFU/cm^2 and for planktonic cultures 2.4 Log_{10} CFU or PFU/ml.

3.2.3 Evidence for reduction of biofilms following bacteriophage treatment

Reductions in the biofilm matrix density following bacteriophage treatment were assessed by measuring the optical density of ethanol solubilized material after staining with crystal violet and comparing these with mock-treated controls. The results showed that bacteriophage-treatment of either *C. jejuni* PT14 or 11168 significantly reduced the attached biofilm material at 2 to 24 h time points after treatment ($P < 0.05$; Figure 3.4C and 3.5C). Whereas, the *C. jejuni* HPC5 was not significantly different ($P > 0.05$; Figure 3.6C).

Bacteriophage treated and untreated *Campylobacter* biofilms were examined by transmission electron microscopy (TEM). Alcian blue staining allowed visualization of the capsular polysaccharide surrounding vegetative cells within the biofilm matrix (Karlyshev *et al*., 2001). Examination of the TEM images revealed that the bacteriophage treatment disrupted the biofilms formed by both *Campylobacter* strains. The TEM images also allowed detailed examination of the structure the *Campylobacter* biofilms which revealed them to consist of few vegetative rod-shaped cells. There are many circular/oval imaged regions stained with alcian blue which could be coccoid or rods in transverse section, which could not be discriminated due to the sectioning of the biofilm embedded bacteria in these TEM images. Interestingly, in the bacteriophage-treated biofilms it was possible to identify *Campylobacter* cells in longitudinal section where the membranes were distorted and swelling outwards as if on the point of lysis (indicated by arrows in Fig.3.7 b, c, e, and f) that were not observable in the non-bacteriophage treated biofilms (Figure 3.8a and 3.8d). The crystal violet staining and TEM images showed that the biofilms after treated with both bacteriophages were decreased when increased incubation time. The trends of decreasing quantities of biofilms were similar by both methods. According to Djordjevic *et al*. (2002) reported that biofilm assay with crystal violet staining and quantitative epifluorescence microscopy showed similar trends. Reuter *et al*. (2010) also reported that the *Campylobacter* biofilms stained with crystal violet and Congo red resulted in the same trends.

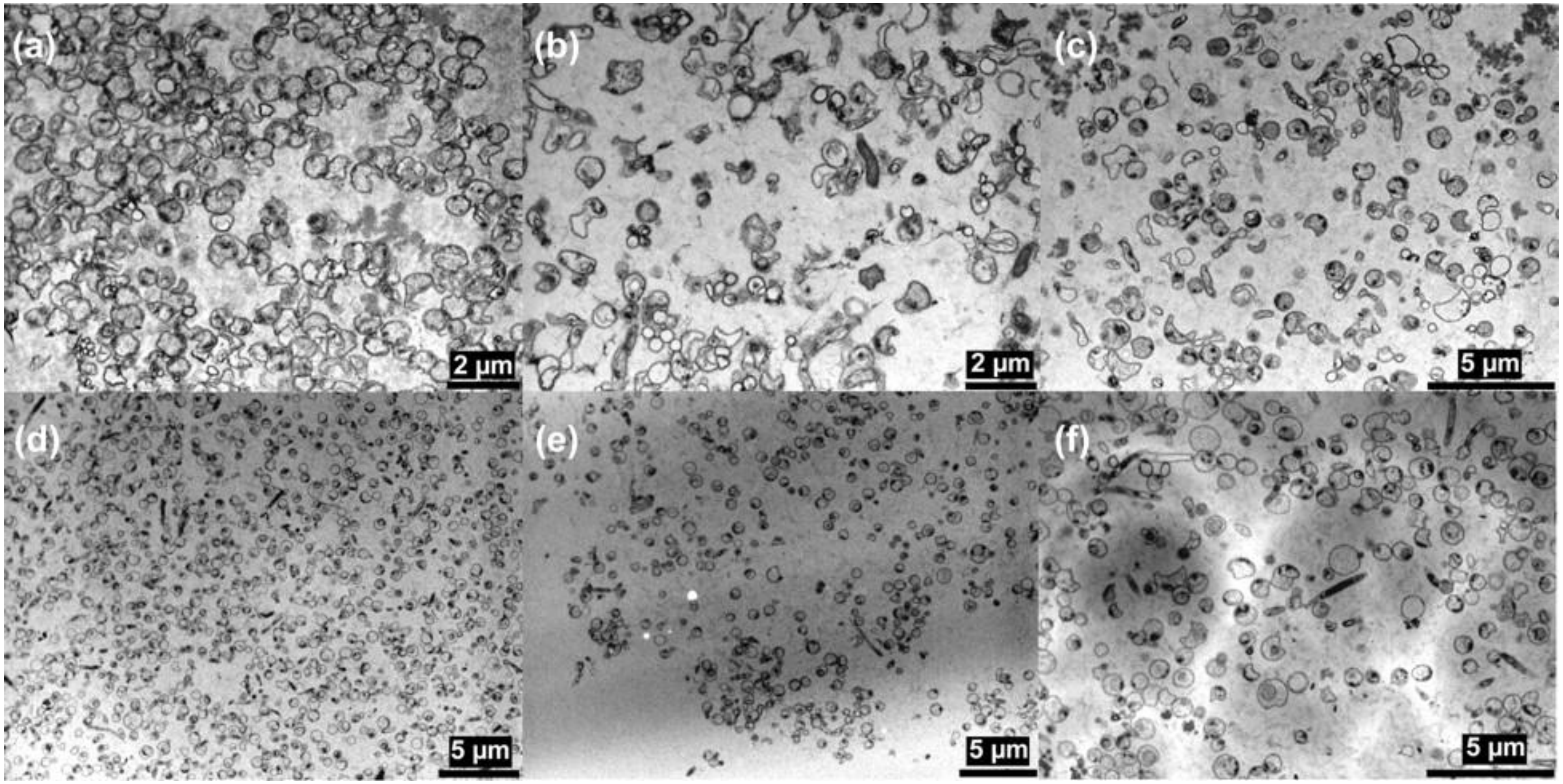


Figure 3.**7**-Transmission microscopy to show the effect of treatment with bacteriophage CP8 and CP30 on biofilms of *C. jejuni* NCTC11168 (a, b, and c) and PT14 (d, e, and f): controls without phage treatment (a and d), bacteriophage CP8 treatment after 2 h (b and e), and bacteriophage CP30 treatment after 2 h (c and f). Section was thinned and stained with alcian blue, indicating the extracellular polysaccharide.

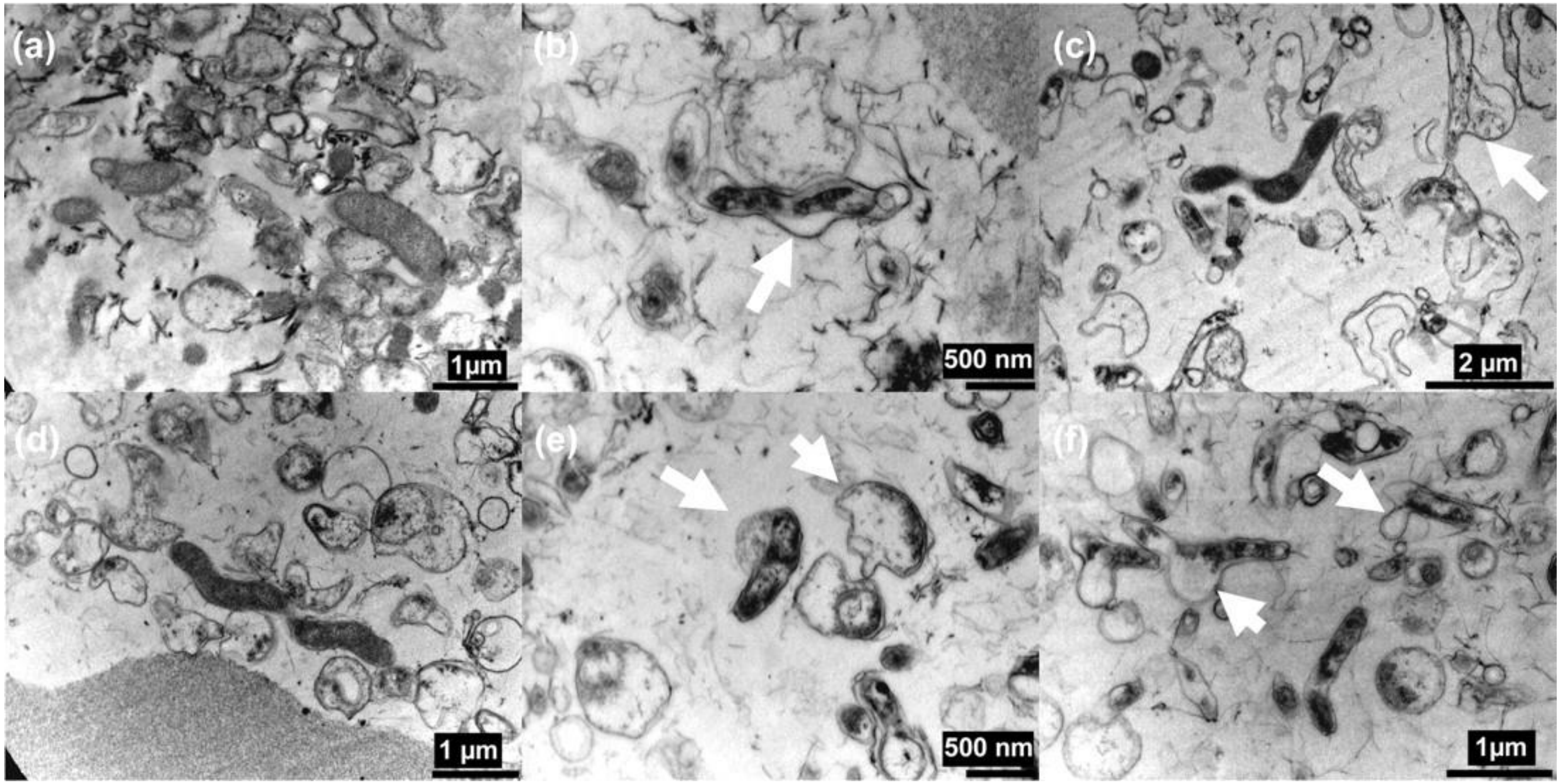


Figure 3.8-Transmission microscopy to show the effect of treatment with bacteriophage CP8 (b and e) and CP30 (c and f) on *C. jejuni* NCTC11168 (a, b, and c) and PT14 (d, e, and f) biofilms after 24 h compared to effects in controls without phage treatment (a and d). Arrows indicate regions of *Campylobacter* cells showing membrane distortion. Section was thinned and stained with alcian blue, indicating the extracellular polysaccharide.

3.2.4 Frequency of phage resistance in bacteriophage treated biofilms.

Viable cells recovered from the biofilms following bacteriophage treatment were tested for acquired resistance. There was very clear disparity between the sensitivity to bacteriophage of the three different strains of *Campylobacter* tested following bacteriophage treatment. All the isolated viable cells from the *C. jejuni* PT14 biofilms remained susceptible to either of the bacteriophages applied. For the *C. jejuni* HPC5 bacteriophage-treated biofilms 7% (2/30) of viable cells recovered were resistant to either bacteriophage CP8 or CP30. In contrast, only 16 % (5/32) and 10 % (3/30) of the viable cells recovered from *C. jejuni* 11168 bacteriophage-treated biofilms remained susceptible to bacteriophages CP8 and CP30 respectively. This difference is not reflected in the planktonic replicates, in which the *C. jejuni* PT14 biofilms retained 8% (8/100) and 9% (9/100) susceptible bacteria for CP8 and CP30 bacteriophage respectively, with 9% (9/100) and 11% (being the 11/100) respective figures for *C. jejuni* 11168.

The motility of the majority of post treatment isolates was not affected on the basis of their ability to swarm on 0.4% Mueller-Hinton agar (Figure 3.9). However, a few isolates recovered after bacteriophage treatment of *C. jejuni* strains PT14 and HPC5 were not motile, which prompted further characterisation, and are the subjects of study in Chapter 4 that determines their status as carrier state life cycle strains (see Table 3.1 and Figure 3.9).

Three of each of the bacteriophage resistant types recovered post CP8 or CP30 treatment of *C. jejuni* 11168 were selected and tested for their ability to make biofilms on glass cover slips. The *C. jejuni* 11168 isolates resistant to CP8 were impaired in the ability to form biofilm based on the observation of significant reductions in the biofilm matrix density (estimated by staining with crystal violet and optical density measurements of the ethanol solubilized material; Figure 3.10A) when compared to the progenitor strain ($P < 0.05$). The *C. jejuni* 11168 isolates resistant to CP30 appeared to form biofilm but upon application of fresh MH broth these lost integrity such that the quantity of crystal violet stainable material recovered from the coverslip was reduced compared to wild type independent of any bacteriophage treatment (Figure 3.10B).

Table 3.1-Example of motility of wild type and isolates after treatment with bacteriophage

Samples	**Diameter (cm)**
C. jejuni NCTC11168	7.1
An isolate from *C. jejuni* NCTC 11168 treated with CP8	6.8
An isolate from *C. jejuni* NCTC 11168 treated with CP30	7.0
C. jejuni PT14	5.3
An isolate from *C. jejuni* NCTC PT14 treated with CP8	4.5
An isolate from *C. jejuni* NCTC PT14 treated with CP30	4.7
An isolate from *C. jejuni* NCTC PT14 treated with CP8	0 (Non-motile)*
An isolate from *C. jejuni* NCTC PT14 treated with CP30	0 (Non-motile)*
C. jejuni HPC5	3.8
An isolate from *C. jejuni* HPC5 treated with CP8	0 (Non-motile)*
An isolate from *C. jejuni* HPC5 treated with CP30	0 (Non-motile)*

* The carrier state life cycle strains

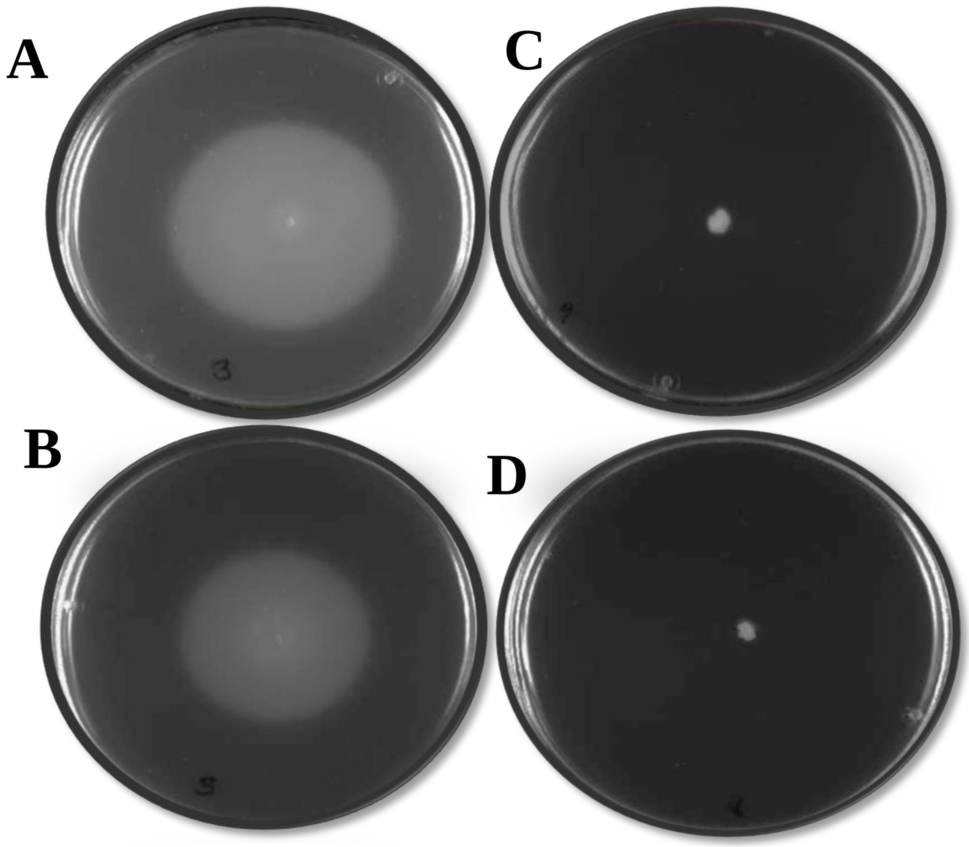


Figure 3.9-Motility of *Campylobacter* isolates recovered post treatment with bacteriophage on 0.4% MHA, (A) An isolate from *C. jejuni* NCTC 11168 treated with CP8, (B) An isolate from *C. jejuni* NCTC 11168 treated with CP30, (C) An isolate from *C. jejuni* HPC5 treated with CP8, (D) An isolate from *C. jejuni* HPC5 treated with CP30.

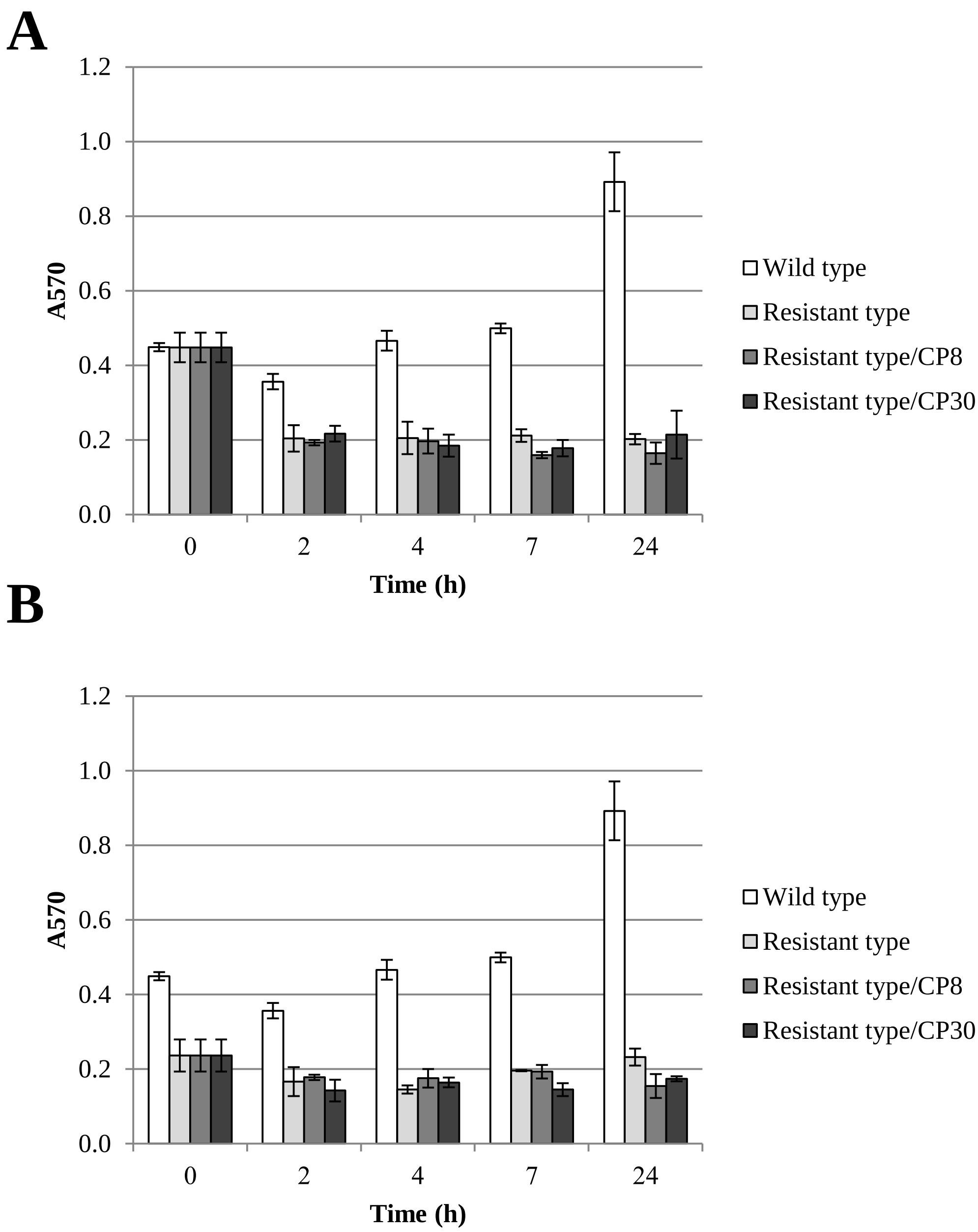


Figure 3.10- Effect of bacteriophages on biofilm formation on glass slides by bacteriophage resistant *C. jejuni* NCTC 11168RCP8 (A) and *C. jejuni* NCTC 11168RCP30 (B), compared to wild type *C. jejuni* NCTC 11168. The bacterial isolates were allowed to form biofilms on glass coverslips at 37 ºC under microaerobic conditions over 5 days and the biofilms quantified using crystal violet staining at harvest and over the time course indicated. The wild type and control resistant type cultures were incubated MH broth whereas the resistant types marked CP8 and CP30 were incubated with MH broth containing the respective bacteriophage (10^6 pfu at an MOI of approximately 1).

3.3 Discussion

Members of the genus *Campylobacter* represent a significant burden to public health, so novel treatments to reduce the spread of infection are of fundamental importance. Most bactericidal treatments are tested on the basis of their ability to kill planktonic cells, but their effects may be reduced where the target bacterium is protected within a biofilm. Bacteriophages have the potential to negate the protection afforded by biofilms either by direct penetration and infection of the cells at the biofilm surface or via infiltration of bacteriophage-infected bacteria into the interior of the biofilm and their subsequent lysis to release bacteriophage *in situ*.

The selection of a particular bacteriophage to use as a treatment is an important issue for phage therapy and bio-sanitization applications, since bacteriophages and their effects are diverse. Not least the bacteriophage should be virulent and not temperate if it is to have a significant effect. Virulent *Campylobacter* bacteriophage can be isolated from chicken excreta and from poultry meat (Atterbury *et al*., 2003; Connerton *et al*., 2004; El-Shibiny *et al*., 2005; Loc Carrillo *et al*., 2007). Bacteriophages CP8 and CP30 are virulent members of the *Myoviridae* with proven stability and efficiency in reducing *Campylobacter* numbers by 2 Log_{10} CFU/g in experimental *Campylobacter*-colonised chickens (Loc Carrillo *et al*., 2005; Scott *et al*., 2007a). These bacteriophages were therefore selected for this study in order to assess if bacteriophage application could reduce or eliminate either *Campylobacter* biofilms or the bacteria within them.

Treatment of *Campylobacter* biofilms attached to glass surfaces with bacteriophages CP8 or CP30 resulted in reductions of viable cells compared to levels in mock-treated controls. Interestingly, the response to bacteriophage treatment was dependent on the host strain forming the biofilm, with differences in both the speed and degree of reduction of the viable count. It is evident that the *Campylobacter* strains have differing capacities for biofilm formation and exhibit a range of cell viabilities, which could influence the effect of the bacteriophages.

