Variation in *Campylobacter* phage and prophage

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ABSTRACT

Campylobacter is a bacterial pathogen commonly responsible for foodborne gastroenteritis worldwide. Due to the rapid development of resistance to antibiotics, many alternative interventions have been studied and bacteriophage therapy is one of them. To maximise the impact of the intervention and to ensure it remains sustainable it important to study the ecology and coevolution of *Campylobacter* and the bacteriophage that infect the pathogen.

Coevolution interactions between *Campylobacter* and bacteriophage drive rapid molecular change and contribute to a higher mutation rates for both parties. In this study genetic modifications were examined in the bacterial host and phage arising either *in vivo* or *in vitro*. We observed the impact of *C. jejuni* containing Mu-like pro-phage on campylobacters populating the caeca of commercial broiler flocks. The Mu-containing campylobacters initially colonised and became the dominant strain, only to be out-competed before depopulation of the broiler house. The presence of the transposable Mu-like prophage ultimately proved to be a limitation in the fitness of the host.

Campylobacter-specific phage CP30 is a T4-like phage of the *Eucampyvirinae* that was isolated from a farm with several other phage showing differences in host range of contemporary farm isolates. After serial passage the phage population acquired sequence variants. One newly characterised sequence type, CP30C, was defective in a tail fibre protein and revealed reduced adsorption ability and sensitivity against *C. jejuni* host strains.

Whole genome sequencing identified host mutation in *C. jejuni* carrier state strains that maintain viability despite the continual production of virulent phage. A point mutation in the flhF gene (*flhF(T368A)*) was hypothesised to contribute to the non-motile phenotype of carrier state strains. Expression of *flhF(T368A)* in a flhF knockout background proved

to have impaired motility, exhibit structural defects in flagella synthesis, were less susceptible to phage infection and show down regulation of σ^{28} dependent and σ^{54} dependent flagellar associated genes. Recombinant protein expression of FlhF demonstrated the protein to have GTPase activity and the FlhF(T368A) to have reduced enzyme activity, greater thermal sensitivity and to be impaired in protein folding compared to wild type FlhF.

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TABLE OF ABBREVIATIONS

A595	Absorbance at 595nm
AHp	Alkyl hydroperoxide reductase
ATP	Adenosine triphosphate
APS	Ammonium persulphate
BA	Blood agar
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CDT	Cytolethal distending toxin
CFU	Colony forming unit
cgMLST	Core genome multi-locus sequence typing
CPS	Capsular polysaccharide
CSLC	Carrier state life cycle
Da	Daltons
ddUTP	2',3'-Dideoxyuridine-5'-Triphosphate
DEPC	Diethyl pyrocarbonate
˚C	Degree Celsius
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
EOP	Efficiency of plating
ETC	Electron transport chain
EU	European Union
FlaA- SVR	*FlaA* Short Variable Region

GST	Glutathione S-transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
KWC	Killed whole-cell vaccines
mRNA	Messenger ribonucleic acid
µl	Microliter
ml	Millilitre
Leg	Legionaminic acid
MβCD	Methyl-β-cyclodextrin
mCCDA	Modified *Campylobacter* blood-free selective agar
MH	Mueller-Hinton
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
NB2	Nutrient broth No. 2
NBT	Nitro-blue tetrazolium chloride
NCTC	National Collection of Type Cultures
nm	Nanometre
NZCYM	New Zealand Casamino Yeast Medium
OD	Optical density
OMV	Outer membrane vesicles
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
% v/v	Percentage volume per volume

% w/v	Percentage weight per volume
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming unit
PMF	Peptide mass fragment
Pse	Pseudaminic acid
RNA	Ribonucleic acid
RNAP	Ribonucleic acid polymerase
RO	Reverse osmosis
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
rSAP	Shrimp Alkaline Phosphatase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
ST	Sequence type
TAE	Tris-acetate ethylene diaminetetraacetic acid
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
Tris	2-Amino-2-(hydroxymethyl)-1,3-Propanediol, tris(hydroxyamino) methane
UV	Ultraviolet
WGS	Whole genome sequencing

CHAPTER ONE

Introduction

1.1 Campylobacter

1.1.1 Early history of *Campylobacter*

Non-culturable, spiral-shaped organisms were first observed in stool samples of children with diarrhoea (Escherich, 1886), which are now believed to be the first observation of *Campylobacter* (Kist, 1986). Later in 1913, these spiral-shaped bacteria were isolated from fetal tissue of an aborted lamb and identified as *Vibrio fetus* (McFadyean and Stockman, 1913). Subsequently, *Vibrio jejuni* was isolated from jejunum of calves (Jones et al., 1931) and *Vibrio coli* was isolated from pigs (Doyle, 1944). In 1957, King stated the isolation of *Vibrio fetus* from blood samples of children with diarrhoea and because of the characteristic variation between the isolates and traditional vibrios, he referred to these isolates as "related *Vibrio*" (King, 1957). In 1963, this organism was altered to the newly proposed genus *Campylobacter* (Sebald and Véron, 1963) with the meaning of curved rod derived from campylos (curved) and baktron (rod) in Greek. In 1972, *Campylobacter* was first isolated from stool samples of patients with diarrhoea and thereafter considered as pathogenic to humans (Dekeyser et al., 1972). In 1977, the development of *Campylobacter* specific selective growth media enabled the detection and isolation of stool samples for *Campylobacter* (Skirrow, 1977).

1.1.2 Characteristics of *Campylobacter*

The *Campylobacter* genus (29 taxa) is the largest constituent of *Campylobacteraceae* family, which also contains the *Arcobacter* (17 taxa) and *Sulfurospirillum* (7 taxa) genera (Lastovica et al., 2014). Currently,

there are 21 species and 8 subspecies of *Campylobacter* as defined by 16s rRNA sequencing (Figure 1.1), of which *C. jejuni* and *C. coli* are the most studied species due to their clinical significance (Lastovica et al., 2014). The species *Helicobacter pylori* and *Helicobacter mustelae*, that were once thought to be campylobacters, have been demonstrated to show differences in morphology (Han et al., 1989), cellular fatty acids composition (Goodwin et al., 1989b) and growth characteristics, which prompted their transfer to a new genus, *Helicobacter* (Goodwin et al., 1989a).

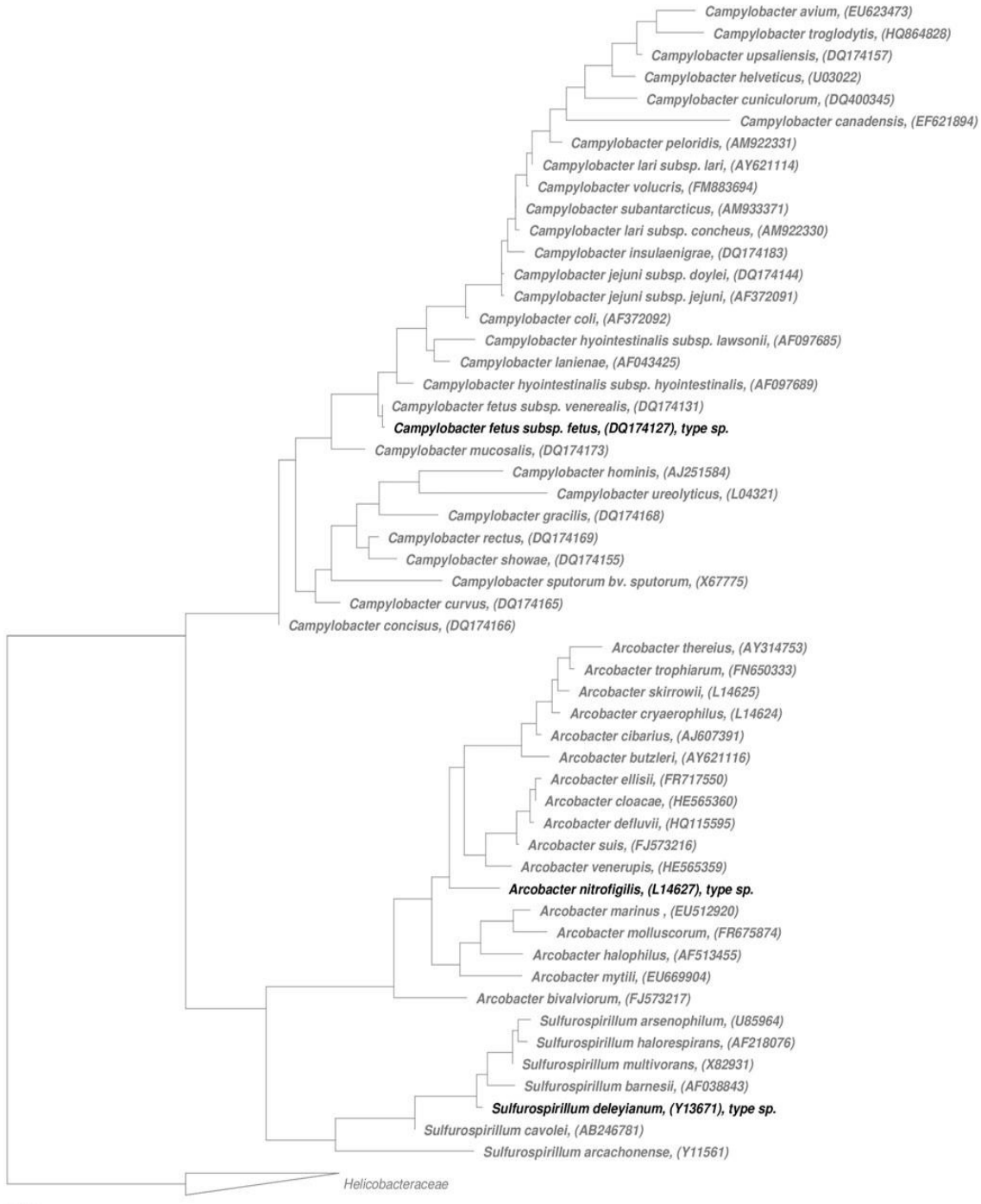


Figure 1.1 The phylogenetic tree of the *Campylobacteraceae* family based on 16s rRNA. The figure is taken from Lastovica et al. (2014)

The *Campylobacter* species are Gram-negative, non-spore-forming bacteria, and the bacterial cells are typically between 0.2 μm to 0.8 μm wide and 0.5 to 5 μm long (Zendehbad et al., 2013; Horrocks et al., 2009;

Vandamme, 2000). The morphology of *Campylobacter* cells include slender rod-shaped, S- spiral shaped or curved-shaped cells, while straighter rods was observed in some *Campylobacter* species like *C. showae* (Wassenaar and Newell, 2006). Moreover, *C. jejuni* may occasionally produce cells with straight rods morphology and Matz et al. (2002) suggested the observation of this straight rod morphology in *C. jejuni* with a *flhB* deletion or long filaments (Ghaffar et al., 2015). Under unfavourable conditions, including temperature stress (reviewed by Ikeda and Karlyshev, 2012), oxidative stress (Moran and Upton, 1986; Boucher and Slater, 1994), starvation on late stationary phase (Rollins and Colwell, 1986; Bovill and Mackey, 1997), the *Campylobacter* cells are able to alter their morphology to a coccoid form (Koike and Shimazaki, 1982). Morphological modification of *Campylobacter* to coccoid form are normally coincident with the decline of colony forming unit counts, Beumer and Devries (1992) stated that coccoid shaped *Campylobacter* are correlated with the viable but non-culturable (VBNC) state, that are less virulent, less likely to colonise and unlikely to cause any health risk. Similar conclusion is drawn in another Gram-negative bacteria species, *Helicobacter pylori* (reviewed by Mazaheri et al., 2015). While later studies suggested that there is potentially no correlation between *Campylobacter* cell morphology and the culturability (Hazeleger, 1995; Ikeda and Karlyshev, 2012). However, even those bacteria in VBNC state are still capable to maintain the metabolic activity, but with a lower metabolic rate (Colwell et al., 1996; Shleeva et al., 2004).

Generally, *Campylobacter* cells exhibit a characteristic darting corkscrew-like motility by means of a single polar non-sheathed flagellum at one or both ends of the cell (Nachamkin et al., 1993). However, several exceptions have been noted, that include the non-motile phenotype for *C. gracilis* due to lack of flagella and the observation of unipolar multiple flagella in *C. showae* (Vandamme, 2000). Non-motile *C. jejuni* and *C. coli* variants could also be produced especially with frequent serial passage *in vitro*.

All members of *Campylobacter* genus are oxidase positive with an exception of *C. gracilis*, and are able to reduce nitrate with *C. jejuni* subspecies *doylei* excluded (Wassenaar and Newell, 2006). The *Campylobacter* species are microaerophilic with optimal oxygen and carbon dioxide concentration of 5-10% and 3-5% respectively, and optimum growth temperatures in the range of 30 - 42˚C. The *Campylobacter* species *C. gracilis, C. hyointestinalis, C. showae* and *C. sputorum* bv. *faecalis* are able to grow under anaerobic conditions, while species *C. concisus, C. mucosalis, C. rectus, C. curvus* and *C. gracilis* require the presence of nitrogen and/ or formate or fumarate for their growth (Lastovica et al., 2014). Generally campylobacters cannot ferment or oxidise carbohydrates, instead, they have a respiratory energy metabolism that utilises amino acids and intermediates from the citric acid cycle as substrates (Mohammed et al., 2004). The majority of *Campylobacter* species are hippurate negative, where only *C. jejuni* and *C.*

avium possess the *HipO* gene, and are able to hydrolyse hippurate to benzoic acid and glycine (Rossi et al., 2009; Lastovica et al., 2014).

1.1.3 Genome analysis of *Campylobacter*

In 2000, the genome sequence of *C. jejuni* NCTC11168 was published as the first available genome sequence within the *Campylobacter* species, and later was re-annotated in 2007 (Parkhill et al., 2000; Gundogdu et al., 2007). Since then, the whole genome sequences of various campylobacters have become available including *C. jejuni, C. coli, C. fetus, C. concisus, C. lari, C. showae, C. ureolyticus, C. curvus, C. hominis, C. rectus* etc. The genome size of *Campylobacter* species is relatively small compared to other bacterial species like *Salmonella, E. coli* and *Pseudomonas.* Typical genome size for *C. jejuni* is between 1.63 and 1.85 Mb but the largest genome that has been sequenced among campylobacters is *C. rectus* strain RM3267, which has the genome size of 2.51 Mb. Campylobacters have genomes of relatively low G+C content, generally in the range of 28% (*C. peloridis* LMG23910) to 47% (*C. gracilis* ATCC33236).

1.1.4 Reservoirs of *Campylobacter*

Campylobacter species are ubiquitous in the environment and have been isolated from soil, mud, sand and environmental water (Chai et al., 2009; Stuart et al., 2010; Bolton et al., 1999; Kemp et al., 2005). Normally, *Campylobacter* species inhabit the mucosal surface of gastrointestinal tracts of human, various warm-blooded animals like cats, dogs, ferrets, hamsters (Inglis and Kalischuk, 2003), and food animals including cattle,

pig, sheep and typically poultry (Schultheiss et al., 1989; Young et al., 2000; Pearson et al., 1996). Whilst some campylobacters are also able to colonise cold-blooded animals, for example *C. fetus* was previously isolated from faecal samples of reptiles including snakes, turtles and lizards (Wang et al., 2013). Campylobacters are frequently host associated but this association is not exclusive. For example, *C. coli*, *C. mucosalis* and *C. hyointestinalis* commonly inhabit the pig intestine and *C. lari* is frequently isolated from wild birds and shellfish (Vandamme, 2000). *C. jejuni* commonly colonise the gastrointestinal mucosa of avian species with a body temperature of 42˚C that coincides with their optimal growth, and as such is identified as a thermophilic species. In the avian intestine, *C. jejuni* has been reported to act as a commensal (Waldenström et al., 2002) and is normally harmless to the host. However, the colonised *C. jejuni* may be recognised by Toll-like receptors 2t2/16 and 21 to result in the initiation of an innate immune response in the intestine of the host and further cause an influx of inflammatory cells such as heterophils (de Zoete et al., 2010; Meade et al., 2009; Smith et al., 2008). Certain chicken lines have been reported to exhibit an inflammatory response to *Campylobacter* colonisation that can result in diarrhoea, poor-quality wet litter and damage to chicken feet and legs (Wearne, 2013). Moreover, *C. jejuni* may also influence the structure of the poultry intestinal epithelium (Awad et al., 2015).

1.2 Prevalence of *Campylobacter*

1.2.1 Prevalence of *Campylobacter* in broiler flocks

Although various kinds of warm-blood animals are frequently colonised by campylobacters, poultry are documented as one of the most significant hosts for *Campylobacter*, and are typically the source of most cases of human infection (de Zoete et al., 2007). It is reported that an average 71.2% of broiler chickens are *Campylobacter* positive at slaughter in European countries (Newell and Fearnley, 2003; EFSA, 2010a), and a high prevalence of campylobacters on broiler carcasses and retail poultry meat have been widely documented worldwide (Kramer et al., 2000; Jørgensen et al., 2002; Atterbury et al., 2003; Cui et al., 2005; Ridley et al., 2011; Wieczorek and Osek, 2015; Silva et al., 2016). Among campylobacters, two thermophilic species, *C. jejuni* and *C. coli* are considered as the most common species isolated from food samples, and have been identified as the causative agent of the majority of human gastroenteritis cases (Lynch et al., 2011; Lastovica et al., 2014). Both species colonise avian intestine with a commensal relationship, where colonised broiler chickens are often reported to carry *Campylobacter* up to 10^9 cfu g^{-1} of their ceca content under experimentally challenged conditions (Heres et al., 2003).

The rate of *Campylobacter* present in broiler flocks varies according to season and geography in developed countries. Jorgensen et al. (2011) determined the prevalence of *C. jejuni* and *C. coli* in the caecal contents from 797 broiler flocks representing 211 farms in the UK from 2004 to 2006, and reported that flocks are generally revealed a high prevalence of

Campylobacter when slaughtered between June and November compared to the rest of the year. Positive flocks were more likely to be reared in northern UK than in the midlands or southern counties. They also suggested that flocks that are slaughtered at the same time would generally have a similar type of *Campylobacter* based on multilocus sequence typing (MLST). Similar findings were stated by Nylen et al. (2002), when analysing the rate of *Campylobacter* positive broiler flocks in nine European countries and New Zealand. The seasonal pattern revealed a high consistency from year to year with the peak of *Campylobacter* isolates being observed in week 22 in Wales, week 26 in Scotland, week 30 in Finland, week 32 in Denmark, and week 33 in Sweden. The prevalence of *Campylobacter* in retail chicken meat and human patients are also reported as following a seasonal pattern. Meldrum et al. (2005) analysed the level of *Campylobacter* isolated from fresh retail chicken and human patients in Wales on a weekly basis from January to December in 2002 and suggested the peak of *Campylobacter* isolates presented between week 22 to 25 in human patients and between week 24 to 26 in chicken samples. Notably, seasonality of *Campylobacter* in developing countries is less prominent or even absent (Coker et al., 2002; Platts-Mills and Kosek, 2014). Moreover, conventionally reared broiler flocks in the UK (Ayling et al., 1996), Sweden (Berndtson et al., 1996) and the Netherlands (Jacobs-Reitsma et al., 1995; Ring et al., 2005) are predominantly colonised with only one or two subtypes of *C. jejuni* . Whilst in other countries including the USA (Hiett et al., 2001; Nesbit et al., 2001) and Austria (Thomas et al., 1997),

multiple *Campylobacter* strains are commonly isolated from broiler flocks.

The prevalence of *Campylobacter* is also generally higher in organic or free range poultry than intensively reared flocks. Heuer et al. (2001) reported a study in Denmark that found 100% of the free range flocks tested were *Campylobacter* positive. This could presumably reflect the high prevalence of *Campylobacter* in the environment to which free range flocks are continually exposed (Heuer et al., 2001). Similar high prevalence is also stated in UK when comparing the prevalence rate of campylobacters in organic and free range flock with conventional intensively reared broilers during 2004 to 2005 (Allen et al., 2011). In this study, 95%- 100% of 41 organic and free range flocks are identified as *Campylobacter* positive, which is significantly greater than the 55% average prevalence rate of *Campylobacter* in tested 40 intensively reared flocks ($p<0.01$).

The evidence suggest most broiler flocks become *Campylobacter* positive at 21 day of age and are rarely isolated in the first two weeks of the rearing period (Newell and Wagenaar, 2000; Shane, 2000), and this period is known as the "lag phase". It is presumably due to the presence of maternal derived antibodies in young chickens to against the colonisation from *Campylobacter* (Sahin et al., 2003). However it is possible to experimentally colonise those chicken when challenged with a high dose of *Campylobacter* (Young et al., 1999).

Once one chicken is colonised, bird to bird transmission is rapid and frequently, major of the flock (>95%) would become contaminated within a few days (Shreeve et al., 2000; Stern et al., 2001). They will remain colonised until slaughtered at 36-42 days of age for intensively reared chickens or typically 56 days for free range chickens and 73 days for organic chickens (El-Shibiny et al., 2005), which subsequently results in contamination of the slaughter facility and retail meat (reviewed by Lin, 2009). The original source of flock infection is still controversial, and due to being low in moisture, poultry feed is less likely to be the source of *Campylobacter* colonisation (Altekruse et al., 1999; Newell and Fearnley, 2003). However, a number of horizontal transmission routes have been suggested including bioaerosol (Posch et al., 2006), water (Shanker et al., 1990) and other potential vectors that could introduce infection (EFSA, 2010a). Whilst the major transmission route for rapid spread of *Campylobacter* within flock is believed as faecal- oral. *Campylobacter* positive chickens are able to shed large numbers of *Campylobacter* in their faeces and through common water, feed source and contact with infected faeces, other flocks on the farm can become rapidly colonised (Newell and Fearnley, 2003; Barrios et al., 2006; Lee and Newell, 2006).

Vertical transmission was initially thought to be uncommon for *Campylobacter* colonisation in broiler flocks (Callicott et al., 2006; Fonseca et al., 2006; Bull et al., 2006). By analysing *Campylobacter* isolates from two independent broiler flocks as well as their progeny with ribotyping and flaA-SVR typing, Cox et al. (2002) described the

consistency of *Campylobacter* subtypes individually isolated from two generations and concluded that vertical transmission could be a source of *Campylobacter* colonisation in broiler flocks. Later, Cox et al. (2012) suggested that the *Campylobacter* transmission from egg could not only mean vertical and transovarian transmission, but also include the possibilities of contamination of egg shell, shell membrane and albumen from faecal reservoirs with the aid of moisture, as well as the newly hatched chicken could ingest *Campylobacter* and become colonised.

Campylobacters are highly motile and relatively small in size (0.2 to 0.8μm wide, 0.5 to 5μm long) which can enable them to pass through the egg shell's pore, which has an average diameter of 11 to 12μm. However, the egg albumen contains several defense systems against invading microorganisms, including the presence of lysozymes and ovotransferrin. Ovotransferrin has a antimicrobial activity based on its ability to deprive the organisms of iron (Baker et al., 1987; Abdou et al., 2013), and therefore makes the environment unfavorable for the growth of *Campylobacter.* The successful penetration of *Campylobacter* through the egg shell's pore was reported by Fonseca et al. (2014), with the successful recovery of *C. jejuni* from fertile specific pathogen free (SPF) eggs after three hours of exposing to wood shavings, which had been artificially infected with *C. jejuni* IAL2383 at a count of 10^5 cfu g^{-1}, with a recovery rate of 20%. In their study, the albumen environment was inhibitory but was not efficient enough to totally inhibit the growth of *C. jejuni,* which was predicted to be correlated with the incubation temperature.

However, their study failed to recover *C. jejuni* at all times from commercial eggs that were infertile suggesting that fertility was required for *C. jejuni* penetration, and also confirmed that commercial eggs are not a potential reservoir responsible for human campylobacteriosis.

1.2.2 The prevalence of *Campylobacter* on broiler carcasses

Besides the high prevalence of *Campylobacter* within broiler flocks, high *Campylobacter* contamination rates have been reported for broiler carcasses. A study carried out in Europe in 2008 to determine the prevalence of *Campylobacter* in broiler flocks and carcasses after slaughter demonstrated the high level of carriage and contamination (EFSA, 2010a). In total 10,132 broiler samples were randomly collected from 561 abattoirs within 26 European countries for analysis. As part of the study, 401 samples were collected from the UK, and it was reported that typically 75.3% of broiler flocks were *Campylobacter* colonised by analysing their presence in pooled caecal contents, whilst 86.3% of broiler carcasses were contaminated. It is clear that when colonised broiler flocks are slaughtered, intestinal campylobacters can be rapidly transferred to the carcasses (Guerin et al., 2010), and a *Campylobacter* positive flock is approximately 30 times more likely to result in a *Campylobacter* contaminated carcass. Notably, there were over 10% higher carcass contamination than *Campylobacter* positive flock populations. Although this significantly varied between countries, higher *Campylobacter* prevalence was observed on broiler carcasses for most of the countries tested (74%). Clearly cross contamination from

Campylobacter-positive to negative carcasses or by contacting with contaminated surfaces or equipment in abattoir contribute to this (Normand et al., 2008).

1.3 Clinical significance of *Campylobacter*

As the major food animal, approximately 60 billion chickens are currently consumed by humans annually, that is numerically more than any other (reviewed by Colles et al., 2016). The high prevalence of campylobacters on broiler carcasses and retail poultry meat brings potential health safety risk to humans.

1.3.1 Epidemiology

Campylobacter is a major cause of gastroenteritis worldwide and the prevalence and incidence of campylobacteriosis have increased during last decade in both developed and developing countries (Kaakoush et al., 2015). *Campylobacter jejuni* and *C. coli* represent the main causative agents of campylobacteriosis (Fhogartaigh and Edgeworth, 2009).

Currently, campylobacteriosis is the most commonly reported foodborne disease in the EU and has been so since 2005 (EFSA, 2015). In 2014, there are 236,851 confirmed campylobacteriosis cases (notification rate of 71.0 per 100,000 population), with an increase of 22,067 cases than that reported in 2013 (notification rate of 64.8 per 100,000 population). This number is nearly 3 times more than the confirmed cases for salmonellosis (88,715 cases with a notification rate of 23.4 per 100,000

population) in 2014, which is the second most reported disease during that year. Although this number is thought to be underreported due to the self-limiting nature of campylobacteriosis, and the actual number of campylobacteriosis cases is estimated to be approximately 9 million annually (EFSA, 2015). A statistically significant increasing trend in number of reported campylobacteriosis has been reported in the EU for eight years, which could in part be correlated with improved surveillance system and diagnostics for campylobacteriosis (EFSA, 2015). The cost of campylobacteriosis associated cases, including the cost of public health systems and the loss of productivity, in EU is estimated to be approximately 2.4 billion annually (EFSA, 2015). Notably, the death number attributed to campylobacteriosis decreased from 56 deaths in 2013 to 25 deaths in 2014. Specific species of *Campylobacter* have been determined for 52.6% of the confirmed campylobacteriosis cases. Amongst these *C. jejuni* is the most prevalent pathogen, and is responsible for 81.8% of the reported incidence, followed by *C. coli* and *C. lari* with incidences of 7.13% and 0.09% respectively. Solely in UK, a decrease in number reported campylobacteriosis cases was observed in 2014 (66,790) and 2013 (66,465) compared with the 72, 560 cases reported in 2012 (EFSA, 2015).