Difficulties in obtaining viable counts from biofilms of *Campylobacter* in monoculture have been reported (Reuter *et al*., 2010; Ica *et al.*, 2012), whereas other authors have noted a range counts that are dependent on the strain, the incubation conditions and the substrate on which the biofilm is established (Reeser *et al*., 2007; Gunther and Chen, 2009). The batch dependent range reported in this thesis of Log_{10} 4 CFU/cm^2 to Log_{10} 6 CFU/cm^2 for the formation of a static biofilm on a glass surface. As an alternative to determining the viable counts from biofilms authors have either examined the shedding of viable bacteria from established biofilms (Reuter *et al*., 2010) or resorted to photomicrographic counting of live/dead stained bacteria (Ica *et al*., 2012). In the latter case the inability to recover viable campylobacters but the continued ability to produce vital metabolic staining has been cited as evidence for a "viable but not culturable state" (VBNC) in biofilms formed of *Campylobacter* monocultures (Ica *et al.*, 2012). The VBNC state of *Campylobacter* has been the subject of debate for many years but essentially relates to the resuscitation of physiologically compromised bacteria (Rollins and Colwell, 1986; Bovill and Mackey, 1997). The failure of several authors to recover viable campylobacters from monoculture biofilms would suggest that the viability of the bacteria is low and/or that the cells are physiologically impaired. In the experiments reported here were successful in recovering the bacteria but it is likely that the biofilms contain many more non-viable cells.

Two hours post-infection of *C. jejuni* NCTC 11168 biofilms a reduction in the viable count of 2.5 Log_{10} CFU/cm^2 was recorded (Figure 3.1A). Bacteriophage CP30 was similarly effective at this time point against *C. jejuni* PT14 despite the increase in the biofilm matrix associated with this strain. However, treatment of *C. jejuni* PT14 with bacteriophage CP8 did not significantly reduce the viable *Campylobacter* count compared to that in the mock-treated control. At 24 h post-infection, CP8 treatment of *C. jejuni* NCTC 11168 biofilm reduced the viable count below the detection limit but CP30 treatment results were not significantly different from the control. Bacteriophages CP8 and CP30 significantly reduced the viable count compared to that in the mock-treated control at 24 h post-infection of *C. jejuni* PT14 biofilm ($P < 0.01$). The initial fall in viable count at 2 h was followed by fast recovery at 4 to 8 h post-infection. An increase >2 Log_{10} CFU/cm^2 in 2 h was recorded for CP8-treated *C. jejuni* NCTC 11168. It is not likely that such a rapid recovery in the number of viable cells could occur by normal cell division alone, and therefore it may be associated with reattachment of *Campylobacter*-containing biofilm that had been detached by the bacteriophage treatment. To account for the recovery in the number of viable count, it may be postulated that not all of the campylobacters were compromised but remained viable and associated with sloughed biofilm material. If left undisturbed for longer periods, this material could become reattached, although in the long term, viability was reduced due to the actions of the bacteriophage irrespective of the initial detachment. TEM images provide support for the contention that biofilms were dispersed in the presence of bacteriophage after 2 h (Figure 3.7). Higher-resolution TEM images at 24 h post-bacteriophage treatment also clearly show that campylobacters are subject to cell lysis within the biofilm matrix (Figure 3.8). Regardless of whether the viability of the campylobacters persists in the short term (4 h) the immediate effect of bacteriophage mediated biofilm dispersal would be to create a window of opportunity for the removal of *Campylobacter* biofilms. Bacteriophage could be used to initiate biofilm dispersion in industrial settings that could be followed by thorough pressure flushing to purge water conduits.

The numbers of viable bacteriophage found in the biofilms and planktonic cultures showed an initial binding phase characterized by a decrease in numbers, then a recovery, followed by a period where numbers appeared to remain static. At first sight it might seem strange that the reduction in viable cells in the biofilms from 4 h to 24 h under treatment by phage CP8 should not be accompanied by a rise in phage numbers. This could be an example of what is called passive (or inundation) therapy (Payne and Jansen, 2000) in which weight of numbers of phage is sufficient to reduce cell counts without need for significant levels of phage replication. Even if this is not the case, phages that infect *Campylobacter* generally have a small burst size of around 2-3 (Cairns *et al*., 2009) and so it would only require a small extra loss of phage to prevent significant amplification of phage numbers. In should be noted that phage amplification depends not only on cell lysis, but also on the proportion of free phage able to successfully reach and infect another susceptible bacterium, which in turn depends on the density of bacteria and the movement within the medium. Mean burst size could be attenuated by the fact that the majority of *Campylobacter* cells in the mature biofilms are likely to be in stationary phase and growing relatively slowly. Moreover, movement through the biofilm may be impaired by proximity to cell debris from the lysis event (Rabinovitch *et al*., 2003), so that debris may be encountered and bound to at a higher rate than in planktonic systems. Experiments where phage were administered to *Campylobacter* colonized chickens also appear to confirm this stasis in phage titres, as there appears to be a threshold above which phage numbers do not increase despite being continually added to the system through replication (Loc Carrillo *et al*., 2005). It is perhaps not surprising that the phage-bacteria kinetics show somewhat different properties in biofilms compared to those observed for planktonic systems or *in vivo*. Clearly a formal mathematical model, based on more refined data, will need to be developed to properly understand the kinetic properties of the system.

Bacteriophages have been shown to diffuse through the biofilms of other bacteria (Briandet *et al*., 2008), and it is also been reported that biofilm formation does not provide additional protection against bacteriophage attack (Sharma *et al*., 2005). Our data support these observations since campylobacters within the biofilm are subject to bacteriophage mediated lysis. However, it is also reported that the effects of bacteriophage on attached and planktonic cells are similar (Sharma *et al*., 2005). In contrast, our work indicates that bacteriophage were actually more effective at reducing numbers of viable cells when campylobacters were associated in a biofilm compared to planktonic cells. However, caution is required in drawing mechanistic interpretations from these contrasting results: phage-bacteria kinetics are determined by density thresholds (Payne and Jansen, 2000) whereas in most empirical studies the standard practice is to control experiments using MOI (which is a ratio not a density). Further experiments, with control of the initial densities, will be needed to untangle the differing balances of processes in biofilm and planktonic populations. This will not be trivial, as it is anticipated that localized high densities of host and bacteriophage may arise within the biofilm, and these could give rise to physiological differences between the bacterial populations. Further, the planktonic cells in the overlaying static culture are likely to be in stationary phase, which may not support phage replication, and notably exhibit a high frequency of phage resistance in these experiments (82 to 92%). The propensity of campylobacters to aggregate/agglutinate in dense suspensions as encountered in the planktonic phase cultures will also impact on the adsorption and lysis by bacteriophage (Miller *et al*., 2000; Guerry *et al*., 2006). These conditions also effect the functional expression of the flagella that will also negatively impact phage replication (Guerry *et al*., 2006, Coward *et al.*, 2006; Scott *et al.*, 2007b).

The mechanism by which the bacteriophages were able to break down the biofilm is unknown. It seems possible that bacteriophage CP8 and CP30 can reduce the extra-cellular matrix produced by *C. jejuni* in biofilms through enzymatic means. Bacteriophages have been shown to encode enzymes that can degrade exopolysaccharide which forms the major component of the biofilm matrix (Hughes *et al*., 1998a; Hughes *et al*., 1998b). Carvalho *et al*. (2012) reported that the *Campylobacter* phage vB_cCom_IBB-35 contained genes encoding enzyme classes capable of extracellular polysaccharide depolymerisation. An alternative to phage-encoded enzymatic degradation is that the *Campylobacter* bacteriophages may break down biofilm indirectly, through bacterial cell lysis with the subsequent release of bacterial enzymes that degrade the biofilm matrix. The TEM images indicate that the bacteriophage not only lysed their targets but also caused a breakdown of the biofilm matrix, as evidenced by the density of the matrix polysaccharide stained by alcian blue in the phage treated biofilms compared with the control (presented in the lower resolution TEM images in Figure 3.7).

In the laboratory, bacteria that survive bacteriophage infection often develop resistance to subsequent attack through mutation. Moreover, phage resistance of bacteria within biofilms has been reported to be due to the loss of phage receptor sites that become incorporated into the cell envelope (Donlan, 2009). This is contrary to what is observed for campylobacters surviving phage therapy in chickens, where the environment of the gut counter-selects resistant types that are physiologically compromised (Loc Carrillo *et al*., 2005; Scott *et al*., 2007b). It was therefore of interest to examine if the bacteria recovered from biofilms were resistant to the test bacteriophage. There were clear differences in the frequency of acquired bacteriophage-resistance dependant on the host strain used. All of the viable *C. jejuni* PT14 isolates recovered from the biofilm retained sensitivity to both bacteriophages. In contrast, only a minority (10 to 16%) of the viable *C. jejuni* NCTC 11168 isolates from the treated biofilm retained sensitivity to the bacteriophages. Treatment of planktonic bacterial cells with bacteriophages *in vitro* results in the selection of resistant mutants (Adams, 1959). Resistant mutants selected following *in vitro* treatment of planktonic cells are often deficient in motility (Cairns *et al*., 2009), and as a consequence would be unlikely to be able to survive outside the laboratory environment. The incidence of resistant phenotypes following bacteriophage treatment *in vivo* was found to be relatively low (4%) (Loc Carrillo *et al*., 2005; Scott *et al*., 2007b), where the isolates were noted to be motile probably due to the requirement of a competent motility phenotype for colonization (Handrixson *et al*., 2004). The frequency of resistance to bacteriophage in *Campylobacter* isolated from the biofilms of the two strains did not therefore match either the observations made for phage infection *in vitro* or *in vivo*. The incidence of resistance to bacteriophage was high for *C. jejuni* 11168 biofilms but unlike the survivors from *in vitro* planktonic infections, the isolates were fully motile. However, the bacteriophage resistant types recovered from *C. jejuni* 11168 biofilms were impaired in their ability to either form or maintain biofilms compared to the parental type strain. No resistant isolates were recovered from bacteriophage-treated biofilms of *C. jejuni* PT14, a pattern more reminiscent of that observed *in vivo*, during phage therapy of chickens (Connerton *et al*., 2004; Loc Carrillo *et al*., 2005; Scott *et al*., 2007b). Resistant bacteria can be resident within populations but there is no evidence that the stock *Campylobacter* cultures give rise to the frequency of non-motile types observed post phage replication in laboratory cultures. These non-motile types retain their phenotype through laboratory subculture implying the changes are not regulatory or otherwise temporary. The rates of phage resistance have been reported recently by Fischer *et al*. (2013) and the characterisation of phage resistant mutations in capsular polysaccharide encoding genes characterised by Sorensen *et al*. (2011 and 2012).

3.4 Conclusion

These results have shown the potential for use of lytic bacteriophages against campylobacters as a treatment to control biofilm formation. The viruses within the biofilm could not only effectively target and lyse campylobacters but were also able to disperse the extracellular matrix forming the biofilm. Further studies to identify the components involved in this action will allow further understanding of the interaction between bacteriophage and host within biofilms. More refined experiments, together with kinetic modelling, will be needed to better understand how the phage-bacteria interactions differ in biofilms and planktonic populations and the kinetic consequences of those differences. The preliminary results show promise, not only because the viruses within the biofilm effectively target and lyse campylobacters but also because they are able to disperse the extracellular matrix forming the biofilm.

CHAPTER 4

FORMATION AND PHYSIOLOGICAL STUDIES OF CARRIER STATE LIFE CYCLE (CSLC) OF *CAMPYLOBACTER JEJUNI* BACTERIOPHAGE

4.1 Introduction

Bacteriophage life cycles are generally ascribed to being either lytic or lysogenic. During the lytic cycle, following injection of nucleic acid, host metabolism is redirected towards the replication of phage nucleic acid and assembly of new phage particles, which are released during cell lysis. In the lysogenic cycle, injected nucleic acid integrates into the host genome or remains as a stable episome, replicating along with the host, but can still enter the lytic cycle following induction, or spontaneously, as a rare occurrence. In addition a spectrum of other types of life cycles, based on either of these two strategies exists (reviewed by Abedon, 2009). A common feature amongst alternate life cycles is their inherent instability making their study problematic. Terminology of these variant life cycles is often confused but the two most documented types are; pseudolysogeny (false lysogeny) and the carrier state (Adams, 1959; Lwoff, 1953).

The term pseudolysogeny has been used to define various different unstable bacteriophage-host interactions, including intersection with the carrier state (reviewed by Abedon, 2009). A specific definition was applied by Ripp and Miller (1997), where pseudolysogeny was defined as a phage-host cell interaction in which the nucleic acid of the infecting phage, neither establishes a long-term, stable relationship (lysogeny) nor elicits a lytic response but simply resides within the cell in a non-active state. This type of pseudolysogeny is usually associated with starvation conditions. As nutrient supplies are replenished, the viral genome either establishes true lysogeny or becomes activated to produce and release viable bacteriophage following lysis of the cell (Ripp and Miller, 1998; Los *et al*., 2003). A different definition of pseudolysogeny is used to describe a situation where the bacterial genome is carried as an unstable episome in the host that is both distinct from lysogeny and can be easily cured through subculture (Lood and Collin, 2011).

The carrier state life cycle (CSLC) describes mixtures of bacteria and of bacteriophages which are in a more or less stable equilibrium (Lwoff, 1953). A proportion of bacteria are resistant; however, the presence of some sensitive variants appears to sustain the phage population so that both thrive. The main difference to the state described as pseudolysogenic (Ripp and Miller, 1997 definition), is that bacteriophage are constantly being generated within the culture at the expense of the sensitive cells. When first isolated, strains exhibiting CSLC, resemble lysogens, in that they appear to be resistant to superinfection and exhibit infrequent plaque formation in soft agar lawns but differ from the majority of lysogens as the nucleic acid does not appear to be integrated into the host. Treatment with specific antiserum, which can inhibit infection by neutralisation with bacteriophage or repeated sub culture leads to the cessation of the CSLC, an important way in distinguishing the CSLC from lysogenisation and possibly indicating that the majority of the active phage population exists externally to the phage-resistant elements of the host population. The CSLC is usually observed in strictly lytic phage and there are numerous examples from various different genera (Li *et al*., 1961; Jones *et al*. 1962; Baess, 1971; Pauling, 1982; Jarling *et al*., 2004; Bastías *et al*., 2010). Bacteriophage life cycle variations are sometimes confused with pseudolysogeny or the carrier state as noted by Abedon (2009).

Biofilms, which comprise a polymeric matrix of extracellular polysaccharide, protein, lipid and nucleic acid to protect the embedded bacterial cells from stressful environments, commonly generate nutrient limiting conditions (O'Toole *et al.*, 2000; Flemming and Wingender, 2010). Chapter 3 describes the bacteriophage treatment of *Campylobacter jejuni* biofilms with virulent bacteriophage CP8 and CP30 isolated from chickens (Loc Carrillo *et al*., 2005; Scott *et al*., 2007a). Our studies on the effect of bacteriophage on biofilms (Siringan *et al*., 2011) during determination of frequency of phage resistance led to the discovery that CSLC was occurring in the biofilms.

The phenomenon of CSLC is poorly understood, probably due to the instability of the relationship, with very few research articles available. In this chapter the isolation and characterization of the first examples of CSLC *Campylobacter* cultures are presented. It is postulated that the establishment of CSLC may grant a survival advantage to both bacteria and bacteriophage within biofilms. CSLC associated with biofilms has not previously been studied. This study therefore aims to investigate the formation and physiology of CSLC in *C. jejuni*. In addition, the phenotypic characteristics of CSLC phages have been studied and compared to the characteristics of the parental phages produced by conventional host lysis.

4.2 Results

4.2.1 Formation of the carrier state life cycle (CSLC) of *Campylobacter jejuni* in biofilms

Genomic DNA from single colonies of *C. jejuni* NCTC 11168, PT14 and HPC5 isolated from either biofilms or planktonic cultures that had been treated with either bacteriophage CP8 or CP30, were subcultured and prepared in agarose blocks as described in section 2.5.5. Uncut and *Sma*I restriction digested genomic DNAs were analysed by PFGE (Section 2.5.4). DNA bands of approximately 140 kb were identified in the genomic DNAs of *C. jejuni* isolates recovered from phage treated-biofilms and planktonic cultures of PT14 and HPC5 strains but not NCTC 11168 (Figure 4.1). The DNA bands were of the size expected of the CP8 and CP30 phage genomes (Loc Carrillo *et al*., 2005). This result indicated that the phage genomes may be present, but not incorporated, into the chromosome of bacterial cells present in biofilm and planktonic cultures. A southern blot of PFGE separated genomic DNAs was hybridized with CP8 bacteriophage DNA labelled by DIG random prime (Section 2.5.6). This result confirmed that the 140 kb bands were indeed phage genomic DNAs (Figure 4.2). Furthermore, DNA sequencing of whole genomes of CSLC-HPC5 and - PT14 isolates did not contain the phage genome integrated into their genome.

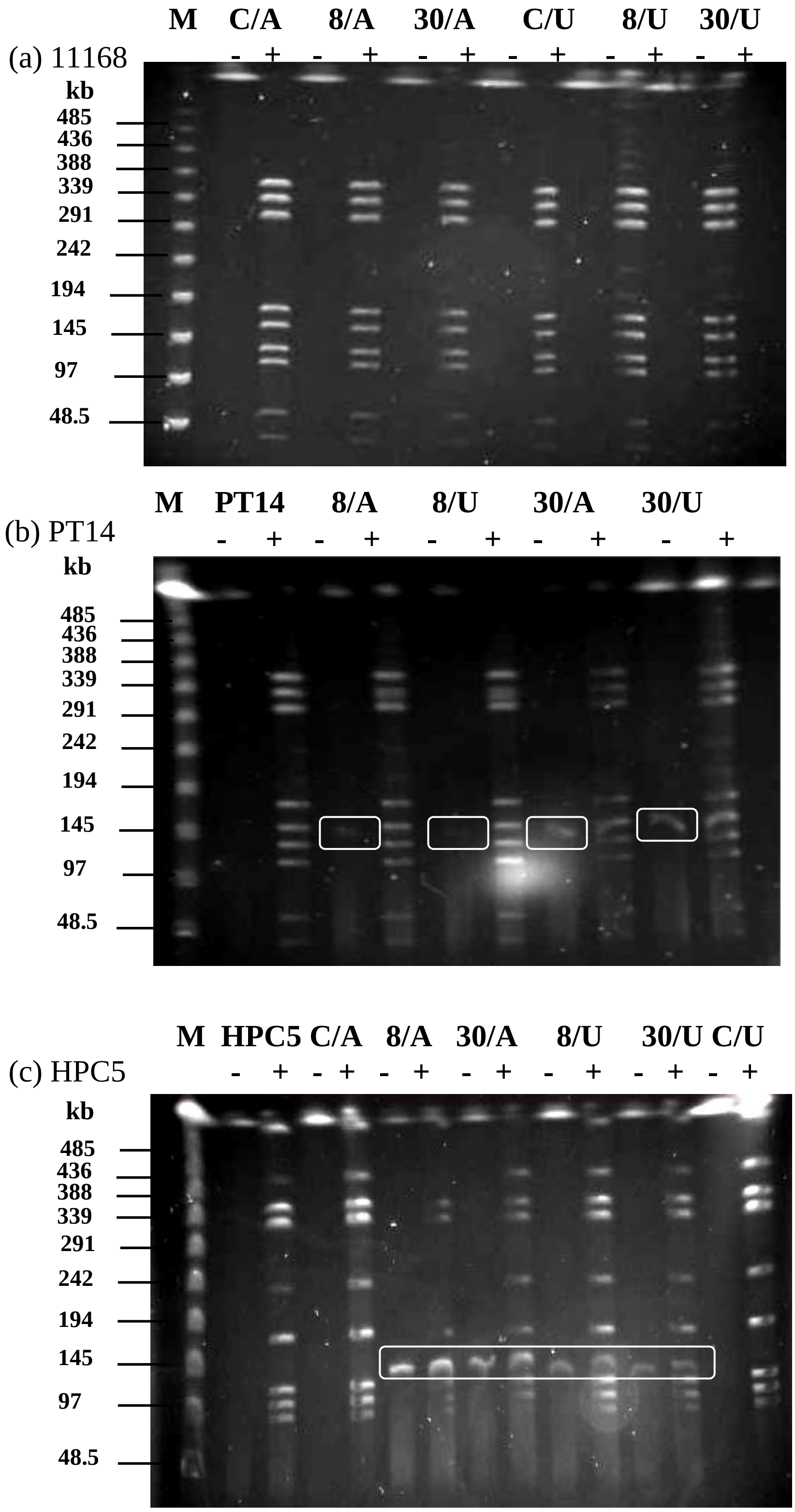


Figure 4.1- Pulsed-field gel electrophoresis of genomic DNA from *Campylobacter jejuni* NCTC 11168 (a), PT14 (b) and HPC5 (c) recovered from biofilms with or without treatment with bacteriophages. C: Control biofilm cells without phage; A: Attached cells (biofilms); U: unattached cells (Planktonic); - : undigested; +: digestion with *Sma*I.

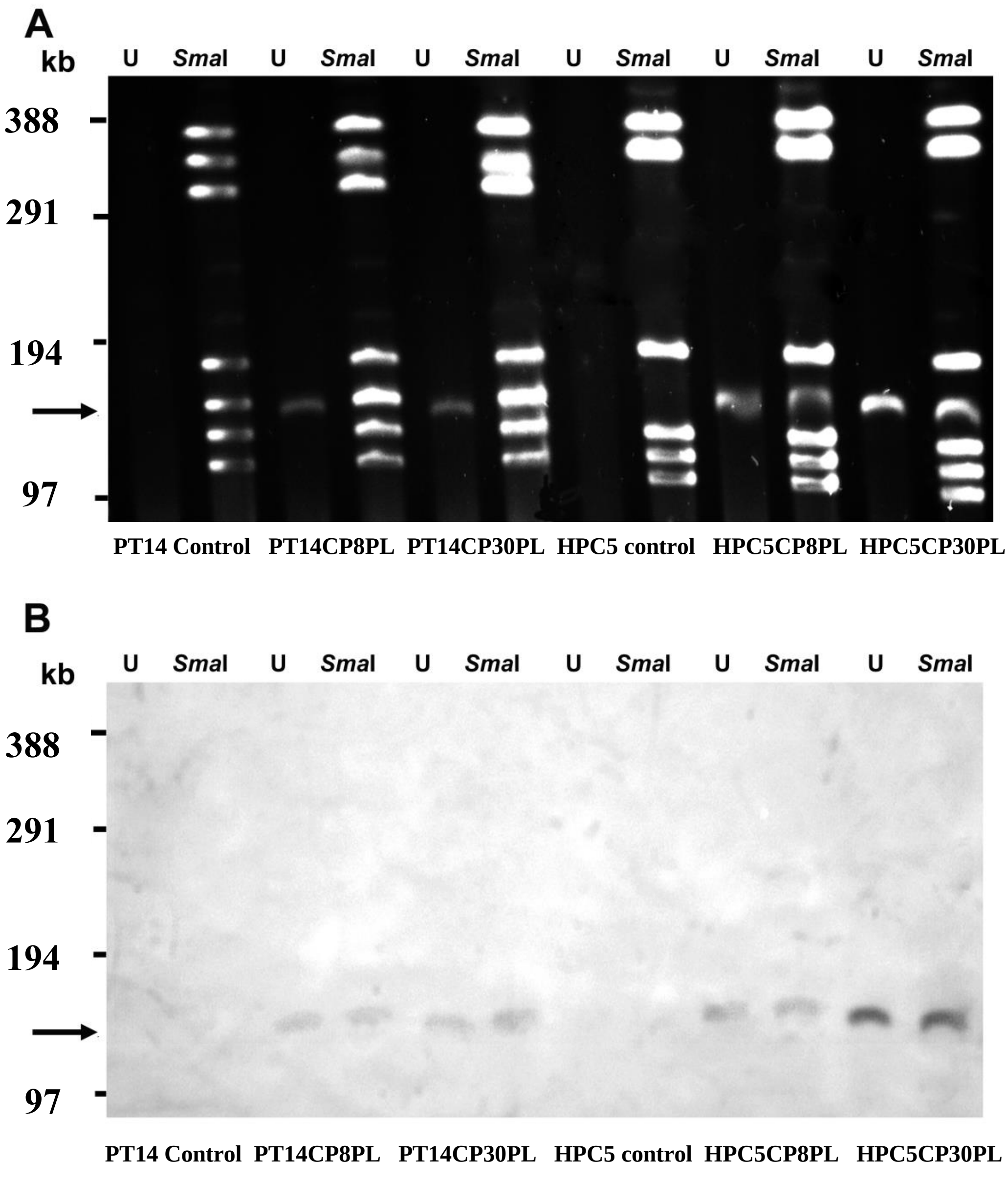


Figure 4.2- PFGE (A) and Southern blot (B) of genomic DNA from HPC5 control and CSLC of biofilm isolates (U: undigested and *Sma*I digested) The Southern blot was hybridized with a bacteriophage DNA probe.