Whilst in the United States, 9.4 million foodborne illness are reported each year, and among them *Campylobacter* is the third most prevalent bacterial pathogen, being responsible for 9% of those cases (Scallan et al., 2011). It is estimated that over 1.3 million people are infected by *C. jejuni*

every year, which results in 76 deaths (reviewed by Shang et al., 2016) at a total estimated annual cost of 1.7 billion dollars (Hoffmann et al., 2012; Batz et al., 2012). For the developing countries, campylobacteriosis incidence can be as high as 0.4 episodes per child per year (Prasad et al., 2011). *Campylobacter* isolation rates are reported between 5% to 20% from patients suffering diarrhoea (Coker et al., 2002; Oberhelman and Taylor, 2000).

Most cases of *Campylobacter* infections are sporadic with rare asymptomatic infection, whilst in developing countries, campylobacteriosis can be endemic and asymptomatic (Platts-Mills and Kosek, 2014; Frost, 2001). It is estimated that consumption or handling of chicken may be responsible for 40% of human infections, whilst up to 80% of campylobacteriosis are correlated with poultry meat as a reservoir (EFSA, 2010b). Raw cow's milk is another food vehicle for *Campylobacter* infection, which is a significant risk when intended for direct consumption (EFSA, 2015).

Although several outbreaks have been recognised, outbreaks are generally not common for *Campylobacter* infections (Inns et al., 2010; Gardner et al., 2011; CDC, 2013; DeFraites et al., 2014). In year 2014, 31 outbreaks with strong-evidence associated with campylobacters were confirmed in the EU, that resulted in 525 human cases, 40 hospitalizations but no deaths. Another 415 outbreaks with weak evidence of *Campylobacter* association have been reported, that lead to

13,83 patients with 149 of them hospitalised (EFSA, 2015). Most of the outbreaks are foodborne whilst only two of the 31 strong-evidence outbreaks identified were water-borne. For those foodborne outbreaks reported with strong evidence, the most frequently reported vehicle was broiler meat (55.2%) and it is also the main vehicle for weak evidence outbreaks (12 outbreaks). Notably, *Campylobacter* is reported as the 5th most commonly detected causative agent for outbreaks in the EU in 2014, and is responsible for 8.5% of all outbreaks (EFSA, 2015).

Whilst in the US, from 1997 to 2008, in total 262 outbreaks were reported with 9135 human cases, 159 hospitalizations and 3 deaths. The data for these outbreaks were analysed by Taylor et al. (2013) and the common vehicles associated with campylobacteriosis outbreaks were reported as water 24 (9%), animal contact 7 (3%) and foodborne transmission 225 (86%). Among the foodborne vehicles, dairy products were the most reported cause responsible for 65 (29%) of the *Campylobacter* outbreaks during the period, followed by poultry 25 (11%) and other produce 12 (5%). Outbreaks caused by the consumption of raw peas (Gardner et al., 2011), mud (Stuart et al., 2010) and transmission from pet animals to humans (Mughini et al., 2013; Davis et al., 2013) and person to person (Blaser, 2000) were also reported infrequently.

Many reports suggested that both sporadic and outbreaks show seasonality in the developed countries (Meldrum et al., 2005), as this could partly be related with the seasonality of *Campylobacter* prevalence

in broiler flock and on retail meat (Cowden and Ruiz-Palacios, 1996). Although campylobacters are capable of infecting patients of all ages, Nielsen et al. (2013) reported that higher prevalence was observed in toddlers from 1 to 4 years and young adults from 15 to 24 years compared to other age groups. In developing countries where *Campylobacter* is commonly endemic, symptomatic infections are generally limited to children, and the prevalence of *Campylobacter* is generally negatively correlated with human age. Rao et al. (2001) suggested that this negative correlation between *Campylobacter* prevalence and patient age could be related with the development of protective immunity against *Campylobacter* due to the exposure to such bacteria at an early age. This may also explain the observation that in developing countries, *Campylobacter* infections are commonly asymptomatic. Interestingly, this hypothesis may possibly be linked with another study. When analysing the free range broiler flocks, Colles et al. (2011) reported that the prevalence rate of *Campylobacter* was not correlated with season, temperature, humidity, but majorly influenced by flock age as the highest prevalence rate of 88% was present between 10 to 13 weeks and for the rest 50 weeks, the prevalence rate varied between 27% and 67% with a mean value of 40%. This could also be explained as the immunological maturity (Newell and Fearnley, 2003; Colles et al., 2011), due to the high prevalence of *Campylobacter* in the rearing environment.

1.3.2 Symptoms of disease

Campylobacter species have a relatively low infection dose as only 500 organisms are required to cause disease (Robinson, 1981). However, later it was speculated that as low as 360 CFU of *C. jejuni* would be sufficient to develop campylobacteriosis (Hara-Kudo and Takatori, 2011).

Following ingestion, it usually takes 24 to 72 hours until the onset of symptoms occur, although this time is infectious dose and host immune status dependent. The manifestations of *Campylobacter* infection are geographically dependent as more severe symptoms are commonly observed in developed countries than that in developing world, where campylobacteriosis is commonly asymptomatic (Oberhelman and Taylor, 2000). Typical symptoms for campylobacteriosis commonly include diarrhoea, malaise and abdominal pain and can last for 3- 4 days with a maximum of two weeks duration (Blaser, 1997; Butzler, 2004; Man, 2011). Most campylobacteriosis cases are self-limiting, however, *Campylobacter* infection, especially *C. jejuni* infection, can be severe and patients may exhibit fever, abdominal cramps and bloody diarrhea (Tracz et al., 2005; Gillespie et al., 2006; Epps et al., 2013). Some *C. jejuni* infections may even result in death for those individuals with immunocompromised conditions including AIDS, cancer and some liver diseases (Lastovica et al., 2014). Older data reported that in England and Wales, diarrhoea (95%), abdominal pain (85%) and fever (78%) are the most commonly reported symptoms, whilst vomiting (35%) and bloody diarrhoea (27%) were relatively less frequent (CDC, 2011).

C. jejuni infection has been reported to be related with Guillain-Barré syndrome (Chen et al., 2010; Jacobs et al., 1997). Guillain-Barré syndrome (GBS) is an autoimmune disorder of peripheral nervous system and commonly characterised by either acute or subacute symmetrical ascending motor weakness as well as areflexia and mild to moderate sensory abnormalities (Asbury and Cornblath, 1990). It is considered as the most frequent cause for acute flaccid paralysis (Adams and Victor, 1993) with an incidence rate of 0.6 to 4 cases per 100,000 population worldwide (Vucic et al., 2009). The disease is self-limiting and patients will over weeks to months recover muscle strength, but during this period approximately 20% of patients may need mechanical ventilation (Rantala et al., 1991). Although most patients can recover uneventfully, approximately 15-20% of the patients are left with severe neurologic deficiency (Winer et al., 1988; Ropper, 1986). The mortality rate of GBS patients vary between 1%-18%, and in developing countries the disease has generally a higher mortality rate than developed countries (Winer and Evans, 1993; Netto et al., 2011). Death commonly results from pneumonia, adult respiratory distress syndrome, and sepsis (Netto et al., 2011). Approximately two thirds of GBS patients were observed with various preceding infections including *C. jejuni,* Cytomegalovirus, Epstein-Barr virus and *Mycoplasma pneumoniae* (Sinha et al., 2004*).* However, among these, *C. jejuni* is the only firmly confirmed causative agent for GBS (Nachamkin et al., 2002a; Godschalk et al., 2004). Poropatich et al. (2010) studied 2,502 cases of GBS and 31% among them were identified as due to *C. jejuni* infection. Nachamkin et al. (1998) also reviewed that general

isolation rate of *C. jejuni* from stool sample of GBS patients soon after the onset of neurologic symptoms is between 8% and 50%.

The onset of GBS neurological symptoms commonly occur 10 to 21 days after the onset of campylobacteriosis. Due to the self- limiting nature of *C. jejuni,* when GBS neurological symptoms are detected, *C. jejuni* may no longer in the host, and this can make it difficult to positively correlate GBS with *C. jejuni* infection (Allos, 1997; Hadden and Gregson, 2001). Rhodes and Tattersfield (1982) were the first to correlate GBS with *C. jejuni* when reporting a GBS following *C. jejuni* infection and since then, observations of GBS with a preceding *C. jejuni* infection have been reported by a number of researchers (Ho et al., 1995; Hariharan et al., 1996; van Koningsveld et al., 2001).

GBS is now believed to be the result of molecular mimicry of sugar moieties on nerve gangliosides by the lipopolysaccharide of *C. jejuni*. A preceding infection that provokes an antibody response can include ganglioside mimics, which can then cross-react with patient nerve gangliosides to cause demyelinization of nerves and the degeneration of axons (Nielsen et al., 2010).

1.3.3 Mechanisms of *Campylobacter* infection

Despite the clinical significance of campylobacters and the access to their whole genome sequence, the pathogenesis of *Campylobacter* infection at molecular level is still poorly understood. This could due to the fact that

Campylobacter lacks homologues of virulence factors in common with other enteric pathogens (Guerry, 2007). However, several factors have been reported as essential for successful colonisation of *Campylobacter,* including motility, chemotaxis, adhesion and invasion. Whilst iron acquisition and oxidase stress defense are required for *Campylobacter* survival within host cells. Moreover, toxin production from *Campylobacter* may also be another potential factor responsible for infection.

1.3.3.1 Motility

During the infection step, *Campylobacter* mainly resides within gastro-intestinal thick mucus layer lining (Berry et al., 1988). The motility of *Campylobacter* increases within the highly viscous solution up to 75µm sec^{-1} (Lee, 1986), and this robust motility is significant for bacteria migrating to and moving within the mucus lining to reach desired microenvironments capable of supporting growth (Lertsethtakarn et al., 2011). It has been reported that only motile *C. jejuni* could be recovered post passage in human volunteers whom had been challenged with a mixture of both motile and non-motile *C. jejuni* (Black et al., 1988), suggesting that motility is essential for colonisation of *Campylobacter.* Similar conclusions were drawn using animal models including piglets and mice that report to mimic human disease (Morooka et al., 1985; Chang and Miller, 2006; Malik-Kale et al., 2007). Non-motile *Campylobacter* normally cannot colonise (Mertins et al., 2013), or in some

cases, only lead to short-term colonisation and cannot persist after seven days post infection (Hendrixson and DiRita, 2004).

1.3.3.2 Chemotaxis

Using a mouse model, Takata et al. (1992) reported that chemotaxis is also an essential factor for *Campylobacter* colonisation since their isolated, actively motile, but non-chemotactic *C. jejuni* mutants failed to colonise. Chandrashekhar et al. (2015) reported a similar finding with a chicken model. In their study, a *Tlp10* knockout *C. jejuni* 81-176 mutant was generated, with decreased chemotaxis towards aspartate and fumarate, but without resulting in any motility change. They challenged chicken with 4 $\log_{10}$ CFU of this *C. jejuni Tlp10* mutant and reported it to be severely defective in colonisation of the chicken cecum, duodenum and jejunum. Chemotaxis is the way flagellar locomotion of campylobacters is directed based on the presence of specific chemoeffectors that was first reported in bacteria in the late nineteenth century (Engelmann, 1881; Pfeffer, 1884). With the aid of chemotaxis, campylobacters are driven to a location where higher concentrations of chemoeffectors are present; or away from where higher concentrations of chemorepellents are detected. Notably, energy sources normally function as chemoattractors whilst toxic substances act as chemorepellents (Zautner et al., 2012). In this way, chemotaxis allows campylobacters to keep fathoming favorable microenvironments within hosts to access relatively high energy sources but with low in toxic substances, and thereby contributes to successful *Campylobacter* colonisation (Chandrashekhar et al., 2015).

1.3.3.3 Adhesion and invasion

Once *Campylobacter* reaches intestinal epithelial cells, it could either result in an asymptomatic colonisation (Christenson et al., 1983; Cawthraw et al., 2000) or cause illness symptoms. The detailed mechanism for symptomatic colonisation in humans remains unclear, however, based on clinical syndromes from human patients, both adhesion and invasion of epithelial cells, and production of toxins could be responsible for the campylobacteriosis *(Wassenaar, 1997; Hu and Kopecko, 2000).*

The campylobacters are capable of crossing the mucus layer covering epithelial cells and adhere to these cells followed by a subpopulation of bacteria that subsequently invade (van Vliet and Ketley, 2001). Several *Campylobacter* adhesion proteins have been analysed using both cell culture and *in vivo* models. The CadF protein of *Campylobacter* was identified as a 37kDa protein able to bind fibronectin, and is required for *in vivo Campylobacter* binding to the chicken cecum and human enterocyte-like INT-407 cells. The campylobacters are also reported to use Peb1 and JlpA for adhesion and the latter, specifically binds to eukaryotic cell surface protein Hsp90 and induces host cell signal transduction (Jin et al., 2003).

Campylobacter invasion of host epithelial cells can result in excessive mucus and cell damage as well as inflammation, and eventually these collectively cause a common fluid loss and diarrhoea symptoms (Bhunia,

2008). Invasion ability varies between different *Campylobacter* strains, and fresh clinical isolates have a higher invasion frequency compared to isolates after serial lab passage (Everest et al., 1992; Konkel and Joens, 1989). Notably, both adhesion and invasion of *Campylobacter* are highly dependent on flagellar motility (Wassenaar et al., 1991; Grant et al., 1993). The studies of Yao et al. (1994) constructed *C. jejuni* 81-176 mutants with either *flaA* or *plfA* deletions, that would respectively result in non-flagella or paralysed flagella phenotypes, and analysed their adherence and invasion ability against INT407 cells. They reported that compared with the parental strain, *C. jejuni flaA* mutant is non-adherent and non-invasive whilst the *C. jejuni pflA* mutant shows decreased adherence and are unable to invade cells. Therefore, motility can be considered as essential for *Campylobacter* invasion, however, for adherence, not only motility but also the major flagellin subunit (FlaA protein) may be needed (Yao et al., 1994; Grant et al., 1993; Nuijten et al., 1992). FlaA protein even as an immobilised filament may be able to mediate adherence (Yao et al., 1994). Moreover, campylobacters are capable of translocating cell layers, and transcytosis is commonly considered as the consequence of invasion (Bras and Ketley, 1999).

1.3.3.4 Toxin production

Cytolethal distending toxin (CDT) is an important virulence factor associated with *Campylobacter* induced acute inflammation involved in enterocolitis (Zheng et al., 2008). The corresponding gene sequences are possessed by almost all *C. jejuni* isolates. However, due to natural

deletions and point mutations, not all *C. jejuni* will produce this toxin (Pickett et al., 1996).

Bacterial CDTs belong to AB_2- type toxins that generally consist three subunits, CdtA, CdtB, and CdtC (Ge et al., 2008). CdtA and CdtC are carriers to deliver CdtB into host cells, whilst CdtB is a catalytic subunit and functions as a DNase I- like protein (Elwell and Dreyfus, 2000; Smith and Bayles, 2006). CdtB is internalised, while CdtA and CdtC are still associated with the receptor on the cell membrane (Lee et al., 2003). Hassane et al. (2003) and Li et al. (2002) reported CdtB induces double stranded cuts within host cell DNA, that would cause activation of DNA repair responses, cell cycle arrest at the G_2/M phase, and in this way lead to cell distention, which eventually results in target cell senescence or death (Whitehouse et al., 1998; Lara-Tejero and Galán, 2001).

In the study of Boesze-Battaglia et al. (2006), the binding site of CdtA and CdtC were stated to be on the cell membranes in association of lipid rafts, which contain abundant cholesterol, phospholipids and sphingolipids (Brown and London, 1998). By treating target cells with methyl-β-cyclodextrin (MβCD), which is a cholesterol-depleting agent that can perturb the structure of lipid rafts, resulted in an attenuated binding activity and reduced cell intoxication (Guerra et al., 2005; Boesze-Battaglia et al., 2006). Using a mouse model, more severe intestinal inflammation caused by CDT induction was observed for mice with higher cholesterol diet intake than normal diet, further suggesting that

cholesterol plays a critical role in facilitating CDT virulence *in vivo* (Lai et al., 2015). Lai et al. (2016) demonstrated that cholesterol provides a critical ligand for the binding of CDT to cell membranes to cause host intoxication, and proposed CdtA and CdtC contain a cholesterol-binding motif.

Instead of secreting virulence factors directly into the surrounding environment that could be subsequently degraded by host proteases, outer membrane vesicles (OMVs) are employed by several Gram-negative bacteria for protein or toxin delivery into the host cells (Kulp and Kuehn, 2010), and the association of CDT with *C. jejuni* secreted OMVs was proposed by Lindmark et al. (2009). Using immunogold-labeling electron microscopy, they observed that all three CDT subunits were closely associated with OMVs, and that the latter were considered as a route for toxin delivery into the cellular environment (Lindmark et al., 2009). Interactions between CDT-related OMVs with the membrane lipid rafts have been reported in several bacteria (Kesty et al., 2004; Kaparakis et al., 2010; Rompikuntal et al., 2012). In *C. jejuni*, Elmi et al. (2012) reported a partially inhibited OMV-induced inflammation caused by the absence of lipid rafts on cell membrane via MβCD treatment of target cells, and suggested that lipid raft on the cell membrane plays a role in OMV-mediated signal transduction in *C. jejuni.*

1.3.3.5 Iron acquisition

Bacterial adhesion to host cells can trigger cytoskeletal rearrangements of the host cell, which can result in bacterial internalization. Iron is at premium for internalised campylobacters. The ability of *Campylobacter* to acquire iron in host cell environment is extremely critical for survival and the development of pathogenesis. Iron is a significant cofactor in many proteins that would be involved in metabolism and basic cellular pathways for campylobacters and the host cells they have entered (Miller et al., 2009). The Fe^{3+} is predominantly present under aqueous aerobic conditions. Siderophores, known as compounds with low molecular mass but high affinity iron binding ability, are commonly required for Fe^{3+} uptake for Gram-negative bacteria including campylobacters (Miethke and Marahiel, 2007). *C. jejuni* was considered as incapable of synthesising siderophores (Field et al., 1986; Pickett et al., 1992), which was consistent with the observation that siderophore biosynthesis genes were not identified within the genome sequences of *C. jejuni* NCTC11168, 81-176 and RM1221 (Parkhill et al., 2000; Fouts et al., 2005). However, they can utilise exogenous siderophores as iron sources. Notably, host iron-binding and transport processes that deliver Fe^{3+} to cells could also be hijacked by campylobacters as another Fe^{3+} source.

Fe^{3+} uptake by campylobacters normally starts with the binding of the iron source with a specific iron-regulated ligand-gated surface receptor protein. After successful binding, a conformational change facilitates the iron transport through the receptor pore. The energy source for this

modification of conformation is thought to be transduced from the cytoplasmic membrane to the outer membrane by the ExbBD-TonB protein complex as described by Chakraborty et al. (2007). Once the Fe^{3+} has reached the periplasmic space, the ferri-siderophore complex is sequestered by a periplasmic binding protein and subsequently delivered to an inner membrane ATP binding cassette protein complex, which functions as a transporter system. This ATP binding cassette complex consists of permease protein subunits, which form an inner membrane pore and is coupled with an ATP binding protein (Davidson and Maloney, 2007). With the energy provided from ATP hydrolysis, the Fe^{3+} is transported across the inner membrane and eventually reaches cytoplasm, where iron may be liberated from siderophores by siderophore degradation (Faraldo-Gómez and Sansom, 2003).

However, for the oxygen reduced intestinal environment, iron is more likely to form its ferrous state and the ability of *C. jejuni* to uptake the ferrous ion is therefore essential. *E. coli feoB* is required for ferrous iron uptake, an orthologue of which can be identified in the genome of *C. jejuni* NCTC11168, a gene (*cj1398*) (Marlovits et al., 2002; Hantke, 2004), showing 29% identity (Miller et al., 2009). Adjacent to *cj1398,* the gene *cj1397* shows limited identity (16%) to *feoA* of *E. coli*, whilst a *feoC* homologue is lacking (Parkhill et al., 2000). Naikare et al. (2006) constructed a *C. jejuni* NCTC11168 *cj1398* knockout mutant, and this mutant shows a defect in the ability to transport $^{55}Fe^{2+}$, and accumulates only half the amount of ferrous iron of its parental strain. In this way,

Naikare et al. (2006) concluded that *cj1398* plays a significant role in the uptake of Fe^{2+} in *C. jejuni* NCTC11168. Moreover, using a chicken colonisation model, they suggested that this *cj1398* mutant could be out-competed by the parental strains, indicating that *feoB*-like *cj1398* is essential for proper ferrous iron uptake as well as proper colonisation. However, the *cj1398* counterpart is non-functional in *C. jejuni* M129 as well as F38011, due to frameshift mutations and the likelihood of presence of alternative Fe^{2+} uptake systems in these strains needs to be further investigated.

1.3.3.6 Oxidative stress defense

As a microaerophilic bacterium, proper growth of *Campylobacter* requires a reduced oxygen environment (Smith et al., 2000), and the low-oxygen intestinal environment contributes to its proper growth. Nevertheless, the exposure of *Campylobacter* to reactive oxygen species (ROS) in the host is inevitable (Palyada et al., 2009; Kim et al., 2015). ROS include superoxide anion, hydrogen peroxide and hydroxyl radicals, and are formed by the stepwise one-electron reduction of molecular oxygen (Imlay, 2008). In a typical host intestinal environment, when cellular Fe^{2+} is present, the ferrous iron can catalyse the transfer of an electron to hydrogen peroxide (H_2O_2), which results in the production of Fe^{3+}, hydroxide (OH^-), and more importantly, an hydroxyl radical ($^{\bullet}OH$), which is known as the strongest oxidant that exists in aqueous environments (Imlay, 2003). The ROS to which campylobacters are exposed include those produced by the host immune system as well as the host intestinal

microflora (Lidbeck and Nord, 1993; Annuk et al., 2003; Mayer-Scholl et al., 2004). Irrespective of the sources, ROS generally leads to the degradation and modulation of protein functions, which results in lipid and DNA damage (Broman et al., 2002; Yamasaki et al., 2004). Although bacteria commonly possess mechanisms to maintain ROS homeostasis, a change in oxygen concentration will influence the generation rate of ROS, and further results in oxidative stress (Lushchak, 2011). Therefore, the development of oxidative stress defense systems that detoxify ROS are important for the survival of *Campylobacter.*

Common ROS detoxification factors for bacteria include superoxide dismutase (SOD), alkyl hydroperoxide reductase (AHp) as well as catalase (Imlay, 2008). SOD is the primary enzyme which is responsible for superoxide detoxification (Winterbourn et al., 1975), whilst AHp is reported as primary scavenger of organic peroxide (Seaver and Imlay, 2001; Parsonage et al., 2008), and catalase would efficiently catalyse hydrogen peroxide into water and oxygen (Imlay, 2008). Unlike other bacteria that often possess redundant forms of these ROS detoxification enzymes, the genome sequences suggest that *C. jejuni* harbors single gene copies of *sodB, ahpC* and *katA* (Parkhill et al., 2000; Atack and Kelly, 2009). Oh et al. (2015) constructed knockout mutants of all three antioxidant genes in *C. jejuni* NCTC11168, and all three *C. jejuni* mutants revealed significant growth reduction under aerobic conditions than their parental strain with the most significant growth defect in the *ahpC* mutant. Higher levels of ROS accumulation in *C. jejuni* were observed under aerobic

conditions compared to microaerobic conditions, and confirmed the positive correlation between ROS generation rate and the oxygen concentration.

Flagellar synthesis of campylobacters may also indirectly influence their superoxide stress defense since the deletion of flagellar biosynthesis genes including *motA, motB, flgR, flhB, flgD*, from *C. jejuni* NCTC11168 increases its susceptibility significantly to superoxide, and slightly to hydrogen peroxide (Flint et al., 2014). Based on the fact that the flagellar motor uses the proton potential of the cell inner membrane to generate rotation (Terashima et al., 2008), the author hypothesised that the increased susceptibility to ROS could be related with a disturbance in the function of the electron transport chain (ETC). As the ETC is a major source of endogenous ROS production (Imlay, 2008), any disturbance in ETC activity could contribute to increased ROS production via electron leakage and eventually lead to the hypersensitive phenotype of flagellum mutants (Terashima et al., 2008).

1.4 Typing methods for *Campylobacter*

A sensitive, reproducible, and well-standardised typing approach is critical for the analysis of either sporadic or outbreak cases of campylobacteriosis. To date, a number of phenotypic and genotypic approaches have been described to discriminate *Campylobacter* species to enable the tracking of sources of infections.

1.4.1 Phage typing

A *Campylobacter* phage typing schemes was firstly described by Grajewski et al. (1985) in the US, including 14 virulent phages isolated from poultry faeces that were able to type most *C. jejuni* isolates of human origin (94.5% and 88.1% for two sets of isolates containing 225 and 51 respectively). Later, a new phage typing scheme was reported by Salama et al. (1990) in UK, including 6 phages from the initial US phage typing scheme in addition with 10 virulent phages previously isolated from pig, poultry manure and sewage effluent (Salama et al., 1989). With this scheme, they successfully identified the causative strain for five outbreaks, that had been biotyped and serotyped (Salama et al., 1990). Two years later, a Canadian phage typing scheme containing 19 virulent phages was formulated by extending the original US scheme with another five phages isolated from chicken litter in Canada (Khakhria and Lior, 1992). Under this scheme, they identified a total of 754 *Campylobacter* isolates from either human or non-human sources from 17 countries, with an average of 80.6% isolates being typable with geographical variation from 61% in Thailand (47/77) to 93.2% in Canada (261/280).

Although major *C. jejuni* isolates are phage typable, some isolates inevitably were not. However, there are many literature reports that have used phage typing to discriminate *C. jejuni* isolates (reviewed by Connerton et al., 2011).

1.4.2 Ribotyping

Ribotyping enables discrimination between bacterial species based on the nucleotide variation in ribosomal operons, that include three highly conserved genes within bacterial species that individually encode the 16S, 23S and 5S rRNAs (Maidak et al., 1997). The approach normally involves bacterial genomic DNA digestion followed by separating by electrophoresis, transferring to a nylon membrane and detecting with rRNA-specific probes (Bouchet et al., 2008). By digesting the genome with different restriction enzymes, the target rRNA type will be discriminated from the resulting fragments that can be detected after hybridising with specific probes. The number of visualised fragment will be a reflection of the multiplicity of rRNA operons present in the species (Bouchet et al., 2008). Based on the differences in number of visualised rRNA operons and distinct restriction digested fragment due to the variation of flanking DNAs surrounding the target rRNA gene between species, bacterial strains could be discriminated. As 16S rRNA gene is often identical among the three copies, it is frequently selected as target gene to classify bacterial species (Kolbert and Persing, 1999). Within *Campylobacteraceae* family, ribotyping is usually applied for *C. jejuni* analysis, where PstI and HaeIII are the commonly used restriction enzymes for genome digestion (Bouchet et al., 2008). However, due to relatively low in copy number of ribosomal operons (3) in both *C. jejuni* and *C. coli*, ribotyping is generally less discriminatory compared with other genotyping approaches (Lastovica et al., 2014).

1.4.3 Pulse-field gel electrophoresis (PFGE)

As one of the first general DNA based typing approaches for *Campylobacter* species (Ribot et al., 2001; reviewed by Taboada et al., 2013), PFGE is able to separate large DNA molecules in a gel matrix by applying periodically a direction change in the electric field (Schwartz and Cantor, 1984). When analysing *C. jejuni* isolates, the chromosome is firstly digested with restriction enzymes, typically KpnI or SmaI, the resulting large fragments are then separated with PFGE based on size (Ribot et al., 2001), eventually, the well-separated fragments are visualised and analysed with commercial software packages (Olive and Bean, 1999). Sails et al. (2003) reported the presence of resistance of *Campylobacter* sporadic isolates to SmaI digestion, and Michaud et al. (2001) also stated that when typing *Campylobacter* species with PFGE, digestion with KpnI would has a better discrimination than SmaI digestion.

Due to the remarkable discriminatory ability as well as the reproducibility, PFGE has been considered as a "gold standard" of genotyping for many bacterial foodborne pathogens including *Campylobacter*, and is used by the PulseNet program in US to classify outbreaks of bacterial foodborne diseases (Swaminathan et al., 2001).

1.4.4 *FlaA* Short Variable Region (*FlaA*- SVR)

Sequencing of a single short locus within *flaA* gene has been suggested as a significant typing scheme (Meinersmann et al., 1997). This particular locus is highly variable among different bacterial species, and thus can be

considered as a genetic marker (Harrington et al., 1997; Nuijten et al., 2000). Although the target sequencing fragment is relatively small in size (321bp), the discriminatory power could be nearly the same as with whole *flaA* sequencing (Fitzgerald et al., 2001).

Due to the relative simplicity and low cost of *FlaA*- SVR typing, it has been successfully applied to investigate a number of campylobacteriosis outbreaks (Clark et al., 2005; O'Reilly et al., 2006). Due to the fact that campylobacters are naturally competent and capable of taking up foreign DNA, in addition to the frequency of intra species transfer, the *flaA* alleles are not stable and may not be species specific (Dingle et al., 2005). The *FlaA*- SVR is commonly applied together with other genotyping methods, such as MLST, to improve the discriminatory power, which is comparable to that of PFGE (Sails et al., 2003; Clark et al., 2005).

1.4.5 Multilocus sequence typing (MLST)

Bacterial genomes show sequence variability as the consequences of mutation and recombination, and the sequence variability within particular genes can be applied as the basis for typing approaches (reviewed by Foley et al., 2009). MLST is one of these typing method and targets specific nucleotide base modifications to determine the genetic correlation between bacterial strains (Spratt, 1999). As house-keeping genes are commonly sufficient to provide variability and further develop distinct alleles for different strains (Kotetishvili et al., 2002), they are commonly sequenced. The house-keeping genes *aspA, glnA, gltA, glyA,*

pgm, tkt and *uncA* are typically selected for discrimination of *Campylobacter* species (Sails et al., 2003). Each of the sequenced PCR products, are assigned with an allele number after matching their sequence with the MLST database. The seven individual allele numbers from each gene are then grouped and the strain is assigned to a specific sequence type (ST) based on the database (Enright and Spratt, 1999). STs sharing four or more alleles belongs to the same clonal complex (Dingle et al., 2001).

Due to the high occurrence of horizontal gene transfer and intra-molecular recombination, *Campylobacter* species generally have a weakly clonal population (Dingle et al., 2005). As MLST targets the house-keeping genes, which show sufficient diversity but are relatively stable, a high discriminatory power can then be achieved. MLST has been suggested as a valuable typing approach to study sporadic campylobacteriosis. However, when investigating campylobacteriosis outbreaks in Canada in 2000, the occurrence of indistinguishable *Campylobacter* strains was reported (Clark et al., 2005). In this case, the MLST scheme was later extended by combining the original seven house-keeping gene with *flaA*, *flaB* and *porA*, to allow an even higher discriminatory (Dingle et al., 2008).

1.4.6 Whole genome sequencing (WGS)

Currently, whole genome sequencing is the most comprehensive method to analyse bacterial genomes. It is capable of distinguishing bacterial isolates with only one base pair variation, and thus provides the highest

discriminatory power for epidemiological studies (Taboada et al., 2013). Unfortunately, the costs of WGS data generation limits this technique to priority strains but the costs are falling with the developments in technology (Parkhill et al., 2000; Pearson et al., 2007). The rapid development of next generation sequencing platforms, has meant genome sequencing is currently widely utilised to study outbreak events (Lahti et al., 2016; Revez et al., 2014; Gardy et al., 2011). WGS also enables access to information that can be extrapolated to gain insights into mechanisms of bacterial virulence, the development of resistance, general biological function and structural variation, that collectively provide a better understanding of the bacterial pathogens and aid the development of new interventions against further outbreaks (Pendleton et al., 2015).

Moreover, based on the bacterial genome data provided by WGS, current MLST schemes have been widely expanded (Colles and Maiden, 2012; Sheppard et al., 2012). Currently, the expanded scheme for the *C. jejuni/ C. coli* core genome MLST (cgMLST) contains 1,343 loci as defined by the re-annotated genome sequence of *C. jejuni* NCTC11168. The cgMLST scheme itself is defined from 2,742 *Campylobacter* isolate genomes from Oxfordshire human patients. This cgMLST scheme could be used to complement the original scheme, and therefore significantly increase the discriminatory power of the typing method (Taboada et al., 2013).

1.5 Interventions against *Campylobacter*

Antibiotics have been used for decades in food production animals to control, prevent, and treat infections, and to enhance growth (Rozynek et al., 2007; Igimi et al., 2008). However, the rapid increase in the development of antibiotic resistance in *Campylobacter*, particular to fluoroquinolone has been reported in many countries (Engberg et al., 2001; Gaudreau and Gilbert, 2003; Alfredson and Korolik, 2007), which has resulted in concern for untreatable severe human *Campylobacter* infection (Nachamkin et al., 2002b). A number of studies have drawn attention to the use of fluoroquinolones in poultry which may lead to the emergence of drug resistance in both humans and poultry (Engberg et al., 2001; Luber et al., 2003). As a consequence, the US Food and Drug administration withdrew approval for the usage of enrofloxacin for prophylaxis and as a growth promotion in poultry (Davidson, 2004). In the EU, the use of fluoroquinolones in poultry was recommended to be limited in 1998 (Gallay et al., 2007), and the routine use of antibiotics as growth promoters is banned. In respect of this, alternative intervention strategies have been sought by researchers against *Campylobacter* (Adzitey et al., 2012).

1.5.1 On-farm intervention

Based on the fact that a 90% reduction in potential risk to public health could be achieved by reducing 3 $\log_{10}$ unit *Campylobacter* in broiler flocks, and even with a 1 $\log_{10}$ unit decrease could reduce the risk to

between 50% and 90% (EFSA, 2011), most interventions focused on *Campylobacter* attempt to exert control at farm level.

1.5.1.1 Hygienic and biosecurity measures

By reducing the environmental exposure of broiler flocks to *Campylobacter* with biosecurity measures, the likelihood of these conventional reared broiler flocks to become colonised would be reduced. The major approach for biosecurity measures is to identify the sources of *Campylobacter* and subsequently disrupt the transmission routes with targeted interventions to avoid horizontal transmission. Several risk factors for introducing *Campylobacter* in broiler flock houses have been investigated, which commonly focus on contaminated drinking water, fecal contaminated litter, contaminated sewage, insects poor ventilation, the close location to other poultry sites, and short empty periods (Jones, 2001; Hald et al., 2008; Workman et al., 2008; Rosenquist et al., 2009).

Positive evidence for using biosecurity measures as a *Campylobacter* intervention approach has been reported in Switzerland. After introducing biosecurity measures to two chicken farms, van de Giessen et al. (1998) reported that the prevalence of *Campylobacter* was decreased from 60% and 100% to 22% and 42% respectively. Similar evidence was reported by Gibbens et al. (2001) in UK with a 50% reduction of *Campylobacter* colonisation was achieved, and by Rosenquist et al. (2009) in Denmark, with *Campylobacter* positive broiler flocks at slaughter decreased from 43% to 27%. However, Newell et al. (2011) stated that

under practical conditions, it is extremely difficult to provide stringent biosecurity measures effectively and consistently in order to exclude *Campylobacter* contamination from poultry houses. Moreover, the application of biosecurity measures to reduce *Campylobacter* prevalence for free-range or organic broiler flocks is not really possible given their exposure to *Campylobacter* in their rearing environments. Nevertheless, biosecurity measures could be applied in combination with other *Campylobacter* interventions.

1.5.1.2 Vaccination

Vaccination of broiler flocks against *Campylobacter* colonisation is thought to be another intervention with potential since the observation of a *Campylobacter*- specific immune response in chicken (Rice, 1997). Various vaccination approaches are recently being developed in order to control *Campylobacter* in poultry and generally can be divided into three strategies: live attenuated vaccines, killed whole-cell vaccines and subunit vaccines.

Based on the theory that infection with *Campylobacter* would induce the anti-*Campylobacter* antibody, it is hypothesised with a live attenuated vaccine, the host could be protected by provoking the immune response. However, Ziprin et al. (2002) reported that colonising chicken experimentally with non-colonising *C. jejuni* F38011 (*dnaJ, cadF, ciaB* and *pldA* mutants) failed to provoke host protective immunity and further

failed to protect the host from subsequent challenge with the parental strain after one week.

Killed whole-cell vaccines (KWC) have been demonstrated in developing countries to prevent diarrhoeal disease (Pace et al., 1998; Walker, 2005), and have the advantage of relatively safe use and are cost effective. Various KWC vaccines have been analysed as vaccines against *Campylobacter.* In the study of Rice (1997), a high dose (9 $\log_{10}$ cfu) of formalin-inactivated *C. jejuni* cells were orally administered to broiler chickens and allowed to develop for a various periods with a minimum of 6 days. After challenging with the homologous strain, number of isolated *C. jejuni* from KWC pre-treated group revealed moderate reduction with the highest variation of 1.5 $\log_{10}$ CFU per g intestinal content observed. Using a similar methodology, Cawthraw et al. (2000) reported that a formalin treated KWC orally administered to chicken on days 3, 10 and 17, showed no reduction in the *Campylobacter* colonisation level after challenging with the homologous strain at day 35.

Although live attenuated vaccines and KWC only achieved limited success, a most encouraging vaccination study was published by Wyszynska et al. (2004) when using CjaA via attenuated *Salmonella* carrier as a vaccine, a 6 $\log_{10}$ reduction in *C. jejuni* colonisation was observed. CjaA is known a subunit of a highly immunogenic protein and is conserved among different *Campylobacter* serotypes (Wyszynska et al., 2004). However, due to lack of *Salmonella* carrier control group in this study, the boost of

host immunity could also be caused by *Salmonella* infection (Wyszynska et al., 2004). More recently, Neal-McKinney et al. (2014) reported to use attenuated *Salmonella* to carrier CadF, FlpA, FlaA and CmeC, which are all known as essential for *Campylobacter* colonisation and are respectively responsible for cell adhesion, flagellin formation, flagella formation and cell membrane structure, as vaccines. Chickens at 16 days of age were administered with these vaccines and seven days later, *C. jejuni* F38011 was orally challenged to chickens with high dose and the colonisation level was subsequently determined. The application of CadF, FlpA and FlaA vaccine resulted in a 1.69 $\log_{10}$, 3.17 $\log_{10}$ and 3.32 $\log_{10}$ cfu g^{-1} significant reduction in *C. jejuni* colonisation, whilst CmeC vaccine failed to reveal any significant difference. Among those, FlpA and FlaA vaccine showed a higher than 2 $\log_{10}$ reduction in *C. jejuni* colonisation and indicates such interventions would significantly lower the public health risk from *Campylobacter.* More recently, reductions up to 10 $\log_{10}$ cfu g^{-1} in *C. jejuni* colonisation has been reported by Nothaft et al. (2016) using an N-glycan antigen that represents an N-linked decoration of glycoproteins located at the *Campylobacter* cell surface of all test *C. jejuni* and *C. coli* strains. The antigen was either combined with a protein carrier, GlycoTag, or fused to an *E. coli* carrier as the vaccine delivery vehicle.

1.5.1.3 Bacteriophage therapy

As the natural predators of bacteria, bacteriophages have been reported as an alternative intervention to control *Campylobacter* colonisation. A growing number of studies have successfully reduced *Campylobacter*

colonisation using phage therapy both *in vivo* and *in vitro*. More details about phage therapy will be introduced in the next section.

1.6 Bacteriophage

Bacteriophage, commonly referred as phages, are viruses that only infect their bacterial host (Kutter et al., 2005). Each individual phage particle contains a DNA or RNA genome, which is located inside of a protein or lipoprotein capsid (Guttman et al., 2005). The target hosts for phage are normally a specific group of bacteria within the same species, whilst it is possible that several related species are infec2ted by the same phage (Harper and Enright, 2011). Although bacteriophages carry all the information to direct reproduction in their target host, they are identified as obligate parasites due to lack of energy generating machinery and protein generating ribosomes. Bacteriophages are often noted the most abundant living entities on planet earth, with populations of bacteriophage in the range of 10^4 to 10^8 virions per millilitre in aquatic systems and approximately 10^9 virions per gram in the soil, and are estimated to be approximately 10^{31} in the biosphere (Weinbauer, 2003; Kutter et al., 2005). In any ecosystem, bacteriophages are capable of maintaining a dynamic balance among the widely varied bacterial species based on their natural characteristics including high specificity, long-term survivability and rapid reproducibility. When appropriate hosts are absent, many bacteriophages can maintain their infection ability for decades in the absence of damage from external agents (Guttman et al., 2005).

1.6.1 Discovery and early history of bacteriophages

Probably the first bacteriophage activity observation was reported by Hankin (1896). In the report, an antiseptic activity against various kinds of bacteria, typically against *Vibrio cholerae*, which was observed in the waters of the Jumna and Ganges Rivers in India and this activity was filterable and destroyed after boiling. At that time, Hankin concluded that the source of the antiseptic activity was some volatile chemical substance. However, the actual beginning of bacteriophage research is thought to be the observation of glassy and transparent spots on the bacterial contaminated agar plates when Twort (1915) was trying to propagate vaccinia virus. He proposed the possible explanations for this as either an unusual manifestation of bacterial life cycle, or an enzyme product by the contaminated bacteria or some ultramicroscopic virus. Shortly thereafter, d'Hérelle (1917) independently discovered a microbe which functioned as a bacterial antagonism, and further caused bacteria to lyse in liquid culture and on the surface of agar overlaid with bacteria, separated patches were observed (he named these patches as plaques). These invisible microbes were conceived as ultra-viruses, that are capable of invading bacteria and to multiply at their expense, and were termed as bacteriophage by d'Hérelle (1917). Later in 1919, while d'Hérelle was investigating an outbreak of bacillary dysentery, he examined the filtered dysentery samples for the presence of invisible viruses, which may potentially alter the bacterial growth and pathogenicity from patients. Similarly, he observed lysis in liquid culture as well as the clear plaques formed in the bacterial confluent culture covering the agar slants

(reviewed by Summers, 1999). He conceived that the invisible microbes were particulate and also noted that they could be multiplied indefinitely, requiring living cells to multiply, cell lysis seemed to be essential for multiplication, and could be enumerated by plaque counting. Moreover, he interpreted the multipication cycles as infection, multiplication, release and reinfection (reviewed by Summers, 2004). He applied bacteriophages to the patients and observed a titre rise of bacteriophage when patients were recoverying. In this case, he reasoned bacteriophages are natural agents that resist infectious diseases and further advocated bacteriophage as therapeutic agents (reviewed by Summers, 2004). Later, with a second research program focusing on the biological nature of bacteriophages, d'Hérelle found that they were obligate intracellular parasites with antigenic properties and their host range specificity appeared to be characterised by types of phages.

The lysogenic cycle of bacteriophage was firstly described by Bordet (1925), and thereafter, Frank Burnet confirmed the viral nature of bacteriophage, suggested different species of phages did exist, and reported the types of intereaction between phage and bacterial host (Burnet, 1933; reviewed by Sankaran, 2010). The nucleoprotein composition of bacteriophage was reported by Schlesinger (1934). Eventually, with the advent of the electron microscope, Helmut Ruska first described the observation of round particles together with sperm-shaped particles adhering to a bacterial membrane in a phage suspension (Ruska, 1940). Shortly after Ruska's findings, Luria and Anderson (1942)

visualised different types of phages and concluded their common structure as a non-homogeneous head that was round shaped attached to a much thinner tail, resulting a peculiar sperm-shapped structure. They further described the phage lysis stages including adsorption to bacterial cell, extensive bacterial damage followed by the presence of a large number of newly formed phages (Luria et al., 1943).

1.6.2 Characteristics of bacteriophage

Bacteriophages vary in genome sizes, with some only a few thousand bases within their genome, whilst on the other hand, the largest phage genomes can be up to 497,513 base pairs (*Bacillus megaterium* phage G). To date, approximately 96% population of known bacteriophages are Caudovirales (tailed phages) and these phages can be further classified into three families based on their distinct tail morphologies: *Siphoviridae* (61%), with long flexible tails; *Myoviridae* (25%), with long double layered contractile trails and *Podoviridae* (14%), with short stubby tails (reviewed by Fokine and Rossmann, 2014). Caudovirales virions are generally half double stranded DNA and half protein by mass. Most virions (75%) within the Caudovirales have icosahedral heads, that are assembled from many copies of one or two proteins, whilst others (15%) have prolate heads (Fokine and Rossmann, 2014). On the other hand, tailless phages are commonly divided into ten families, and each family contains but a few members. They vary by shape, including rods, spherical, pleiomorphic or lemon-shaped; by whether having a lipid coat;

and by the way they are released from the host cell either by lysis or continual extrusion from the host cell surface.

Besides the classification based on tail characteristics, bacteriophages have been divided into two classes based on their life cycle: lytic or lysogenic phages.

1.6.2.1 Lytic bacteriophages

Lytic bacteriophages, or virulent phages, are a type of phages have a lytic life cycle, which will obligately lyse the infected bacterial cell, and subsequently release progeny (Connerton et al., 2011).

Infection by tailed phages commonly starts with adsorption, which is the binding step of a specialised adsorption structure, like fibres or spikes, to the receptor located on the surface of the target host bacterial cell. Typically, in Gram-negative bacteria, proteins, oligosaccharides and lipopolysaccharides all can function as receptors for infection from different phages (Rakhuba et al., 2010). However, since lacking of specific structures responsible for the motion of virion particles, bacteriophages cannot move independently, and the adsorption is based on random collision between phage and host cell as first described by Schlesinger (1932). Therefore, the adsorption rate is positively correlated with the concentration of both phages and bacterial host cells, and is also dependent on several other factors including pH, temperature, the presence of ions and cultural conditions (Delbrück, 1940; Quiberoni and

Reinheimer, 1998; Sillankorva et al., 2004). The phage adsorption steps include reversible and irreversible, and only irreversible binding would be able to initiate subsequent penetration of nucleic acid (Rakhuba et al., 2010).

After the irreversible adsorption, the phage genome is injected into the host cell, and the mechanisms involved are specific for each phage group. Here we will focus on the mechanisms for T4 phages, as one of the most extensively studied *Myoviridae* phage, and also because most phages applied in this thesis are within the T4-like phage superfamily. T4 phage contains 6 long tail fibres and 6 short tail fibres attached to its baseplate (Fokine and Rossmann, 2014). In the initial step, the long tail fibres of T4 phages reversibly bind to specific receptors on the surface of host cell outer membrane. The successful attachment of over three tail fibres on the cell surface is essential to trigger the conformational modification of phage tail for subsequent DNA penetration (Arscott and Goldberg, 1976; Crawford and Goldberg, 1977). After the reversible adsorption via long tail fibres, a signal is transmitted to the baseplate that results in the extension of the 6 short tail fibres followed by the irreversible attachment of these 6 short tail fibres to the heptose moiety in the core domain of LPS (Riede et al., 1985; Montag et al., 1987).

The central hub of baseplate is formed by a trimer of gp27 protein that binds to the tail spike made by a trimeric gp5 protein. The gp5 protein contains three domains including the N-terminal domain with an

oligosaccharide- oligonucleotide binding function; the central domain functions as a lysozyme; and the C-terminal domain forms an intertwined β-helix structure with the tip capped by a monomeric protein, gp5.4, which sharpens the central spike (Shneider et al., 2013). Upon irreversible attachment, the phage baseplate modifies its shape from hexagonal-shape to a star-like conformation, in this way, simultaneously triggers the contraction of the tail sheath. This causes the $(gp5)_3$ –gp5.4 spike to puncture the outer membrane of the target bacterial cell, and the central lysozyme domain of gp5 lyses the peptidoglycan in the periplasm space in order to facilitate DNA migration along the tail tube into the cell (Moody, 1973; Kanamaru et al., 2002; Kostyuchenko et al., 1999).

The T4-like phage does not have its own RNA polymerase (RNAP), but instead, they are capable to redirect RNAP from host to express their own genes. They also encode multiple factors that are responsible for the modification of the specificity of the polymerase when infection is taking place. These modifications are dependent on the temporal regulation of three classes of transcription: early, middle and late (reviewed by Hinton, 2010). Based on the commonly shared high sequence homology, the double stranded DNA recognition elements present within phage promoters are recognised by portions of the host RNA polymerase immediately after phage DNA penetration, and in this way the phage can initiate early transcription (reviewed by Hook-Barnard and Hinton, 2007). Evidence indicates that T4 phage has 40 or so early genes with extended -10 and -35 promoter nucleotide sequences that optimise the

interaction with host RNAP, and compete extremely well for the available RNAP with other host promoters (Wilkens and Ruger, 1996). Besides the sheer strength of phage early promoters, an internal phage head protein is injected with the phage DNA to assist this process. A mono-ADP-ribosyltransferase protein ensures transcriptional dominance of phage genes, based on its ability to impair host promoters by ADP- ribosylating a host specific residue Arg265, which is known to be crucial for proper transcription from host promoters (Horvitz, 1974; Koch et al., 1995; Depping et al., 2005). Since phage promoters out-compete host promoters for RNAP binding, a burst of immediate early transcription occurs within the first minute after DNA penetration. Meanwhile, the phage encoded nucleases, including *denA* encoding EndoII and *denB* encoding EndoIV, start degrading DNA containing unmodified cytosines (Carlson et al., 2004; Hirano et al., 2006). As cytosines contained in phage DNA are commonly hydroxymethylated and glucosylated (Kashlev et al., 1993; Severinov et al., 1994), this DNA degradation is selective for host DNA, and in this way, removes the host DNA template for RNAP, and further eliminates host transcription. With appropriate infection temperature, after approximately one minute post penetration, some of the early products start mediating the transition from early to middle transcription. As phage early products, activator MotA and co-activator AsiA are significant in this transition, and serve to change the specificity of RNAP from early to middle promoters.

To date, nearly 60 middle promoters have been identified, and most of the middle genes commonly encode proteins required for replication, recombination, nucleotide metabolism and certain transcription factors which program the switch from middle to late promoter activation (Hinton, 2010). There are two mechanisms that are involved in transcription of middle genes: sigma dependent activation of the middle promoters; and extension of early gene transcripts to the downstream middle genes (Berdygulova et al., 2011; Miller et al., 2003). Unlike early promoters, phage middle promoters contain a σ70 dependent host element as well as a MotA box phage element. The co-activator AsiA is identified as a 10kDa protein that binds to the C-terminal portions of σ70 subunit of RNAP (Ouhammouch et al., 1994; Lambert et al., 2004; Sharma and Chatterji, 2006). The AsiA forms a homodimer in solution, and upon binding, one monomer of AsiA is simply replaced by σ70 (Lambert et al., 2004). The AsiA protein has the ability to inhibit most host promoters and expression of plasmid encoded AsiA is also toxic to the bacteria. Therefore, AsiA is a specific anti-sigma 70 factor that functions as an inhibitor of host transcription (Hinton, 2010). However, AsiA inhibition does not affect early promoter dependent transcription (Pene and Uzan, 2000). MotA is an activator that tightly binds to the MotA box DNA recognition element, and subsequently activates transcription when AsiA associated RNAP is present (Hinton, 1991; Ouhammouch et al., 1995). On the other hand, middle transcripts can also be generated by extension of early transcripts into middle genes due to the fact that most middle genes are located downstream of early genes (Hinton, 2010). However, this

middle RNA generation is time delayed and cannot be synthesised until the elongating RNAP reaches the downstream middle genes.

Late transcription is responsible for expression of the phage head, tail and tail fibre proteins (Williams et al., 1994). The conserved sequence TATAAATA in the late promoter shares homology with the host canonical promoter sequences within the bacterial chromosome. A phage sigma factor, σ55, is encoded for RNAP recognition of late promoters (Williams et al., 1994), and specifically recognises the host promoter via the common sequence. However, the initiation of late transcription also requires the co-activator, gp33, to mediate interactions between σ55 and the phage gp45 that functions as a sliding clamp, which is important for processivity of the DNA replication complex, and functions as a mobile enhancer in late transcription (Wong and Geiduschek, 1998). Notably, activated late promoters are able to outcompete middle promoters on the same plasmid *in vitro* (Kolesky et al., 1999; Williams et al., 1994).

Once late genes are expressed, the viral DNAs are packaged and assembled to form phage progeny particles. Eventually the lytic cycle ends with cell lysis, which is known as a tightly time controlled event (Wang, 2006). At the desired time point, a phage encoded membrane protein (holin) forms a lesion in the host cell membrane. Upon the cell membrane disruption, a phage encoded endolysin attacks the cell wall, and results in cell lysis within a few seconds (Gründling et al., 2001).

1.6.2.2 Lysogenic bacteriophages

Lysogenic bacteriophages describe those phages under lysogenic life cycle, which can integrate their DNA into the host bacterial chromosome as a prophage, or sometimes maintained as plasmid, and do not produce new bacteriophages. The lysogenic life cycle can remain indefinitely where prophage DNA is replicated along with their host cell replication. However, these prophages are capable to come out from their quiescent condition and subsequently enter a lytic cycle occasionally. Unlike lytic phages that encode many host-lethal proteins, lysogenic phages always encode a repressor that will function at certain operator sites to prevent the gene transcription from the prophage genome and potentially other related phages. Superinfection by lytic phages can also be halted where genes of the superinfection bacteriophage are regulated by the same repressor, and in this way prophages block lytic infection thereby preventing their genetic material from being lost as a casualty of secondary infection. This repressor may be the only phage encoded protein expressed during the lysogenic stage, however, some other genes that are potentially beneficial to host cell survival may also be expressed from prophages. Therefore, lysogenic bacteriophages generally protect their bacterial host against infection from several kinds of phages (Guttman et al., 2005).

1.6.2.4 Bacteriophages susceptibility to environmental conditions

Bacteriophages have been found in all environments where bacteria are present and those environments include but are not limited to ground/

surface water, soil, hot spring, food, sewage, sludge (Yoon et al., 2002; Lucena et al., 2006; Kumari et al., 2010), as well as feces, urine, saliva, spit, rumen and serum from human and animal sources (Gantzer et al., 2002; Bachrach et al., 2003; Nigutová et al., 2008; reviewed by Jończyk et al., 2011). Bacteriophages are a significant factor in limiting bacterial populations, and are estimated responsible for 10- 80% of bacterial mortality in total (Weinbauer, 2003).