To further identify that the phage DNAs within the *Campylobacter* were derived from the infecting phage and not the result of prophage excision, DNAs extracted from phage containing cultures, wild type *C. jejuni* HPC5 and conventional CP30 propagated by lysis were PCR amplified using primers designed about a region distinguishable from all other available DNA sequences from *Campylobacter* bacteriophage (deposited in the GenBank database). Figure 4.3 shows the PCR products separated by agarose gel electrophoresis. A contemporary preparation of *Campylobacter* genomic DNA did not constitute a template for the amplification and confirmed the extraction and PCR reagents did not contain CP30 or CP30 bacteriophage DNA but bacteriophage cultures derived from CP30 infection produced PCR amplicons of the correct size along with CP30 genomic DNA. The amplified DNA sequence of CSLC isolates were identical (presented in appendix 1) to confirm the persistence CP30 bacteriophage and not the excision of a prophage.

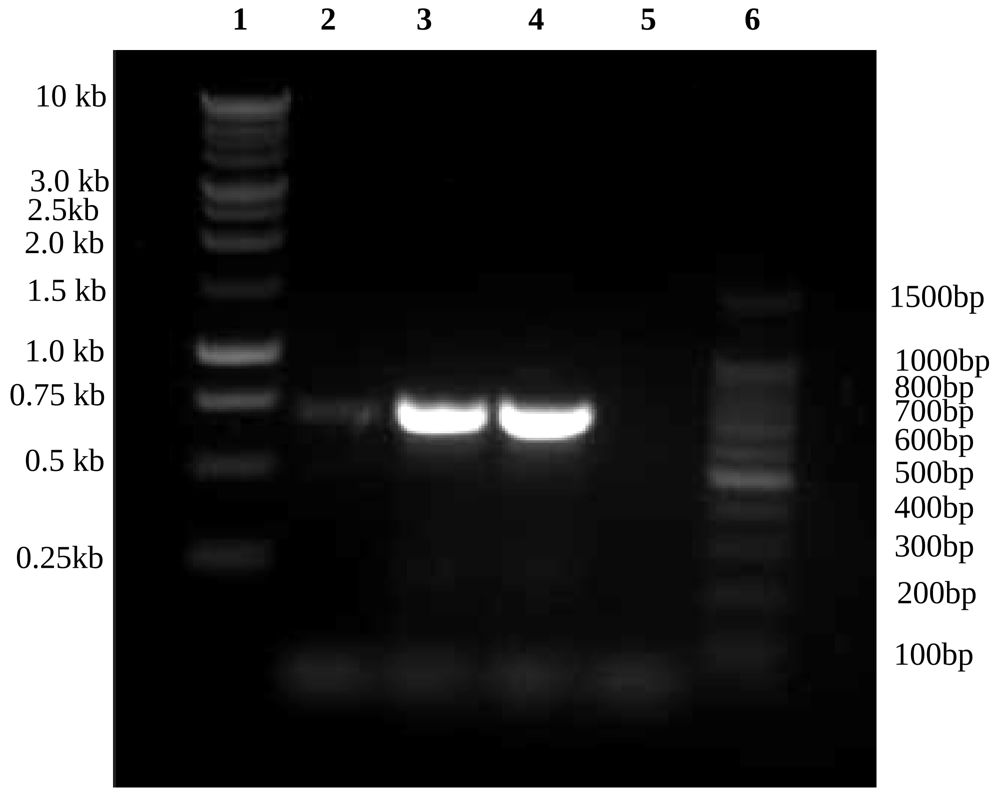


Figure 4.3- PCR products amplified from bacteriophage CP30 DNA (2), *Campylobacter jejuni* PT14CP30PL DNA (3), *Campylobacter jejuni* HPC5CP30PL DNA (4), *Campylobacter jejuni* HPC5 DNA (5) and DNA markers 1 kb (1), 100 bp (6) (Promega). Primers (CP30tp in section 2.11) were designed on genomic DNA of CP30.

Bacteriophage CP8 and CP30 are classified within the family *Myoviradae* that have double-stranded DNA genomes of approximately 140 kb. In order to differentiate these bacteriophage and ensure the phage recovered from CSLC cultures were those used for the initial therapy, primer pairs were designed based on a region of the bacteriophage CP8 genome that contained a DNA sequence insertion as compared with all class III bacteriophage sequences available in all nucleotide sequence databases including CP30. The bacteriophage DNA preparations produced different sized PCR amplicons using the primer pair CP853B and CP854B (see section 2.12) of 200 and 280 bp respectively from CP30 and CP8 that are resolved by agarose gel electrophoresis in Figure 4.4A. PCR amplification of *Campylobacter jejuni* genomic DNAs extracted from PT14CP30PL and PT14CP8PL produced DNA fragments of 200 and 280 bp, which are consistent with the presence of phage DNAs corresponding with the phages used in the treatment of the biofilms that created them. Control *C. jejuni* PT14 DNA did not produce a DNA fragment corresponding with the size of either of the phage DNA amplicons using the same extraction and PCR amplification reagents within the same experiment (Figure 4.4B). Instead an 800 bp DNA fragment was amplifies as visualised upon agarose gel electrophoresis. Control PCR reactions with single or no primer additions identified the DNA fragment as a single primer product of *C. jejuni* genomic DNA. Similarly PCR amplification with the CP853B and CP854B primer pair of HPC5CP30PL and HPC5CP8PL genomic DNAs produced fragments of 200 and 280 bp (Figure 4.4B), that were consistent with the presence of phage DNAs corresponding to the phages used in the treatment of the biofilms that created them. Control *C. jejuni* HPC5 genomic DNA did not produce a fragment size corresponding with either of the phage DNA amplicons but an 800 bp fragment corresponding with the single primer amplification product noted above. The *C. jejuni* genomic DNA controls confirmed that neither the extraction or PCR reagents contained CP30 or CP30 bacteriophage DNAs. The source of the amplifiable DNA was the genomic DNA preparations that gave rise to them, which had been subcultured from single colony isolates a minimum of three times. The identities of the phage DNA products were confirmed by DNA sequencing (presented in appendix 2). It is clear that these bacteriophages retain the genotypes of the parent phage from all host sources, and are not the consequence of prophage excision or contamination with alternative bacteriophages. Additionally, PCR amplification of total DNAs extracted from CSLC cultures produced PCR products and the amplified DNA sequence similar to those of the parental phage DNAs (Figure 4.4B). These data provide further evidence that the CSLC isolates contain phage DNAs of the same genotype as the phage used to treat the biofilms from which they were recovered.

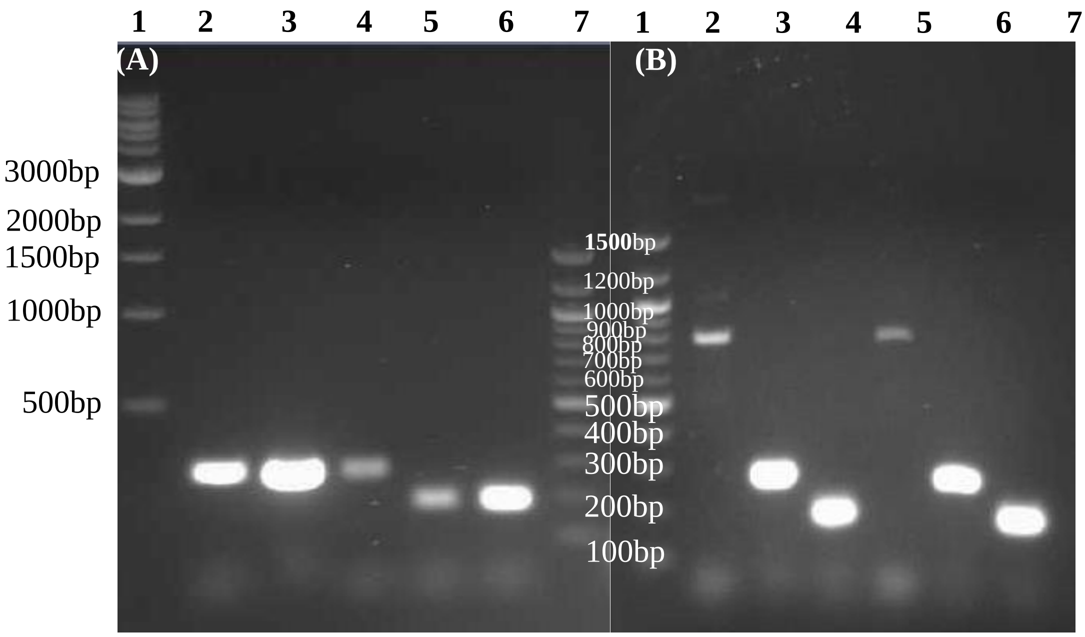


Figure 4.4- (A) PCR products amplified from CP8 and CP30 DNAs. CP8 was propagated from either *Campylobacter jejuni* PT14 (2), or *C. jejuni* HPC5 (3), or *C. jejuni* 11168 (4) and CP30 DNA propagated from either *C. jejuni* PT14 (5) or *C. jejuni* HPC5 (6), DNA marker 1 kb ladder(1) and 100 bp (7) (Biolabs); (B) PCR products amplified from *C. jejuni* PT14 DNA (2), *C. jejuni* PT14CP8PL DNA (3), *C. jejuni* PT14CP30PL DNA (4), *C. jejuni* HPC5 DNA (5), *C. jejuni* HPC5CP8PL DNA (6), *C. jejuni* HPC5CP30PL DNA (7), 100 bp (Biolabs) (1). Primers were designed on the genomic DNA of CP8 (CP853B-CP854B Section 2.12). Note: the band migrating at 800 bp is the unexpected product of single forward primer amplification.

The continued presence of bacteriophage appearing as propagatable clear plaques in bacterial lawns of single colony subcultures coupled with evidence for the presence of bacteriophage DNA in these cultures support the idea that the bacteriophage virulent life cycle had been interrupted, and that phage DNA is intimately associated with the host bacteria either as internalised DNA or pre-absorbed phage particles. The host bacteria are released from nutrient limitation upon subculture in rich media but the associated phage persist. Based on the definitions outlined by Abedon (2009) these observations are consistent with the formation of a carrier state life cycle. Moreover, the treatment with specific antiserum raised against bacteriophage CP8 that was able to neutralise the bacteriophage CP8 indicated the CSLC bacteriophages to be protected either as internalised particles or adsorbed on the bacteria. The antiserum was able to inhibit the phage CP8 alone or when mixed with parent bacteria but not when associated with the CSLC strains (Table 4.1).

Table 4.1-Treatment of C. *jejuni* HPC5 and PT14 parent bacteria, CP8 phage, HPC5CP8PL and PT14CP8PL carrier strain bacteria with pre-immune and anti-phage sera

Combination	**Pre-immune serum**		**Anti-phage serum**	
	Bacteria count Log_{10} CFU/ml	Phage count Log_{10} PFU/ml	Bacteria count Log_{10} CFU/ml	Phage count Log_{10} PFU/ml
CP8 phage only	0	5.3	0	0
HPC5 parent (bacteria only)	7.5	0	7.8	0
HPC5 parent bacteria mixed with phage	7.3	4.8	6.9	0
HPC5CP8PL	7.6	7.5	7.5	7.6
PT14 parent (bacteria only)	7.5	0	7.6	0
PT14 parent bacteria mixed with phage	6.5	6.6	6.6	0
PT14CP8PL	7.5	7.4	7.5	6.7

4.2.2 Frequency of CSLC isolates from *Campylobacter* phage treatment of biofilms

The frequency of CSLC of bacterial isolates recovered from *C. jejuni* PT14 biofilms after treatment with either bacteriophage CP8 or CP30, was approximately 10 %. In contrast, 40% and 90% of isolates from CP8 and CP30 treated *C. jejuni* HPC5 biofilms were respectively found to be CSLC cultures. For planktonic cells, the results showed that 10% of isolates recovered from PT14 were CSLC strains. The CSLC isolates obtained from the phage treatment of biofilms were designated as *C. jejuni* PT14CP8PL, PT14CP30PL, HPC5CP8PL and HPC5CP30PL. Interestingly, the CSLC strains were found to be non-motile compared to wild types on 0.4% Mueller-Hinton agar (Table 3.1 and Figure 3.8C-D). Transmission electron microscopy (TEM) showed bacteriophage associated with *Campylobacter* cells and no flagella attached to the cells but were associated with phage particles (Figure 4.5).

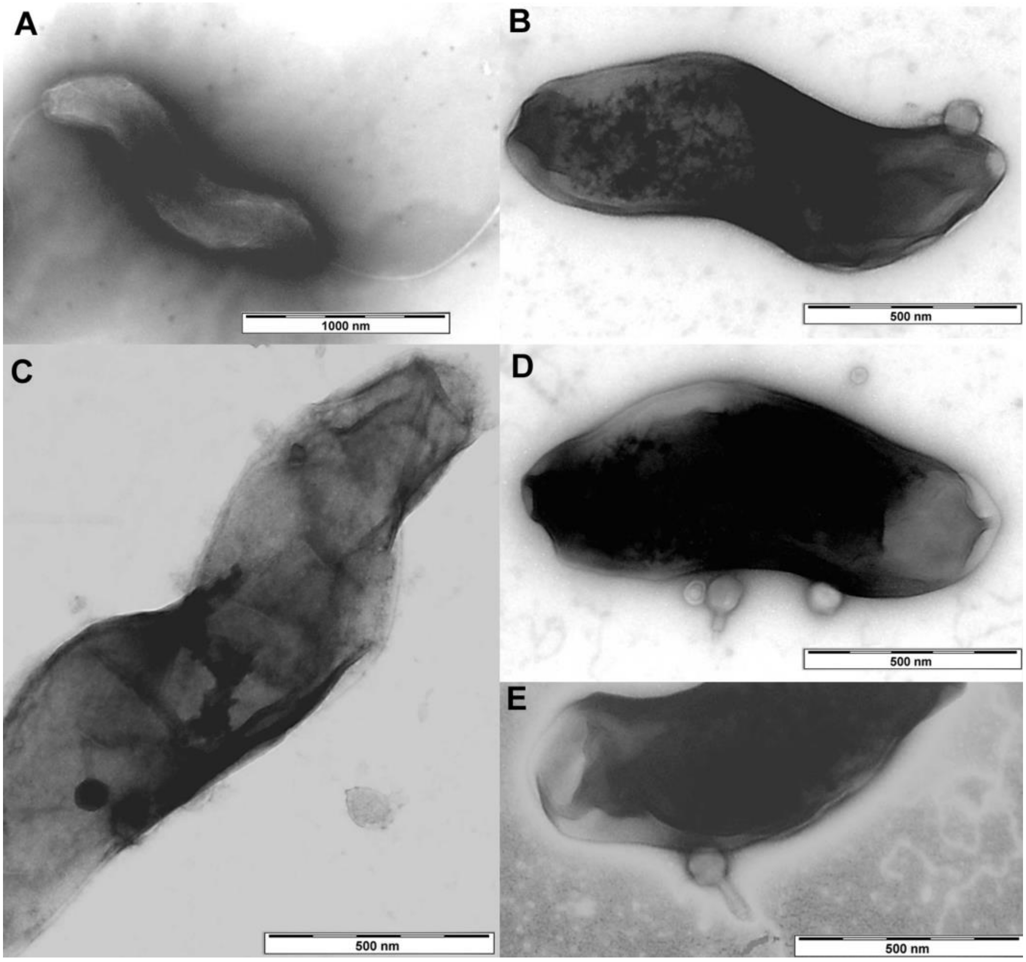


Figure 4.5-Transmission electron microscopy showing *Campylobacter jejuni* (A), pseudolysogenic *Campylobacter jejuni* as the carrier state life cycle (B,C,D,E).

4.2.3 Growth curves of *C. jejuni* and CSLC strains

Growth curves were performed to identify if the CSLC cultures differed from the parental strain in their ability to replicate under different atmospheric conditions. Growth curves were prepared for *C. jejuni* PT14, PT14CP8PL, PT14CP30PL, HPC5, HPC5CP8PL, and HPC5CP30PL that had been grown in Mueller-Hinton broth at 42 °C under microaerobic (Figure 4.6), low oxygen (<1% O_2) (Figure 4.7) and atmospheric (Figure 4.8) conditions, with samples taken over 24 h (Section 2.7.1). The mean ratio of free bacteriophage and bacterial cells (Log_{10} PFU/ Log_{10} CFU) of *C. jejuni* PT14CP8PL, PT14CP30PL, HPC5CP8PL, and HPC5CP30PL were 0.792 ± 0.101 (n=19), 0.722 ± 0.133 (n=19), 0.780 ± 0.134 (n=16) and 0.939 ± 0.084 (n=18), respectively after bacterial cells were sub-cultured on blood agar for all experiments. The doubling time or generation times (g) and growth rate constants (μ) are shown on Table 4.2. The doubling time and growth rates of the *Campylobacter* controls and all of the CSLC strains showed no significant difference (*P*>0.05, t-test) under microaerobic conditions. The CSLC strains were therefore, not affected by the presence of phage and grew as well as the non-CSLC bacteria under these conditions (Figure 4.6). These growth curves showed typical log exponential phase during 2-12 h followed by a stationary phase. There were however, reductions in numbers of viable CSLC cells compared to the parental strain between 10 and 14 h for *C. jejuni* PT14CP30PL (Figure 4.6A), while the numbers of viable *C. jejuni* HPC5CP8PL and HPC5CP30PL were decreased slightly between 8 and 12 h (Figure 4.6B) compared to the parental strain. During this period phage titres from CSLC of *C. jejuni* PT14 rose substantially, when the numbers of viable bacterial cells reached to 10^7 CFU/ml (Figure 4.6A). In contrast, the CSLC of *C. jejuni* HPC5 produced very high titres of phages when the numbers of viable bacterial cells reached 10^5 CFU/ml and the phage titres continued to increase rapidly along with the growth of bacteria (Figure 4.6B). The numbers of bacteriophages reached a maximum 12 h post-incubation for both CSLC of *C. jejuni* PT14 and HPC5.

Table 4.2- The doubling time and growth rate constant of *C. jejuni* PT14, HPC5 and CSLC strains

Strains	The generation time, g (h)		The growth rate constant, μ (h^{-1})	
	Microaerobic	Low oxygen	Microaerobic	Low oxygen
C. jejuni PT14	0.916	1.254	0.757	0.552
C. jejuni PT14CP8PL	0.949	1.239	0.730	0.560
C. jejuni PT14CP30PL	0.976	1.142	0.710	0.607
C. jejuni HPC5	0.894	1.058	0.775	0.656
C. jejuni HPC5CP8PL	0.952	1.241	0.728	0.558
C. jejuni HPC5CP30PL	0.934	1.028	0.742	0.674

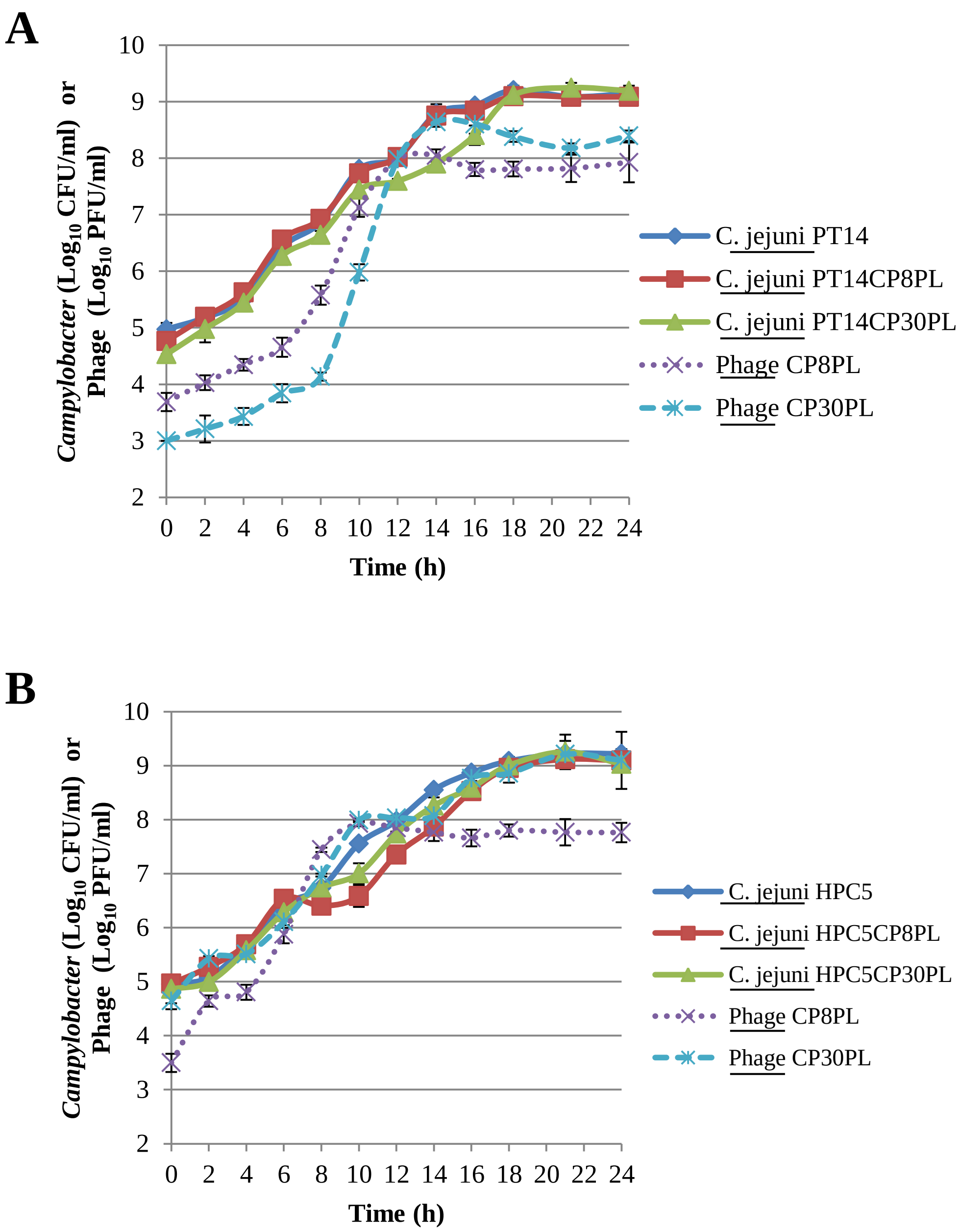
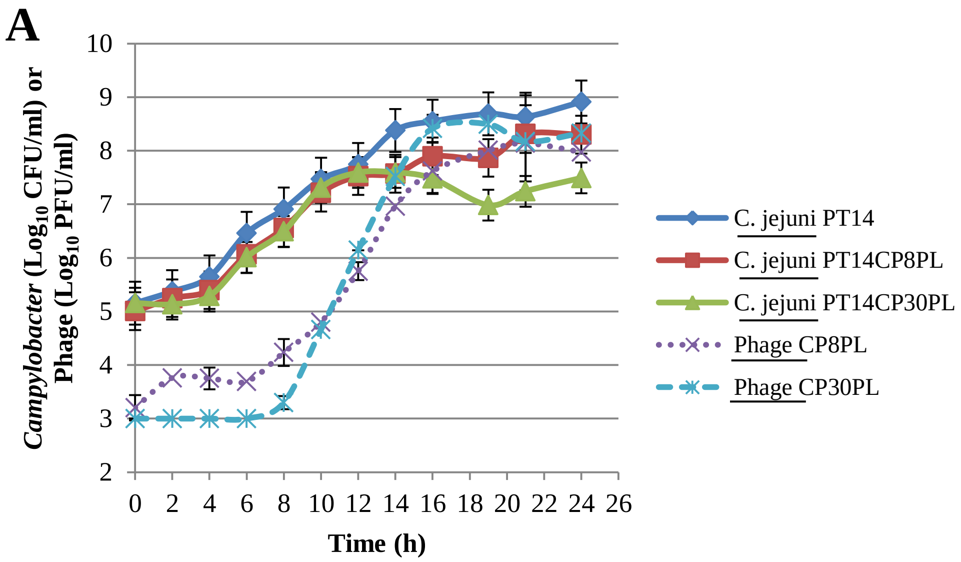


Figure 4.6- (A) Growth curve of *C. jejuni* PT14 and CSLC strains, (B) *C. jejuni* HPC5 and CSLC strains. Growth curves were performed at 42 °C under microaerobic (5% O_2) conditions in Muller-Hinton broth. The curves are representatives of two biological replicates where the points are the means and SD of triplicate viable count determinations.