Some bacteriophages are reported as able to survive extreme conditions. Breitbart et al. (2004) reported the successful isolation of phage from hot springs at temperatures between 40 - 90˚C. In sauerkraut fermentation tanks where pH can be less than 3.5, Lu et al. (2003) reported the recovery of 24 phage isolates after 60 and even 100 days. Seaman and Day (2007) isolated phage from the great salt plains in Oklahoma, US, with the salinity of groundwater and soil reported in the range of 4%-37% and 0.3%-27% respectively (Wilson et al., 2004). One phage isolate, ΦgspC, which is a member of the *Myoviridae* family, had an uncommonly large genome of 340kb, and the author suggested that this large genome may encode several environmental adaption genes that probably contribute to its high survivability in high salinity conditions. Prigent et al. (2005) reported the isolation of phages from extremely dry surface sand in the Sahara Desert, where it is described as highly deficient in humidity, highly exposed to UV radiation with large temperature shifts (Heulin et al., 2003).

Although some phages remain infectious under extreme conditions, the susceptibility of bacteriophages varies greatly between environmental conditions or exposure to chemical or physical agents, and are generally unpredictable often needing to be experimentally determined. However, there are some general principles. All phages are sensitive to UV light in the wavelength of 260nm, and generally are stable in the pH range between 5 to 8. Phages are commonly sensitive to protein denaturing agents including urea and urethane, and the susceptibility to these agents depend on the concentration, temperature as well as the phage type (Guttman et al., 2005). Detergents generally have less influence on phage than they do on bacteria, whilst chelating agents have strong influence on some of phages. Several mutagenic agents include nitric oxide and UV light, could inactivate phage and could also induce the lytic cycle of lysogenic phages.

1.6.3 Bacteriophage therapy

Although phage therapy was firstly developed in early 1900s after their independent discovery by Twort (1915) and D'Hérelle (1917), due to the wide use of antibiotics in last century, the scientific interests related to phage were founded on their biotechnological potential as vectors and sources of enzymes, and their specificity to enable phage-based detection and the typing of pathogenic bacteria. However, the exception to this was in the former Soviet Union, where bacteriophages had been routinely used as a bacterial infection therapies (reviewed by Chan et al., 2013). There has been an expansion in research on bacteriophage largely driven

by the increasing number of multi-drug resistance pathogenic bacteria and the prohibition by the European Union of including antibiotics in animal feed to promote growth. Bacteriophage therapy as a sustainable treatment against pathogenic bacteria has regained the general interest (Wagenaar et al., 2005).

As natural predators of bacteria, the use of bacteriophages as an intervention against the prevalence of *Campylobacter* provides several advantages that include: ease of isolation, nothing new is added as they are already present in the same environment as their host bacteria; host specificity to prevent damage to the normal microbiota; and self-replicating and self-limiting, as they can only multiply with the presence of their host bacteria (reviewed by Connerton et al., 2011). However, lysogenic phages are unsuitable for phage therapy, as integration produced phage-encoded repressor will possibly render the host bacteria immune to further infection.

Loc Carrillo et al. (2005) and Wagenaar et al. (2005) first reported using bacteriophage to reduce the number of *Campylobacter* in chicken ceca, and later plenty of studies have come to the same conclusion as the application of bacteriophage to chicken will result in a 2 $\log_{10}$ cfu g^{-1} decline of *Campylobacter* count in chicken intestinal contents with 5 $\log_{10}$ cfu g^{-1} reported as the highest reduction (Fischer et al., 2013). Therefore, bacteriophage therapy applied at farm level to reduce the number of *Campylobacter* in chickens could be meaningful intervention. Moreover,

bacteriophages may be directly applied to the surfaces of foods or processing facilities, as a bio-sanitisation tool, to reduce the number of pathogenic bacteria in food and potentially prevent cross-contamination occurring in the abattoir or from poultry meat when prepared by the consumer (Sulakvelidze and Barrow, 2005). It was determined that a 1-2 $\log_{10}$ CFU cm^{-2} reduction would be present after the application of bacteriophages on experimentally contaminated chicken skin and a greater reduction could be achieved if combined with freezing (Connerton et al., 2011).

1.7 *Campylobacter* resistance to bacteriophages

Although phage application could significantly reduce the number of *Campylobacter* present in the poultry intestinal content, as reported by El-Shibiny et al. (2009), 2% of *Campylobacter* exposed to virulent bacteriophage developed phage resistance and in some cases, this resistance rate has been reported up to 13% according to the study of Carvalho et al. (2010). Common mechanisms of bacteriophage resistance in bacteria include the prevention of phage adsorption via blockage of phage receptors, the production of a protective extracellular matrix or the production of competitive inhibitors; the adoption of superinfection exclusion (Sie) systems to prevent phage DNA entry; cutting phage nucleic acids via restriction-modification system; CRISPR-Cas system recognition; and abortive infection (Abi) systems, which lead to the death of the infected cell and therefore halt replication within the bacterial population (reviewed by Labrie et al., 2010).

Although bacteria can become resistant to phage infection, this resistance is not as worrisome as antibiotic-resistance (reviewed by Keen, 2012). Similar to bacteria, phages can also mutate to counter phage resistant bacteria (Matsuzaki et al., 2005). Notably, the mutation frequency of phage is generally higher than that of bacteria, so when phage resistant bacteria are present, the phage responds quickly (Bernhardt et al., 2001). This potentially endless cycle of bacterial defense and phage counter defense constitutes a typical host-parasite coevolution interaction (reviewed by Koskella and Brockhurst, 2014).

As reported by Humphrey et al. (2005), ciprofloxacin resistant *Campylobacter* in chicken emerged rapidly after antibiotic treatment and the resistant strain could persist in chicken for up to four weeks even without the presence of ciprofloxacin. However, according to the study of Scott et al. (2007b), phage resistant *Campylobacter* were subject to a severe cost in fitness, which made these resistant mutants become non-competitive in chickens in the absence of phage. Loc Carrillo et al. (2005) reported a similar result as phage resistant *Campylobacter* isolates recovered from phage treated chicken reverted to a phage sensitive phenotype when they were used to colonise chickens in the absence of phage. In this case, reducing the use of phage after the observation of resistant strains could prevent the spread of resistant mutants. It was also reported that phage resistant bacteria occurred at a significantly lower frequency compared with the occurrence of antibiotic resistant bacteria (Carlton, 1999). Moreover, the bacterial resistance issue could be

controlled by the application of a combination of various phage with different receptor specificity, and in addition to bacterial resistance limitation, this phage cocktail treatment could provide a broader host range (Scott et al., 2007b). Therefore, bacteriophage therapy is still a promising intervention for controlling *Campylobacter* in meat production.

1.8 Objectives

The main objective of this study is to analyse the interactions between *Campylobacter* and its bacteriophage, including: Prophage acquisition of *C. jejuni* in broiler flocks, competition between different *C. jejuni* strains within the gut environment and bacteria/phage coevolution; An investigation of how genetic mutations present in *Campylobacter* or phage can modify their interactions; Exploration of host phenotypes evident in the alternative carrier state life cycle for *Campylobacter* specific phage and analyses of the resulting genetic modifications and mechanisms associated with the coexistence of *Campylobacter* and what are otherwise virulent bacteriophage.

CHAPTER TWO

Materials and Methods

2.1 Culture and storage media preparation

All culture and storage media described in this thesis were prepared in reverse osmosis (RO) water followed by sterilization via autoclaving on liquid cycle at 121°C and 15 psi for twenty minutes. Prior to addition of supplements or selective antibiotics, agar media were tempered to 50°C in a water bath, and then poured into sterile Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet. Liquid media were stored at room temperature and agar media were stored at 4°C for a maximum of four weeks.

2.1.1 Blood agar

Blood agar base No. 2 (Oxoid, Basingstoke, UK) was prepared by suspending 40g in 1 litre of RO water. After autoclaving, a final concentration of 5% v/v defibrinated horse blood (TCS Biosciences, Buckingham, UK) was added to the liquid agar medium, cooled to 50°C, and then poured into Petri dishes.

2.1.2 Mueller-Hinton (MH) agar with blood

MH agar was first prepared by adding 21g Mueller-Hinton broth (Oxoid) to 1 litre of RO water with the addition of 15g of bacteriological agar No. 1 (Oxoid) to a final concentration of 1.5 w/v. After autoclaving, 5% v/v defibrinated horse blood was added to the liquid agar medium cooled to 50°C prior to dispensing into Petri dishes.

2.1.3 *Campylobacter* blood-free selective agar (mCCDA)

Campylobacter blood-free selective agar (Oxoid), was prepared by adding 45.5g into 1 litre of RO water with additional 0.8% w/v bacteriological agar No. 1 (Oxoid) added to give a final concentration of 2% w/v agar. After autoclaving, *Campylobacter* selective supplement (Prolab, Cheshire, UK) was added to a final concentration of 32mg l^{-1} cefoperazone and 10mg l^{-1} amphoteracin.

2.1.4 New Zealand casamino yeast medium (NZCYM) basal agar

NZCYM basal agar was prepared by adding 22g of NZCYM broth (ThermoFisher, Leicestershire, UK) to 1 litre of RO water with the addition of 15g of bacteriological agar No. 1 (Oxoid) to a final concentration of 1.5% w/v.

2.1.5 NZCYM overlay agar

NZCYM overlay agar was prepared by adding 22g of NZCYM broth (ThermoFisher) to 1 litre of RO water with the addition of 6g of bacteriological agar No. 1 (Oxoid) to a final concentration of 0.6% w/v. Prior to use, the agar was melted in a microwave and tempered to 50°C in a water bath.

2.1.6 *Campylobacter* motility agar

Motility agar was prepared by adding 21g of Mueller-Hinton broth (Oxoid) to 1 litre of RO water with the addition of 4g of bacteriological agar No. 1 (Oxoid) to a final concentration of 0.4% w/v.

2.1.7 Luria-Bertani (LB) agar

LB agar was prepared by adding 20g of LB broth (Lennox; Difco from ThermoFisher) to 1 litre of RO water. 15g of bacteriological agar No. 1 (Oxoid) was then added to a final concentration of 1.5% w/v.

2.1.8 Nutrient broth No. 2

Nutrient Broth No.2 was prepared by adding 25g of Nutrient broth No. 2 (Oxoid) to 1 litre of RO water. After autoclaving, Nutrient Broth No. 2 broth was stored at room temperature for a maximum of eight weeks.

2.1.9 MH broth

MH broth was prepared by adding 21g of Mueller-Hinton broth (Oxoid) to 1 litre of RO water. After autoclaving, MH broth was stored at room temperature for a maximum of eight weeks.

2.1.10 LB broth

LB broth was prepared by adding 20g of LB broth (ThermoFisher) to 1 litre of RO water. After autoclaving, LB broth was stored at room temperature for a maximum of eight weeks.

2.1.11 Maximum recovery diluent (MRD)

MRD was prepared by suspending 9.5g of maximum recovery diluent (Oxoid) in 1 litre of RO water. After autoclaving, MRD was stored at room temperature for a maximum of eight weeks.

2.1.12 Phosphate buffered saline (PBS)

PBS was prepared by dissolving one phosphate buffered saline tablet (ThermoFisher) in 200ml of RO water. After autoclaving, PBS buffer was stored at room temperature.

2.1.13 Salt magnesium (SM) buffer

SM buffer was prepared by dissolving sodium chloride (ThermoFisher) to a final concentration of 10mM; magnesium sulphate heptahydrate (ThermoFisher) to a final concentration of 8mM; tris (hydroxymethyl) aminomethane (Sigma-Aldrich, Dorset, UK) to a final concentration of 50mM and buffer pH was adjusted to 7.5. SM buffer was sterilised by autoclaving and then stored at room temperature for a maximum of eight weeks.

2.1.14 Bacterial storage medium

Bacterial storage medium was prepared by adding glycerol to nutrient broth No.2 (2.18) to a final concentration of 40% v/v. After autoclaving, the bacterial storage medium was stored at room temperature for a maximum of eight weeks.

2.1.15 S.O.C. medium

S.O.C. medium was prepared with ingredients obtained from Fisher, with the final medium containing 2% w/v tryptone, 0.5% w/v yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium sulphate and 20mM glucose. Once prepared, the medium was filter

sterilised through a 0.2µm filter (Sartorius, Goettingen, Germany) and stored at room temperature for a maximum of eight weeks.

2.1.16 Antibiotics

Selected antibiotics were prepared in appropriate solvent according to manufacturer's instructions. After preparation, antibiotics were aliquoted as 1ml stock solutions and stored at -20°C. Stock concentrations for antibiotics used in this thesis are listed in Table 2.1

Table 2.1 Antibiotics used in this thesis.

Antibiotic	Supplier	Solvent	Stock concentration	Working Concentration
Kanamycin	Sigma-Aldrich	RO water	50 mg ml^{-1}	50 µg ml^{-1}
Ampicillin	ThermoFisher	RO water	100 mg ml^{-1}	100 µg ml^{-1}
Chloramphenicol	Sigma-Aldrich	100% Ethanol	30 mg ml^{-1}	30 µg ml^{-1}

2.2 *Campylobacter*

2.2.1 Growth conditions

Selected *Campylobacter* were cultured, either on plates or in broths, in a rubber sealed 3.5 litre anaerobic jars (Oxoid). To obtain a microaerobic atmosphere the jar was firstly evacuated to -22 psi and then the gas pressure was restored with gas mix containing 5% v/v hydrogen, 85% v/v nitrogen and 10% v/v carbon dioxide (BOC limited, Surrey, UK). This provided a microaerobic atmosphere containing approximately 7% v/v

oxygen, 3% v/v hydrogen, 83% v/v nitrogen and 7% v/v carbon dioxide. Alternatively, *Campylobacter* was cultured in a Modular Atmospheric Controlled System (MACS) cabinet (Don Whitley Scientific, Shipley, UK) with a gas mixture of 5% v/v oxygen, 2% v/v hydrogen, 88% v/v nitrogen, 5% v/v carbon dioxide (BOC Limited). *Campylobacter* cells were normally cultured at 42°C unless otherwise stated.

2.2.2 Strains

Campylobacter strains used in this thesis were listed in Table 2.2

Table 2.2 *Campylobacter* strains used in this thesis.

***Campylobacter* species**	**Strain**	**Source**
C. jejuni	NCTC11168	NCTC
C. jejuni	HPC5	Laboratory stock
C. jejuni	PT14	Laboratory stock
C. jejuni	PT14CP30CS	Laboratory stock
C. jejuni	BIII C3	Laboratory stock
C. jejuni	TIF 2	Laboratory stock
C. jejuni	OK VIII C3	Laboratory stock
C. jejuni	CII C3	Laboratory stock
C. jejuni	OKIX C3	Laboratory stock
C. jejuni	OC I F3	Laboratory stock
C. jejuni	DV IV C1	Laboratory stock
C. jejuni	OK X C2	Laboratory stock
C. jejuni	FDIC2	Laboratory stock
C. jejuni	HP I F9	Laboratory stock
C. jejuni	OCII F1	Laboratory stock
C. jejuni	OCII F3	Laboratory stock
C. jejuni	OCII C10	Laboratory stock
C. coli	NCTC12668	NCTC

2.2.3 Storage conditions

For long term storage, overnight grown fresh *Campylobacter* cells were harvested from blood agar plates into 10ml nutrient broth No. 2 (2.1.8) using a sterile cotton swab. A 500µl aliquot of the bacterial suspension was mixed with 500µl of bacterial storage medium (2.1.14) in a cryovial and thoroughly mixed using a bench top vortexer. The cryovial was then stored at -80°C. When in use, frozen stock *Campylobacter* was quickly thawed and a small amount of cell suspension was transferred onto a blood agar plate using a 1µl inoculation loop. The plate was incubated at 42°C overnight under microaerobic conditions (2.2.1) to provide a stock of *Campylobacter* cells and could be stored at 4°C under microaerobic conditions for a maximum of four weeks.

2.2.4 *Campylobacter* Enumeration

Campylobacter enumeration was carried out using a modified Miles and Misra method (Miles et al., 1938). Briefly, a bacterial suspension was ten-fold serially diluted into PBS (2.1.12) with a maximum dilution factor of 10^{-8}. Triplicate aliquots of 10µl for each dilution were dispensed onto the surface of a mCCDA plate (2.1.3) and allowed to dry into the agar next to a Bunsen burner. Once dried, the mCCDA plate was incubated under microaerobic conditions (2.2.1) for 48 hours. After incubation, for the dilution that gave a colony number between 3 and 30, the number of colonies was counted and the CFU ml^{-1} was calculated using following equitation,

$$CFU\ ml^{-1} = (number\ of\ colonies \times dilution\ factor) / sample\ volume \quad (1)$$

2.2.5 Confirmatory tests for Campylobacter

2.2.5.1 Gram stain

Gram stain reagents were prepared according to manufacturer's instructions before use. Bacterial colonies to be tested were aseptically removed from a blood agar plate (2.1.1), and then emulsified into 10µl PBS (2.1.12) on a glass microscope slide (BDH Lab supplies, Leicestershire, UK) using a 1µl inoculation loop. The bacterial suspension was air-dried followed by heat fixing using a Bunsen burner. The slide was immersed in crystal violet solution (Prolab diagnostics, Bromborough, UK) for one minute and the excess solution was rinsed off with RO water. The slide was then placed in Lugol's iodine (Prolab) for thirty seconds and excess solution was rinsed off with RO water. After that, the slide was immersed in 70% ethanol (ThermoFisher) for one minute in order to decolourise the stained bacterial cells, followed by rinsing off excess ethanol with RO water. Finally, slide was immersed in carbol fuschin counterstaining solution (Prolab) for thirty seconds and then excess solution was rinsed off with RO water. The slide was air-dried and examined under immersion oil using a light microscope at 100x magnification.

2.2.5.2 Oxidase test

Oxidase test solution was prepared by dissolving N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Acros Organics) in RO water to give a final concentration of 0.01 g ml^{-1}, immediately prior to use. A sterile swab, which was already moistened with oxidase test solution, was used to harvest the cells from the surface of a blood agar plate (2.1.1). Within ten seconds, the development of a deep blue colour suggested positive and light blue or colourless indicated a negative result.

2.2.5.3 Catalase test

A small amount of overnight grown bacteria was harvested aseptically using a 1µl inoculation loop and transferred onto a microscope slide. A drop of 3% hydrogen peroxide was placed onto the bacteria using a pipette and observed for immediate bubble formation. Immediate bubble formation was indicative of a positive catalase reaction and no bubble formation was considered to be a catalase negative reaction.

2.2.5.4 Hippurate hydrolysis test

A final concentration of 5% w/v sodium hippurate (Sigma-Aldrich) was prepared with RO water. The solution was filter sterilised and aliquoted into 500µl portions and stored at -20°C until required. A 7% w/v 1,2,3-triketohydrindene monohydrate (ninhydrin reagent) solution was freshly prepared in RO water prior to the test. The hippurate hydrolysis test was carried out by addition of a heavy inoculum from an overnight *Campylobacter* culture to the thawed sodium hippurate solution. This

suspension was incubated at 37°C under microaerobic conditions (2.2.1) for two hours followed by the addition of 100µl of ninhydrin solution and another ten minutes' further incubation. After incubation, a solution with a deep violet colour was considered as positive for hippurate hydrolysis and a light purple colour indicated a negative reaction.

2.2.6 Motility assay

A semi-solid motility agar plate (2.1.6) was inoculated with *Campylobacter* using a sterilised pipette tip to stab the centre of the plate. The plates were incubated at 42°C under microaerobic conditions (2.2.1) for 48 hours and then diameter of the motility halo was measured.

2.2.7 Natural transformation of *Campylobacter*

Overnight grown *Campylobacter* was harvested into 10ml MH broth (2.1.9) using sterile cotton swab and an OD_{600} reading of the suspension was measured using a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer with MH broth as blank. The bacterial concentration was then estimated based on optical density using equation (2), which was previously stated by A. Scott (2006).

$$\textit{Campylobacter}\ \text{concentration (CFU ml}^{-1}) = (OD_{600} \times 2 \times 10^{9}) - 6 \times 10^{6} \quad (2)$$

Approximately 7.5 $\log_{10}$ CFU *Campylobacter* cells were dispensed onto the centre of a MH blood agar plate (2.1.2) and incubated at 42°C under

microaerobic conditions (2.2.1) for 6 hours. A 1µg aliquot of DNA were dispensed onto the surface of the bacteria halo and allowed to air dry. The plate was then incubated for another 18 hours at 42°C under microaerobic conditions. After incubation for three to five days, the transformants were selected using MH blood agar with appropriate antibiotics, and further confirmed by polymerase chain reaction (PCR).

2.2.8 *In vitro* growth curves

The *Campylobacter* strain was cultured on blood agar (2.1.1) at 42°C under microaerobic conditions (2.2.1) overnight. After incubation, *Campylobacter* cells were harvested into 10ml MH broth (2.1.9) using a sterile cotton swab. An OD_{600} reading of the bacterial suspension was measured using a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer with MH broth as blank. The *Campylobacter* concentration was estimated using equation (2), which was stated in section 2.2.7.

The bacterial suspension was diluted to contain approximately 7 $\log_{10}$ CFU ml^{-1} with MH broth and 100µl of diluted suspension was transferred into a sterile conical flask containing 100ml of MH broth with appropriate antibiotics to give a final concentration of 4 $\log_{10}$ CFU ml^{-1}. This flask was incubated at 42°C with 100rpm orbital shaking under microaerobic conditions for 24 hours. A 1ml aliquot was removed every two hours for 24 hours to determine the *Campylobacter* viable count (2.2.4). Where *Campylobacter* were cultured with the presence of bacteriophage,

Campylobacter was firstly grown for two hours prior to the addition of bacteriophage to give a multiplicity of infection (MOI) of 0.01. The time at which the bacteriophage was added was recorded as time zero. After the addition of bacteriophage, a 1ml aliquot was removed every two hours for 24 hours to determine the viable count of *Campylobacter* and titre of bacteriophage respectively. The bacteriophage titration was carried out by firstly filtering the suspension through a 0.2µm filter in order to remove bacterial cells and then the titre of bacteriophage was determined as described in section 2.4.3.

2.2.9 *Campylobacter* isolation from broiler chickens

Chickens were sacrificed according to Home Office Schedule I procedure with CO_2 asphyxiation followed by neck dislocation. The caecal contents were squeezed into a sterile plastic universal bottle and weighed. The contents were then diluted 1 in 10 w/v in MRD (2.1.11) and mixed by vigorously vortexing. This suspension was considered as 10^{-1} dilution and *Campylobacter* was then enumerated on mCCDA plates (2.2.4).

2.3 *Escherichia coli*

2.3.1 Growth conditions

The *E. coli* strains were cultured in either LB broth (2.1.10) or on LB agar plates (2.1.7) with appropriate antibiotics, at 37°C, overnight unless otherwise stated. For growth in broth, the cultures were shaken at 150rpm in an orbital shaker.

2.3.2 Strains

The *E. coli* strains used in this thesis are listed in Table 2.3.

Table 2.3 *E. coli* strains used in this thesis

***E. coli* strain**	**Genotype**	**Source**
TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
BL21DE3 Star	F-*omp*T *hsd*S_B (r_B-, m_B-) *galdcmrne*131 (DE3)	ThermoFisher
Origami 2 DE3 pLysS	*Δ(ara-leu)7697 ΔlacX74 ΔphoA* *Pvu*II *phoR araD139 ahpC galE galK* *rpsL* F′[lac^+ $lacI^q$ *pro*] (DE3) *gor522*::Tn*10 trxB* pLysS (Cam^R, Str^R, Tet^R)	MerckMilipore

2.3.3 Transformation of *E. coli*

2.3.3.1 Preparation of chemically competent E. coli

The *E. coli* strain was cultured in LB broth (2.1.10) supplemented with 20mM magnesium sulphate and incubated at 37°C with 150rpm shaking until the OD_{600} reached 0.5. The bacterial suspension was centrifuged in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g,* at 4°C, for 10 minutes and the supernatant was removed. The pellet was resuspended into 25ml of ice-cold 30mM calcium chloride and stored on ice for 10 minutes. This suspension was centrifuged in a Beckman JS-21 centrifuge

with JA10 rotor at 6,000 x *g* at 4°C for 5 minutes and the supernatant was discarded. The pellet was resuspended into 15ml of ice-cold 30mM calcium chloride and incubated on ice for one hour. This suspension was centrifuged in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g* at 4°C for 5 minutes and the supernatant was removed. The pellet was resuspended in 2ml of ice-cold 30mM calcium chloride. The cell suspension was then dispensed into 50µl aliquots and stored at -80°C until required.

2.3.3.2 Transformation of chemically competent E. coli

Prior to the transformation, a water bath was equilibrated to 42°C. One aliquot of chemically competent *E. coli* was thawed on ice for each reaction. LB plates (2.1.7) with appropriate antibiotics were pre-warmed at 37°C for 30 minutes. Approximately 1µg of DNA was transferred into the thawed chemically competent *E. coli* aliquot (2.3.3.1) and mixed by gently tapping. This tube was incubated on ice for 30 minutes. The cells were heat shocked at 42°C in the pre-warmed water bath without shaking for exactly 45 seconds and then immediately transferred to an ice bath. After which, 250µl of S.O.C. medium (2.1.15) was added to the cells and further incubated at 37°C with 150rpm shaking for one hour. After incubation, 10µl, 20µl, 50µl and 100µl of cell suspension were individually spread onto pre-warmed LB plates with appropriate antibiotics to ensure well-spaced colonies would be obtained. Plates were incubated at 37°C, without shaking, overnight and colonies were analysed using colony PCR (2.5.6.3). For TOPO pCR2.1 (ThermoFisher)

transformation, LB plates to which 40µl of 40 mg ml^{-1} 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; Sigma-Aldrich) had been applied were used. After overnight incubation, white or light blue colonies were assumed to contain inserts and were picked for PCR analysis.

2.3.3.3 Preparation of electroporation competent E. coli

The *E. coli* strain was cultured in 100ml of LB broth (2.1.10) with appropriate antibiotics at 37°C with 150rpm shaking for approximately 2 hours until the OD_{600} reached 0.6. The bacterial suspension was centrifuged in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g* at 4°C, for ten minutes and the supernatant was removed. The pellet was resuspended in 100ml of ice-cold RO water and the cell suspension was then centrifuged in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g* at 4°C for ten minutes. The supernatant was carefully removed and the pellet was resuspended in 100ml of ice-cold RO water and the cell suspension was centrifuged again in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g* at 4°C, for ten minutes. The supernatant was then discarded and the pellet was resuspended in 40ml of ice-cold RO water. The cell was pelleted by centrifuging in a Beckman JS-21 centrifuge with JA10 rotor at 6,000 x *g* at 4°C for ten minutes and the supernatant was discarded. The pellet was then resuspended in 1ml of 10% v/v ice-cold glycerol and transferred into a fresh microcentrifuge tube. The tube was centrifuged in a Beckman JS-21 centrifuge with JA18.1 rotor at 6,000 x *g* at 4°C, for 5 minutes. The pellet was finally resuspended in 1.5ml 10% v/v

ice-cold glycerol and dispensed into 50µl aliquots. Cell aliquots were stored in -80°C until required.

2.3.3.4 Electroporation transformation of E. coli

Prior to electroporation transformation, LB broth (2.1.10) was pre-warmed at 37°C in a water bath and an aliquot of electroporation competent *E. coli* (2.3.3.3) was thawed on ice. The cuvette holder was pre-chilled at -20°C and LB plates (2.1.7) with appropriate antibiotics were pre-warmed at 37°C for 30 minutes. Approximately 1µg of DNA was added into the electroporation competent *E. coli* cells (2.3.3.3) and gently mixed using a pipette tip. This mixed suspension was incubated on ice for 10 minutes and transferred into an electroporation cuvette (Bio-rad, Hemel Hempstead, UK). Electroporation transformation was carried out using a Bio-rad Gene Pulser set at 2.5kV, 25 micro-faradays and 100Ω resistance according to manufacturer's instructions. After transformation, the time constant was recorded and was usually 4.8 milliseconds. Transformed *E. coli* cells were immediately diluted with 200µl of pre-warmed LB broth. Aliquots of 10µl, 20µl, 50µl and 100µl of the cell suspension were individually spread onto pre-warmed LB plates, with appropriate antibiotics, to ensure well-spaced colonies would be obtained. Plates were incubated at 37°C overnight and colonies were analysed using colony PCR (2.5.6.3). For TOPO pCR2.1 transformation, LB plates to which 40µl of 40 mg ml^{-1} X-gal had been applied were used for blue /white screening. After overnight incubation, white or light blue

colonies were assumed to contain inserts and were picked for PCR analysis.

2.3.3.4 Transformation efficiency determination

For non-commercially prepared *E. coli* competent cells, the transformation efficiency was determined by transforming 1ng of highly purified supercoiled plasmid pUC19 (Merckmilipore, Watford, UK) into either chemically or electroporation competent cells as described in section 2.3.3.2 and 2.3.3.4. After incubation, colonies from the plate that gave rise to 30 to 300 colonies were counted and the transformation efficiency was determined using equation 3,

Transformation efficiency (CFU ml^{-1}) = Colonies number/ applied DNA amount (μg)/ dilution factor/ plated volume (ml) (3)

2.4 Bacteriophage

2.4.1 Strains

Bacteriophages used in this thesis are listed in Table 2.4 together with their host strain.

Table 2.4 Bacteriophages used in this thesis

Bacteriophage	Propagating Strain	Accession No.	Source
CP8	*C. jejuni* PT14	NC018861.1	Dr. P. Connerton
CP30A	*C. jejuni* PT14	KF148616.1	Dr. P. Connerton
CP30B	*C. jejuni* PT14		Dr. P. Connerton
CP30C	*C. jejuni* PT14		This thesis
CPX	*C. jejuni* PT14	NC106562.1	Dr. P. Connerton
Φ3	*C. jejuni* PT14	KT887559.1	NCTC
Φ15	*C. jejuni* PT14	NC015208.1	NCTC
CLP6	*C. jejuni* PT14		Dr. P. Connerton
CLP47	*C. jejuni* PT14		Dr. P. Connerton

2.4.2. Bacteriophage propagation

Propagation of bacteriophage was carried out using the full plate lysis method. Prior to the propagation, NZCYM overlay agar (2.1.5) was melted in a microwave, dispensed in 5ml aliquots in sterile universal bottles and tempered to 50°C in a water bath. Overnight grown *Campylobacter* host strain was harvested from a blood agar plate (2.1.1) into 10ml 10mM magnesium sulphate solution and the OD_{600} was determined using a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer with 10mM magnesium sulphate as the blank. The cell concentration of this bacterial suspension was adjusted to contain 8 $\log_{10}$ CFU ml^{-1} with 10mM magnesium sulphate solution and dispensed into 500µl aliquots. An aliquot of 100µl of bacteriophage at approximately 7 $\log_{10}$ PFU ml^{-1} was mixed with one aliquot of host cell suspension and left on bench for ten minutes. This *Campylobacter* / bacteriophage mixed suspension was then transferred into 5ml of melted NZCYM overlay agar and thoroughly mixed. This was poured onto the surface of a NZCYM agar plate (2.1.4)

and allowed to dry next to a Bunsen burner. The Plate was incubated at 42°C under microaerobic conditions, overnight. To harvest propagated bacteriophage, 5ml of SM buffer (2.1.13) was transferred onto the surface of the plate, and incubated at 4°C on a gyratory shaker with 60 cycles minute^{-1} overnight. After incubation, SM buffer containing bacteriophage was recovered, and bacterial cells and cell debris were removed by filtering the sample through a 0.2µm filter. Finally, the filtered bacteriophage sample was stored at 4°C in sterile plastic universals.

2.4.3 Efficiency of plaquing (EOP)

Bacteriophages were enumerated by determining their efficiency of plaquing against their host strain. Prior to the propagation, NZCYM overlay agar (2.1.5) was melted in a microwave, dispensed in 5ml aliquots in sterile universal bottles and tempered to 50°C in a water bath. Overnight growth from the *Campylobacter* host strain was harvested from a blood agar plate (2.1.1) into 10ml 10mM magnesium sulphate solution and adjusted to 8 $\log_{10}$ CFU ml^{-1} with 10mM magnesium sulphate solution as described in 2.4.2. An aliquot of 500µl of bacterial suspension was transferred into the melted NZCYM overlay agar and thoroughly mixed. This was poured onto the surface of NZCYM agar plate (2.1.4) to form a bacterial lawn and allowed to dry next to a Bunsen burner. The bacteriophage suspension was ten-fold serial diluted with SM buffer (2.1.13) down to a dilution factor of 10^{-8}, and 10µl of each dilution was dispensed onto the bacterial lawn, in triplicate and allowed to dry next to a Bunsen burner. The plates were incubated at 42°C under microaerobic

conditions (2.2.1) overnight and the number of plaques were counted for dilutions that gave rise to between 3 and 30 plaques. Finally, the average plaque number was used to calculate the efficiency of plaquing using the equation 4.

PFU ml^{-1} = number of plaques/ dilution factor/ volume of spotted sample (4)

2.4.4 Bacteriophage typing

Bacteriophage typing was carried out in order to determine if a bacteriophage was able to replicate on a particular *Campylobacter* strain. NZCYM overlay agar (2.1.5) and the *Campylobacter* host strain were prepared as described in 2.4.2., and bacterial lawn plates prepared as described in 2.4.3. The bacteriophages were diluted to contain 7 log_{10} PFU ml^{-1} and 10µl of these dilutions were dispensed onto the surface of the bacterial lawn and allowed to dry next to a Bunsen burner. The plates were incubated at 42°C under microaerobic conditions (2.2.1) overnight. After incubation, bacteriophages that gave clear plaques were considered as able to replicate on the tested *Campylobacter* strain.

2.4.5 Bacteriophage concentration

Bacteriophages were concentrated using a modification of the method of Sambrook et al. (1989). Briefly, 20ml of bacteriophage sample was firstly filtered through a 0.2µm filter and centrifuged in a Beckman JS-21 centrifuge at 4°C with 40,000 x *g* using a JA20 rotor for two hours. After centrifugation, the supernatant was discarded carefully, and the pellet

was resuspended in 2ml of SM buffer (2.1.13) using vigorous vortexing. Concentrated bacteriophage samples were stored at 4°C in a sterile plastic universal bottles.

2.5 Nucleic acid isolation and analysis

2.5.1 *Campylobacter* genomic DNA isolation

2.5.1.1 GenElute Bacterial Genomic DNA kit

Genomic DNA was isolated from cells using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) according to manufacturer's instructions. Overnight grown *Campylobacter* cells were harvested from a blood agar plate (2.1.1) into 1.5ml PBS (2.1.12) using a sterile cotton swab. The bacteria suspension was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute. The supernatant was discarded and the pellet was resuspended into 180µl of lysis solution T supplemented with 20µl of 20mg ml^{-1} DNase-free RNase. Then, 20µl of 20mg ml^{-1} proteinase K (Sigma-Aldrich) was added and this suspension was incubated at 55°C for 30 minutes. After incubation, 200µl of lysis solution C was added to this suspension and thoroughly mixed by vortexing for 15 seconds followed by incubating at 55°C for 10 minutes. During incubation, the column was prepared by adding 500µl of column preparation solution to the column, centrifuging the column at 12,000 x *g* in a Biofuge Pico bench top centrifuge and discarding the flow through. After incubation, 200µl of 100% ethanol was added into the cell mix and thoroughly mixed by vortexing for 10 seconds. This suspension was then

loaded into the column via a wide-bore pipette and the column was then centrifuged in a Biofuge Pico bench top centrifuge at 6,500 x *g* for one minute. The flow through was discarded and 500µl of wash solution was transferred into the column. The column was then centrifuged in a Biofuge Pico bench top centrifuge at 6,500 x *g* for one minute and the flow through was discarded. Another 500µl of wash solution was added into the column and the column was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for three minutes. The collection tube was discarded and a new tube was replaced. An aliquot of 200µl of elution solution was loaded into the column and left for one minute in order to elute the DNA. The column was then centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute and the flow through was collected. Concentration of isolated DNA was determined using a Nano-drop spectrophotometer (ThermoFisher). Finally, isolated genomic DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.1.2 Wizard Genomic DNA purification Kit

Genomic DNA from *Campylobacter* was isolated using Wizard Genomic DNA purification kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Briefly, overnight cultured *Campylobacter* cells were harvested from a blood agar plate (2.1.1) into 1.5ml PBS (2.1.12) using a sterile cotton swab. This bacterial suspension was centrifuged in a Biofuge Pico bench top centrifuge at 13,000 x *g* for two minutes. The supernatant was carefully removed and the pellet was resuspended in

600µl of Nuclei Lysis Solution by gently pipetting. This was incubated at 80°C for 5 minutes to lyse bacterial cells and then cooled to room temperature. Then, 3µl of RNase Solution was added and mixed by tube inversion for 5 times. The tube was incubated at 37°C for one hour and then cooled to room temperature. An aliquot of 200µl of Protein Precipitation Solution was added to the cell lysate and mixed by vortexing for 20 seconds. This was further incubated on ice for 5 minutes and centrifuged in a Beckman JS-21 centrifuge at 4°C with 13,000 x *g* for 3 minutes using JA18.1 rotor. The supernatant containing DNA was transferred into a fresh microcentrifuge tube and 600µl of isopropanol was added and mixed by gentle inversion. The tube was centrifuged in a Beckman JS-21 centrifuge at 4°C with 13,000 x *g* for 3 minutes using JA18.1 rotor and the supernatant was carefully removed. To wash the pellet, 600µl of 70% v/v ethanol was added and gently mixed by inversion for several times. The tube was further centrifuged in a Beckman JS-21 centrifuge at 4°C with 13,000 x *g* for 3 minutes using JA18.1 rotor. The ethanol was removed and the pellet was allowed to air dry for approximately ten minutes. Finally, 100µl of DNA Rehydration Solution was added to the pellet and the DNA rehydrated at 4°C overnight. Isolated genomic DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.2 *E. coli* plasmid DNA isolation

2.5.2.1 Mini preparation

Small amounts of plasmid DNA from *E. coli* cells was prepared using a GeneElute plasmid miniprep kit (Sigma-Aldrich). Briefly, *E. coli* cells were grown overnight in 10ml of LB broth (2.1.10) with appropriate antibiotics at 37°C, with 150rpm shaking and after incubation, 3ml of the culture was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute. The supernatant was discarded and the bacterial pellet was completely resuspended in 200µl of resuspension solution by pipetting up and down until homogeneous. The *E. coli* cells were then lysed by adding 200µl of lysis solution to the bacterial suspension and immediately mixed by gently inverting the tube until the mixed suspension became clear and viscous. After lysing *E. coli* cells, cell debris were precipitated by addition of 350µl of neutralization/ binding solution. This was mixed by gently inverting the tube 4 – 6 times and the cells debris were pelleted by centrifuging in a Biofuge Pico bench top centrifuge at 12,000 x *g* for ten minutes. During centrifugation, the column was prepared by adding 500µl of column preparation solution, then centrifuging the column in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute and discarding the flow through. The supernatant from the *E. coli* cell suspension was then loaded into the prepared column. The column was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute and the flow through was discarded. An aliquot of 750µl of the wash solution was transferred to the

column and the column was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute and the flow through was discarded. The empty column was then centrifuged again in a Biofuge Pico bench top centrifuge at 12,000 x *g* for 2 minutes with lid open in order to remove excess ethanol contained in wash solution, and the collection tube was discarded. The column was inserted into a fresh collection tube and 100µl of molecular biology reagent water was loaded to the column. This tube was centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for one minute and the plasmid DNA flow through was collected. Concentration of isolated plasmid DNA was determined using a Nano-drop spectrophotometer. Finally, isolated plasmid DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.2.2 Midi preparation

Larger amounts of plasmid DNA from *E. coli* cells were prepared using, a plasmid midi kit (Qiagen). Briefly, *E. coli* cells were cultured in 50ml of LB broth (2.1.10) with appropriate antibiotics at 37°C, with 150rpm shaking, overnight. After incubation, *E. coli* cells were harvested by centrifuging in a Beckman JS-21 centrifuge at 6,000 x *g* for 15 minutes, at 4°C, using JA20 rotor. The supernatant was carefully removed and the pellet was resuspended in 4ml of buffer P1, supplemented with DNase-free RNase, at a final concentration of 2mg ml^{-1}. An aliquot of 4ml of buffer P2 was then added to the bacterial suspension and mixed thoroughly by vigorously inverting the tube 4 - 6 times. This was incubated at room temperature

for five minutes. An aliquot of 4ml of chilled buffer P3 was transferred to the tube and immediately mixed thoroughly by vigorous inversion 4 – 6 times. The tube was then incubated on ice for 15 minutes and centrifuged in a Beckman JS-21 centrifuge at 13,000 x *g* for 30 minutes at 4°C using JA20 rotor. The supernatant containing plasmid DNA was then collected. Concentration of isolated plasmid DNA was determined using a Nano-drop spectrophotometer. Finally, isolated plasmid DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.3 Bacteriophage DNA isolation

2.5.3.1 Phage DNA isolation kit

DNAs from bacteriophages were isolated using a phage DNA isolation kit (Norgen biotek corp, Thorold, Canada). Briefly, 200µl of bacteriophage sample was transferred into a sterile microcentrifuge tube at a titre of approximately 9 $\log_{10}$ PFU ml^{-1}. An aliquot of 500µl of lysis buffer was added and the tube was vigorously vortexed for ten seconds. The tube was then incubated at 65°C for 15 minutes and the lysate was occasionally mixed by inverting the tube during incubation. After incubation, 320µl of isopropanol was added to the lysate and mixed by brief vortexing. A spin column was attached to the collection tube and up to 650µl of the lysate was loaded onto the column. The column was centrifuged in a Biofuge Pico bench top centrifuge at 6,000 x *g* for one minute and the flow through was discarded. The column was washed by adding 400µl of wash buffer, centrifuging the column in a Biofuge Pico bench top centrifuge at 6,000 x *g* for one minute and discarding the flow

through, and the wash step was repeated three times. The empty column was further centrifuged in a Biofuge Pico bench top centrifuge at 12,000 x *g* for two minutes in order to dry the resin. The collection tube was discarded and a new collection tube was replaced. An aliquot of 75µl of elution buffer was added to the column and the column was centrifuged in a Biofuge Pico bench top centrifuge at 6,000 x *g* for one minute and the flow through containing bacteriophage DNA was collected. The concentration of bacteriophage DNA was determined using a Nano-drop spectrophotometer. Bacteriophage DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.3.2 Bacteriophage DNA isolation – PEG precipitation

Larger amounts of bacteriophage DNA were prepared using a polyethylene glycol (PEG) precipitation method. A precipitant solution was prepared with 30% w/v PEG-8000 (Sigma-Aldrich) and 3M sodium chloride. An aliquot of 10ml of bacteriophage lysate was transferred into a sterile centrifuge tube. To this 5µl of 10µg ml^{-1} RNase- free DNase was added and incubated at 37°C for 30 minutes. After incubation, precipitant solution (2.1.17) was added to bacteriophage lysate at a ratio of 1:2 in order to obtain a final concentration of 10% w/v PEG-8000 and 1M sodium chloride. This was mixed by gentle inversion and incubated at 4°C overnight. The precipitated lysate was centrifuged in a Beckman JS-21 centrifuge at 4°C with 10,000 x *g* for ten minutes using JA20 rotor and the supernatant was carefully removed. The pellet was resuspended in 500µl of 5mM magnesium sulphate by pipetting up and down, and transferred

into a sterile microcentrifuge tube. The tube was centrifuged briefly in a Biofuge Pico bench top centrifuge in order to pellet any insoluble particles and the supernatant was transferred into a fresh microcentrifuge tube. Proteinase K (Sigma-Aldrich) was added to the supernatant to a final concentration of 100µg ml^{-1}. This was incubated at 50°C for 30 minutes and then allowed to cool to room temperature. To purify the bacteriophage DNA, a wizard plus minipreps DNA purification system (Promega, Southampton, UK) was used. Briefly, 1ml of minipreps DNA purification resin was added to the bacteriophage DNA suspension and mixed by gentle inversion. The plunger was removed from a sterile 3ml syringe and the syringe barrel was attached to the minicolumn from the wizard plus minipreps DNA purification system. Resin/ lysate mix was pipetted into the syringe and the plunger was attached to the syringe. Resin/ lysate mix was slowly pushed through the minicolumn and the flow through was discarded. After this, the minicolumn was firstly detached from the syringe, and the plunger was removed. To wash the column, the syringe barrel was reattached to the minicolumn, and 2ml of 80% v/v isopropanol was loaded into the syringe. The syringe barrel was then attached and isopropanol was pushed through the minicolumn. The minicolumn was detached from the syringe, and placed on top of an opened sterile microcentrifuge tube. The column was then dried by centrifuging in a Biofuge Pico bench top centrifuge at room temperature with 13,000 x *g* for two minutes. The microcentrifuge tube was discarded and the minicolumn was placed on a fresh microcentrifuge tube. An aliquot of 100µl of RO water, which has been heated to 80°C, was loaded

onto the column, and the column was immediately centrifuged at room temperature with 13,000 x *g* for one minute. The flow through containing bacteriophage DNA was collected. Concentration of isolated bacteriophage DNA was determined using a Nano-drop spectrophotometer. Finally, isolated bacteriophage DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.4 DNA concentration

2.5.4.1 Vacuum centrifuge

Vacuum centrifugation was used to concentrate DNA samples. Briefly, microcentrifuge tubes containing DNA sample were loaded into the Jouan RC 1022 vacuum concentrator (ThermoFisher) and centrifuged in vacuum at 50°C at 10,000 x *g*. The concentration of DNA was measured every 15 minutes using a Nano-drop spectrophotometer until the desired concentration was reached. Finally, concentrated DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.4.2 Ethanol precipitation

A 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of ice cold 100% ethanol were added to the DNA sample and mixed well by pipetting up and down. The sample was incubated at -20°C for two hours and then centrifuged in a Beckman JS-21 centrifuge at 4°C with 13,000 x *g* for 15 minutes using JA18.1 rotor. After centrifugation, the supernatant was carefully removed and 1ml of 70% v/v ethanol was added to the pellet. This was briefly centrifuged in a Biofuge Pico bench top centrifuge and

the supernatant was carefully removed. The pellet was air dried and then resuspended in appropriate volume of RO water. The concentration of DNA was determined using a Nano-drop spectrophotometer. Finally, concentrated DNA was stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.5 RNA isolation

2.5.5.1 RNase removal

Before carrying out any RNA related work, the ubiquitous existing RNase needed to be completely removed from the working environment and all buffers. Sterile disposable plasticware were used whenever possible and were considered to be RNase-free. Glassware used for RNA work were cleaned by diethyl pyrocarbonate (DEPC; Sigma-Aldrich). Briefly, glassware to be cleaned was fully filled with 0.1% v/v DEPC in RO water, shaken vigorously, allowed to stand overnight at 37°C, and then autoclaved on a liquid cycle at 121°C and 15 psi, for twenty minutes, to eliminate residual DEPC. After autoclaving, DEPC treated RO water and glassware were considered as RNase-free. RNase from buffer solutions was removed using 0.1% v/v DEPC and autoclaving as described above. For those buffer solutions that are sensitive to DEPC treatment, such as Tris buffer, solutions were prepared using DEPC treated RO water and glassware to avoid the contamination from RNase. Elimination of RNase from the work surface or any other glass or plastic surfaces was achieved by wiping the surface to be cleaned with 1% w/v sodium dodecyl sulphate (SDS; ThermoFisher), followed by wiping with DEPC treated RO

water, absolute ethanol, 3% v/v hydrogen peroxide and finally with DEPC treated RO water. Alternatively, RNase ZAP (Sigma-Aldrich) spray was used for complete removal of RNase contamination from these surfaces.

2.5.5.2 RNA Isolation with TRIzol reagent

Overnight growth of *Campylobacter* was harvested from a blood agar plate (2.1.1) into 5ml PBS (2.1.12) using a sterile cotton swab and centrifuged in a Beckman JS-21 centrifuge at 4°C with 10,000 x *g* for 15 minutes using JA20 rotor. The supernatant was carefully removed and the pellet was resuspended in 1ml of PBS then transferred to a fresh microcentrifuge tube and centrifuged using an Eppendorf 5415R benchtop centrifuge (Fisher) at 4°C with 10,000 x g for 15 minutes. This was repeated three times with PBS to remove all traces of growth medium. The total RNA was then isolated using a Trizol Max Bacterial Isolation kit (Invitrogen, Paisley, UK). After centrifugation, the supernatant was discarded and the pellet was resuspended in 200µl of max bacterial enhancement reagent preheated to 95°C, by pipetting up and down. This suspension was incubated at 95°C for 4 minutes, and 1ml of TRIzol reagent was added to the lysate and mixed by inversion. The tube was then incubated at room temperature for five minutes and 0.2ml ice cold chloroform (Sigma-Aldrich) was added. This was mixed by vigorously shaking the tube for 15 seconds and further incubated at room temperature for three minutes. The tube was then centrifuged in a Beckman JS-21 centrifuge at 4°C with 12,000 x *g* for 15 minutes using JA18.1 rotor. After centrifugation, the mixture was separated into three

phases, a red phenol-chloroform lower phase, an interphase and a colourless upper aqueous phase containing bacterial RNA. The upper aqueous phase was carefully transferred to a fresh microcentrifuge tube and 0.5ml of ice cold isopropanol was added in order to precipitate RNA. The tube was incubated at room temperature for ten minutes and then centrifuged in a Beckman JS-21 centrifuge at 4°C with 15,000 x *g* for ten minutes using rotor JA18.1. The supernatant was carefully removed and the pellet was resuspended in 1ml 75% v/v ethanol and mixed well by vortexing. This was further centrifuged in a Beckman JS-21 centrifuge at 4°C with 7,500 x *g* for five minutes using rotor JA18.1 and the supernatant was discarded. The pellet was air dried and resuspended in 50µl of RNase-free water by pipetting up and down. The concentration of RNA was measured using a Nano-drop spectrophotometer. The quality of RNA was determined by running an RNA agarose gel. Isolated RNA samples were stored at -80°C to avoid degradation.

2.5.5.3 Phase separation using hot phenol

Larger amounts of RNA from *Campylobacter* were prepared using the hot phenol method. Prior to the experiment, phenol (Sigma-Aldrich) and lysis buffer, which containing 100mM Tris-HCl (pH 7.5), 40mM Na_2EDTA, 200mm NaCl, 0.5% w/v SDS, was heated to 60°C. Overnight growth of *Campylobacter* was harvested from a blood agar plate (2.1.1) using a sterile cotton swab to 3ml of RNase-free water and the OD_{540} was adjusted to approximately 0.5 using RNase-free water. An aliquot of 1ml of this bacterial suspension was transferred to a 2ml microcentrifuge tube

containing 200µl of stop mix solution (95% v/v ethanol, 5% v/v phenol) and mixed by gentle inversion. This was centrifuged in a Biofuge Pico bench top centrifuge at room temperature with 3,500 x *g* for three minutes. The supernatant was carefully discarded and the pellet was resuspended into 500µl of pre-heated lysis buffer followed by incubating at 60°C for two minutes. After incubation, 500µl of pre-heated phenol was added to the suspension and mixed by vigorous vortexing for ten seconds. This was incubated at 60°C for one hour with occasional vigorous vortexing and placed on ice for ten minutes. The tube was then centrifuged in a Beckman JS-21 centrifuge at 13,000 x *g* for ten minutes at 4°C using JA18.1 rotor. The upper phase was carefully transferred into a fresh microcentrifuge tube and equal amount of pre-heated phenol was added. This was incubated once more at 60°C for one hour with occasional vigorous vortexing and placed on ice for ten minutes. The tube was then centrifuged in a Beckman JS-21 centrifuge at 13,000 x *g* for ten minutes at 4°C using JA18.1 rotor. The upper phase was carefully transferred into a fresh microcentrifuge tube and equal amount of chisam (Sigma-Aldrich), containing chloroform and isoamyl alcohol at a ratio of 24:1, was added. The upper phase containing bacterial RNA and the chisam was mixed by vortexing and the tube was centrifuged in a Biofuge Pico bench top centrifuge at room temperature with 13,000 x *g* for five minutes. The upper phase was transferred into a fresh microcentrifuge tube and the RNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol, and incubating at -20°C overnight. After incubation, the tube was centrifuged in a Beckman JS-21

centrifuge at 13,000 x *g* for 30 minutes at 4°C using JA18.1 rotor. The supernatant was carefully removed and 1ml of 70% v/v ethanol was added to the pellet. The tube was then centrifuged in a Beckman JS-21 centrifuge at 13,000 x *g* for 20 minutes at 4°C using JA18.1 rotor. The supernatant was carefully removed and the pellet was air dried. An aliquot of 100µl of RNase-free water was added to suspend the pellet. The concentration of isolated RNA was measured using a Nano-drop spectrophotometer. The quality of the RNA was determined by running an RNA agarose gel. Isolated RNA samples were stored at -80°C to avoid degradation.

2.5.6 cDNA synthesis

Selected *Campylobacter* strain were grown on blood agar plates (2.1.1) containing appropriate antibiotics at 42°C under microaerobic conditions (2.2.1) overnight. Bacteria were then harvested into MH broth (2.1.9) using a sterile cotton swab and the total RNA was isolated with Trizol reagent (2.5.5.2). First strand cDNA was then synthesised using a protocol modified from Untergasser (2008). Firstly, 20µl of enzyme mix containing 8µl of 5x First Strand Buffer (ThermoFisher), 4µl of Dithiothreitol (DTT; ThermoFisher), 2µl of dNTPs (10mM each; ThermoFisher), 1µl of SUPERase inhibitor (ThermoFisher), 1µl of SuperScript II (ThermoFisher) and 4µl of water was prepared and stored at room temperature. Annealing mix was prepared in 20µl volume containing 1µg of total RNA, 25ng μl^{-1} random hexamers and the primers were annealed in a BIOER XP thermal cycler (Alpha Laboratories, Hampshire, UK) at 70°C for ten

minutes followed by 25°C for ten minutes. 20µl of enzyme mix was then added into the reaction and the cDNA was synthesised in a thermocycler at 25°C for ten minutes, 37°C for forty-five minutes, 42°C for forty-five minutes and 70°C for fifteen minutes. The cDNA sample was diluted one in five and stored at -20°C until required.