For growth curves produced under low oxygen conditions (<1% O_2), there was no significant differences ($P > 0.05$) in the generation times and growth rate constants between the control and CLSC cultures (Table 4.2). However, the growth curves revealed a delay in the onset of the exponential phase in the CSLC of *C. jejuni* PT14 cultures by 6 h compared to the parent strain, which was followed by a rapid increase in the number of viable cells and a corresponding increase in phage titre after this time (Figure 4.7A). The growth of *C. jejuni* HPC5 and its CSLC variants under low oxygen conditions were similar. The generation times under low oxygen conditions were notably longer than those observed under microaerobic conditions (Table 4.2, Figure 4.7B). The numbers of phages remained constant until 4 h and then increased substantially after this time. After 12 h the phage titres did not increase any further. These data suggest that the controls and CSLC bacteria could grow under low oxygen conditions and that the CSLC cultures could produce phage but the titres produced were lower than that produced by these cultures under microaerobic conditions.



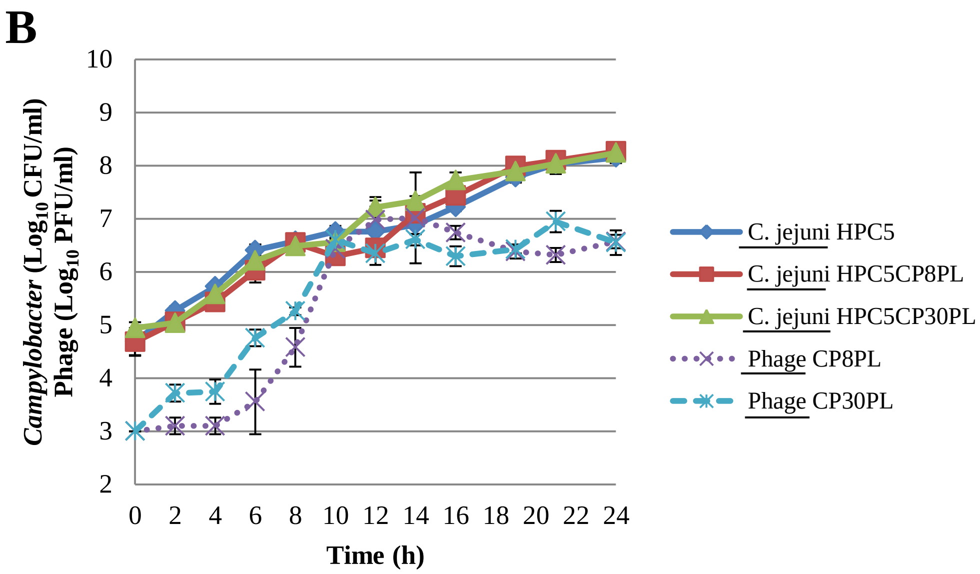
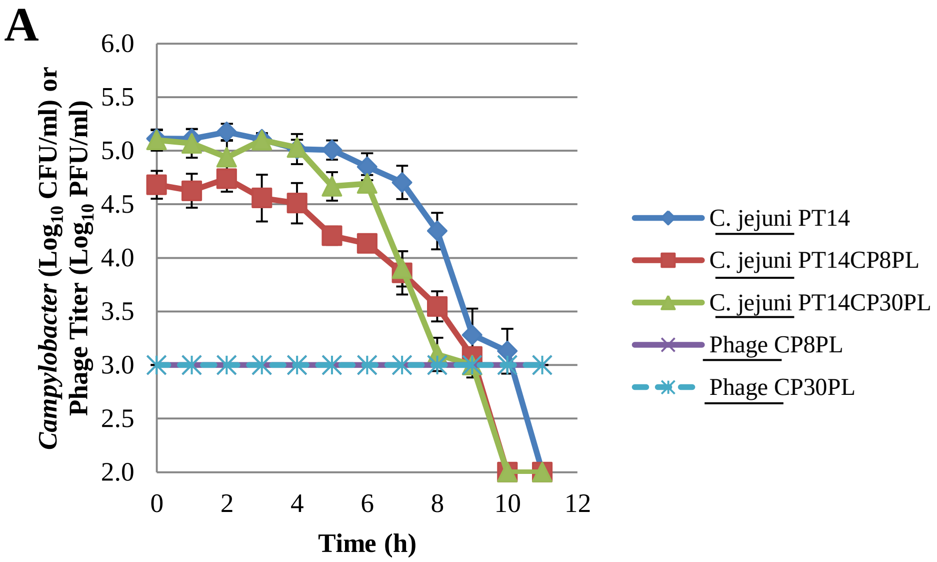


Figure 4.7- (A) Growth curve of *C. jejuni* PT14 and CSLC strains, (B) *C. jejuni* HPC5 and CSLC strains. Growth curves were performed at 42 °C under low oxygen (<1% O_2) conditions in Muller-Hinton broth. The curves are representatives of two biological replicates where the points are the means and SD of triplicate viable count determinations.

When *C. jejuni* PT14 and HPC5 cultures were incubated under atmospheric conditions the numbers of viable cells remained constant for 4 h but dramatically decreased thereafter (Figure 4.8). The decay constant rates of *C. jejuni* PT14, PT14CP8PL and PT14CP30PL strains during 6-9 h were $-0.523h^{-1}$, $-0.352h^{-1}$, $-0.564h^{-1}$, respectively. Additionally, the decay constant rates of HPC5, HPC5CP8PL and HPC5CP30PL strains during 5-10 h were $-0.341h^{-1}$, $-0.297h^{-1}$, $-0.296h^{-1}$, respectively. The phage titres obtained from each sample point for both of the CSLC-PT14 strains was approximately 10^3 PFU/ml and these titres persisted throughout the experiment (Figure 4.8A). In contrast the phage titres obtained from both the CSLC-HPC5 strains increased by 1-2 Log_{10} PFU/ml after incubation for 2 h and thereafter remained constant up to 11 h (Figure 4.8B). These results indicate that phage titres do not increase under atmospheric conditions for PT14, probably because the host was unable to replicate so neither could the phage. However, for HPC5 increased numbers of phage particles were released in the first 2 h of aerobic incubation and thereafter the titres remained constant. The increased phage numbers may have been a result of the presence of pre-formed phage particles that were then released rather than being a direct result of replication. Neither phage showed any viability reduction as a result of incubation under atmospheric conditions.



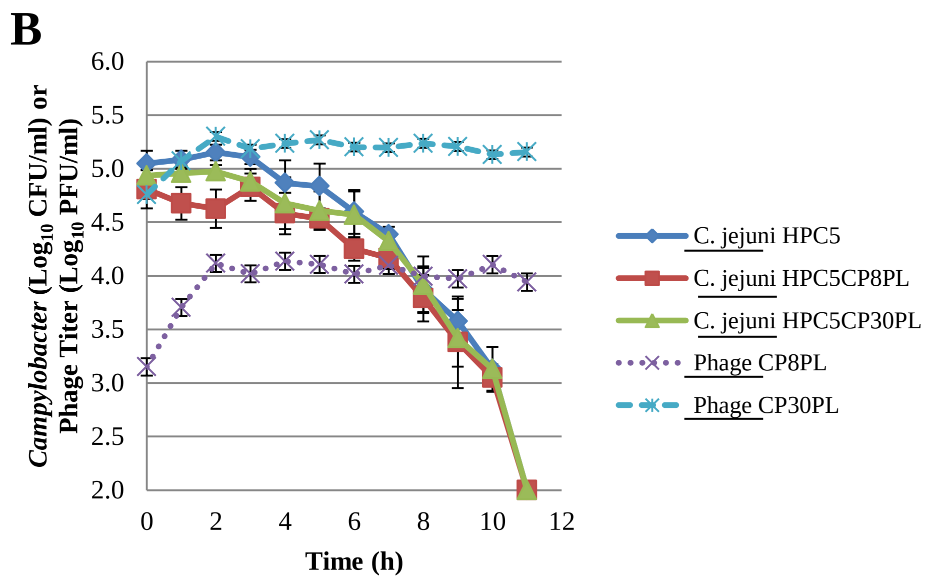


Figure 4.8-(A), Viability of *C. jejuni* PT14 and CSLC strains (B) *C. jejuni* HPC5 and CSLC strains. Growth curves were performed at 42 °C under atmospheric conditions in Muller-Hinton broth. The curves are representatives of two biological replicates where the points are the means and SD of triplicate viable count determinations.

4.2.4 Survival of *C. jejuni* and CSLC strains under nutrition limitation

Survival studies under nutrition limitation conditions were performed in order to determine if the CSLC cultures differed from their parental strains in their ability to persist under different atmospheric conditions. Survival of *C. jejuni* and CSLC variants under nutrition limitation (incubated in maximum recovery diluent) were assessed at 42 °C under aerobic, low oxygen, and microaerobic conditions (Section 2.7.2). The results showed that all of the cultures were able to survive (100%) throughout the experiment (6 h) under microaerobic and low oxygen conditions (Figure 4.9B-C and 4.10B-C). Moreover, the bacteria were able to survive and could be detected at 24 h under these conditions. The number of bacteriophages also tended to remain constant throughout the experiment for all conditions. Interestingly however, under atmospheric conditions, the percentage survival rate of CSLC of *C. jejuni* PT14 (~95%) was higher than for non-CSLC bacteria (~79%) at 2 h as shown as Figure 4.9A. The viable count fell below the limit of detection (>2 Log_{10} CFU/ml) at 4 h for *C. jejuni* PT14 and the CSLC of *C. jejuni* PT14CP8PL, and 6 h for PT14CP30PL.

Similarly, for the CSLC-HPC5 strains, at 2 h the percentage survival rate of CSLC of *C. jejuni* HPC5CP8PL and HPC5CP30PL strains was approximately 75% and 92% respectively, compared to the parent strain, which had fallen below the detection limit (recorded as 0%). The CSLC of *C. jejuni* could therefore survive longer than the wild type strains under aerobic conditions in nutrient deficient medium (Figure 4.10A).

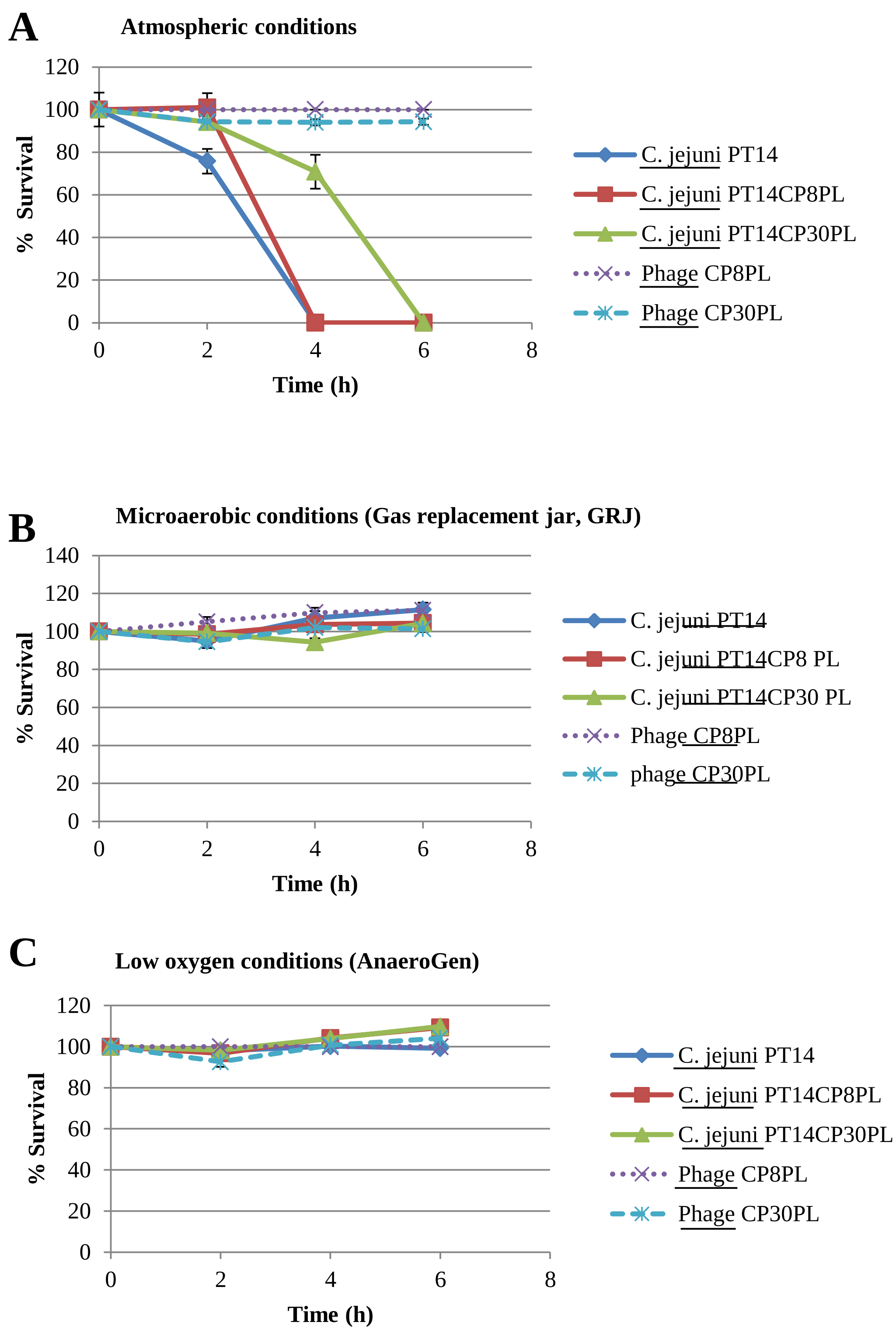


Figure 4.9- Survival of *Campylobacter jejuni* PT14 and CSLC strains under nutrient limitation incubated in atmospheric (A), low oxygen (<1% O_2) (B), and microaerobic (5% O_2) (C) conditions. The data are the means and SD of three independent experiments (n=3).

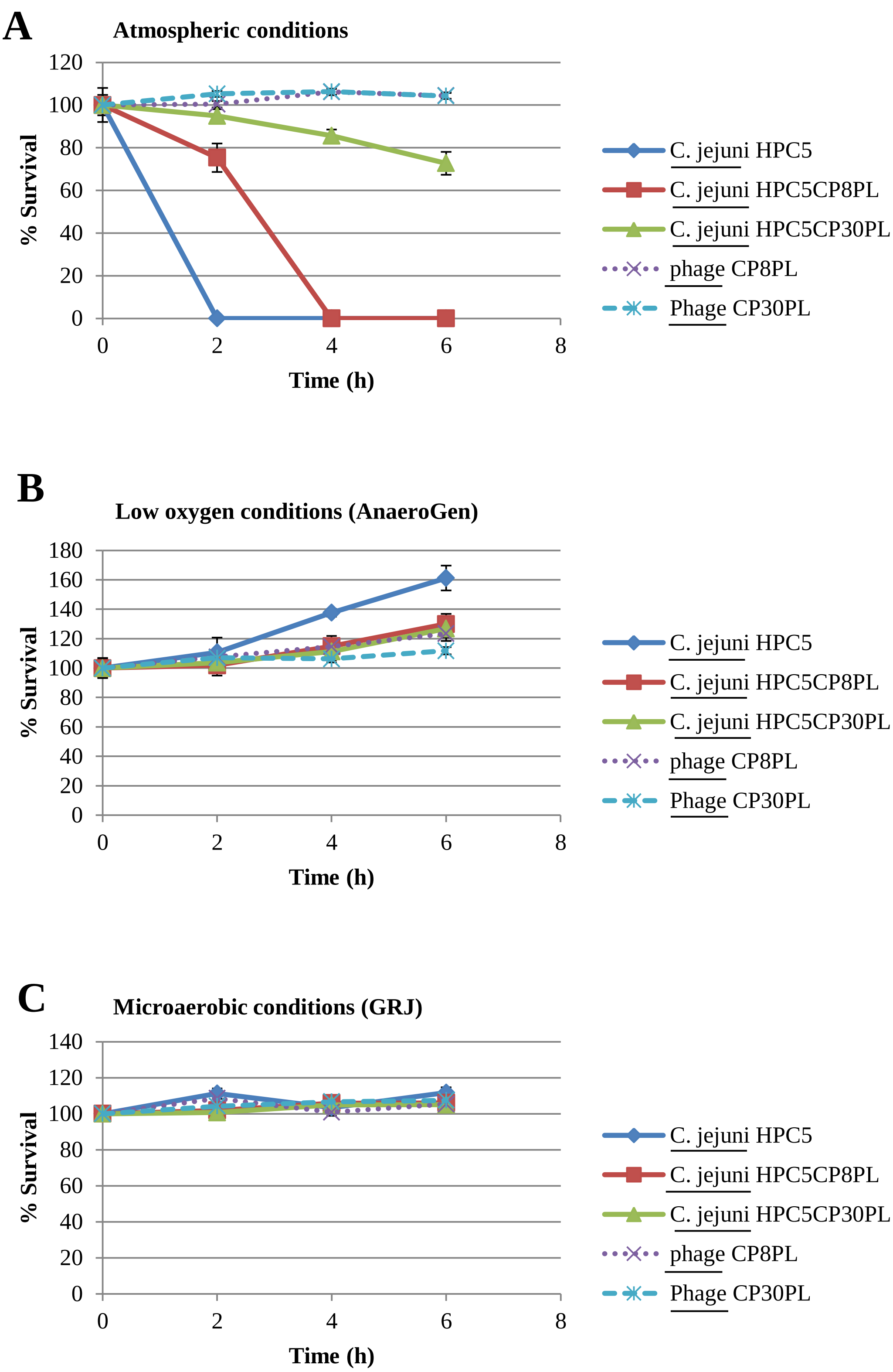


Figure 4.10- Survival of *Campylobacter jejuni* HPC5 and CSLC strains under nutrient limitation incubated in atmospheric (A), low oxygen (<1% O_2) (B), and microaerobic (5% O_2) (C) conditions. The data are the means and SD of three independent experiments (n=3).

4.2.5 Effect of temperature on the survival of *C. jejuni* and CSLC strains

The survival of CSLC strains and parent bacteria under ambient atmosphere in the absence of nutrition at low temperature (4 °C) and high temperature (50 °C) were examined to evaluate the effect of cold and heat stresses on the viability of the bacteria and bacteriophage (Section 2.7.3).

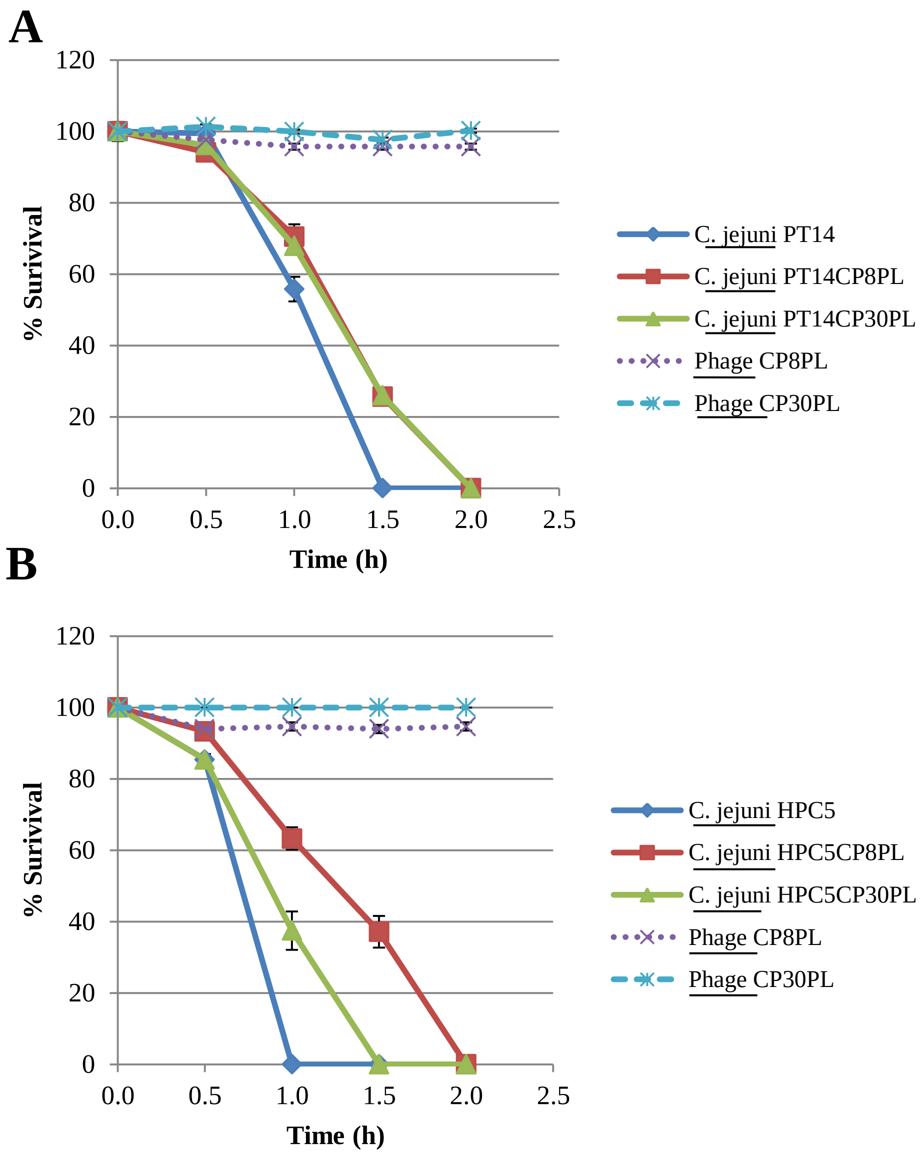


Figure 4.11- Survival of *C. jejuni* PT14 and CSLC strains (A), survival of *C. jejuni* HPC5 and CLSC strains (B) at 50 °C under ambient atmospheric conditions in the absence of nutrients. The data are the means and SD of three independent experiments (n=3).

The viability of CSLC and parent bacteria decreased after incubation at 50 °C (0.5 h) at ambient atmosphere, whereas the numbers of bacteriophages remained constant throughout the experiment (Figure 4.11). The percentage of survival of the CSLC strains was approximately 70% (PT14CP8PL and PT14CP30PL), 63% (HPC5CP8PL) and 38% (HPC5CP30PL), while the parent strains were approximately 56% (PT14) and 0% (HPC5) at 1 h post-incubation. Interestingly, the percentage of survival of CSLC cultures was higher than the parent bacteria after incubation for 1 h. The decay constant rates of *C. jejuni* PT14, PT14CP8PL and PT14CP30PL strains during 0.5-1 h were $-7.168h^{-1}$, $-3.68h^{-1}$, $-4.348h^{-1}$, respectively. Additionally, the decay constant rates of HPC5, HPC5CP8PL and HPC5CP30PL strains during 0.5-1 h were $-14.158h^{-1}$, $-4.712h^{-1}$, $-6.418h^{-1}$, respectively. These results suggest that the CSLC strains are more likely to persist at the higher temperature than their parent strains.

At 4 °C parent and CSLC bacteria survived better than at 42 °C. However, there was no significant difference ($P > 0.05$) between the behaviour of the parent and CSLC bacteria throughout the experiment (Figure 4.12). Additionally, the bacteriophage titres were largely unaffected over the 9 day course of the experiment. These data indicate that the CSLC and parent strains survive at refrigeration temperature under atmospheric oxygen conditions.

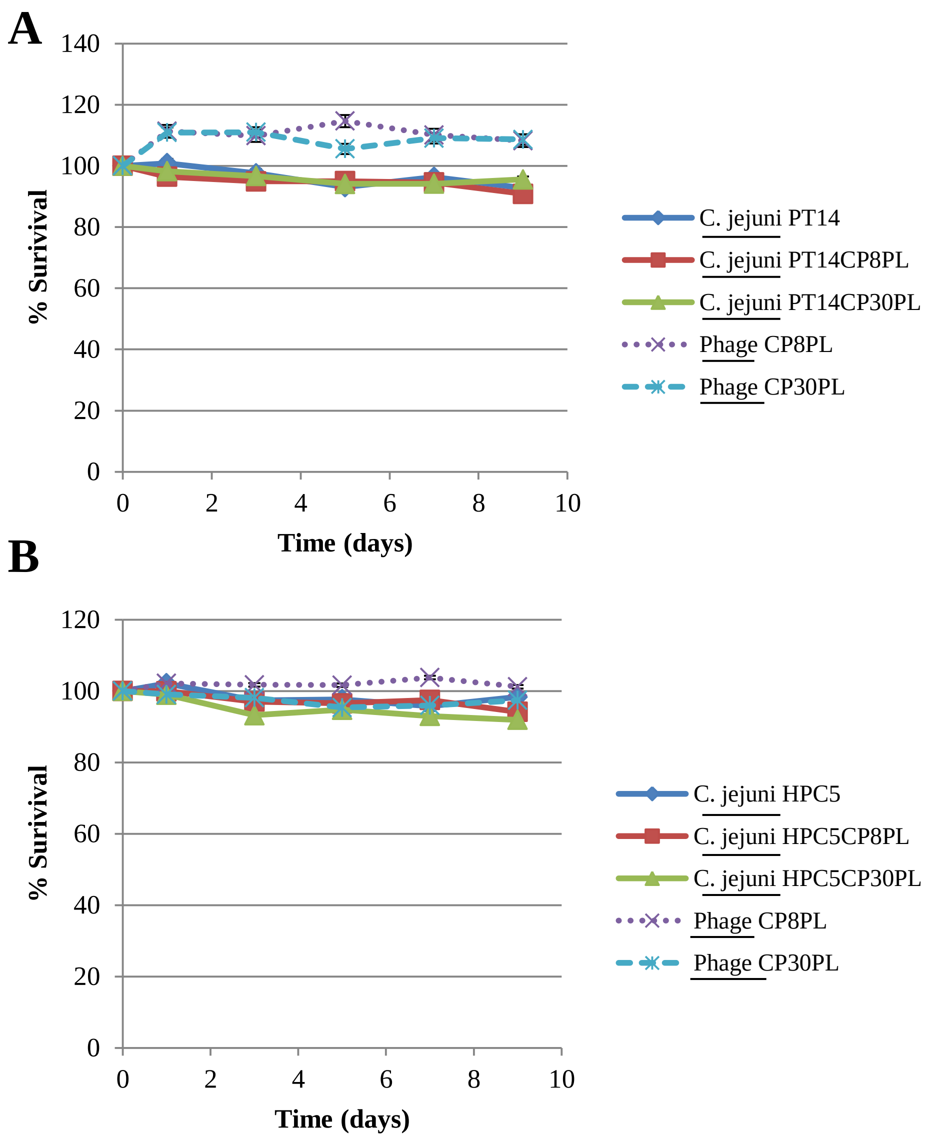


Figure 4.12-Survival of *C. jejuni* PT14 and CSLC strains (A), survival of *C. jejuni* HPC5 and CSLC strains (B) at 4 °C under ambient atmospheric conditions in the absence of nutrients. The data are the means and SD of three independent experiments (n=3).

4.2.6 Transduction of chloramphenicol resistance gene via phages and CSLC phages

Transduction is the process by which DNA is transferred from one bacterium to another mediated by bacteriophages during the lytic cycle of growth (Miller, 2001). The transfer of antibiotic resistance genes and pathogenic traits are undesirable in terms of phage therapy. The transfer of antibiotic resistance genes was used to assess if transduction could be occurring in *Campylobacter* via conventional phages or CSLC phages by using *C. jejuni* PT14 constructed with either *yfp* (encoding yellow fluorescent protein) or *gfp* (encoding green fluorescent protein) adjacent to a chloramphenicol resistance gene (*cam*R). Bacteriophages CP8 and CP30 (approximately 10^9 PFU/ml) that were propagated on *C. jejuni* PT14YFP and PT14GFP, containing either *yfp* or *gfp* and the chloramphenicol resistance gene (*cam*R), were mixed with wild type *C. jejuni* PT14 and NCTC 11168 at a multiplication of infection (MOI) approximately 1, for 1 h at 42 °C under microaerobic conditions (Section 2.6). No chloramphenicol resistant *Campylobacter* were recovered suggesting that if transduction had occurred then the frequency was less than 10^{-9}. The CSLC bacteriophages (CP8PL and CP30PL, approximately 10^{10} PFU/ml) obtained from *C. jejuni* PT14YFPCP8PL and PT14YFPCP30PL were mixed with wild type *C. jejuni* PT14 and NCTC 11168 at an MOI of 1, for 1 h at 42 °C under microaerobic conditions. No chloramphenicol resistant *Campylobacter* isolates were obtained after treatment with these CSLC phages. This suggests that the transduction of the chloramphenicol resistance gene via phages or CSLC phages either did not occur or was at a frequency less than 10^{-10}.

4.2.7 Comparison of the ability of conventional propagated bacteriophages and CSLC phages to plaque on different hosts

A comparison of the host profiles of the parent phages and CSLC phages was carried out to determine if the CSLC phage were altered in their host range. In addition, the effect of the propagation host on the host ranges of the phages was compared to detect possible host modification effects. Routine phage titres (approximately 10^6 PFU/ml) were used to examine the ability of parent bacteriophages and CSLC phages to form plaques on the different *C. jejuni* strains, including the CSLC strains (Section 2.7.4; Table 4.3). All the parent phages and CSLC phages were able to efficiently lyse PT14 irrespective of host used to propagate them. A similar result was obtained for 11168 but with a reduction in plaque number for CP30PL phage propagated on PT14CP30PL host indicating a possible host restriction effect.

Table 4.3- Ability of bacteriophages and CSLC phages to form plaques on different *Campylobacter* strains

Phages	Propagating strains	Hosts		
		PT14	11168	HPC5
CP8	PT14	CL	CL	CL
CP8PL	PT14CP8PL	CL	+++	CL
CP8	HPC5	CL	CL	CL
CP8PL	HPC58PL	CL	+++	CL
CP30	PT14	CL	++++	CL
CP30PL	PT14CP30PL	CL	++	++++
CP30	HPC5	CL	++++	CL
CP30PL	HPC5CP30PL	CL	CL	CL

CL, complete lysis; +, < 30 plaques; ++, mean number of plaques = 30-100; +++, mean number of plaques = 100-200; ++++, mean number of plaques = 200-500.

In contrast, the plaque forming ability of these phages and CSLC phages on 11168 strains was markedly different, particularly for the CP8 phages, depending on whether they originated from lysis of a parental strain or were of CSLC origin. Notably CP8PL exhibited a reduction in the number of plaques irrespective of the propagation host. The CP30 parent phage propagated on PT14 could not affect complete lysis of *C. jejuni* 11168, whilst the CSLC phage, CP30PL, produced less plaque on the same host indicating a reduction in potency for the CSLC derived phage. However, the CSLC derived phages, CP30PL, propagated on the CSLC host, and HPC5CP30PL in contrast was more potent than the parental phage propagated on the parental strain. These results suggest that the ability of CSLC phage CP8PL and CP30PL to form plaques are distinctly different from the parent bacteriophages released upon completion of the lytic cycle when applied to *C. jejuni* 11168. In general being derived from a CSLC host was associated with a reduction in plaque forming ability.

4.2.8 Efficiency of plating of parent phages and CSLC phages

In order to quantify the changes observed in 4.2.6 the efficiency of plating for parent and CSLC strains was determined. Efficiency of plating (EOP) is the ratio of the number of plaques formed by phage infected strains (HPC5 and 11168) cells to the number of plaques assayed on strain *C. jejuni* PT14 (Table 4.4) using the routine phage calculation (Section 2.7.5). The strain *C. jejuni* PT14 was used as reference due to its broad susceptibility to bacteriophage infection.

Table 4.4-Efficiency of plating (EOP)

Phages	**Propagating strains**	**EOP(HPC5/PT14)**	**EOP(11168/PT14)**
CP8	PT14	0.761 (SD±0.190)	0.041 (SD±0.018)
CP8PL	PT14CP8PL	0.633 (SD±0.526)	0.022 (SD±0.002)
CP8	HPC5	0.953 (SD±0.513)	0.934 (SD±0.130)
CP8PL	HPC5CP8PL	0.624 (SD±0.615)	0.113 (SD±0.107)
CP30	PT14	0.724 (SD±0.050)	0.022 (SD±0.027)
CP30PL	PT14CP30PL	0.318 (SD±0.034)	0.006 (SD±0.008)
CP30	HPC5	0.760 (SD±0.368)	0.480 (SD±0.355)
CP30PL	HPC5CP30PL	1.544 (SD±0.382)	0.155 (SD±0.017)

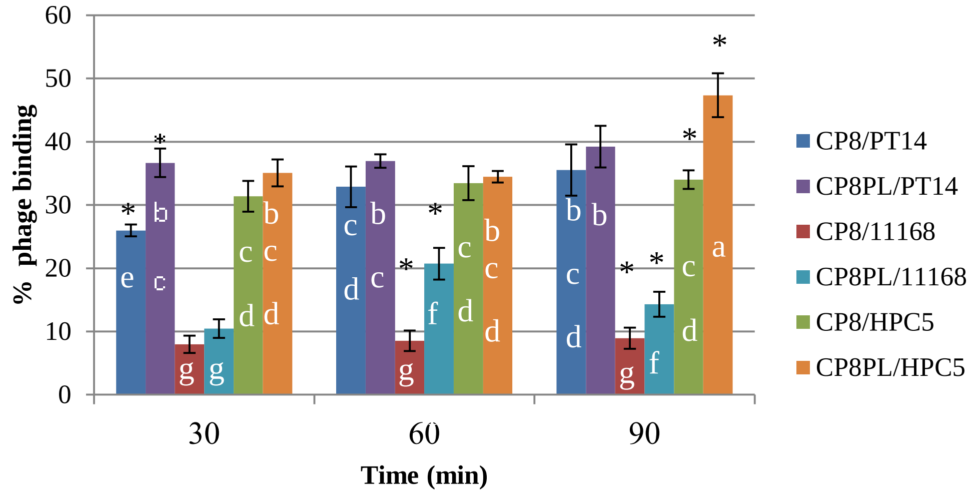
Mean and SD of three independent experiments (n=3).

In general the EOP of *C. jejuni* HPC5 over PT14 strains did not reveal any marked differences (*P*> 0.05, t-test) between the parental bacteriophage and CSLC phages. The exception to this was CP30PL, which conversely plaqued more efficiently on HPC5 than PT14 (1.544). Comparison of the EOP ratios between *C. jejuni* 11168 and PT14 hosts showed that for the EOP of the parental phages, varied more than the HPC5/PT14 comparison, with the most efficient phage being CP8, propagated on HPC5 (0.934), whilst the least efficient was an order of magnitude lower for CP30 propagated on PT14 (0.022). The CSLC phages however, revealed reductions in EOP (0.006-0.022) compared to the parental phages, the exception being CP30PL phage, which propagated on the HPC5CP30PL host (0.155 compared to 0.480 for CP30 parental phage on the HPC5 host). The lack of “reduced efficiency” for this CSLC phage compared to the parental phage had already been observed in Table 4.3.

4.2.9 Binding assay of bacteriophages and CSLC phages on different hosts under microaerobic conditions

The ability of phages (CP8, CP30, approximately 10^5 PFU/ml) and CSLC phages (CP8PL and CP30PL, approximately 10^5 PFU/ml) to bind to different *C. jejuni* strains (11168, PT14 and HPC5) at 42 °C under microaerobic conditions were examined by determination of the percentage of bacteriophage binding (Section 2.7.6; Figure 4.13 and 4.14). Whilst binding was determined after 30, 60 and 90 min, there was actually little change after the first 30 min for the majority of phage host combinations indicating that most binding occurred during this time. Bacteriophage CP8 and CP8PL propagated on PT14 and PT14CP8PL hosts respectively, bound equally well to HPC5 and PT14, while these phages bound significantly less well to the 11168 host (P< 0.05, paired t-test) (Figure 4.13A). Similarly, the binding ability of CP30 and CP30PL replicated on PT14 and PT14CP30PL hosts was not significantly different on HPC5 and PT14 (P> 0.05, paired t-test). However, whilst CP30 was found to bind approximately 20 % of the 11168 cells at each sample point, CP30PL was only found to bind to approximately 5% of the *C. jejuni* 11168 cells at 30 and 60 min rising to approximately 15% at 90 min (not significantly different to CP30), indicating a retardation of binding for this phage host combination (Figure 4.13B). Importantly, the CSLC phages obtained from CSLC of *C. jejuni* HPC5 were able to bind more effectively (30-35%) to PT14 and HPC5 strains than the parent phages (20%, Figure 4.14A-B). These results indicate that the binding ability of CSLC phage CP8PL and CP30PL on distinct hosts were significantly different from phage CP8 and CP30 (P< 0.05, ANOVA). With the exception of the comparison of CP30 and CP30PL propagated from PT14 which was not significantly different, the binding of CSLC phage and parental phage to different hosts show significant differences ($P < 0.05$, ANOVA). A pair wise comparison of CSLC phage and parent phage are shown in Figures 4.13 and 4.14 over the time course.

A



B

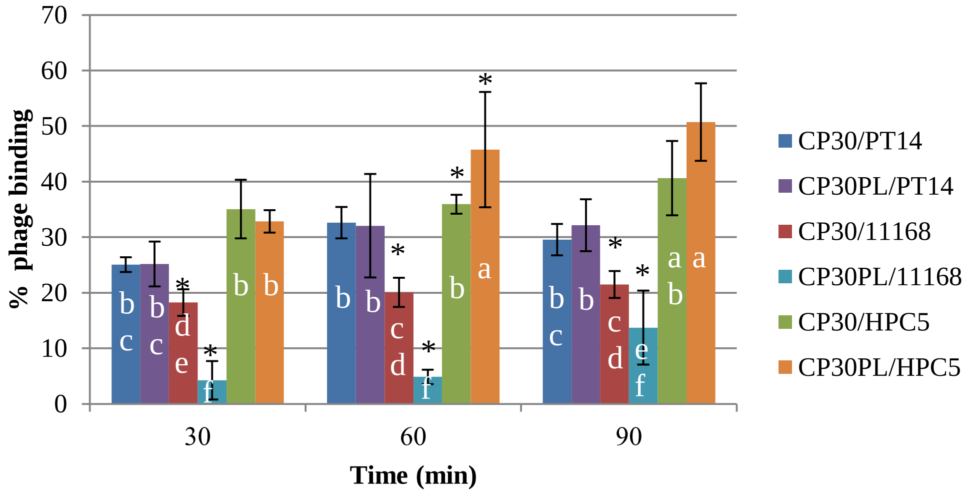
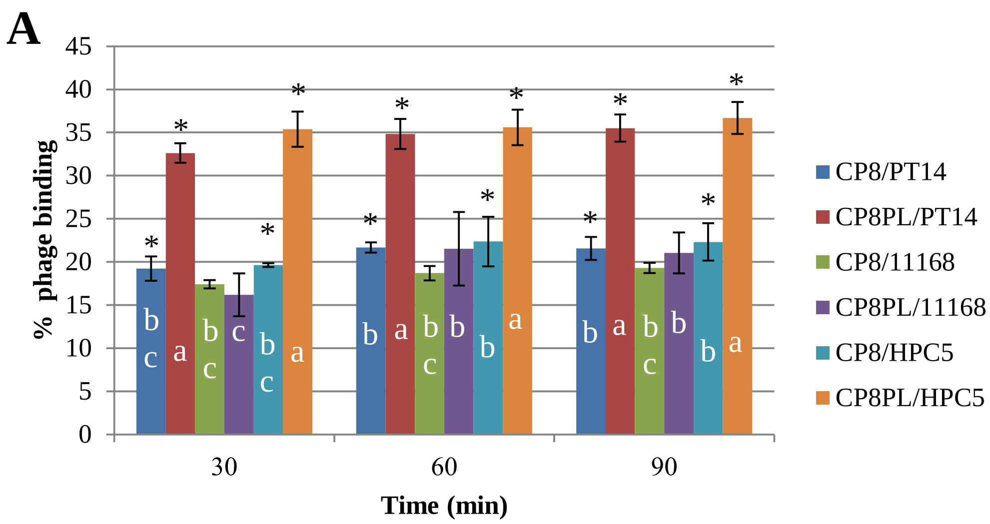


Figure 4.13-Binding ability of CP8, CP8PL (A); CP30 and CP30PL (B) on *C. jejuni* PT14, 11168 and HPC5. These phages were propagated on PT14 and CSLC isolates. The letters on each bar demonstrate the grouping for the mean values derived from Tukey's 95% confidence intervals and the different letter indicate significant differences (P< 0.05). The * indicate significant differences P < 0.05, between pairs of CSLC phage and parent phage using ANOVA. These data are the means and SD of triplicate determinations of phage binding based on three independent post-binding phage titre determinations, and are a single representative of two biological replicates.



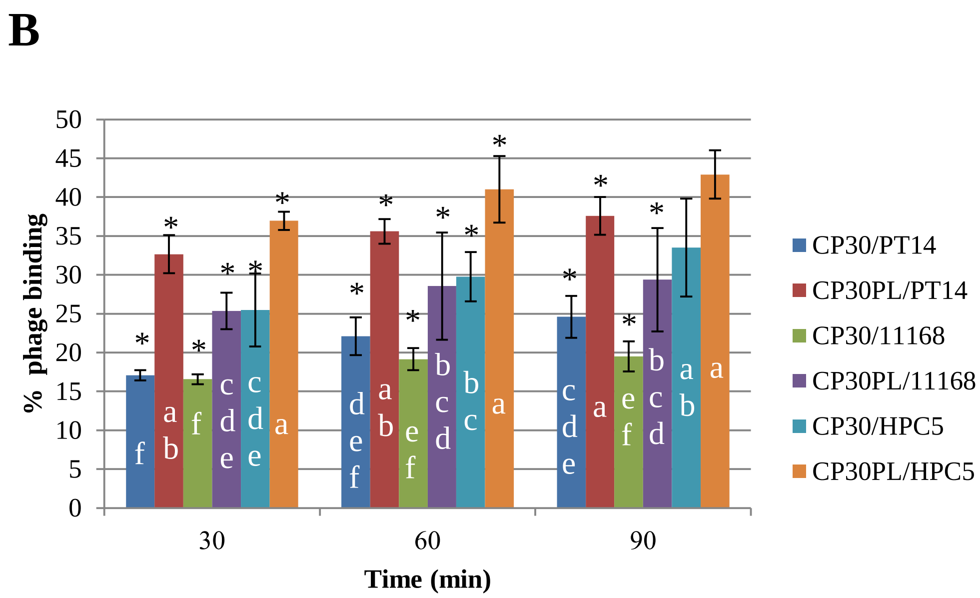


Figure 4.14- Binding ability of CP8, CP8PL (A); CP30 and CP30PL (B) on *C. jejuni* PT14, 11168 and HPC5. These phages were propagated on HPC5 and CSLC isolates. The letters on each bar demonstrate the grouping for the mean values derived from Tukey's 95% confidence intervals and the different letter indicate significant differences ($P< 0.05$). The * indicate significant differences $P < 0.05$, between pairs of CSLC phage and parent phage using ANOVA. These data are the means and SD of triplicate determinations of phage binding based on three independent post-binding phage titre determinations, and are a single representative of two biological replicates.

Table 4.5- Comparison of binding ability of CSLC phage and parent phage on different hosts using t-test: unpaired two samples for means.

Comparison	Propagating strains	Probability
CP8 and CP8PL	**PT14**	**0.002***
CP30 and CP30PL	**PT14**	**0.1**
CP8 and CP8PL	**HPC5**	**<0.001***
CP30 and CP30PL	**HPC5**	**<0.001***

*Significant difference at *P*< 0.05

Table 4.6-Summary of phenotypic differences of bacteria and phages from CSLC cultures

CSLC isolates	**CSLC phages**
▪ Non-motile	▪ Host Changes
▪ Normal growth under microaerobic and low oxygen conditions	▪ EOP differences
▪ Greater decay in rich medium with atmospheric oxygen	▪ Binding differences
▪ Increased survival under nutrition limitation at ambient atmosphere	
▪ Normal survival at 4 °C under nutrition limitation and ambient atmosphere conditions	
▪ Increased thermo-tolerance (50 °C) under nutrition limitation and ambient atmosphere conditions	

4.3 Discussion

Alternative phage life cycles such as CSLC and pseudolysogeny are a phenomenon that impact on the viability of bacteria and their viruses in the environment (Ripp and Miller, 1997). This chapter describes the occurrence of CSLC in *Campylobacter* biofilms after phage treatment. Experiments were carried out to investigate the characteristics of CSLC bacteria. These included an analysis of the genomic DNA by PFGE that showed that the genomic phage DNA was established in the bacterial cells. The presence of the phage genome (approximately 140 kb) was confirmed by hybridization with phage DNA. This evidence indicated that the interaction of bacteriophage and biofilm caused some bacteria to be in a carrier state life cycle. An explanation for this may be that the full replication cycle of bacteriophage may be suppressed during phage treatment on biofilms due to starvation within the biofilm comparable to the observations on pseudolysogeny (Ripp and Miller, 1998; Hunt *et al*., 2004). This situation means that the CSLC strains may then infect and release phage particles when bacterial cells are subsequently able to grow under suitable conditions such as nutrient addition (Ripp and Miller, 1997 and 1998). The frequency of occurrence of CSLC in both CP8 and CP30 phage-treated *C. jejuni* PT14 biofilm was approximately 10%. However, 40% and 90% of the isolates recovered from *C. jejuni* HPC5 biofilms treated respectively with CP8 and CP30 were CSLC isolates. The tendency to form CSLC strains varies according to the host bacteria and probably the nutrient associated stress environment responsible for their formation. Additionally, CSLC isolates was found in planktonic cells in approximately 10% of the PT14 isolates. None of the *C. jejuni* 11168 recovered from phage-treated biofilms proved to be CSLC strains, most probably because almost all of these isolates were resistant to both the bacteriophages examined. In contrast, none of the bacterial isolates from PT14 treated biofilms were resistant to the phages that they had been treated with, and very few isolates recovered from the *C. jejuni* HPC5 biofilms were resistant, again indicating differing host encoded factors, and potentially that selection for resistance was not compatible with survival of these two strains in biofilms. *C. jejuni* 11168 in contrast was able to become resistant and successfully be retained in biofilm. The mechanisms of phage resistance such as restriction-modification systems, the CRISPR-Cas system and phage immunity proteins may differ between strains and may prevent the CSLC in *C. jejuni* 11168 derived strains (Labrie *et al*., 2010). The interaction of environmental factors, such as nutrient limitation, and inherent host encoded factors are likely to govern the propensity of cultures to form CSLC strains. These combinations may elicit variable selective pressures that either drive phage lifecycle arrest and possible gene expression or lead to phage resistance or simply arrest cell division.