2.5.6 Polymerase Chain Reaction (PCR)

2.5.6.1 PCR Primers

The PCR Primers used in this thesis are listed in Table 2.5. All primers were designed ideally with a length of 20–30 base pair, melting temperature in the range from 56 to 64°C, melting temperature difference within forward and reverse primer did not exceed 4°C, GC content between 40 and 60%, and terminated with a guanine or cytosine residue at the 3' end. Possibilities of formation of stable primer secondary structures, hairpins, self-dimer and cross dimer were also considered by calculating the Gibbs free energy (ΔG) of these structures via oligo analyser (Integrated DNA Technologies). Unless otherwise stated, primers within this thesis were all designed based on *C. jejuni* PT14. Melting temperature for each primer was calculated using equation below,

$$T_m = 4°C\ (G+C) + 2°C\ (A+T) \quad (5)$$

Primers applied in this thesis were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Table 2.5 List of Primers

Primer Name	Primer Sequence (5' to 3')	T_m (°C)	Amplicon size (bp)
flhF-KO-F	CGTCTCACATGGTGATAAGTGGTGTGAGGTG	60	1466
flhF-KO-R	GCCGGCTCATCTGGCACTTCTTGTCC	60	
flhF- KI- F	CGTCTCACATG/GTGATAAGTGGTGTGAGGTG	60	1747
flhF- KI- R	GCCGGC/GCCAAGCCAATATCAGCATC	60	
KanR- F	TCTAGA/GCAAGGAACAGTGAATTGGAG	62	943
KanR- R	TCTAGA/GTGCGTAAGAACATAGAAAGG	60	
flhF- His- F	AACATATG/GGACAACTTATACATACTT	50	1472
flhF- His- R	AAGGATCC/ATTGCGAAGTTTATTTGCTTGG	60	
CRISPR- F	TCGCAACCTCCTTTTAGTGGA	62	422
CRISPR- R	ATTGTAACCCCGCAGAGTCC	62	
Pseudo gene 0046- F	CTATTGCCATAGTAGCTCTTAG	62	1407 (11168) /1289 (PT14)
Pseudo gene 0046- R	CCAACAGCTATCATCACTAC	58	
L-lactate permease- F	CTTAAAGGCTATCAAGCAGG	58	643
L-lactate permease- R	CAGGTAATTCCGCTCCTAG	58	

CP30-A/BC-F	TGTGTTAATTGTTTTTCATTTTC	56	881 (CP30-A)/ 948 (CP30-BC)
CP30-A/BC-R	TAAGTGCTTTAGTAGCTGCTC	60	
CP30-AB/C-F	GTGTCTTTATGTTCTTTCGG	56	1493 (CP30-AB)/ 717 (CP30-C)
CP30-AB/C-R	CAAACATCATCTATCGTAGC	56	
RT PCR flhF- F	CCGTTGAAGATACAGAACAAAT	60	131
RT PCR flhF- R	GGCTACCATAACCTCATAAAG	60	
RT PCR pkg- F	TAGACGCATAAGATCAGCTATTCC	68	144
RT PCR pkg- R	AAGTCTAGCAAGACGCTTAGC	62	
RT PCR flaA- F	CAGCTGAGTCACAAATCCGT	60	96
RT PCR flaA- R	CCATGGCATAAGAGCCACTT	60	
RT PCR flaB- F	GTTAAAGCAGCAGAATCAACCA	62	105
RT PCR flaB- R	ACTCATAGCATAAGAACCTGACTG	68	
RT PCR flgD- F	AATGGCTGGACAAGAAGTTCC	62	125
RT PCR flgD- R	CTCCATCGCTTGAACCACCA	62	
RT PCR motB- F	ACGTATGGCTGAGCTAACAACA	64	115
RT PCR motB- R	CGCTCTTTGGTAGGCGAGTT	62	
RT PCR pflA- F	CCAAGCATGCGTGATCAACAA	62	146
RT PCR pflA- R	ACATCAGCACGCTGAGCTAAA	62	
RT PCR flhG- F	AGCGCGAATCTAGCCAATGT	60	146
RT PCR flhG- R	AAGGAGCATTCTCCGCGTAA	60	
RT PCR flaA-11168- F	ACAAAATCAAGTTACATCAACTA	58	146
RT PCR flaA-11168- R	AAGAGCCGCTTTGAGCTAAG	60	
RT PCR flaB-11168- F	GCAAAATCAACTTCAAGTTACTA	60	146
RT PCR flaB-11168- R	AAGAACCTGATTGCGCAAGG	60	
RT PCR pflA-11168- F	TGCCTTATGTTGGAGCTTTGG	62	133
RT PCR pflA-11168- R	TGTGCATCAATCACCACTTGA	60	

2.5.6.2 PCR running conditions

PCR reactions were carried out using DreamTaq Green 2x PCR Master Mix (ThermoFisher) in a BIOER XP thermal cycler. Master Mix containing 2U of DreamTaq DNA polymerase, 2x DreamTaq Green buffer, 0.4mM of dNTP each and 4mM of magnesium chloride. PCR samples were prepared by mixing 12.5µl of PCR master mix with 30ng of template DNA, 10pmol of forward and reverse primer in a final volume of 25µl. The PCR reaction was carried out with an initial denaturation at 95°C for three minutes; 35 cycles of PCR amplification including denaturation at 95°C for 30 seconds, annealing at 56-60°C for 30 seconds, extension at 72°C for 1-3 minutes; and the reaction ended with a final extension at 72°C for 5 minutes. The extension time during PCR amplification cycles were dependent on the size of the PCR product, with 30 seconds per kilo base pair of PCR product. Amplified PCR product was verified by running an agarose gel electrophoresis (2.5.7.1) and then stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.6.3 Colony PCR

Colony PCR was applied to determine the presence or absence of insert DNA in plasmid constructs during gene cloning. PCR buffer was prepared by mixing 12.5µl of DreamTaq Green PCR Master Mix (2x) with 10pmol of forward and reverse primer in a final volume of 25µl. A small amount of overnight grown bacterial colony was transferred into the PCR buffer using a sterile pipette tip. The PCR reaction was carried out with an initial denaturation at 95°C for 15 minutes; 35 cycles of PCR amplification

including denaturation at 95°C for 30 seconds, annealing at 56-60°C for 30 seconds, extension at 72°C for 1-3 minutes; and the reaction ended with a final extension at 72°C for 5 minutes.

2.5.6.4 Digoxigenin labelling by PCR

The PCR method could also be used to create short probes for northern and southern blots. Specific primers were designed to amplify the probe sequence with reverse primer 5' end DIG labelled. The DIG pre-labelled primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The PCR reaction were carried out under conditions as described in 2.5.6.2 to synthesise negative strand 5' end DIG labelled probe.

2.5.6.5 Cleaning PCR products

A Wizard SV Gel and PCR clean up system (Promega) was used remove excess nucleotides and primers. Briefly, an equal volume of membrane binding solution was added to the PCR product and this mixture was transferred into the SV minicolumn. The column was incubated at room temperature for one minute and then centrifuged in a Biofuge Pico bench top centrifuge at 13,000 x *g* for one minute. The flow through was discarded and 700µl of membrane wash solution was loaded onto the SV minicolumn. The column was centrifuged in a Biofuge Pico bench top centrifuge at 13,000 x *g* for one minute and the flow through was discarded. Another 500µl of membrane wash solution was added to the column and the column was centrifuged in a Biofuge Pico bench top

centrifuge at 13,000 x *g* for five minutes. The flow through was discarded and the column was centrifuged in a Biofuge Pico bench top centrifuge at 13,000 x *g* with the lid open to evaporate any residual ethanol contained in the membrane wash solution. The SV minicolumn was then transferred into a new 1.5ml microcentrifuge tube and 50µl of nuclease free water was applied to the column. The column was allowed to stand at room temperature for one minute followed by centrifuging in a Biofuge Pico bench top centrifuge at 13,000 x *g* for one minute. The flow through containing pure PCR product was collected. The DNA concentration of the purified PCR product was measured using a Nano-drop spectrophotometer. The quality of the purified PCR product was determined by running an agarose gel electrophoresis. Purified PCR products were stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.6.6 Addition of 3' A-overhang using PCR

A 3' A-overhang could be added to blunt-ended DNA fragments using PCR and a Platinum Taq DNA Polymerase High Fidelity enzyme (ThermoFisher). Briefly, a PCR sample was prepared by mixing approximately 1µg of blunt-ended DNA fragment with 1 unit of Platinum Taq polymerase, 1x High Fidelity PCR Buffer, 1.5mM magnesium chloride and 0.2mM dATP in a final volume of 20µl. This was incubated at 72°C for 20 minutes and used quickly as the 3' -overhang may be lost during storage.

2.5.6.7 Quantitative reverse transcription PCR (RT-qPCR)

RT-qPCR samples were prepared using PowerUp SYBR Green Master Mix (ThermoFisher) according to manufacturer's instructions. Briefly, samples were prepared in a 96 well microtitre plate by mixing 10µl of PowerUp SYBR Green Master Mix with 10pmol of gene specific forward, reverse primer, and 2µl of cDNA template (2.5.6) in a total volume of 20µl. The RT- PCR was carried out using a Light Cycler 480 instrument (Roche) with settings of pre-incubation at 95°C for 6 minutes; followed by 40 cycles of amplification including incubation at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for one minute; and then one cycle of melting curve including incubation at 45°C for 5 seconds and 65°C for one minute; and the run was finished by cooling at 40°C for 30 seconds. The resulting data was analysed using LightCycler 480 software to determine the cycle threshold value (Ct value) and relative expression value was then calculated based on it.

2.5.7 Gel electrophoresis

2.5.7.1 Agarose gel electrophoresis

DNA in this thesis was routinely analysed by agarose gel electrophoresis (0.8% agarose) unless otherwise stated. To prepare an agarose gel, 0.8% w/v agarose (Invitrogen) was firstly added to 50ml of 1x TAE buffer (40 mM tris-acetate adjusted with glacial acetic acid to pH 8, 1 mM disodium ethylenediaminetetraacetic acid) in a 250ml sterile conical flask. This was melted completely in a microwave and allowed to cool to approximately 50°C. Ethidium bromide (Fisher) was added at a final concentration of

0.4µg ml^{-1} and mixed by gently swirling the flask. This solution was poured into a pre-assembled casting tray and the comb was then inserted. The gel was allowed to set at room temperature for approximately 30 minutes, and the end pieces and comb were carefully detached from the casting tray. The gel was then placed in a gel electrophoresis tank containing TAE buffer, and DNA samples mixed with 1x blue/ orange loading dye (Promega) were loaded into the wells. Either a 100bp or 1kb DNA ladder (Promega) were also loaded to wells to estimate the size of DNA samples. These samples were run at 75V for approximately 45 minutes until bromophenol blue dye reached ¾ length of the gel. Finally, samples were visualised under UV light using a Gel Doc XR+ System (Bio-Rad, Hertfordshire, UK).

2.5.7.2 DNA purified from agarose gel

DNA was separated in an agarose gel electrophoresis as described in 2.5.7.1. DNA bands were visualised using a long-wavelength UV transilluminator. The DNA fragment of interest was excised from the gel using a sterile scalpel and transferred into a weighed microcentrifuge tube. The weight of the excised gel slice was determined using a digital balance and the DNA was then extracted from gel slice using wizard SV Gel and PCR clean up system (Promega). Membrane binding solution was added at the ratio of 10µl of solution per 10mg of agarose gel and this was incubated at 60°C until the gel was completely melted. After incubation, dissolved gel mixture was transferred into the SV minicolumn. The DNA

was then purified as described in 2.5.6.5 and stored at either 4°C for a maximum of four weeks or -20°C for long term storage.

2.5.7.3 Formaldehyde gel electrophoresis

Prior to northern blot analysis, RNA samples were denatured and separated using formaldehyde gel electrophoresis and a NorthernMax 10x Denaturing Gel Buffer (ThermoFisher). The running buffer, casting tray and electrophoresis tank were cleaned to get rid of RNase as described in 2.5.5.1. Then 1% w/v agarose was added in 45ml nuclease-free water in a sterile conical flask and melted in a microwave. This was allowed to cool to approximately 50°C. Under the fume hood, 5ml of NorthernMax 10x Denaturing Gel Buffer was added and mixed by swirling. This mixture was then poured into a pre-assembled casting tray under the fume hood and the comb was then inserted. This was allowed to set at room temperature under the fume hood for approximately one hour and then submerged in the gel electrophoresis tank containing 1x MOPS buffer. The RNA samples were firstly denatured by heating at 95°C for five minutes and chilling on ice. Denatured RNA samples were mixed with an equal volume of 2x RNA Gel Loading Dye (ThermoFisher) and were loaded into the wells as well as an RNA marker (Promega). The gel was then electrophoresed in 1x MOPS buffer at 75V for approximately 1-2 hours until the bromophenol blue dye reached ¾ length of the gel. To examine the quality of the target RNA, this formaldehyde gel could be stained in 0.25–0.5 μg ml^{-1} ethidium bromide solution and then visualised under UV light using a Gel Doc XR+ System.

2.5.7.4 Denaturing polyacrylamide gel for RNA

A mini-protean II electrophoresis cell was used for denaturing RNA polyacrylamide gel electrophoresis. Briefly, glass plate sandwiches were prepared by placing the longer rectangular glass plate at the bottom, laying two 0.4mm spacer along the short edges of the glass plate base and placing the shorter glass plate on top of the spacers. This glass plate sandwich was then transferred into the clamp and secured with four screws. The longer glass plate should be the same side with the screws. The clamp was placed into a casting slot. Then, a 12.5% gel mixture was prepared by mixing 4.8g urea, 3.125ml 40% v/v acrylamide/ Bis solution (Bio-Rad), 1ml of 10x TBE buffer (1M tris-acetate adjusted with boric acid to pH 8, 20mM disodium ethylenediaminetetraacetic acid) in a final volume of 10ml. 33µl of 30% w/v ammonium persulphate (APS) and 4µl of tetramethylethylenediamine (TEMED) was added immediately before pouring the gel mixture to the middle of the two layers of glass plates. The comb was then inserted and the gel was allowed to set at room temperature for approximately 45 minutes. Once set, the comb was detached and the clamp was released from the casting slot and assembled to the cooling core. If only one gel was used, the other clamp was filled with a short glass plate to avoid leakage. The unit was placed in the buffer chamber and then 1x TBE buffer was added as running buffer to a level where the wells were submerged. The wells were rinsed with running buffer and the gel was pre-run at 75V for 30 minutes. During this time, RNA samples were denatured by heating at 95°C for five minutes and chilling on ice. Denatured RNA samples were mixed with an equal volume

of RNA Gel Loading Dye (2x) and were then loaded into the wells together with an RNA marker. The gel was electrophoresed at 75V for 45 minutes until bromophenol blue dye reached ¾ length of the gel and then ready for downstream applications.

2.5.8 DNA sequencing

2.5.8.1 PCR product sequencing

Purified PCR fragments (2.5.6.5) were diluted to the concentration of 5ng μl^{-1} and 15µl of diluted PCR product was transferred into a microcentrifuge tube. An aliquot of 15µl of each primer at exactly 10pmol μl^{-1} was transferred to another microcentrifuge tube and 5µl extra volume of primer was required for each additional sequencing reaction. PCR samples and primers were sent to Eurofins genomics for sequencing.

2.5.8.2 Bacteriophage whole genome sequencing

Bacteriophage DNA was first isolated as described in 2.5.3.1. After purification, approximately 10mg of bacteriophage DNA sample was transferred into a microcentrifuge tube and the tube was sealed with parafilm (Sigma-Aldrich). Samples were sent to Next Generation Sequencing Facility (Queen's Medical Centre, Nottingham, UK) for whole genome sequencing via MiSeq platform. Briefly, bacteriophage genomic DNA sample was first sheared into 200-300 bp fragments. The adapters and additional motifs, such as sequencing binding sites and regions complementary to flow cell oligonucleotides, were then attached to create the sequencing library. Each fragment molecule was then isothermally

amplified to simultaneously generate millions of clusters in a flow cell, and this process is known as bridge amplification. A flow cell is a glass slide with lanes which are coated with a lawn composed of two types of oligonucleotide to allow the binding of template fragment. The sequencing starts with the binding of the first sequencing primer to generate the read. In each sequencing cycle, one fluorescent tagged nucleotide is incorporated into the synthesised complementary chain of the template fragment. The fluorescent label also serves as the terminator for polymerization. After incorporation of one nucleotide, the fluorescent dye is excited by a laser and the fluorescent signal is emitted. The signals generated are used to produce images which are then analysed by the MiSeq image control software. After this, the fluorescent label is enzymatically cleaved to enable the incorporation of next nucleotide. In this way, the fragment length is determined by the number of the sequencing cycles, and the base calling is done based on the emission wavelength together with the signal intensity. After sequencing, paired end data were de novo assembled via the CLC genomic workbench by Prof. Ian Connerton.

2.5.8.3 Campylobacter jejuni carrier state whole genome sequencing

Genomic DNA from carrier state *Campylobacter jejuni* was isolated as described in 2.5.1. After purification, approximately 5mg of DNA was transferred into a microcentrifuge tube and the tube was then sealed with parafilm. The sample was sent to DNA Sequencing Facility (University of Northumbria, Newcastle, UK) for whole genome sequencing via MiSeq

platform as described in 2.5.8.2. After sequencing, the paired end data were de novo assembled via the CLC genomic workbench by Prof. Ian Connerton.

2.6 Manipulation of DNA

2.6.1 Plasmid DNA

The *E. coli* plasmid DNA applied in this thesis are listed in Table 2.6 together with their host strains. Plasmid DNA was stored at -20°C and for those within their host strains were stored at -80°C.

Table 2.6 *E. coli* plasmids used in this thesis

Plasmid DNA	Description	Source
pUC4K	Cloning vector	N. Cummings
TOPO pCR2.1	Cloning vector	Invitrogen
pCfdxA::perR	Cloning vector	L. Liz
pET28b	Expression vector	N. Cummings
pUC4KΔ*Kan* (pUC4)	Knocking-out *Kan*R gene to form a EcoRI-pUC4K-EcoRI fragment	This thesis
pCR2.1::*flhF*	*flhF* amplified from *C. jejuni* PT14 using primers containing BsmBI (5') and NgoMIV (3') overhang, and then cloned into pCR2.1 vector	This thesis
pCR2.1::*flhF*(T368A)	*FlhF*(T368A) amplified from *C. jejuni* PT14CP30-2 using primers containing BsmBI (5')	This thesis

	and NgoMIV (3') overhang, and then cloned into pCR2.1 vector	
pCR2.1::*flhF-2*	*flhF* amplified from *C. jejuni* PT14 using primers containing NdeI (5') and BamHI (3') overhang, and then cloned into pCR2.1 vector	This thesis
pCR2.1::*flhF*(T368A)-2	*FlhF*(T368A) amplified from *C. jejuni* PT14CP30-2 using primers containing NdeI (5') and BamHI (3') overhang, and then cloned into pCR2.1 vector	This thesis
pCR2.1::*Kan*	Kanamycin resistance gene was amplified from pUC4K vector and cloned into pCR2.1	This thesis
pUC4::*flhF*	*flhF* obtained by digesting pCR2.1::*flhF* with EcoRI followed by ligating into pUC4 backbone	This thesis
pUC4::*flhF*::*Kan*	Kanamycin resistance gene was obtained by digesting pCR2.1::*Kan* with XbaI followed by inserting into pUC4::*flhF* through XbaI site	This thesis
pCfdxA	Knocking-out perR gene by digesting pCfdxA::perR vector with BsmBI and NgoMIV	This thesis
pCfdxA::*flhF*	*flhF* was obtained by digesting pCR2.1::*flhF* with BsmBI and NgoMIV followed by ligating into pCfdxA backbone	This thesis
pCfdxA::*flhF*(T368A)	*flhF*(T368A) was obtained by digesting pCR2.1::*flhF* with	This thesis

	BsmBI and NgoMIV followed by ligating into pCfdxA backbone	
pET28b::*flhF*	*flhF* was obtained by digesting pCR2.1::*flhF-2* with NdeI and BamHI followed by ligating into pET28 that was pre-digested with NdeI and BamHI	This thesis
pET28b::*flhF*(T368A)	*flhF*(T368A) was obtained by digesting pCR2.1::*flhF-2* with NdeI and BamHI followed by ligating into pET28 that was pre-digested with NdeI and BamHI	This thesis

2.6.2 Restriction digestion of DNA

Restriction enzymes from New England Biolabs (Hitchin, UK) were used in this thesis for restriction digestion of DNA according to manufacturer's instructions and they are listed in Table 2.7. Briefly, the restriction digestion reaction was set up by mixing appropriate amount of target DNA with 10 units of restriction enzyme, 2µl of appropriate 10x buffer in a final volume of 20µl using a pipette tip. This mixture was incubated at appropriate temperature for one hour and the restriction enzyme was then heat inactivated at 65°C for 20 minutes unless otherwise stated. After digestion, DNA was verified (2.5.7.1) and purified (2.5.7.2) by agarose gel electrophoresis and stored at 4°C for a maximum of eight weeks or at -20°C for long term storage.

Table 2.7 Restriction enzymes used in this thesis

Restriction enzyme	Cleavage sequence	Buffer	Incubation temperature	Heat inactivation
BsmBI	CGTCTCN$_1$*N$_5$	NEBuffer 3.1	55°C	80°C
EcoRI	G*AATTC	NEBuffer EcoRI	37°C	65°C
EcoRV	GAT*ATC	NEBuffer 3.1	37°C	80°C
NaeI	GCC*GGC	CutSmart Buffer	37°C	No
NdeI	CA*TATG	CutSmart Buffer	37°C	65°C
NgoMIV	G*CCGGC	CutSmart Buffer	37°C	No
XbaI	T*CTAGA	CutSmart Buffer	37°C	65°C

2.6.3 Dephosphorylation of DNA

Restriction digested DNA was normally dephosphorylated to avoid re-circularization during downstream ligation steps, Shrimp Alkaline Phosphatase (rSAP; New England Biolabs) was used to remove the 5' and 3' end phosphate of the digested DNA according to manufacturer's instructions. Since rSAP was compatible with all NEBuffers applied in restriction digestion (2.6.2), it could be applied directly to digested DNA without any buffer exchange. Briefly, after restriction digestion, 1µl of rSAP was directly applied to the reaction, and incubated at 37°C for 30 minutes. After dephosphorylation, rSAP was inactivated by incubating at 65°C for five minutes and dephosphorylated DNA could be gel purified if

appropriate. Finally, dephosphorylated DNA sample was stored at 4°C for a maximum of eight weeks or at -20°C for long term storage.

2.6.4 DNA Ligation

Linearised DNA fragments with complemented ends were ligated using T4 DNA Ligase (New England Biolabs) according to manufacturer's instructions. Briefly, gel purified DNA insert (2.5.7.2) and dephosphorylated vector backbone (2.6.3) were mixed in a ligation solution containing 1µl of 10x ligase buffer and 1 unit of T4 DNA ligase in a final volume of 10µl. The amount of insert DNA added into the reaction was calculated using equation (6), and unless otherwise stated, 3 insert: 1 vector was applied as standard molar ratio in this thesis.

ng of insert = [(ng of vector x kb size of insert) / kb size of vector] x (molar ratio of insert: vector) (6)

This was incubated at 16°C overnight and the T4 DNA ligase was then heat inactivated at 65°C for ten minutes. Ligated DNA was stored at 4°C for a maximum of eight weeks or at -20°C for long term storage.

2.7 *In situ* hybridisation

2.7.1 Probes

Probes used in this thesis were labelled using 3' end labelling (2.7.1.1), PCR labelling (2.5.6.4) or commercial pre-labelled oligonucleotide (Eurofins), and they were listed in Table 2.8.

Table 2.8 Probes used in this thesis

Name	Sequence (5' to 3')	Melting temp
Prophage probe	CCTTCGCCTACACAATCTTC	60°C
hipO probe	TTCTTTTGCAACCTCACTAGCAAAATCCAC	60°C
cdtA probe	AAAATTTCCCGCCATACCACTTGTCCC	62°C
Campy probe	GGTAAGCTACTAAGAGCG	54°C

To design a commercial pre-labelled oligonucleotide probe, its length was normally in the range between 18 to 50 base pairs to give optimum hybridisation times and binding specificity. The GC content was ideally between 40% and 60%, for optimum specificity. Hairpin structure needed to be avoided by preventing the presence of complementary regions within the probe sequence and long stretches of a single base were also avoided.

2.7.1.1 DIG Oligonucleotide 3' End Labelling

A DIG Oligonucleotide 3' – End Labelling Kit, 2nd Generation (Roche, Welwyn Garden City, UK) was applied as an alternative method to label oligonucleotide according to manufacturer's instructions. Briefly, 100pmol of oligonucleotide was transferred into a microcentrifuge tube along with RNase-free RO water to make a final volume of 10µl. Then, 4µl of Reaction Buffer (5x), 4µl of $CoCl_2$ Solution, 1µl of DIG-ddUTP solution and 1µl of Terminal Transferase (20 Unit μl^{-1}) was added to the tube, and mixed by centrifuging briefly. This mixture was incubated at 37°C for 15

minutes and then cooled on ice. The labelling reaction was stopped by adding 2µl of 0.2M EDTA (pH 8.0). The labelled probe was stored at 4°C for a maximum of eight weeks or stored at -20°C for long term storage.

2.7.1.2 Labelling efficiency determination

A portion of the labelled oligonucleotide was diluted to a concentration of 2.5pmol μl^{-1} and a five-fold serial dilution was prepared until the down to 1fmol μl^{-1}. An aliquot of 1µl of each dilution was dispensed on an Amersham Hybond-N+ membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) and allowed to air dry. Oligonucleotides were then fixed to the membrane by exposing to long wavelength UV light for three minutes and then visualised using chromogenic methods (2.7.2.7).

2.7.2 Northern blot

2.7.2.1 Capillary transfer

RNA samples were denatured and separated using a formaldehyde gel electrophoresis (2.5.7.3) and the gel was then soaked in 20x SSC (3M sodium chloride, 0.3M sodium citrate, pH 7.0) twice each for 15 minutes to removed formaldehyde. A capillary transfer sandwich was then assembled. Briefly, a piece of Whatman 3MM paper (Sigma-Aldrich), which was pre-soaked with 20x SSC transfer buffer, was placed on top of the support with both ends submerged in transfer buffer. The formaldehyde agarose gel was then placed atop the soaked Whatman 3MM paper and formation of any air bubbles avoided. An Amersham Hybond-N+ membrane was cut to exact size of the gel and after pre-

wetting with 20x SSC transfer buffer, the membrane was placed on top of the formaldehyde gel. Another two pieces of pre-wet Whatman 3MM paper were then placed on top of the membrane and the blot assembly was finally completed by adding a stack of paper towels, a glass plate and a 500g weight sequentially on top of the Whatman 3MM paper. The blot was allowed to be transferred overnight in 20x SSC transfer buffer. The transferred RNA was then fixed while still damp by firstly rinsing the membrane with 2x SSC buffer, exposing the wet membrane under long wave UV light for three minutes with RNA side facing UV light source, rinsing membrane briefly in RNase-free RO water, and finally air drying the membrane. The membrane was then immediately processed to downstream applications or stored at 4°C between two pieces of Whatman 3MM paper in a sealed bag.