Transmission electron microscopy allowed visualization of CSLC isolates and revealed *Campylobacter* cells associated with bacteriophage together with some infected bacterial cells. CSLC bacteria were non-motile evidently due to loss of their flagella (Figure 4.5), while the wild type controls remained motile and retained their flagella. The CSLC clearly impacts on the motility of *C. jejuni*. Cell motility is critically important in colonization of hosts such as poultry by *C. jejuni* (Karlyshev *et al*., 2002; Jones *et al*., 2004), and therefore its loss must be considered disadvantageous for colonization but advantageous to avoid phage infection and lysis.

An analysis of growth characteristics showed that the growth of CSLC isolates was generally similar to the growth of the parental strains under microaerobic conditions with indistinguishable generation times and growth rate constants. Phage titres were observed to increase markedly when the viable counts of *Campylobacter* cells reached 10^7 CFU/ml for the PT14 strain and approximately 10^6 CFU/ml for the HPC5 strain. This observation is consistent with the proliferation threshold required to support productive phage replication in the lytic lifecycle, i.e. the cell concentration required to increase in phage titres (Cairns *et al*., 2009). Phage particles were released spontaneously from CSLC bacteria without induction, when they were grown on normal growth media. These phages could therefore potentially infect the progeny cells, resulting in an increase in numbers of phages once the permissive host population had achieved the proliferation threshold. A typical latent period and burst size were observed, indicating that a process similar to the one-step growth of normal phage infection of the host could be achieved once nutrient limitation had been lifted (Cairns *et al*., 2009).

The growth of *C. jejuni* and their pseudolysogens under low oxygen (<1%) conditions were observed. Generally, *C. jejuni* has been considered to be an obligate aerobe as a microaerophile that is unable to grow in the complete absence of oxygen but requiring oxygen at approximately 5% for respiration (Weingarten *et al*., 2008). Nevertheless, *C. jejuni* was able to grow under oxygen environment of less than 1% possibly due to the availability of an alternative respiration pathway that can contribute to energy conservation (Sellars *et al*., 2002). However, the generation times under these conditions were longer than those observed in a microaerobic environment and featured an extended lag phase. Interestingly, the latent period of CSLC phage under low oxygen conditions was also extended compared with that of the microaerobic environment. This may be because the energy for phage propagation was not adequate at lag phase and growth was therefore delayed until bacterial cells had started the exponential growth phase before an increase in phage propagation was possible.

No growth of any *C. jejuni* strain or their CSLC derivatives was observed under aerobic conditions. The absence of growth did not enable phage propagation under these conditions. The CSLC bacteria remain obligate microaerophiles like their parental strains. However, the persistence of the CSLC bacteria and the parental strains in atmospheric oxygen was 4-5 h before their viability was decreased. The phage numbers were not increased due to the fact there was no growth of *Campylobacter* under these conditions. However, phage numbers remained constant under atmospheric oxygen conditions.

The survival of *C. jejuni* parental strains and their CSLC derivatives were examined under atmospheric, low oxygen and microaerobic conditions combined with nutrition limitation. All of the isolates and parental bacteria could persist under nutrient limitation throughout the experiments carried out under low oxygen and microaerobic conditions. Interestingly, the numbers of survivors of the CSLC isolates under aerobic conditions were significantly higher than parental strains at 2 h post incubation ($P<0.05$). The results also showed that the survival times for the CSLC strains under nutrition limitation conditions were shorter (2 h) than those incubated in Mueller-Hinton broth (4 h) under atmospheric conditions. The survival of *C. jejuni* in the environment depends on many factors such as temperature, nutrient availability, oxygen stress, biofilms formation and biotic interactions (Jackson *et al*., 2009; Sanders *et al.,* 2008; Davis and Conner, 2007; Snelling *et al*., 2005; Buswell *et al*., 1998). It is therefore notable that nutrient and CSLC phage affected the survival of *C. jejuni* in an atmospheric environment. Importantly, in terms of food safety, CSLC may help *C. jejuni* to survive for longer periods in environments where non-CSLC would be impaired.

The survival of CSLC derivatives under nutrient limitation and ambient atmosphere at 50 °C and 4 °C was investigated. The CSLC were able to survive heat shock (50 °C) better than their parent bacteria under nutrient limitation and atmospheric conditions. The CSLC derivative strains exhibited enhanced thermotolerance and therefore should be examined for the induction of their heat shock response (Holmes *et al*., 2010; Bǽk *et al*., 2011). However, the survival of the CSLC strain at 4 °C was not significantly different to that of the parent strains over a 9 days incubation period. Similarly, previous studies have demonstrated the survival of *C. jejuni* at refrigeration temperatures (4 °C) with 77% viability after a week on cooked chicken meat (Bhaduri and Cottrell, 2004; Eideh and Al-Qadiri, 2011). Haddad *et al*. (2009) reported that the survival of *C. jejuni* in nutrient-rich media at 4 °C ambient atmosphere was 70% after an incubation period of 7 days. It is noted that the CSLC variants reported here were able to survive prolonged exposure to low temperature just as well as the parental strains.

The transduction of the chloramphenicol resistance gene from campylobacters carrying the resistance determinant alongside either green or yellow fluorescent cell markers was examined. CSLC phages recovered from either the lysis of genetically marked bacteria or those recovered from CSLC host bacteria were unable to mediate the transduction of chloramphenicol resistance. It is therefore clear that the CSLC phages and free bacteriophages do not mediate generalized transduction and are therefore unlikely to be able to disseminate pathogenic traits via this process (Konkel *et al*., 2001; Kelly *et al*., 2009).

The ability of CSLC phages to form plaques on different hosts was notably different from the parental phages particularly using *C. jejuni* 11168 as the host. Similarly, the ability of CSLC phage to bind to distinctive hosts was significantly different to phage propagated by lysis of the parental bacteria. However, comparison of the host binding ability of CSLC phage and conventionally propagated phage on *C. jejuni* 11168 was not significantly different. In contrast, the plaque forming ability and EOP of CSLC phage and their parent phage on 11168 were very different, in particular CSLC phage derived from the *C. jejuni* PT14 strain. The production of variant progeny phage particles could arise by the interactions between the host and the phage that can manifest through modification of the phage particles themselves and/or changes in genetic or physiological processes within the host cells (Maio and Zahler, 1958). The type strain *C. jejuni* 11168 has been reported to be able to adapt to stressful environments through changes in the expression of cell surface antigens either via transcription or unusually as a consequence of sequence slippage placing key components in and out of reading frame (Parkhill *et al*., 2000; Gaynor *et al*., 2004; Dorrell *et al*., 2001; Wilson *et al*., 2010). These variations in surface structure can include capsular polysaccharide and flagella, which are candidate structures for the efficient recognition and binding of host cells by *Campylobacter* bacteriophage (Linton *et al*., 2001; Taboada *et al*., 2004; Hiett *et al*., 2008). According to Coward *et* *al*. (2006), surface structures such as capsular polysaccharide and flagella are required for bacteriophage infection of *C. jejuni* based on the interaction of typing phage with mutated host derivatives. In addition, mutation of genes involved in variation of the sugar moiety on capsular polysaccharide showed a reduction in plaque formation by *Campylobacter* bacteriophage (Sørensen *et al*., 2011). It is notable that bacteriophage infection of CSLC hosts might result in phage modification and phage diversity leading to the loss of plaque forming ability and ability to adsorb to the host. This is particularly notable for *C. jejuni* 11168 strains but not using *C. jejuni* PT14 or HPC5 hosts as propagation strains. The diversity among distinctive strains may therefore affect the ability of phage infection and host induced modification of the bacteriophage because of the variation in surface structure and metabolic profiles of each *Campylobacter* strain (Fouts *et al*., 2005; Duong and Konkel, 2009). The insertion or deletion of restriction/modification systems of distinctive species has been observed in comparative genomic hybridization surveys, implying that the different hosts have the capacity to encode the apparatus to support distinguishable mechanisms for the prevention of bacteriophage replication (Furuta *et al*., 2010).

4.4 Conclusion

The formation of CSLC in *C. jejuni* biofilms is likely dependent on several factors but these include nutrient limitation and the physiological state of the host bacteria. The CSLC isolates had similar viability under microaerobic and low oxygen conditions to their parental strains. However, higher numbers of phage particles were propagated under microaerobic conditions than under low oxygen conditions. Of significant note the CSLC strains could survive under nutrient limited conditions in an ambient atmosphere longer than their parent bacteria, conditions which are likely to occur frequently in the environment. The CSLC cultures needed to produce growth to enable phage propagation, likely due to the metabolic needs required to support the process. Additionally, the CSLC cultures exhibited greater thermotolerance than their parental counterparts under atmospheric oxygen but exhibited similar survival rates under conditions of atmospheric oxygen, low temperature and nutrient limitation. The CSLC phages were found to be different from the parental phages in their plaque forming ability, host binding ability and efficiency of plating on 11168 strains but not on the propagating strains. The transduction of chloramphenicol resistance did not occur by conventionally propagated bacteriophage or CSLC phages. Host encoded factors such as phage resistance mechanisms and changes of surface structures warrant further study to demonstrate their influence on the characteristics of the CSLC phages and formation of CSLC within biofilms.

CHAPTER 5
TRANSCRIPTOMIC STUDY OF THE CARRIER STATE LIFE CYCLE OF *CAMPYLOBACTER JEJUNI*

5.1 Introduction

DNA microarray technologies have been used to monitor the gene expressions during the infection cycle bacteriophage. The transcriptional profiles of bacteriophage genes during infection of their respective hosts were able to classify the majority of genes into early, delayed early, middle and late, based on their expression profiles (Poranen *et al*., 2006; Su *et al*., 2010). For instance, Frye *et al*. (2005) reported using a DNA microarray to measure temporal changes in phage and host genomic DNA expression during lytic growth of temperate bacteriophages of *Salmonella enterica* serovar Typhimurium LT2. Duplessis *et al*. (2005) studied temporal gene expression of the two groups of phages infecting the Gram-positive lactic acid bacterium *Streptococcus thermophilus*.

Chapter 4 described the formation of the carrier state life cycle (CSLC) of *Campylobacter jejuni* isolated post phage treatment of *Campylobacter* biofilms. The growth of the CSLC strains was similar to their parent bacteria under microaerobic and oxygen-limited conditions. However, the CSLC strains were better able to survive under nutrient limited conditions in atmospheric oxygen compared to their parent strains. Additionally, phages recovered from these cultures showed host range changes associated with host dependent differences in EOP and binding. The CSLC cultures may therefore have a critical influence on the ecology of *Campylobacter* and the bacteriophages that infect it. Phenotypic modification of the host bacteria in CSLC cultures would not only confer an increased capacity for the host to survive atmospheric oxygen but also play an important role in the protection and evolution of bacteriophages in these environments.

Transcriptomic studies of the CSLC strains may provide a useful insight into phage-host interactions of the CSCL strains. Transcriptional changes associated with the phage-host interaction during the growth of the CSLC strains have not been previously studied in any species. This study therefore aims to determine the transcript profiles of the *C. jejuni* CSLC strains growing at 42 °C under microaerobic conditions.

5.2 Results

Data collected on the differential transcription of the CSLC strains were compared to the parent bacteria during early exponential phase of growth under microaerobic conditions at 42 °C. The cultures were harvested at a time to maximise growth yield for RNA extraction but before reaching the phage proliferation threshold of $\log_{10}$ 7 CFU/ml, and therefore before the increase in the phage titres that are likely to be the result of orthodox infection and lysis (Figure 5.1) (Section 2.9). Transcript levels were determined using microarray hybridizations under conditions where equal quantities of cDNA were labelled with the same dye and competed against a common labelled cDNA control (Section 2.10). Genes exhibiting significant differential expression between the parent and CSLC strains (*P*<0.05 by ANOVA and using the Benjamini Hochberg False Discovery Rate correction) were classified into functional groupings according to the Sanger Institute database (www.sanger.ac.uk/resources/downloads/bacteria/campylobacter-jejuni.html) compiled with respect to the re-annotation of the *C. jejuni* 11168 genome (Gundogdu *et al.*, 2007). The oligonucleotide microarray contains the gene repertoires (locus tags) from *C. jejuni* 11168 (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000), *C. jejuni* RM1221 (Fouts *et al.*, 2005), *C. coli* RM2228 (Fouts *et al.*, 2005), and all new non-redundant locus tags associated with *C. jejuni* and *C. coli* genomes available in the GenBank database at the time of construction (May 2009). In addition, oligonucleotides representing all the *Campylobacter* bacteriophage genes for representative class II (CP220 Accession No: FN667788.1; Timms *et al*., 2010) and class III (CPX Accession No: JN132397.1; Al-kandari *et al*., unpublished) bacteriophage.

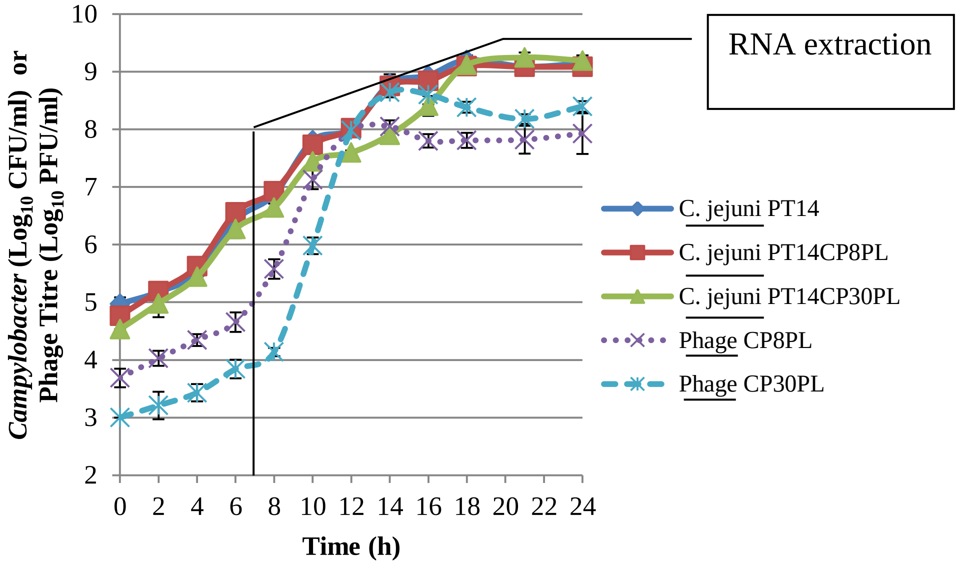


Figure 5.1- The growth curve of the CSLC strains and parent bacteria at 42^{o} C under microaerobic conditions. This graph shows the time point of RNA extraction and represents where the transcriptome of the CSLC strain was compared to the parent strains before an increase in phage titre.

5.2.1 Transcriptome analysis of CSLC strains of *C. jejuni* HPC5

Transcriptomic analysis of the CSLC strain HPC5CP8PL compared to the parent strain showed 2-fold or greater changes in the levels of transcription for 22 host genes with 7 genes showing up-regulation (Table 5.1) and 15 genes down-regulation (Table 5.2). Four bacteriophage genes also showed a significant increase in expression over background, which are also recorded in Table 5.1. The CSLC strain HPC5CP30PL showed a 2-fold change in host gene expression in a total of 24 genes that included 8 genes showing up-regulation (Table 5.3) and 16 genes showing down-regulation (Table 5.4). HPC5CP30PL also showed a significant increase over the background signal of the bacteriophage gene CPT_0011 (P=0.0003), a situation that it shares with HPC5CP8PL (Table 5.3).

Table 5.1– Gene expression at least 2-fold up-regulation of the CSLC of *C. jejuni* (HPC5CP8PL) at exponential phase of growth under microaerobic conditions at 42 °C. * The phage genes are measured over background.

locus_tag	gene name	function	fold change
Phage-encoded genes			
CPT_0011		RNaseH	3.4*
CPT_0084		Hypothetical (radical SAM domain protein)	3.3*
CPT_0111		Hypothetical (possible membrane lipoprotein)	3.0*
CPT_0148		UvsW RNA-DNA and DNA-DNA helicase, ATPase	9.2*
Biosynthesis of cofactors, prosthetic groups and carriers			
Cj1347c	cdsA	phosphatidate cytidylyltransferase	2.0
Cj1044c	thiH	thiH protein	2.1
Synthesis and modification of macromolecules			
Cj0847	psd	Putative phosphatidylserine carboxylase	9.4
Cj0342c	uvrA	excinuclease ABC subunit A	2.5
Cj0322	perR	peroxide stress regulator	4.8
Conserved hypothetical proteins			
CCOA0173		conserved hypothetical protein	3.3
Unknown			
CCOA0077		hypothetical protein	2.8

Table 5.2 – Gene expression at least 2-fold down-regulation of the CSLC of *C. jejuni* (HPC5CP8PL) at exponential phase of growth under microaerobic conditions at 42 °C

locus_tag	gene name	function	fold change
Amino acid biosynthesis			
Cj1716c	leuD	putative 3-isopropylmalate dehydratase	5.3
Synthesis and modification of macromolecules			
Cj0018c		small hydrophobic protein	3.3
Cj1035c		possible transferase	4.4
Cj0493	fusA	elongation factor G	2.7
Cell envelope			
Cj0830		putative integral membrane protein	3.6
Cj0906c		putative periplasmic protein	2.4
Cj1414c	kpsC	possible polysaccharide modification protein	2.3
Chaperones, chaperonins, heat shock			
Cj1034c		possible dnaJ-like protein	3.5
Cj0518	htpG	hsp90 family heat shock protein	2.9
Conserved hypothetical proteins			
Cj0073c		hypothetical protein	2.2
CCOA0164		conserved hypothetical protein	3.0
Unknown			
Cj0249		hypothetical protein	3.8
CJE0202		hypothetical protein	4.4
Misc			
CCO_CctmRNA1			6.5
CJE_tRNA-Trp-1			2.4

Table 5.3- Gene expression at least 2-fold up-regulation of the CSLC of *C. jejuni* (HPC5CP30PL) at exponential phase of growth under microaerobic conditions at 42 °C. * The phage genes are measured over background.

	locus tag	gene name	function	fold change
Phage				
	CPT_0011		RNaseH	2.5*
Biosynthesis of cofactors, prosthetic groups and carriers				
	Cj1347c	cdsA	phosphatidate cytidylyltransferase	3.2
	Cj1044c	thiH	thiH protein	2.0
Synthesis and modification of macromolecules				
	Cj0847	psd	phosphatidylserine decarboxylase	4.1
	Cj0342c	uvrA	excinuclease ABC, A subunit	2.0
Protein and peptide secretion				
	Cj1092c	secF	protein-export membrane protein SecF	2.7
	Cj0322	perR	transcriptional regulator, Fur family	3.5
Conserved hypothetical proteins				
	CCOA0173		conserved hypothetical protein	3.3
Unknown				
	CCOA0077		hypothetical	9.0

Table 5.4- Gene expression at least 2-fold down-regulation of the CSLC of *C. jejuni* (HPC5CP30PL) at exponential phase of growth under microaerobic conditions at 42 °C.

locus tag	gene name	function	fold change
Amino acid biosynthesis			
Cj1716c	leuD	3-isopropylmalate dehydratase, small subunit	3.8
Synthesis and modification of macromolecules			
Cj0018c		small hydrophobic protein Cj0018c -related	2.1
Cj1035c		probable transferase Cj1035c	4.0
Cj0493	fusA	translation elongation factor G	2.0
Degradation of macromolecules			
Cj0980		probable peptidase Cj0980	2.2
Cell envelope			
Cj0830		probable integral membrane protein Cj0830	3.0
Cj0906c		conserved hypothetical secreted protein	2.0
Cj1414c	kps C	capsule polysaccharide biosynthesis protein	2.2
Cj0060c	fliM	flagellar motor switch protein FliM	2.4
Chaperones, chaperonins, heat shock			
Cj1034c		DnaJ domain protein	3.7
Cj0518	htpG	hsp90 family heat shock protein Cj0518	2.4
Conserved hypothetical proteins			
Cj0073c		conserved hypothetical protein	2.5
Cj0249		conserved hypothetical protein	3.7
Unknown			
CJE0202		hypothetical	3.8
CCOA0164		hypothetical	3.3
Misc			
CCO_CctmRNA1			6.2

5.2.2 Transcriptome analysis of CSCL strains of *C. jejuni* PT14

Gene transcription of the CSLC *C. jejuni* PT14CP8PL showed a large number of transcriptional differences in genes representing all functional categories with 21 genes exhibiting more than 2-fold significant ($P < 0.05$) differences in transcript levels between the CSLC strains and parent strains. Of these, 13 genes exhibited a two-fold or more increase in the host CSLC strains compared to the parent bacteria (Table 5.5) with 8 showing the opposite trend (Table 5.6). Two bacteriophage genes exhibited a significant increase in expression against the background hybridization levels and these are recorded in Table 5.5. The microarray data indicate the transcript levels of CSLC *C. jejuni* PT14CP30PL show at least a 2-fold change in 65 host genes with 26 host genes showing a relative increase in transcription (Table 5.7) and 37 genes a decrease in expression in the CSLC strains (Table 5.8). The phage gene CPT_0172 in common with PT14CP8PL shows a significant increase in expression against the background hybridization (P=0.0005).

Table 5.5- Gene expression at least 2-fold up-regulation of the CSLC of *C. jejuni* (PT14CP8PL) at exponential phase of growth under microaerobic conditions at 42 °C. * The phage genes are measured over background.

locus_tag	gene name	function	Fold change
Phage			
CPT_0144		hypothetical	13.8*
CPT_0172		hypothetical	7.7*
Central intermediary metabolism			
Cj1366c	glmS	Glucosamine-fructose-6-phosphate	2.2
Purines, pyrimidines, nucleosides and nucleotides			
Cj0233c	pyrE	putative orotate phosphoribosyltransferase	5.1
Synthesis and modification of macromolecules			
Cj0207	infC	translation initiation factor IF-3	73.3
Cj1673c	recA	recA protein	3.8
Cell envelope			
Cj0818	-----	putative lipoprotein	93.8
CCO1443	flaB	flagellin Cj1338c	4.4
Conserved hypothetical proteins			
CCO0807	-----	conserved hypothetical protein	16.1
Unknown			
Cj1232	-----	hypothetical protein	119.5
Cj1242	-----	hypothetical protein	7.5
Cj0550	-----	hypothetical protein	6.4
Cj0794	-----	hypothetical protein	10.3
Cj1216c	-----	hypothetical protein	4.7
CCOA0165	-----	hypothetical protein	32.2
CJE1462	-----	hypothetical protein	118.8

Table 5.6- Gene expression at least 2-fold down-regulation of the CSLC of *C. jejuni* (PT14CP8PL) at exponential phase of growth under microaerobic conditions at 42 °C.

locus_tag	gene name	function	Fold change
Biosynthesis of cofactors, prosthetic groups and carriers			
Cj0443	accA	acetyl-coenzyme A carboxylase	2.9
Cell envelope			
Cj1424c	gmhA2	phosphoheptose isomerase	3.5
CCO1330		probable periplasmic protein Cj1240c	6.2
Conserved hypothetical proteins			
Cj0761		hypothetical protein	3.3
CCOA0201		conserved hypothetical protein	5.8
Unknown			
Cj1309c		hypothetical protein	3.4
Cj0873c		hypothetical protein	2.1
Cj0738		hypothetical protein	13.9

Table 5.7- Gene expression at least 2-fold up-regulation of the CSLC of *C. jejuni* (PT14CP30PL) at exponential phase of growth under microaerobic conditions at 42 °C. * The phage genes are measured over background.

locus_tag	gene name	function	Fold change
Phage			
CPT_0172		Hypothetical	62.5*
Prophage			
CJE1458		major capsid protein, HK97 family	2.2
Degradation			
Cj1624c	sdaA	L-serine dehydratase	4.6
Energy metabolism			
Cj0465c		truncated haemoglobin	4.5
Cj1586		putative bacterial haemoglobin	25.3
Central intermediary metabolism			
Cj1366c	glmS	glucosamine--fructose-6-phosphate	11.5
Biosynthesis of cofactors, prosthetic groups and carriers			
Cj1183c	cfa	putative cyclopropane-fatty-acyl-phospholipid	3.7
Synthesis and modification of macromolecules			
Cj0207	infC	translation initiation factor IF-3	409.2
Cj1673c	recA	recA protein	3.6
Cell envelope			
Cj0672		putative periplasmic protein	2.2
Cj1456c		putative periplasmic protein	2.1
Cj0818		putative lipoprotein	450.0
Cj0672		putative periplasmic protein	2.7
Cj0320	fliH	putative flagellar assembly protein	3.2
Transport/binding proteins			
Cj0238		putative integral membrane protein	2.2
Protein and peptide secretion			
Cj1470c		pseudogene	3.4
Conserved hypothetical proteins			
Cj0761		hypothetical protein	3.5
CCO0807		conserved hypothetical protein	174.0
Unknown			
Cj1232		hypothetical protein	572.3
Cj1242		hypothetical protein	40.7

Table 5.7 continued

locus_tag	gene name	function	Fold change
Cj0550		hypothetical protein	37.0
Cj1216c		hypothetical protein	14.6
CCOA0032		hypothetical protein	8.2
CCOA0165		hypothetical protein	249.7
CJE0139		hypothetical protein	3.4
CJE1462		hypothetical protein	831.9
CJE_tRNA-Arg-3		tRNA-Arg	3.3
CJE_tRNA-Asn-1		tRNA-Asn	3.9
CJE_tRNA-Cys-1		tRNA-Cys	2.4

Table 5.8- Gene expression at least 2-fold down-regulation of the CSLC of *C. jejuni* (PT14CP30PL) at exponential phase of growth under microaerobic conditions at 42 °C

locus_tag	gene name	function	Fold change
Amino acid biosynthesis			
Cj0346	trpD	anthranilate synthase component II	2.4
Cj0345	trpE	putative anthranilate synthase component I	2.2
Cj0348	trpB	tryptophan synthase beta chain	3.7
Cj0347	trpF	N-(5'-phosphoribosyl) anthranilate isomerase	3.8
Synthesis and modification of macromolecules			
Cj1705c	rplW	50S ribosomal protein L23	3.9
Cj1690c	rpsE	30S ribosomal protein S5	2.2
Cj1700c	rplP	50S ribosomal protein L16	2.5
Cj1697c	rplN	50S ribosomal protein L14	2.4
Cj1699c	rpmC	50S ribosomal protein L29	3.2
Cj1702c	rplV	50S ribosomal protein L22	2.5
Cj1701c	rpsC	30S ribosomal protein S3	2.5
Cj0473	nusG	putative transcription antitermination protein	2.2
Cj0765c	hisS	histidyl-tRNA synthetase	2.0
Cj1704c	rplB	50S ribosomal protein L2	2.7
Cj1706c	rplD	50S ribosomal protein L4	3.0
Cj1691c	rplR	50S ribosomal protein L18	2.5
Cj0476	rplJ	50S ribosomal protein L10	2.4
Cell envelope			
Cj1316c		hypothetical protein	3.0
Cj1424c	gmhA2	putative phosphoheptose isomerase	2.8
Cj1339c	flaA	flagellin	5.1

Table 5.8 continued

locus_tag	gene name	function	Fold change
Cj0549	fliS	flagellar protein	3.0
Cj0547	flaG	possible flagellar protein	2.5
Cj1316c		hypothetical protein	2.7
Cj1338c	flaB	flagellin	4.6
Cj1211		putative integral membrane protein	2.3
Cj0548	fliD	putative flagellar hook-associated protein	2.6
Cj1127c	wlaE	putative glycosyltransferase	2.0
CCO1443	flaB	flagellin Cj1338c	5.0
CCO1444	flaA	flagellin Cj1339c	4.9
Transport/binding proteins			
Cj0679		truncated KdpD protein	2.2
Conserved hypothetical proteins			
Cj1465		hypothetical protein	2.4
Cj1463		hypothetical protein	2.1
Unknown			
Cj0873c		hypothetical protein	7.7
Cj1656c		hypothetical protein	4.3
Cj0738		hypothetical protein	9.3
Misc			
CCO_tRNA-Gly-2		tRNA-Gly	2.3
CJE_tRNA-Gly-1		tRNA-Gly	2.5
CJE_tRNA-Trp-1		tRNA-Trp	2.5

5.3 Discussion

Transcriptome analyses of the CSLC strains obtained after phage treatment of *Campylobacter jejuni* biofilms were performed. The transcript analysis identified differential host gene expression between parent and CSLC derivative strains. Phage gene expression was examined over background because the parent cultures did not have phage present. Using this criterion at the specific point in the growth period before the rise in phage titre resulted in a limited set of phage genes that were observed to produce significant expression. It is possible that early stage CSLC cultures may not contain phage DNA inside the host cells and that the gene expression recorded represents the start of phage replication or non-synchronous replication before achieving the bacterial density of susceptible cells required for mass infection. However, the expression of phage genes is evident from *C. jejuni* HPC5CP8PL, with the genes CPT_0011, CPT_0084, CPT_0111 and CPT_0148 all show significant levels over background. The hypothetical functions of CPT_0084 and CPT_0111 are not specifically phage related, encoding respectively a putative radical *S*-adenosyl-*l*-methionine (SAM) domain protein (paralogous genes for which are prevalent in *Campylobacter* bacteriophage), and a membrane lipoprotein. It is notable that the database contains an analogue of CPT_0084 located in the chromosome of *C. fetus* subspecies fetus and annotated as a haem biosynthesis protein (Timms *et al*., 2010), an observation that is consistent with the contention that these genes are not strictly required for bacteriophage replication. In contrast the putative function ascribed for CPT_0148 is that of the phage DNA repair helicase UvsW. The product of *uvsW* gene in T4 phage acts in DNA replication to reactivate stalled DNA replication forks leading to the formation of double-stranded DNA ends (Nelson and Benkovic, 2010), which could relate to the stalled replication of the phage genomes in HPC5CP8PL CSLC cultures. Expression of phage gene CPT_011 that encodes ribonuclease H (RNase H) was up-regulated in both HPC5CP8PL and HPC5CP30PL. RNase H functions in the T4 multienzyme replication system to remove ribopentamer primers from nascent DNA chains during synthesis by specific hydrolysis of the phophodiester bond of RNA-DNA and DNA-DNA dulplexes. Evidence suggests RNaseH has an important role in T4 phage replication since a T4 null mutation of this gene reduced the burst size two fold (Hollingsworth and Nossal, 1991; Hobbs and Nossal, 1996; Bebenek *et al*., 1999). This gene may contribute to the control of phage replication in the CSLC isolates. The CSLC of *C. jejuni* PT14CP8PL and PT14CP30PL exhibited an increase in phage gene expression of CPT_0172 that shows a significant BLAST match with a hypothetical protein of *C. jejuni* RM1221. The hypothetical gene CPT_0144 also showed expression in PT14CP8PL (Timms *et al*., 2010).

Chapter 4 demonstrated that CSLC cultures were able to grow at similar growth rates to their parent bacteria under microaerobic conditions but notably showed discontinuities in their growth that were contemporary with the observed increases in phage titres. Under the experimental conditions adopted (microaerobic) for the transcriptional studies, perhaps the most notable phenotypic change common to all CSLC cultures was their loss in motility. TEMs presented in chapter 4 indicate the flagella are not just paralysed but absent. Consistent with this observation is the 5-fold down regulation of the flagellin genes *flaA* and *flaB* together with genes encoding several flagellar structural proteins (*flaG*, *fliD* and *fliS*) in PT14CP30PL. Although it should be noted the putative flagellar assembly protein *fliH* is 3-fold up-regulated. In PT14CP8PL the alternative minor flagellin component encoded by *flaB* is also up-regulated. These differences could reflect the active growth and cell division if the culture in early exponential phase with the daughter cells of the CSLC strains reverting to phage sensitivity, which could account for the sharp rise in the phage titre, observed in the PT14 derived CSLC cultures. Deficiency in flagellar synthesis can lead to phage resistance (Coward *et al*., 2006; Scott *et al*., 2007b), and reinstating flagellar synthesis could be one of the triggers to enable orthodox bacteriophage infection of daughter cells.

The CSLC cultures derived from *C. jejuni* HPC5 (HPC5CP8PL and HPC5CP30PL) show up-regulation of *cdsA* and *psd*. CdsA encodes phosphatidate cytidylyltransferase that catalyses the synthesis of CDP-diacylglycerol from cytidine triphophate and phosphatidate, which act as an intermediate in the biosynthesis of lipids and complex carbohydrates. In *E. coli* and *Bacillus subtilis* these are essential for phospholipid biosynthesis, and likely many other organisms (Weber *et al*., 1999; Sparrow and Raetz, 1985). Significantly, phosphatidylserine decarboxylase encoded by *psd* catalyses the synthesis of phosphatidylethanolamine (PE), which plays an important role in the motility and chemotaxis functions of *E. coli*. Mutant strains of *E. coli* deficient in *psd* are reported to be non-motile and to lack flagella, and thereby link the functional expression of the flagellar apparatus with the availability of PE (Shi *et al*., 1993).

The peroxide stress regulator of *C. jejuni* encoded by *perR* plays a vital role in maintenance the redox balance within cell, and specifically at reduced oxygen tensions where 104 genes have been reported to show associated regulatory changes (Palyada, *et al*., 2009). Campylobacters lack the global *oxyR* regulator present in many other Gram-negative bacteria, and therefore rely upon the metalloregulator *perR*. Notably mutation in *perR* also results in significantly reduced motility (Palyada *et al*., 2009). The *perR* gene is up-regulated in the CSLC strains HPC5CP8PL and HPC5CP30PL, however, reasons behind this are unclear because PerR is not only a repressor of the genes involved in oxidative stress but is also autoregulatory (Kim *et al.*, 2011). Increased production of PerR could result in the tight regulation of the oxidative stress response genes but the increased transcription could also be indicative of the absence of PerR leading to derepression of the *perR* gene. PerR uses iron as a co-repressor, which could also have role in the induction of perR if iron was limited. The genes encoding catalase (*katA*) and superoxide dismutase (*sodB*) also show modest up-regulation but not sufficiently so to conform to the selection criteria imposed on the data. Consistent with this observation it has been noted that under circumstances of iron depletion the classic oxidative stress genes are already transcribed and therefore do not often show additional transcriptional changes in microarray data (Palyada *et al*., 2009). This interpretation would favour the hypothesis that PerR is indeed absent or not fulfilling its role as a repressor of oxidative stress related genes. Presumably, the greater aerotolerance and non-motility of the CSLC isolates may be related with the function of PerR and associated regulatory network.

Independent of *perR* regulation but also indicative of an oxidative stress response is the up-regulation of *uvrA* in the CSLC strains HPC5CP8PL and HPC5CP30PL. The role of UvrA as subunit A of excinuclease ABC is to control excision repair of DNA in *E. coli*, suggesting a response to DNA damage may occur in the CSLC HPC5 isolates (Orren and Sancar, 1989). The genes Cj1586 and Cj0465c encode bacterial haemoglobin (*cgb*) and a truncated form (*ctb*) in *Campylobacter*, which are reported to be co-regulated by NssR in response to nitrosative stress, and that are up-regulated at higher oxygen levels (Monk *et al*., 2008; Smith *et al*., 2011). The CSLC strain PT14CP30PL shows 25-fold and 4.5-fold up-regulation of the *cgb* (*P*=0.00002) and *ctb* (*P*=0.02) genes respectively that likely contribute to the oxygen tolerance observed.

The ThiH protein encoded by *thiH* is a member of the radical S-adenosylmethionine protein superfamily involved in biosynthesis of thiamine pyrophosphate, which is an essential cofactor for all organisms (Martinez-Gomez *et al*., 2004). The *thiH* gene was up-regulated in the CSLC strains HPC5CP8PL and HPC5CP30PL in contrast to the lysogenic lifestyle of Shiga toxin-converting bacteriophage of *E. coli* for example, which down-regulate the *thiH* gene (Su *et al*., 2010). It is also noteworthy that *Campylobacter* bacteriophage genomes contain multiple copies of *S*-adenosylmethionine domain encoding proteins but their significance remains obscure at this time (Timms *et al*., 2010).

Genes showing differential reductions in their transcript levels of the CSLC-HPC5 isolates included: *leuD, cj0018c, cj1035c, cj0493, cj0830, cj0906c, cj1414c, cj1034c, cj0518, cj0073c, CCOA0164*, and *CCO-CctmRNA1*. The *leuD* gene putatively encodes 3-isopropylmalate dehydratase, a gene in the *leuABCD* operon involved in leucine synthesis (Vartak *et al*., 1991). The DnaJ protein is a requirement for motility in *E. coli* as result of the fact that cells deleted for *dnaJ* affect a reduction in the transcription of flhD and fli4 leading to non-motility and no flagella (Shi *et al*., 1992). Remarkably, the suppressed dnaJ-like protein encoded by *cj1034c* may reflect the lack of motility evident in the CSLC cultures. HtpG (cj0518), a member of the heat shock protein 90 family, is a molecular chaperone for folding and regulation, suggesting that repression of *htpG* may have an effect on the house-keeping functions (Picard, 2002). FusA (cj0493), elongation factor G (EF-G), has a function involved in the regulation of mRNA translocation through the ribosome following peptide bond formation (Hou *et al*., 1994). Suppression of *fusA* may effect protein synthesis, suggesting that protein synthesis may be interrupted by phage in preparation for making its own proteins. The repression of *kpsC* (cj1414c), involved in capsular polysaccharide biosyntheis is of interest as capsular polysaccharide has been implicated as a phage receptor or co-receptor (Coward *et al*., 2006; Sørensen *et al*., 2011). A change in capsular polysaccharide in the CSLC isolates could alter the cellular surface of the CLSC cultures leading to resistance to bacteriophage infection or the retention of bacteriophage particles without infection. The genes *gmhA2* and *wlaC* were down-regulated in, and encode components responsible for the synthesis of the cell surface carbohydrates lipooligosaccharide and capsular polysaccharide (Karlyshev *et al*., 2005).

The differential transcription of host genes recorded for the CSLC strains PT14CP8PL and PT14CP30PL showed some marked changes compared to the CSLC cultures derived from HPC5 despite growth under similar conditions. An increase in the expression of *glmS* (glucosamine-frutose-6-phophate aminotransferase) that has an essential function involved in the production of UDP-N-acetylglucosamine was observed in both CSLC cultures of PT14. Up-regulation of this essential component for cell metabolism could be indicative of robust growth of the CSLC cultures. Similarly, an increase in the transcription of *sdaA* (L-serine dehydratase) involved in gluconeogenesis by deamination L-serine to gain pyruvate is a vital component of the metabolism of *Campylobacter* to yield the energy for growth. These findings suggest that the CSLC strains may be able to exploit the prevalent cell metabolism to maintain the growth of cells and energy for phage production. Translation initiation factor IF3 encoded by *infC* is one of three factors for initiation of protein synthesis in bacteria by interaction with 30S ribosomal subunit (Petrelli *et al*., 2001), importantly showing that bacteriophage may take in advance to initiate the phage protein synthesis. Transcription studies of the development of bacteriophage infection demonstrate that phage require host promoters at early stages of infection, thus an increase in the transcription of IF3 may be related with the early host-gene subversion by the phage associated with CSLC isolates in preparation for the efficient translation of phage transcripts (Berdygulova *et al*., 2011). However, the host 50S and 30S ribosomal proteins were repressed in the PT14CP30PL. The *recA* gene of PT14CP30PL shows a 3.6-fold increase over the parent strain. A similar increase in the expression of the RecA protein has been reported when bacteriophage infect *Salmomella enterica,* a finding that has been related to the degradation of bacterial chromosome and/or the production of a significant amount of ssDNA within the cell (Campoy *et al*., 2006). Finally the tryptophan operon (*TrpB, TrpD, TrpE* and *TrpF*) in was down-regulated in its entirety in PT14CP30PL.

5.4 Conclusion

A differential transcription analysis of the CSLC strains growing under microaerobic conditions at exponential phase of growth was conducted by comparison with parent bacteria. These studies demonstrate that a number of genes show 2-fold or greater up-regulation and down-regulation in the CSLC cultures. Interestingly, there were significant changes in host gene expression between distinctive strains, whereas there were fewer differences between CSLC strains arising from the association of different bacteriophage. The different physiology of the CSLC strains may lead to alterations of transcription in the CSLC cultures and subsequently modification of phage progeny. In this study, the transcription of the CSLC isolates showed a few phage gene expressions at early exponential growth before the number of *Campylobacter* achieved the proliferation threshold. It is notable that capsular polysaccharide, lipooligosaccharide, protein may be modified or adapted by the CSLC phage associated with the CSLC strains. Notably, the suppression of a set of flagellar associated genes including flagellin (*flaA* and *flaB*) with other structural and regulatory components (*flaG*, *fliD* and *fliS*) correlated with flagellar modulation that may have an effect on the motility of the CSLC cultures leading to the flagella deficient phenotype and non-motility. Many of the genes identified in this study have also been implicated in the ability of *Campylobacter* to colonise chickens. It would therefore be of interest to examine the colonisation competence of CSLC strains. However, validation of microarray results using quantitative real-time PCR (qRT-PCR) needs to be undertaken to confirm the transcriptional changes observed for the CSLC strains.

CHAPTER 6

FINAL DISCUSSION AND FUTURE WORK

6.1 Final discussion

Campylobacter is an important pathogen causing gastroenteritis worldwide. *Campylobacter jejuni* is typically able to form biofilms on various surfaces under microaerobic and aerobic conditions. Biofilms are a common mechanism employed by bacteria to protect them from harsh environments. Generally, natural biofilms are comprised of single or multiple bacterial species embedded in a surface extracellular polymeric matrix. In the case of *C. jejuni* these structures are likely to play an important role in the spread and transmission of the bacterium into the food chain and thereby cause human disease. Importantly, bacteria within biofilms often exhibit an increase in resistance to antibiotics and other chemical antimicrobials. Thus, methods that avoid the use of antibiotics need to be developed to eradicate biofilms without giving rise to antimicrobial resistance. Bacteriophages are a natural predator of bacteria that are potential biological agents with the prospect to control and disperse bacteria in biofilms. The application of *Campylobacter*-specific bacteriophage in the form of phage therapy has been reported as an effective biological control of *Campylobacter* colonising the intestinal tracts of chickens (Loc Carillo *et al*., 2005). The findings presented in chapter 3 showed that reductions in the viability of *C. jejuni* were observed in biofilms after treatment with group III bacteriophage (CP8 and CP30). Additional evidence showed that the quantity of extracellular polysaccharide was reduced after phage treatment compared to mock treatment following measurements of absorbance change of ethanol-solubilised biofilms stained with crystal violet. These data suggest that bacteriophage can disperse *Campylobacter* biofilms. The mechanisms by which bacteriophage can disperse surface biofilms have still to be studied in *Campylobacter* but are associated with the action of polysaccharide depolymerases in bacteriophage infecting other species (Hughes *et al*., 1998a and b; Lu and Collins, 2007). However, the genome sequences of *Campylobacter* bacteriophage did not identify any candidate gene encoding a polysaccharide depolymerase (Timms *et al*., 2010; Hammerl *et al*., 2011; Kropinski *et al*., 2011). However more recently a putative polysaccharide depolymerase was identified in the genome sequence of the group II phage VB_CcoM-IBB_35 that infects *Campylobacter coli* (Carvalho *et al*., 2012). Polysaccharide depolymerase enzymes encoded within bacteriophage would have to be translated during infection and therefore assist in the release of bacteriophage upon lysis. Alternatively bacteriophage also feature enzymes fused in frame with their structural proteins. For example, the tailspike protein of bacteriophage P22 infecting *Salmonella* contains a rhamnogalacturonase (Steinbacher *et al*., 1996), and the tailspike proteins of the Vi-like phage feature beta-parallel coil structures that resemble the pectin lyase protein family (Hooton *et al*., 2011). The primary action of a bacteriophage polysaccharide depolymerase would be to degrade extracellular polysaccharide that constitutes a major component of biofilms. Subsequently, the post action of bacteriophage would occur after phage infection of the bacteria where upon the lysis process may destroy the structure of biofilms due to the production of lysins or lysozyme that degrade bacterial cell membranes and subsequently release the cell contents, which will either degrade biofilm components and/or kill bacteria within biofilms leading to the loss of biofilm structure and bacterial viability. However, these mechanisms require further study to understand the processes involved in phage-biofilm interactions.

Phage resistance is a potential limitation to phage applications. However, the resistance is often reversible to bacteriophage sensitivity because the resistant types are at a selective disadvantage. The bacteriophage resistant types recovered from the phage treatment of *Campylobacter* biofilms largely depended on the physiological characteristics of the initial *Campylobacter* strains under treatment. The type strain *C. jejuni* NCTC 11168 exhibited a higher frequency of phage resistance than either *C. jejuni* PT14 or HPC5. Mechanisms of bacterial defence to infection by bacteriophage may involve modification of cell surface components that have been demonstrated to have phase variable expression in *C. jejuni*, which could accordingly mediate against the adsorption of bacteriophage (Coward *et al*., 2006). In addition, restriction/modification mechanisms and the CRISPR/Cas system of each strain may affect variation in the acquisition of phage resistance (Abedon, 2011).

Interestingly, the formation of carrier state life cycle (CSLC) of *C. jejuni* occurred during phage treatment of biofilms (described in Chapter 4). The CSLC cultures are mixtures of bacteriophage and bacteria that contain uninfected bacteria, uninfected but phage associated bacteria, infected bacteria and free phage (Abedon, 2009). The evidence presented in chapter 4 indicated that phage DNA did not integrate into bacterial chromosomal DNA based on southern hybridisation of total extracted DNAs separated by PFGE using phage DNA as a control to identify the bacteriophage DNA associated with the CSLC cultures. It is clear that phage DNA present in CSLC cultures is similar to the phage used to treat the biofilms and is not prophage DNA excised from the genome. Bacteriophages are preserved and maintained within biofilms, and thereby possibly promote the occurrence of the carrier state life cycle alongside the normal lytic cycle. CSLC formation may involve many factors such as nutrition limitation and the physiological state of growth.