2.7.2.2 Semi-dry transfer

For RNA samples denatured and separated using a urea-polyacrylamide gel (2.5.7.4), a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-rad) was used to transfer RNA onto membrane blot according to manufacturer's instructions. Briefly, a piece of Whatman 3MM paper, that was pre-wetted with 0.5x TBE transfer buffer, was placed onto the platinum anode. Formation of any air bubbles was avoided. A piece of pre-wetted Amersham Hybond-N+ membrane was placed on top of the filter paper. The gel was carefully placed on top of the membrane. Another piece of pre-wetted Whatman 3MM paper was placed on top of the gel and then the cathode was carefully placed onto the stack. Finally,

the blot was transferred at 10- 15V for 30 minutes. The transferred RNA was then fixed onto blot while the blot was still damp by firstly rinsing the membrane with 0.5x TBE buffer, exposing the wet membrane under long wave UV light for three minutes with the RNA side facing UV light source, rinsing membrane briefly in RNase-free RO water, and finally air drying the membrane. The membrane was then immediately processed to downstream applications or stored at 4°C between two pieces of Whatman 3MM paper in a sealed bag.

2.7.2.3 Hybridisation

Prior to hybridisation, DIG Easy Hyb Granules (Roche) was reconstituted by adding 64ml of nuclease-free water and incubating at 37°C with shaking. The fixed blot (2.7.2.1, 2.7.2.2) was placed in either a plastic sealable bag or a plastic Petri dish, and 10ml of DIG Easy Hyb at 37°C was added for each blot. Bags or Petri dishes containing blots were sealed by impulse easysealer (Packer, Essex, UK) or parafilm accordingly. The blot was pre-hybridised at 37°C in a shaking incubator with 75rpm for 30 minutes. During pre-hybridisation, 100pmol of specific probe was firstly transferred to a fresh microcentrifuge tube along with 50µl of DIG Easy Hyb, and this was denatured at 95°C for five minutes. The tube was chilled on ice, and then 10ml of DIG Easy Hyb, at 37°C, was added. This was mixed by gentle inversion to form the hybridisation solution. After pre-hybridisation, the DIG Easy Hyb was poured out and immediately replaced with hybridisation solution containing specific probe. The blot

was hybridised in a sealed container at 37°C in a shaking incubator with 75rpm overnight.

2.7.2.4 Stringency wash

After hybridisation, the blot was stringently washed to remove non-specifically bound probe and undesired low homology hybrids. Briefly, the blot was placed in a container with 20ml of Low Stringency Buffer (2x SSC containing 0.1% SDS) and incubated at room temperature for five minutes with gentle shaking. The stringency buffer was poured off and another 20ml of fresh Low Stringency Buffer was added and the blot was washed at room temperature for five minutes. The blot was then transferred to another container and washed with High Stringency Buffer (0.1x SSC containing 0.1% SDS) twice at 37°C each for 15 minutes with gentle shaking.

2.7.2.5 Blot preparation for probe detection

Prior to probe detection, the blot was washed, blocked and bound with DIG-antibody using a DIG Wash and Block Buffer Set (Roche). Briefly, the blot was transferred to a container with 20ml of Washing Buffer and incubated at room temperature with gentle shaking for two minutes. The Washing Buffer was discarded and 20ml of Blocking Solution was added. This was incubated at room temperature with gentle shaking for 30 minutes. During the blocking step, the antibody solution was prepared by centrifuging Anti-Digoxigenin-AP (Roche) at 10,000 x *g* for five minutes and diluting in Blocking Solution to give a final concentration of 75mU ml-

[1]. After blocking, the Blocking Solution was discarded and 20ml of Antibody Solution was transferred to the container. This was incubated at room temperature for 30 minutes with gentle shaking and the Antibody Solution was discarded. The blot was then washed in 20ml Washing Buffer twice, each for 15 minutes, and equilibrated in 20ml of Detection Buffer at room temperature for three minutes.

2.7.2.6 Chemiluminescent methods for probe detection

The blot, which was bound with DIG-antibody (2.7.2.5), was placed in the middle of two pieces of plastic sheet and 1ml of CDP- *Star* (Roche) was applied for every 100 cm^2 blot to make sure the blot surface was evenly soaked. Air bubbles were eliminated and the blot was incubated at room temperature for five minutes and excess liquid was squeezed out. The side of the plastic sheet was then sealed by an impulse easysealer and the sealed envelope containing the blot was then visualised with a digital camera.

2.7.2.7 Chromogenic methods for probe detection

Prior to the detection reaction, a Colour Substrate Solution was prepared by adding 200µl of NBT/BCIP solution (Roche) to 10ml of Detection Buffer. The blot, which was bound with DIG-antibody (2.7.2.5), was placed in a container and the Colour Substrate Solution was immediately added to completely submerge the blot. The blot was stored at room temperature in the dark, overnight to develop the signal and the reaction

was stopped by rinsing the blot with 50ml TE buffer at room temperature for five minutes.

2.7.2.8 Stripping the blot

Before stripping the blot, a Stripping Buffer was prepared with 0.2M sodium hydroxide containing 0.1% SDS. For blots detected using chemiluminescent detection, the probe was stripped off by rinsing the blot thoroughly in RNase-free RO water for one minute, washing the blot in stripping buffer at room temperature twice each for 15 minutes and finally rinsing the blot with 2x SSC for five minutes. For blot detected using the chromogenic method, the blot was transferred into a large beaker containing 50ml of dimethylformamide, which was heated to 55°C. The blot was incubated at 55°C for one hour until the blue colour was removed and then rinsed thoroughly with RNase-free RO water. The blot was washed in Stripping Buffer at 37°C each for 20 minutes and then rinsed with 2x SSC at room temperature for five minutes. After stripping the blot was stored, wet, in 2x SSC or the hybridisation was repeated with a different probe.

2.7.3 Southern blot

2.7.3.1 Colony lifting

Campylobacter colonies (2.2.9) were lifted to a transfer membrane using a method modified from Sambrook et al. (1989). Briefly, 100µl different dilutions of *Campylobacter* suspension were spread on mCCDA plates (2.1.3) and incubated overnight at 42°C under microaerobic conditions (2.2.1). After incubation, the plates that give rise to 30 – 300 colonies were cooled to 4°C and circular hybond- N+ nylon membranes (Pharmacia) were placed onto the surface of plates. Even pressure was applied to the membranes and allowed to stand for five minutes. The DNA side of the membranes was marked using a pencil. The membranes were then placed with colony side up on top of 2 pieces of Whatman 3MM paper saturated with 0.5M NaOH and 1.5M NaCl for five minutes in order to release DNA. The membranes were then neutralised by placing them on 2 pieces of Whatman 3MM paper saturated with 1M Tris-HCl, pH 8.0 for five minutes followed by washing with 2x SSC briefly. Nucleic acids were immobilised by exposure to long-wavelength UV light for five minutes. The membranes were then washed in 1x TBS and allowed to dry. Membranes were stored under -20°C until required.

2.7.3.2 Hybridisation

The fixed colony DNA membrane (2.7.3.1) was placed in a plastic petri dish, and 10ml of DIG Easy Hyb heated to 42°C was added for each blot. Petri dishes containing blots were sealed with parafilm and the blot was

pre-hybridised at 42°C in a shaking incubator with 75rpm for 30 minutes. During pre-hybridisation, 100pmol of specific probe was first transferred to a fresh microcentrifuge tube along with 50µl of DIG Easy Hyb, and this mixture was denatured at 95°C for five minutes. The tube was chilled on ice, and then 10ml of DIG Easy Hyb, which was heated to 42°C, was added. This was mixed by gentle inversion to form the hybridisation solution. After pre-hybridisation, the DIG Easy Hyb was poured out and immediately replaced with hybridisation solution containing specific probe. The blot was hybridised at 42°C in a sealed container in a shaking incubator with 75rpm overnight.

2.7.3.3 Stringency wash

After hybridisation, the blot was stringently washed to remove non-specifically bound probe and undesired low homology hybrids. Briefly, the blot was placed in a container containing 20ml of Low Stringency Buffer (2x SSC containing 0.1% SDS) and incubated at room temperature for five minutes with gentle shaking. The stringency buffer was poured off and another 20ml of fresh Low Stringency Buffer was added and the blot was washed at room temperature for five minutes. The blot was then transferred to another container and washed with High Stringency Buffer (1x SSC containing 0.1% SDS) twice at 50°C, each for 15 minutes, with gentle shaking.

2.7.3.4 Probe detection

The blot, which was bound with DIG-antibody (2.7.3.3), was placed in a container and the Colour Substrate Solution (2.7.2.7) was immediately added to completely submerge the blot. The blot was stored at room temperature in the dark, overnight to develop the signal and the reaction was stopped by rinsing the blot with 50ml TE buffer at room temperature for five minutes. The blot was stripped as described in 2.7.1.8. The blot after stripping was stored wet in 2x SSC or the hybridisation repeated with a different probe.

2.7.4 Dot blot

A dot blot was carried out to determine if the specific designed probe could efficiently bind to the target sequence, and further be detected. Briefly, nucleic acid samples were serial diluted to appropriate concentration and 1µl of each dilution was spotted to an Amersham Hybond-N+ membrane. Nucleic acid samples were then fixed to membrane by exposure to long-wavelength UV light for three minutes and visualised using chromogenic methods (2.7.2.7).

2.8 Protein Isolation and Analysis

2.8.1 Addition of Histidine (His)

Target genes along with their promotors were amplified by PCR with primers designed with specific overhang (2.5.6.2), and resulting in an NdeI and BamHI site being added to 5' and 3' ends of the PCR fragment. An A-overhang was also added to the ends of this PCR fragment (2.5.6.6)

and this fragment was then cloned into TOPO pCR2.1 (2.6.4). The NdeI – BamHI fragment was obtained by digesting the plasmid (2.6.2) with NdeI and BamHI restriction enzyme, and then purifying the insert fragment in a gel (2.5.7.2). The NdeI – BamHI fragment was then ligated into the backbone of pET28b, which was previously digested with NdeI and BamHI, followed by gel purification, and by doing so, the fragment was N terminal His_6- tagged. The His_6- tagged fragment was then transformed into appropriate expression host strain (2.3.3.2).

2.8.2 Small scale protein isolation

The Ni- NTA Spin Kit (Qiagen) was used for small scale protein isolation according to manufacturer's instructions. Briefly, an *E. coli* colony harboring the expression plasmid was incubated in 10ml of LB broth (2.1.10) containing appropriate antibiotics at 37°C, with 150rpm shaking, overnight. The overnight culture was then diluted 1:30 with fresh LB broth containing appropriate antibiotics. This culture was further incubated at 37°C with 150rpm shaking until the OD_{600} reached 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to give a final concentration of 0.4mM and the culture was grown at 37°C with 150rpm shaking for another 4 hours. After incubation, the cells were harvested by centrifuging in a Beckman JS-21 centrifuge at 4°C 4000 x *g* for 15 minutes using JA18.1 rotor. Prior to protein isolation, Binding Buffer and Elution Buffer were prepared with Tris-buffer saline (TBS; pH 7.2) containing 10mM imidazole and TBS (pH 7.2) containing 250mM imidazole respectively. The pellet was resuspended in 630µl of Binding

Buffer supplemented with 70µl Lysozyme Stock Solution (10mg ml^{-1}) (Merckmillipore) and 250 units of Benzonase Nuclease (Sigma-Aldrich). This culture was incubated on ice for 30 minutes and centrifuged in a Beckman JS-21 centrifuge at 4°C with 12,000 x *g* for 30 minutes using a JA18.1 rotor. During centrifugation, the Ni-NTA spin column was equilibrated with 600µl of Binding Buffer and centrifuged in a Sigma 1-16K refrigerated microfuge at 4°C with 890 x *g* for two minutes. The culture supernatant was collected and up to 600µl of clear lysate containing the His_6 –tagged protein was loaded onto the equilibrated Ni-NTA spin column. The column was centrifuged in the refrigerated microfuge at 4°C with 270 x *g* for five minutes and the flow through was collected for protein analysis. The Ni- NTA spin column was then washed twice with 600µl of Binding Buffer and centrifuged at 890 x *g* in the refrigerated microfuge at 4°C with 890 x *g* for two minutes. The protein was finally eluted with 300µl Elution Buffer and centrifuged in a bench-top centrifuge at 4°C with 890 x *g* for two minutes and the elution was collected. The protein sample was stored at 4°C until required.

2.8.3 Large scale protein isolation

HisTrap HP, 5 x 1ml (GE Healthcare) was used for large scale protein isolation according to manufacturer's instructions. Briefly, an *E. coli* colony containing the expression plasmid was incubated in 10ml of LB broth (2.1.10) containing appropriate antibiotics at 37°C, with 150rpm shaking, overnight. The overnight culture was then transferred into 2 litre of fresh LB broth containing appropriate antibiotics. This culture was

further incubated at 37°C with 150rpm shaking until the OD_{600} reached 0.6. IPTG was then added to give a final concentration of 0.4mM and the culture was grown at 37°C with 150rpm shaking, for another 6 hours. After incubation, the cells were harvested by centrifuging in a Beckman JS-21 centrifuge at 4°C 4000 x *g* for 15 minutes using JA20 rotor. Prior to isolation, Binding Buffer and Elution Buffer was prepared as described in 2.8.2. A peristaltic pump p-1 (Pharmacia, New Jersey, US) was used to load buffers and clear lysate to the column at a flow rate 1ml $minute^{-1}$. The pump tubing was filled with distilled RO water. The stopper and the snap-off end were removed and the column was connected to the pump. The column was washed with distilled RO water for 5 column volumes and then equilibrated with Binding Buffer for 5 column volumes. The clear lysate was then applied using the pump followed by washing column with Binding buffer for approximately 15 column volumes until the absorbance reached a steady baseline. The protein was subsequently eluted using a linear gradient for ten column volumes and eluted protein samples were then stored at 4°C until required.

2.8.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was used to analyse protein samples. A Mini-Protean II electrophoresis cell was assembled and used as described in 2.5.7.4. Prior to electrophoresis, 5ml of separating gel buffer was prepared by mixing 1.6ml nuclease-free RO water, 2ml 40% acrylamide/ Bis solution, 1.3ml Tris- HCl (pH 8.8) and 50µl of 10% SDS. Immediately after adding 50µl 10% w/v APS and 5µl TEMED to the mixture, this gel

mixture was mixed using pipette tip and poured between the glass plates. Approximately 200µl of Tris saturated butanol was then added to even the surface of the separating gel. This was allowed to set at room temperature for approximately 45 minutes and then the butanol was removed. The surface of the separating gel was rinsed with nuclease-free RO water to remove excess of butanol. During this time, stacking gel was prepared by mixing 1,487.5µl nuclease-free RO water, 625µl 0.5M Tris-HCl (pH 6.8), 250µl of 40% acrylamide/Bis solution and 25µl of 10% SDS. Immediately after adding 25µl of 10% w/v APS and 2.5µl of TEMED, this gel mixture was mixed using a pipette tip and poured on top of the separating gel. The comb was then inserted and the stacking gel was allowed to set for approximately 45 minutes. Meanwhile, protein samples were prepared by mixing with equal volumes of loading buffer (62.5mM Tris-HCl, pH 6.4, 2% w/v SDS, 0.05% w/v bromophenol blue, 20% v/v glycerol, 0.05% v/v β-mercaptoethanol) and denaturing the mix at 95°C for five minutes. After heating, protein samples were cooled on ice and centrifuged briefly in a Biofuge Pico bench top centrifuge. Once the gel was set, it was transferred to the electrophoresis tank and protein samples were loaded together with the Precision Plus Protein Dual Colour Standards (Bio-rad). The gel was run with running buffer (25mM Tris-HCl, pH 8.3, 250mM Glycine, 0.1% w/v SDS) at 150V for one hour, until protein markers were well separated. Following electrophoresis, the SDS-PAGE gel was stained with either coomassie blue or colloidal coomassie blue stain.

2.8.5 SDS-PAGE gel staining

2.8.5.1 Coomassie blue staining

SDS-PAGE gel was stained with coomassie blue as stated by Sambrook *et al.* (1989). Briefly, SDS-PAGE gel was first immersed in 20ml of coomassie blue staining solution (40% v/v methanol, 10% v/v glacial acetic acid, 0.1% w/v brilliant blue R) overnight at room temperature with gentle shaking on an orbital shaker. The coomassie blue staining solution was removed and the gel was washed briefly with RO water twice. The gel was subsequently immersed in destaining solution (40% v/v methanol, 10% v/v glacial acetic acid) at room temperature, with gentle shaking for approximately four hours until the protein bands were clearly visible.

2.8.5.2 Colloidal blue staining

For greater sensitivity the SDS-PAGE gel was stained using Brilliant Blue G-Colloidal Coomassie (Sigma-Aldrich) according to manufacturer's instructions. Briefly, the gel after electrophoresis was fixed in fixing solution (40% v/v methanol, 7% v/v acetic acid) at room temperature for one hour. Immediately prior to stain, 1x Brilliant Blue G- Colloidal Coomassie working solution was mixed with methanol in a ratio of 4:1 to form the staining solution. The SDS-PAGE gel was immersed in staining solution overnight at room temperature with gentle shaking on an orbital shaker. The staining solution was then discarded and gel was washed with destaining solution (25% v/v methanol, 10% v/v acetic acid) for one minute with gentle shaking followed by rinsing with 25% v/v methanol

and destaining in 25% v/v methanol for up to 24 hours until the protein bands were clearly visible.

2.8.6 Protein purification

2.8.6.1 Size exclusion chromatography

A Superose 12 10/300 GL was used for size exclusion chromatography and was connected to AKTA Prime Liquid Chromatography System. The column was equilibrated with 70% v/v ethanol when not in use. Before purification, the column was washed with 50ml of filtered Milli-Q water at a flow rate of 0.5ml $minute^{-1}$ at room temperature. The protein sample was loaded into the system via a sterile syringe and the column was further eluted with 50ml of eluent (TBS, pH 7.2 containing 250mM imidazole) at a flow rate of 0.5ml $minute^{-1}$ at room temperature. Fractioned filtrate was collected with a volume of 500µl for each and each peak on the chromatogram indicated a separated protein sample. Protein samples were analysed on an SDS-PAGE gel (2.8.4) and stored at 4°C until required.

2.8.6.2 Centrifugal filter

An Amicon Ultra 30K device was used to purify protein samples after isolation according to manufacturer's instructions. Briefly, the device column was pre-rinsed with filtered Milli-Q water and up to 500µl of protein sample was loaded. The column was centrifuged in a Sigma 1-16K refrigerated microfuge at 4°C with 13,000 x *g* for ten minutes. The filtrate was discarded and the retentate was processed with up to 500µl of

sample for each run. The purified protein sample was recovered by placing the Amicon Ultra 30K Device upside down in a fresh microcentrifuge tube and centrifuging in a refrigerated microfuge at 4°C with 1,000 x *g* for two minutes. Purified protein was analysed on an SDS-PAGE gel (2.8.4) and stored at 4°C until required.

2.8.7 Bradford assay

2.8.7.1 Standard curve

Bovine serum albumin (BSA; Bio-Rad) was used as a protein standard and a serial dilution series was prepared with a starting concentration of 2 mg ml^{-1} in the same buffer as protein samples. In a 96-well Microtitre plate (ThermoFisher), 100µl of Bradford reagent was mixed with 100µl of protein standard for each dilution in triplicate. After mixing briefly with pipette tip, the absorbance of this mixture was immediately measured in a microtitre plate reader (Tecan, Reading, UK) at a wavelength of 595nm and a standard curve of absorbance against protein concentration was prepared.

2.8.7.2 Protein concentration determination

Protein samples were 5-fold serial diluted in appropriate buffer. In a 96-well micro-titre plate, 100µl of Bradford reagent was mixed with 100µl of protein sample for each dilution in triplicate. After mixing briefly with pipette tip, the absorbance of this mixture was immediately measured in a Microtitre plate reader at a wavelength of 595nm and the stock protein concentration was obtained by plotting the net absorbance against the

protein standard curve (2.8.7.1) and further dividing by the dilution factor.

2.8.8 GTP hydrolysis assay

The GTP hydrolysis assay was carried out using a High Throughput Colorimetric GTPase Assay Kit (Innova Biosciencs, Babraham, UK). In this thesis, temperature assay, heat stability assay and kinetic assay were included.

2.8.8.1 Standard curve

Prior to the GTP hydrolysis assay, a standard curve was prepared to convert the absorbance reading into released free phosphate (P_i) from the hydrolysis reaction. A set of P_i standards was prepared to give a final concentration of 1, 2, 5, 7.5, 10, 20, 50, 75 μM. Triplicate samples were prepared for each P_i concentration by mixing 200μl of each standard with 50μl of P_iColorLock in a microtitre plate (Sigma-Aldrich). After 2 minutes, 20μl of stabiliser was added and mixed thoroughly with pipette tip followed by 30 minutes incubation at room temperature. The absorbance at wavelength 612nm was subsequently measured and the standard curve of absorbance against P_i concentration was plotted.

2.8.8.2 Temperature assay

The substrate buffer mix was first prepared containing 100mM Tris buffer, 5mM $MgCl_2$ and 0.25mM of GTP. An aliquot of 100μl of protein enzyme was mixed with equal volume of substrate buffer mix and

incubated immediately at desired temperature for thirty minutes. An aliquot of 100µl of His_6 purification elution buffer (TBS, pH 7.2, 250mM imidazole) was used as the blank enzyme sample. An aliquot of 50µl of P_iColorLock solution containing 0.5µl Accelerator solution was added to stop the enzyme reaction. After two minutes incubation at room temperature, 20µl of stabiliser was added and mixed thoroughly to prevent colour development from background P_i. The colour reached a maximum signal after incubation at room temperature for 30 minutes and the absorbance of samples was read at 612nm in a microtitre plate reader. The amount of released P_i was then calculated using the standard curve (2.8.8.1).

2.8.8.3 Thermal stability assay

Prior to the temperature assay, protein enzyme was pre-heated at desired temperature for five minutes and an equal volume of substrate buffer was immediately added after pre-heat treatment. His_6 purification elution buffer (TBS, pH 7.2, 250mM imidazole) was used as blank enzyme sample. This mixture was heated at this temperature for another 30 minutes and 50µl of P_iColorLock solution containing 0.5µl Accelerator solution was added to stop the enzyme reaction. After two minutes, 20µl of stabiliser was added and mixed thoroughly to prevent colour development from background P_i. The colour reached a maximum signal after 30 minutes and then the absorbance of samples was read at 612nm in a microtitre plate reader. The amount of released P_i was then calculated using the standard curve (2.8.8.1).

2.8.8.4 Kinetics assay

Protein enzyme sample was individually mixed with equal volume of substrate buffer with different substrate concentrations and assayed at 42°C. At time points of 0, 2, 4, 6, 8, 10 minutes, 200µl of enzyme sample was removed and added to 50µl of P_iColorLock solution containing 0.5µl Accelerator solution was added to stop the enzyme reaction. After two minutes incubation at room temperature, 20µl of stabiliser was added and mixed thoroughly to prevent colour developed from background P_i. The colour reached a maximum signal after incubation at room temperature for 30 minutes and then the absorbance of samples was read at 612nm in a microtitire plate reader. The amount of released P_i for each substrate concentration with different assay time was then calculated using the standard curve (2.8.8.1). A curve with absorbance against assay time for each substrate concentration was plotted and the initial velocity for the protein sample with different substrate concentration was individually determined. Eventually, a Lineweaver-Burk and Michaelis-Menten plot was prepared with initial velocity against substrate concentration.

2.8.9 Protein location determination

E. coli cells after induction were pelleted in a Beckman JS-21 centrifuge at 13,000 x *g* for 15 minutes, at 4°C, using JA18.1 rotor and the pellet was resuspended in 300µl of Solution 1, that containing 20% w/v sucrose, 30mM Tris-HCl, pH 8.0, 1mM EDTA and 1mg ml^{-1} lysozyme. This suspension was left on ice for ten minutes and centrifuged in a Biofuge

Pico bench top at 13,000 x *g* for 15 minutes, at 4°C. The supernatant containing soluble periplasmic protein was collected. The remaining pellet was resuspended in 300µl of 0.1M Tris-HCl, pH 8.0 and frozen at -80˚C for 5 minutes followed by thawing at room temperature for 5 minutes and this cycle was repeated 3 times. The suspension was centrifuged at 13,000 x *g* for 15 minutes, at 4°C and the supernatant containing cytoplasmic protein was collected. Eventually, the remaining inclusion body pellet was resuspended in 100µl 1% v/v Triton X-100 and incubated at 4˚C for ten minutes. Samples collected from each step were analysed with SDS-PAGE gel (2.8.4).

2.9 Transmission Electron Microscopy

Prior to sample preparation, fixative solution was prepared by adding 2.5ml 0.2M cacodylate buffer (pH 7.4), 1.9ml distilled water into 600µl 25% v/v EM glutaraldehyde. Overnight grown *C. jejuni* cells were harvested into 600µl of fixative solution using a 10µl inoculating loop. The bacterial pellet was fixed in the fixative solution at room temperature for one hour and then centrifuged in a Biofuge Pico bench top at room temperature at 10,000 x *g* for one minute. The supernatant was discarded and 1ml of 0.1M cacodylate buffer was added to gently wash the pellet. The pellet was left at 0.1M cacodylate buffer for ten minutes and then centrifuged in a Biofuge Pico bench top at room temperature with 10,000 x *g* for one minute. The supernatant was removed and the pellet was then resuspended gently into 600µl of 0.1M cacodylate buffer. An aliquot of 14µl of fixed *C. jejuni* suspension was transferred onto the formvar/

carbon film on copper 200 mesh grid and left for thirty seconds. The suspension was removed by lens paper and 14μl of 0.5% w/v uranyl acetate was added onto the grids to negative stain the *Campylobacter* cells for one minute. After staining, uranyl acetate was removed by lens paper and the grid was ready to be examined by TEM.