The physiological characteristics of the CSLC isolates were generally similar to their parent bacteria. Interestingly, the growth of CSLC strains under microaerobic and oxygen limited conditions (<1% O_2) were accompanied by an increase in the phage titres. Importantly, the increase in number of phage was related to the phage proliferation threshold, which relates to the minimal density of bacteria required to give rise to an increase in the number of phage particles (Cairns *et al.*, 2009). This would suggest in this phase of growth that phage infection of sensitive bacteria is occurring as it might in any orthodox culture. As observed for *C. jejuni* generally the CSLC isolates did not grow under atmospheric conditions leading to a constant phage titre throughout the incubation time. The growth of CSLC cultures therefore plays an important role in phage production and propagation, without which the bacteria would lack an adequate energy supply for phage production. Transcriptome analysis of the CSLC cultures at early exponential phase of growth (described in chapter 5) showed a differential increase in host gene expression involved in the energy metabolism, in particular energy metabolism related to amino acid utilisation. *C. jejuni* is a non-saccharolytic species and typically utilises the amino acid L-serine and TCA cycle intermediates for energy requirements by using oxygen as a terminal electron acceptor. In this context transcription analysis of the CSLC growth under microaerobic conditions in this study showed up-regulation of *sdaA*, *glmS* which would benefit the cell to gain energy for cell growth and phage production.

Notably, the survival of the CSLC isolates under nutrient limitation in atmospheric conditions was longer than the parent bacteria. Several genes involved in oxidative defence mechanisms of *Campylobacter* were up-regulated in CSLC strains. For example, the up-regulation of *perR* in CSLC cultures is consistent with changes in oxygen tolerance as it is responsible for maintaining redox potential within the cell (Palyada *et al*., 2009). In addition, the oxygen binding bacterial globin genes *ctb* and *cgb* were up-regulated (Monk *et al*., 2008; Smith *et al*., 2011). These genes could play an important role in oxygen tolerance of CSLC strains. The priming of CSLC cultures ready for oxidative stress may also extend to DNA damage since *uvrA* is notably up-regulated in these cultures. Transcriptome analysis of the CSLC cultures should therefore be additionally performed under either low oxygen or atmospheric conditions with controlled nutrition to understand how they respond to oxygen and how this may be related to the interaction of phage and host.

Interestingly, the CSLC strains were non-motile and no flagella were observed by TEM, while the parent bacteria have normal flagella and are motile. Flagella have been implicated as structures necessary for phage replication and required for chicken colonisation and virulence (Coward *et al*., 2006; Scott *et al*., 2007b; Wessenaar *et al*., 1993). Transcription analysis of CSLC cultures demonstrated changes in flagellin expression and flagella biosynthesis.

Factors affecting the formation of CSLC may involve nutrition limitation of the bacterial host, a condition that occurs within mature biofilms. CSLC phages were released when the CSLC cultures were grown under suitable conditions. Physiological characteristics of CSLC phages derived from CSLC cultures were distinguishable from the parental phage. Different plaque forming and binding abilities of bacteriophages were observed on distinct hosts that may be dependent on genotypic modification of the host strains producing them. The data confirmed that CSLC phages recovered from the CSLC cultures were genetically related to the initial phage used to treat the biofilms and are not prophage or a result of contamination with another bacteriophage. However, changes in host modification of CSLC phages could affect a change in physiology of these phages.

In conclusion, in this thesis it has been demonstrated that bacteriophage are a potential biological agent for use in the biocontrol of biofilms. Bacterial viability and biofilm matrix content were reduced after treatment with bacteriophage. Perhaps surprisingly the bacteriophage treatment of biofilms gave rise to the formation of CSLC cultures. This study is the first to show phage-host interactions leading to the formation CSLC within phage treated biofilms. The physiological characteristics of CSLC cultures were typically similar to their parent bacteria cultures in terms of growth rate under microaerobic and low oxygen conditions. Interestingly, however, differences in the physiologies observed between CSLC cultures and parent bacteria included non-motility, greater aerotolerance and thermotolerance when subject to nutrient limitation under atmospheric conditions. Indeed, transcriptome analysis of CSLC cultures under microaerobic conditions provided further support for changes in gene expression related to these phenotypes. CSLC cultures in early exponential phase of microaerobic growth featured up-regulated genes involved in respiration, oxidative stress response, and were down-regulated in specific flagellar biosynthesis functions. CSLC phages could be modified by CSLC cultures, reflecting distinguishable plaque forming and binding abilities on distinctive host strains as well as the efficiency of plating.

6.2 Future work

Further work after this thesis study should be carried out to gain a greater understanding of phage and biofilm interactions and in particular the circumstances under which the phage may be applied. These conditions could include the examination of non-static biofilms and a range of surfaces as substrates for biofilm formation. Bacteriophage treatment of biofilms could be used to further investigate the kinetics of phage infection and how this may effect populations of biofilm bound bacteria and planktonic cells. The influence of the host genotype and phenotype should be investigated with respect to the above processes and the development of phage resistance. The action of bacteriophage to disperse biofilms could also be investigated, and in particular the potential for the bacteriophage to either produce or elicit from the host biofilm matrix depolymerising enzymes. The transcriptome of CSLC cultures under atmospheric conditions in the absence of nutrients to support the growth of *Campylobacter* should be performed to yield a greater understanding of why the CSLC cultures exhibit increased aerotolerance and thermotolerance over their parental bacteria. Additionally, the transcriptional profiles and proteome analysis during the growth of CSLC cultures and development of CSLC phages could provide an understanding of the status of the phages associated with CSLC strains.

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Appendix 1

The DNA sequenced of PT14CP30PL DNA identical to CP30 (Contig 8)

```
tcaatgcggtttgataaaaagcgaatatctttacctatatttaataattc
acacaatttattataattataattttgtaaaagtattctaccagctaatt
ctatattaccatcatacatacaaaacgagtatttcacattattattactt
tccaatatacctataaaacatctatttgtcatcttaatcctctgtgtagt
taataaaacttagttttattaactacaaaatatcatcataaattgagtaa
cctacctatatttacatcattttgttattcccaaaatatttcacacaaat
aattcattttagtttccttctttattaaatacagctaatgaaacattatc
tgggcaaccttttttgtagtttttcctttaaaaaaattataattgagtata
aattaataacattttcaaaataatcatcattatatgaatatcttccattt
gcaccataatacacaaccttatatccaatgctatagacatctccgatata
atgaagaagataacaccaagaaactgttccataagttttgtcttcattaa
gagtttctatttctttagaaatttttaatacttcctcaacttctttaata
ttctttttagttgttatttcaggaaattttagttcaatacattttaacat
taaatcatatatttgtagttttttgttatcatcgcttttttgtagttcca
ta
```

Appendix 2

The distinct DNA sequence of bacteriophage CP8 and CP30

AAATTCCTTTAATATATTGTTTGTTTTTTGAATACCCTCATTTACAATAG	CP8
AAATTCCTTTAATATATT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	CP30
ATTCAACATATTCTCTTTCTTTTCTTATTTCTTCTAATTTTTCATCAGTA	CP8
~~	CP30
TATTCTTTTTCTTTTTCTTTTAAAACTTTTTCA	CP8
~~CTTTTTCTTTTAAAACTTTTTCA	CP30

Appendix 3

Validation of 10 µl spot lawn technique compared to addition of 100 µl to soft top agar technique

A) 100 µl of 10^{-5} dilution of CP30 phage added to whole plate [1]

Replicate	Count	Times dilution	$\log_{10}$	Ave	SD
1	364	364000000	8.56	8.57	0.04
2	429	429000000	8.63		
3	352	352000000	8.55		
4	380	380000000	8.58		
5	408	408000000	8.61		
6	321	321000000	8.51		
7	354	354000000	8.55		
8	360	360000000	8.56		
9	358	358000000	8.55		
10	363	363000000	8.56		

[1]Aliquots of 100 µl of the 10^{-5} dilution of CP30 phage were added to each of 10 eppendorf tubes containing 0.5 ml of 10^{9} CFU/ ml C. *jejuni* HPC5. These were mixed and then added to each of 10 vials of 5 ml molten NZCYM top agar, mixed and dispensed onto each of 10 NZCYM plates and allowed to set. The plates were incubated for 24 h at 42^{o} C under microaerobic conditions before all the plaques for each plate were counted.

B) Spot technique using 10 µl of 10^{-6} dilution of CP30 phage dispensed onto lawn[2]

Rep.	Count 1	Count 2	Count 3	Count 4	Count 5	Sum	Times dilution	$\log_{10}$	Ave	SD
1	4	7	3	5	5	24	4.8×10^8	8.68	8.69	0.04
2	8	4	2	4	6	24	4.8×10^8	8.68		
3	3	6	5	6	5	25	5.0×10^8	8.70		
4	4	8	3	4	2	21	4.2×10^8	8.62		
5	4	4	7	4	8	27	5.4×10^8	8.76		
6	5	6	4	4	3	22	4.4×10^8	8.62		
7	3	1	9	6	7	26	5.2×10^8	8.72		
8	6	3	6	6	6	27	5.4×10^8	8.78		
9	4	4	5	4	5	22	4.4×10^8	8.64		
10	4	7	5	5	5	26	5.2×10^8	8.76		

[2]Lawns (10 in total) were prepared using 0.5 ml of 10^{9} CFU/ ml C. *jejuni* HPC5 which was added to 5 ml of molten NZCYM top agar, mixed and dispensed onto NZCYM plates. The plates were allowed to set then dried for 20 min at 42^{o} C. Each plate was inoculated with 5 droplets of 10 µl and left until the droplets were absorbed into the agar. The plates were incubated for 24 h at 42^{o} C under microaerobic conditions. Plaques were counted from each individual spot then combined.

C) ANOVA: Single Factor Analysis

ANOVA: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Spotting	10	86.972	8.6972	0.0033
Top agar	10	85.656	8.5656	0.0012

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.08662	1	0.08662	37.9150	8.18E-06	4.41387
Within Groups	0.04112	18	0.00228			
Total	0.12775	19				

The bacteriophage CP30 was selected because it is not notably impaired in plaque formation when placed in top agar. This is not true of all *Campylobacter* bacteriophage, which can aggregate upon mixing with the high concentrations of host bacteria encountered in the top agar overlay method. The methods have similar standard deviations suggesting they are of similar precision, and that the pipetting operations are reproducible and accurate. However, the means determined by the methods differ by $\log_{10}$ 0.12 pfu/ml, and are significantly different due to their high precision. Nano particle counts estimated from Nanoparticle Tracking Analysis (NanosightTM) of the clarified phage preparation suggests there are $\log_{10}$ 8.9 particles of 200 nm radius.

Reference: Anonymous in www.nanosight.com. 2009. Review: Applications of Nanoparticle Tracking Analysis (NTA) in Nanoparticle Research.

Appendix 4

Removal attached cells of *C. jejuni* NCTC 11168 on the glass coverslip were re-determined the viable count to make sure removal all the attached bacterial cells on a glass slide.

#1 to detach the attached bacterial cells using 20 times pipette up and down

#2 to detach again with the same method after washed the glass coverslip with MRD 2 times

Rep.	**Bacterial Count after detached # 1 ($\log_{10}$CFU/cm^2)**	**Bacterial Count after detached # 2 ($\log_{10}$CFU/cm^2)**
1	5.176	ND
2	5.204	ND
3	5.116	ND
4	5.322	ND
5	5.415	ND
6	5.462	ND
7	5.255	ND
8	5.845	ND
9	5.778	ND
10	5.342	ND

ND: not detection at dilution 10 and 10^{-1}

Appendix 5

To pipette 10 µl of water

Replicate	Weight of water (mg)	Average	SD
1	0.0098	0.0099	0.000229
2	0.0099		
3	0.0097		
4	0.0100		
5	0.0098		
6	0.0099		
7	0.0101		
8	0.0095		
9	0.0096		
10	0.0099		
11	0.0099		
12	0.0103		
13	0.0100		
14	0.0099		
15	0.0098		
16	0.0097		
17	0.0096		
18	0.0105		
19	0.0101		
20	0.0098		
21	0.0104		
22	0.0102		
23	0.0097		
24	0.0099		
25	0.0100		
26	0.0100		
27	0.0101		
28	0.0100		
29	0.0099		
30	0.0098		

Appendix 6

Reproducible of enumeration of viable biofilm cells of *C. jejuni* NCTC 11168 under microaerobic condition at 37 °C for 5 days was analysed using ANOVA.

Enumeration of viable count

Experiment (Log_{10} CFU/cm^2)			
1	2	3	4
5.267	5.831	4.665	4.744
5.267	5.831	4.693	4.603
5.334	5.768	4.665	4.665
5.489	5.719	4.811	4.635
5.530	5.665	4.904	4.885
5.489	5.635	4.951	4.603
5.665	6.188	4.744	4.392
5.530	6.091	4.744	4.603
5.634	6.392	4.744	4.188

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Experiment 1	9	49.209	5.468	0.022
Experiment 2	9	53.124	5.903	0.068
Experiment 3	9	42.925	4.769	0.010
Experiment 4	9	41.322	4.591	0.040

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.080	3	3.360	95.801	4.53E-16	2.901
Within Groups	1.122	32	0.035			
Total	11.203	35				

Conclusion – low variance within batches but significant batch-to-batch differences <0.05.

Reproducible of enumeration of viable biofilm cells of *C. jejuni* HPC5 under microaerobic condition at 37 °C for 5 days was analysed using ANOVA.

Enumeration of viable count

Experiment (Log_{10} CFU/cm^2)

1	2	3
4.694	5.079	5.443
4.720	4.954	5.392
4.832	5.060	5.392
4.666	5.000	5.334
4.444	4.845	5.392
4.531	4.845	5.568
4.699	5.204	5.267
4.903	5.176	5.603
4.845	5.114	5.489

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	9	42.334	4.704	0.022
Column 2	9	45.277	5.031	0.017
Column 3	9	48.884	5.432	0.012

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.392	2	1.196	70.862	8.51E-11	3.403
Within Groups	0.405	24	0.017			
Total	2.796	26				

Conclusion – low variance within batches but significant batch-to-batch differences <0.05.

Reproducible of enumeration of viable biofilm cells of *C. jejuni* PT14 under microaerobic condition at 37 °C for 5 days was analysed using ANOVA.

Experiment (Log_{10} CFU/cm^2)			
1	2	3	4
6.268	6.694	6.335	6.954
6.092	6.489	6.531	6.954
6.335	6.694	6.603	6.845
6.118	6.444	6.981	7.204
6.092	6.444	6.967	7.000
6.092	6.335	6.981	7.204

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	36.997	6.166	0.012
Column 2	6	39.100	6.517	0.021
Column 3	6	40.398	6.733	0.079
Column 4	6	42.161	7.027	0.021

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.367	3	0.789	23.70	8.61E-07	3.098
Within Groups	0.666	20	0.033			
Total	3.033	23				

Conclusion – low variance within batches but significant batch-to-batch differences <0.05.

Appendix 7

Reproducibility of crystal violet staining measurement of 3 independent *C. jejuni* HPC5 biofilms formed on 6 replicate coverslips under microaerobic conditions at 37 ºC for 5 days analysed using ANOVA.

Crystal violet staining A570 nm

1	2	3
0.097	0.110	0.113
0.109	0.073	0.104
0.113	0.076	0.080
0.070	0.093	0.100
0.084	0.062	0.137
0.090	0.105	0.075

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	0.563	0.093833	0.000257
Column 2	6	0.519	0.0865	0.000366
Column 3	6	0.609	0.1015	0.000513

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000675	2	0.000338	0.891	0.43082	3.682
Within Groups	0.005682	15	0.000379			
Total	0.006357	17				

Conclusion – not low variance within batches and no significant batch-to-batch differences

Reproducibility of crystal violet staining measurement of 3 independent *C. jejuni* NCTC 11168 biofilms formed on 6 replicate coverslips under microaerobic conditions at 37 ºC for 5 days analysed using ANOVA.

Crystal violet staining A570 nm

1	2	3
0.513	0.642	0.739
0.508	0.618	0.672
0.670	0.836	0.636
0.737	0.914	0.536
0.656	0.884	0.655
0.686	0.720	0.659

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	3.770	0.628	0.009
Column 2	6	4.614	0.769	0.016
Column 3	6	3.897	0.650	0.004

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.069	2	0.03451	3.51746	0.055892	3.68232
Within Groups	0.147	15	0.00981			
Total	0.216	17				

Conclusion – low variance within batches but batch-to-batch variation evident but not significant >0.05.

Reproducibility of crystal violet staining measurement of 3 independent *C. jejuni* PT14 biofilms formed on 6 replicate coverslips under microaerobic conditions at 37 ºC for 5 days analysed using ANOVA.

Crystal violet staining A570 nm

1	2	3
1.227	1.438	1.878
1.614	1.396	1.552
1.569	1.374	1.544
1.488	1.846	1.926
1.340	1.662	1.204
1.227	1.628	1.370

Anova: Single Factor

SUMMARY

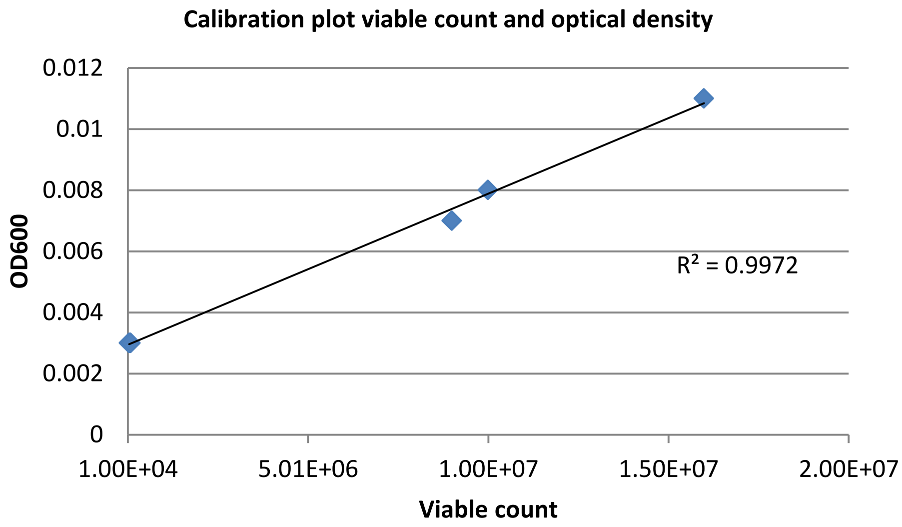
Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	8.465	1.411	0.029
Column 2	6	9.344	1.557	0.035
Column 3	6	9.474	1.579	0.079

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.100423	2	0.050212	1.054747	0.37274	3.682
Within Groups	0.714082	15	0.047605			
Total	0.814506	17				

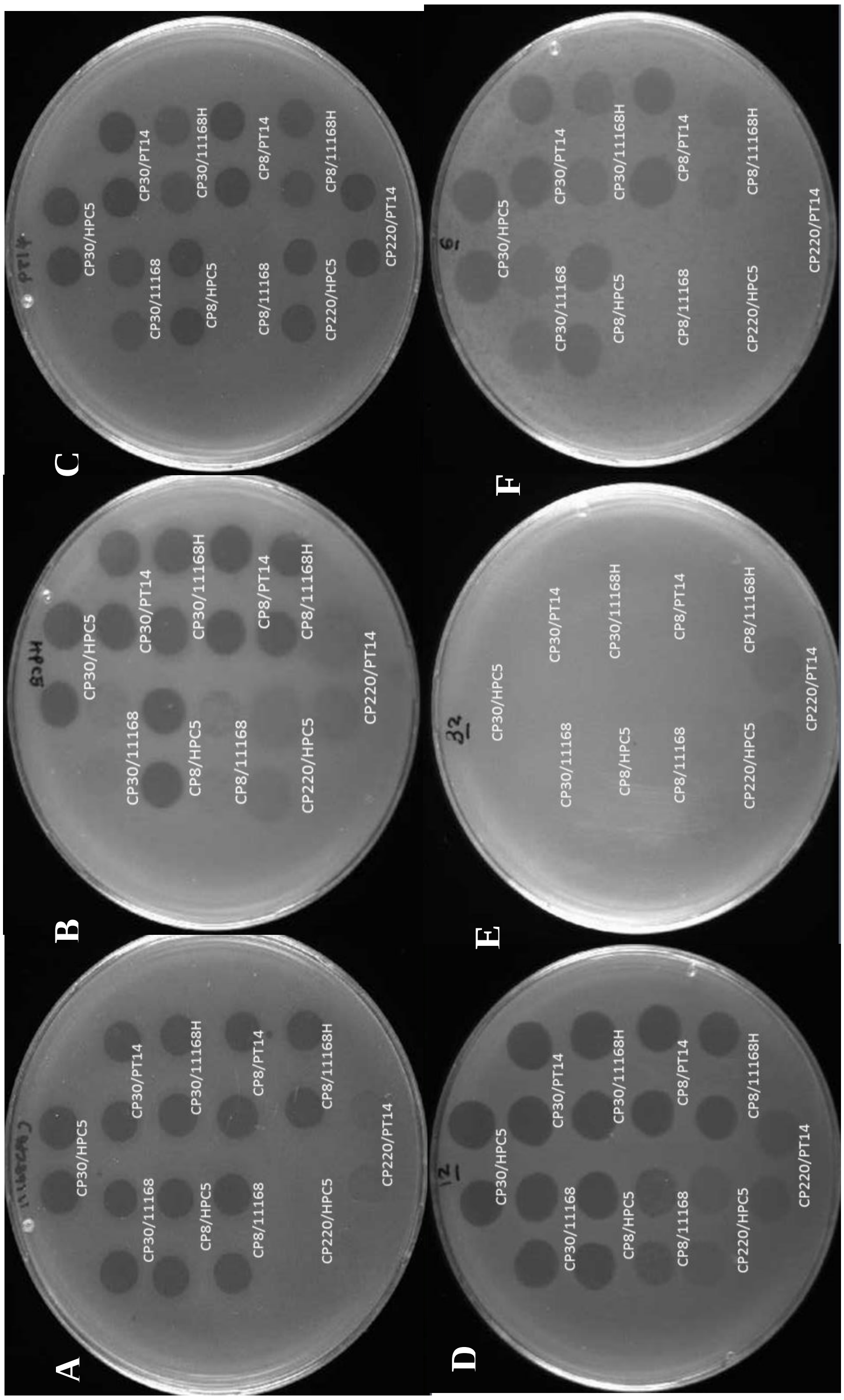
Conclusion – not low variance within batches and no significant batch-to-batch differences

Appendix 8


Calibration plot viable count and optical density
0.012
0.01
0.008
0.006
0.004
0.002
0
OD600
R² = 0.9972
1.00E+04
5.01E+06
1.00E+07
1.50E+07
2.00E+07
Viable count

Appendix 9

Frequency of phage resistance (section 3.2.4)



A: *C. jejuni* NCTC11168, B: *C. jejuni* HPC5, C: *C. jejuni* PT14, D: Isolate no. 12 of *C. jejuni* HPC5 biofilm treated with CP30 that referred a sensitive isolate, E: Isolate no. 32 of *C. jejuni* HPC5 biofilm treated with CP30 referred a resistant isolate, F: Isolate no. 6 of *C. jejuni* HPC5 biofilm treated with CP30 that referred a carrier state life cycle isolate (note the background appearance of phage plaques). Phage CP8 and CP30 are recovered from different replicating strains as indicated were applied at concentration 10^5-10^6 PFU/ml.