CHAPTER 3

Detection of the presence of prophage in campylobacters colonising broiler chickens

3.1 Introduction

Bacteria are ubiquitous in almost all terrestrial and aquatic environments and genetic diversity acts as a significant role for their survival and adaptability (Loc Carrillo and Janež, 2013). The concept of lysogeny leading to the integration of phage DNA into bacterial chromosome in the form of prophages is well established largely on the basis of the paradigm of phage lambda (Casjens and Hendrix, 2015). It is now evident that prophages are common and are represented in many bacterial genomes lodged in public databases (Canchaya et al., 2003; Casjen, 2003). Prophages of enteric bacteria confer functional characteristics that significantly affect their biology, these include survival, virulence niche and host adaption (Clark et al., 2012). Prophages have played a significant role in the evolution of bacterial pathogenicity in many species (Boyd and Fischetti, 2003), the study of which has developed our understanding of the relationship between bacteria and its associated phage from a host-virus interaction to genome co-evolution interactions (Canchaya et al., 2004). As mobile DNA elements, prophage are able to translocate from one site to another within and between genomes of bacteria, causing insertion mutation and other genetic rearrangements, and therefore are considered as a significant source of genetic diversity (Jakhetia and Verma, 2015). In addition, prophage are also vectors for lateral gene transfer between bacteria (Bushman, 2002), with a variety of evidence for the transfer of virulence factors in the development of bacterial pathogenicity (Boyd and Brussow, 2002; Waldor, 1998; Wanger and Waldor, 2002).

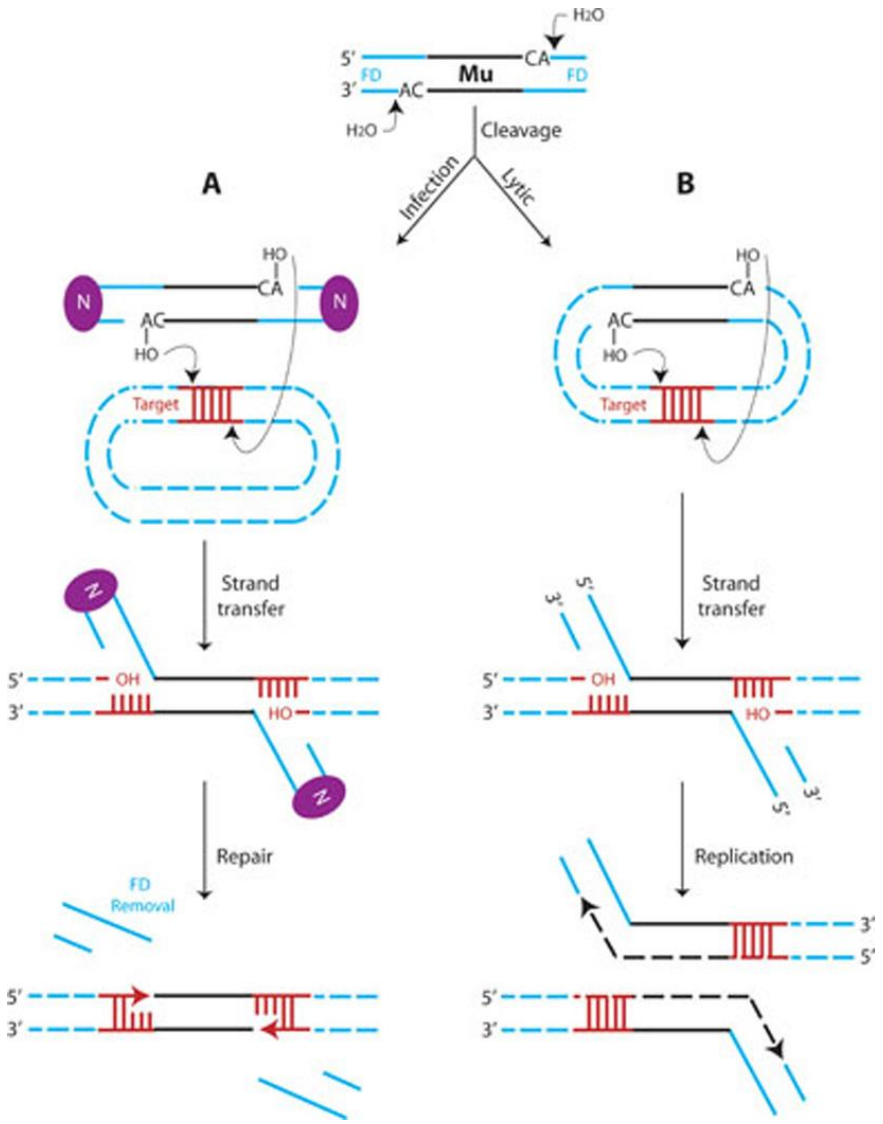


Figure 3.1 Transposition mechanism of Mu prophage that containing two pathways. A. Infection phase, B. Lytic phase. Source (Harshey, 2014)

The *E. coli* phage Mu is a transposable phage, and was the first element observed to cause DNA insertions into a bacterial genome as described by Larry Taylor in late 1950s (Taylor, 1963; reviewed by Harshey, 2012), and since that time the study of phage Mu has never stopped. Phage Mu is described as able to randomly integrate into its host chromosome under not only lysogenic but also lytic life cycles, and further to result in genetic mutations in the host bacterium (Faelen and Toussaint, 1976; Wang and

Higgins, 1994). According to the donor substrate configuration as well as the fate of transposition products, the Mu life cycle can be separated into two distinct phases, an infection phase and a lytic phase (Symonds et al., 1987) as illustrated in Figure 3.1. During the infection phase, Mu DNA is injected into cells, which has a linear DNA configuration in the phage heads with flanking non-Mu host DNA (Symonds et al., 1987). The linear ends are put together with the aid of an injected phage protein N, that are attached to both ends of the flanking host DNA, to form a non-covalently closed supercoiled circle structure before integration occurs. For the lytic phase, Mu is already integrated into a large covalently closed circle of the host genome, which revealed a different donor configuration to that during the infection phase. When transposition starts, the transposase MuA will initiate a water mediated endonucleolytic cleavage of specific Mu-host phosphodiester bonds, and by doing so, produces 3'-OH ends on both ends of the Mu DNA (Au et al., 2006). The Mu DNA will then transpose to the phosphodiester bonds located 5 bp apart from target DNA via the 3'-OH ends. Notably, this transposition step is intermolecular during infection phase whilst intramolecular in the lytic phase (Harshey, 2014). After transposition, the newly formed branched intermediate product needs to be resolved and this occurs via different pathways for phage infection or lysis phases. For the infection phase, the flanking DNA is removed upon transposition and the 5 bp gaps are repaired by limited DNA replication. However, for the lytic phase, the intermediate product is resolved by target primed replication through Mu (Choi et al., 2014).

Prophage in *Campylobacter* were first suggested in the 1960s and 1970s, however, it was not until the last decade, with the help of whole genome sequencing, that the presence of prophage sequences in the genomes of *C. jejuni*, *C. coli* and *C. lari* were confirmed (Fouts et al., 2005). Similar to other prophages, *Campylobacter* prophage also appear to be mobile within the genomes (Parker et al., 2006) and can act as substrate for recombination to cause genome rearrangements (Scott et al., 2007b), and affect molecular typing results including PFGE patterns (Barton et al., 2007). It has been reported that the Mu prophage carries the genes for extracellular DNases that are thought to be the reason for the observation that the frequency of *Campylobacter* natural transformation in Mu-containing strains is significantly reduced (Gaasbeek et al., 2009; Gaasbeek et al., 2010). The Mu-like prophage sequence was first discovered in the genome of *C. jejuni* RM1221 and later similar prophage sequences were observed in many other *C. jejuni* strains (Parker et al., 2006; Scott et al., 2007b). Clark (2011) reported that Mu-like prophage sequences were widely distributed in 52 out of 365 *Campylobacter* isolates when analysing for the presence of such sequences. Mu-like insertions have been demonstrated to be the sites of genomic rearrangements as compared with parental strains (Scott et al., 2007). Such rearrangements have been observed in response to bacteriophage predation, and lead to the development of resistance to the infecting virulent bacteriophage that is accompanied by inefficient chicken gut colonisation. These studies also constitute the first demonstration of

active prophage excision to spontaneously produce infectious *Campylobacter* phage as CampMu virions (Scott et al., 2007b).

In this chapter, the prevalence of *Campylobacter* spp. in the caecal contents of a commercial broiler chicken flock was monitored though the rearing period, and the appearance of a *Campylobacter* subpopulation harboring the Mu-like prophage identified and enumerated within the flock.

3.2 Results

For days 8, 13, 18, 23, 30, 34 and 37 after hatching, 8-10 chickens were collected from a total 8 farms during summer/autumn of 2012, and the spring/summer of 2013. Farms were selected on the basis of their proximity to Sutton Bonington to enable a fast return (Figure 3.2) in order to rapidly process the samples and maximise the recovery of campylobacters from the intestinal contents of chickens collected on farm or at the processing plant. Farm chickens were selected from 8 areas of the barn and culled on-farm before collection at the farm gate to maintain biosecurity. All flocks tested were *Campylobacter* culture negative until sampling days 21-23 post hatch.

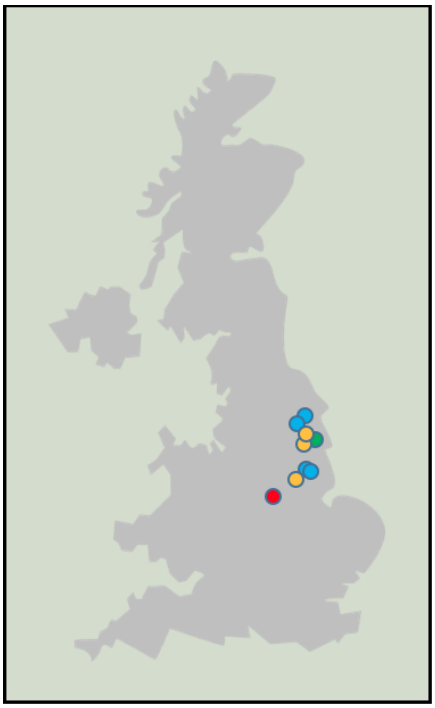


Figure 3.2 Geographical spread of farms. ●, samples collected from summer 2012; ●, samples collected from autumn 2012; ●, samples collected from spring/ summer 2013; ●, Sutton Bonington.

Chicken caecal contents were obtained and campylobacters were subsequently isolated using the mCCDA plating methods described in section 2.2.9. The samples collected in summer of 2012 contained campylobacters with Mu-like prophage from one of three farms shown in Figure 3.2, and constitute the basis for this analysis.

3.2.1 *Campylobacter* confirmation test

Isolated *Campylobacter* samples were analysed with gram stain, oxidase test, motility, *flaA*-SVR typing and hippurate test to confirm they belong to the *Campylobacter* genus and the species. These results are listed in Table 3.1.

The isolated *Campylobacter* strains were named with either HF5 or BA5, referred to the broiler house name, followed by the flock number, chicken number and sacrificed date. As shown in Table 3.1, all bacterial strains grown on mCCDA plates proved to be Gram negative, spiral shaped, positive for oxidase and catalase tests; highly motile and were therefore considered as *Campylobacter* sp. The *flaA*-SVR typing yielded in 4 *flaA*-SVR alleles, allele 2 and 121 for *Campylobacter* isolates from broiler house HF5 and allele 1360 and 467 from broiler house BA5 isolates. Moreover, with the exception of strains BA5-7-1-230712 and BA5-7-2-230712 that revealed negative results in hippurate test and were concluded as *C. coli,* all other samples were designated as *C. jejuni* since they were all hippurate positive.

Table 3.1 *Campylobacter* confirmation of samples isolated from chicken intestinal contents

Strain NAME	Gram	Oxidase	Catalase	Motility (mm)[1]	*flaA*[2]	Hippurate Test
HF5-7-1-120712	-ve spirals	+ve	+ve	30	2	+ve
HF5-7-2-120712	-ve spirals	+ve	+ve	30	2	+ve
HF5-8-1-120712	-ve spirals	+ve	+ve	31	2	+ve
HF5-8-2-120712	-ve spirals	+ve	+ve	39	2	+ve
HF5-9-1-120712	-ve spirals	+ve	+ve	13	2	+ve
HF5-9-2-120712	-ve spirals	+ve	+ve	>43	2	+ve
BA5-2-1-120712	-ve spirals	+ve	+ve	>43	1360	+ve
BA5-2-2-120712	-ve spirals	+ve	+ve	>43	1360	+ve
BA5-3-1-120712	-ve spirals	+ve	+ve	35	1360	+ve
BA5-3-2-120712	-ve spirals	+ve	+ve	36	1360	+ve
BA5-7-1-120712	-ve spirals	+ve	+ve	33	1360	+ve
BA5-7-2-120712	-ve spirals	+ve	+ve	>43	1360	+ve
HF5-2-1-160712	-ve spirals	+ve	+ve	29	2	+ve
HF5-2-2-160712	-ve spirals	+ve	+ve	34	2	+ve
HF5-9-1-160712	-ve spirals	+ve	+ve	>43	2	+ve
HF5-9-1-160712	-ve spirals	+ve	+ve	34	2	+ve
BA5-6-1-160712	-ve spirals	+ve	+ve	>43		+ve
BA5-6-2-160712	-ve spirals	+ve	+ve	38		+ve
BA5-7-1-160712	-ve spirals	+ve	+ve	>43		+ve
BA5-7-2-160712	-ve spirals	+ve	+ve	>43		+ve
HF5-4A-1-190712L	-ve spirals	+ve	+ve	23	2	+ve
HF5-4A-2-190712L	-ve spirals	+ve	+ve	>43		+ve
HF5-4A-3-190712S	-ve spirals	+ve	+ve	36	121	+ve
HF5-4A-4-190712S	-ve spirals	+ve	+ve	39	121	+ve
HF5-4B-1-190712L	-ve spirals	+ve	+ve	36		+ve
HF5-4B-2-190712L	-ve spirals	+ve	+ve	34	2	+ve
HF5-4B-3-190712S	-ve spirals	+ve	+ve	36	121	+ve
HF5-4B-4-190712S	-ve spirals	+ve	+ve	31	121	+ve
BA5-6-1-190712	-ve spirals	+ve	+ve	>43		+ve
BA5-6-2-190712	-ve spirals	+ve	+ve	>43		+ve
BA5-6-3-190712	-ve spirals	+ve	+ve	30	1360	+ve
BA5-9-1-190712	-ve spirals	+ve	+ve	37		+ve
BA5-9-2-190712	-ve spirals	+ve	+ve	>43	1360	+ve
BA5-9-3-190712	-ve spirals	+ve	+ve	>43	1360	+ve
HF5-5-1-230712L	-ve spirals	+ve	+ve	34	2	+ve
HF5-5-2-230712L	-ve spirals	+ve	+ve	38	2	+ve
HF5-5-3-230712S	-ve spirals	+ve	+ve	31	121	+ve
HF5-5-4-230712S	-ve spirals	+ve	+ve	29	121	+ve
HF5-7-1-230712	-ve spirals	+ve	+ve	31	121	+ve
HF5-7-2-230712	-ve spirals	+ve	+ve	30		+ve
BA5-4-1-230712	-ve spirals	+ve	+ve	>43	1360	+ve

BA5-4-2-230712	-ve spirals	+ve	+ve	>43		+ve
BA5-7-1-230712	-ve spirals	+ve	+ve	19	467	-ve
BA5-7-2-230712	-ve spirals	+ve	+ve	19		-ve
BA5-5-1-260712	-ve spirals	+ve	+ve	32	1360	+ve
BA5-5-2-260712	-ve spirals	+ve	+ve	37		+ve
BA5-6-1-260712	-ve spirals	+ve	+ve	35	1360	+ve
BA5-6-2-260712	-ve spirals	+ve	+ve	35		+ve

1. The motility is presented as the diameter of the bacterial halo formed on the motility agar plates.
2. *FlaA* refers to *flaA*- SVR allele nucleotide number. The *flaA* gene was PCR amplified and sequenced for individual *Campylobacter* isolates. The *flaA*- SVR alleles, which are 321 nucleotide bases from coordination 283 to 603 in *flaA* gene, were then calculated via the pubmed MLST database (http://pubmlst.org/campylobacter).

Whole genome sequences of *Campylobacter* isolates HF5-7-1-120712 (2), HF5-4A-4-190712S (121), HF5-5-1-230712L (2) were determined using the MiSeq platform in order to establish the degree of genetic diversity amongst the *flaA*-SVR types (indicated in brackets) within the HF5 broiler house populations. Provisional analysis of contigs from the unassembled genome sequences identified the presence of Mu-like prophage DNA sequences within the genome of HF5-4A-4-190712S but not the others, which provided an opportunity to assess the carriage of mixed *Campylobacter* populations in the broiler chicken gut through the rearing period of a single flock and to study impact of the prophage on the *Campylobacter* populations.

3.2.2 Probe binding efficiency test

Prior to the colony blot, DIG pre-labelled oligonucleotide probes purchased from Eurofins MWG were analysed to determine the efficiency of probe binding and anti-DIG antibody binding for colorimetric detection. Isolated DNA from *C. jejuni* strains HF5-4A-4, HF5-5-1, HF5-7-1

and *C. coli* strain NCTC12668 were quantified using the nanodrop spectrophotometer, and aliquots of 100ng, 10ng, 1ng and 0.1ng were individually spotted onto a positively charged nylon membrane before hybridisation with specifically designed DIG-labelled DNA probes. The prophage probe was designed to bind the gene for Mu-like prophage I protein, the *Campylobacter* probe to bind to the 23s ribosomal RNA gene, whilst the *C. jejuni* and *C. coli* discriminatory probes were designed to target the *hipO* and *cdtA* genes respectively.

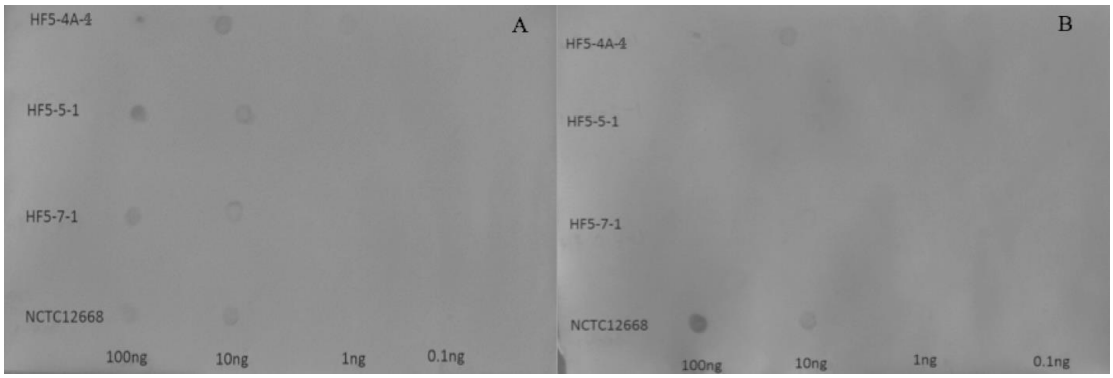


Figure 3.3 Dot blot to determine the probe efficiency. A. Dot blot hybridised with *Campy* probe, B. Dot blot hybridised with the *C.* coli-specific probe

Figure 3.3-A shows a dot blot after hybridisation with the *Campylobacter* probe, and as expected the probe signals were clearly evident for all the *Campylobacter* samples and the signal intensities were proportional with the concentrations of spotted DNA. Notably, signal was not observed for the DNAs spotted with quantities less than 10ng, indicating the minimum quantity of DNA required for the test. After stripping the *Campylobacter* probe, the blot was re-hybridised with the *C. coli* probe followed by colorimetric signal detection. Clear signals were only observed for *C. coli*

NCTC12668, and were again proportional to the DNA quantity applied. However, signals were absent in all other *C. jejuni* samples, suggesting that the *C. coli* probe successfully bound to the *C. coli* specific *cdtA* gene and could be used to distinguish *C. coli* samples from *C. jejuni.* However, no signal was detectable for the prophage probe for any of the samples on the blot (data not shown). This suggests that the prophage probe either failed to bind to the target sequence in the bacterial DNA or the successfully bound probes had been washed off during the stringency washes. Since the melting temperature for the oligonucleotide probes were relatively low due to their short length, we decided to use a PCR reaction to label a prophage fragment with 5' DIG- labelled primers and use the amplicon as the probe.

3.2.3 Binding efficiency test for probe with PCR DIG- labelling

The PCR labelled prophage probe was prepared by PCR amplification of a 521 base pair fragment from Mu-like prophage I encoding gene of strain *C. jejuni* HF5-4A-4 using a pair of primers in which the reverse primer had been 5' end labelled with DIG. The binding specificity was determined using a dot blot under the standardised southern blot protocol. DNA samples were first spotted onto a nylon membrane at their stock concentration followed by spotting individual dilutions with concentrations down to 60ng μl^{-1}. The results are shown in Figure 3.4.

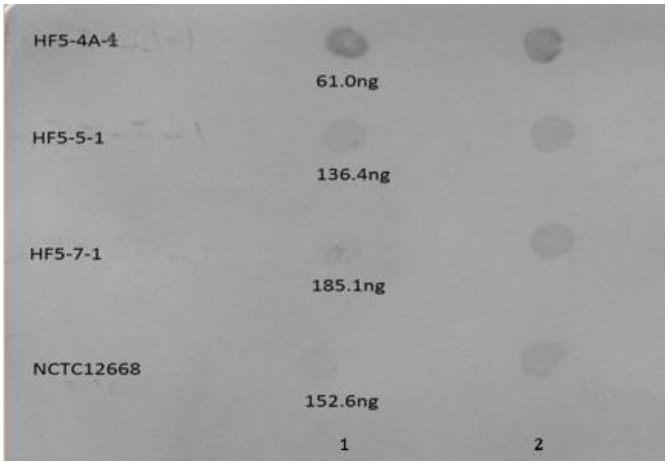


Figure 3.4 Dot blot hybridised with prophage probe. 1µl of bacterial DNA from each strain were spotted. Column 1, samples were spotted without dilution, the amount of spotted DNA was individually labelled; column 2, the amount of spotted DNA was balanced to 60ng for each sample. Stringency washes were carried out at 42˚C.

As illustrated in Figure 3.4, the PCR-labelled prophage probe successfully bound to target in *C. jejuni* HF5-4A-4 strain DNA, which could be bound by the anti-DIG antibody conjugated to alkaline phosphatase, and be visualised using an enzyme linked assay to generate an insoluble colour complex using the NBT/BCIP liquid substrate system for alkaline phosphatase detection. However, positive signals were also observed in strain *C. jejuni* HF5-5-1, HF5-7-1 and *C. coli* 12668, which should not contain any prophage DNA sequences, based on genome sequence data. The reason for the false positive signal was considered as either the PCR synthesised probe was non-specifically bound to other *Campylobacter* DNA sequences or the formation of undesired hybrids of low homology that were not removed following the stringency washes which were not strong enough to remove non-specifically bound probe.

3.2.4 PCR amplification to examine the presence of prophage DNA sequences

As the *Campylobacter* strains applied in this experiment were isolated from chicken caecal content, it is possible that integration of phage DNA into the bacterial chromosome could have occurred in a subset of the campylobacters used for DNA isolation. This can occur because single colonies of thermophilic campylobacters have been demonstrated not to be always of clonal origin (Miller et al., 2000). Since as demonstrated in section 3.2.3, positive signals were detected for *C. jejuni* HF5-5-1, HF5-7-1 and *C. coli* NCTC12668, and should not contain any prophage DNA sequences based on genome sequencing data, the methodology for the detection of the prophage sequence in these *Campylobacter* strains needed to be investigated.

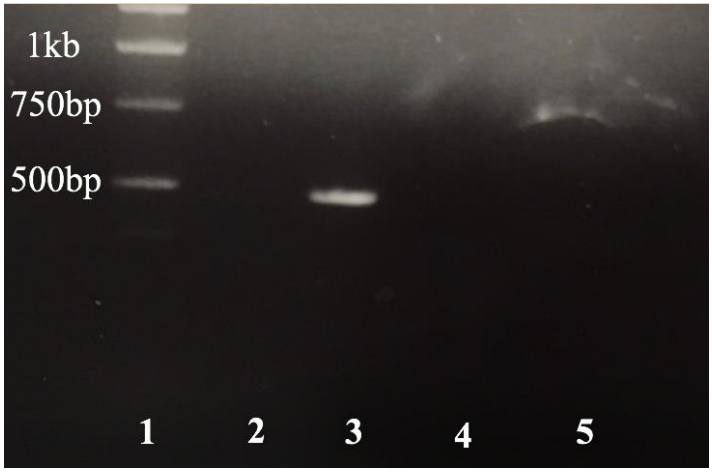


Figure 3.5 PCR amplification using the Mu1 primers to examine the presence of prophage sequence, lane 1. 1kb DNA ladder, 2. *C. coli* NCTC12668, 3. *C. jejuni* HF5-4A-4, 4. *C. jejuni* HF5-5-1, 5. *C. jejuni* HF5-7-1.

With the same primer pair used for the prophage probe synthesis, DNAs of strains *C. jejuni* HF5-4A-4, HF5-5-1, HF5-7-1 and *C. coli* NCTC12668 were PCR amplified. As expected, agarose gel electrophoresis produced a band with a fragment size of approximately 500bp for strain *C. jejuni* HF5-4A-4, suggesting the presence of the prophage (Figure 3.5). However, no bands were detected for any other strain, indicating those strains do not contain any prophage sequences, and this was consistent with our previous sequencing result. Therefore, the positive hybridisation signal observed for *C. jejuni* HF5-5-1, HF5-7-1 and *C. coli* NCTC12668 could only have arisen due to a problem with the method.

3.2.5 Stringency wash temperature determination

The likely reason for the false positive signal in the dot blot experiment was either non-specific binding of the probes or the failure to remove undesired low-homology hybrids. Whichever the case, the false positive signal could be eliminated by increasing the stringency of the washes. The stringency of wash step could be increased by either increasing the temperature or decreasing the salt concentration of the buffer.

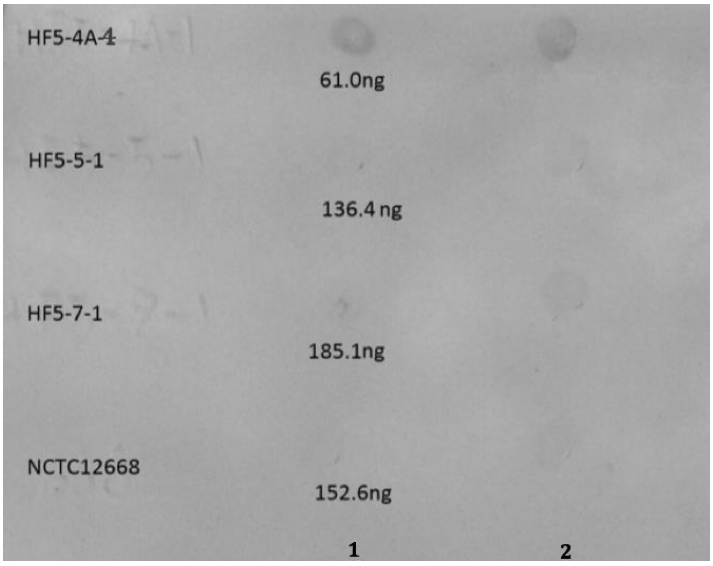


Figure 3.6 Dot blot hybridised with prophage probe. 1µl of bacterial DNA from each strain were spotted. Column 1, samples were spotted without dilution, the amount of spotted DNA was individually labelled; column 2, the amount of spotted DNA was balanced to 60ng for each sample. Stringency washes were carried out 1x SSC containing 0.1% SDS at 58˚C.

After several optimizations, it was determined that low stringency washes carried out at 58˚C with 1x SSC containing 0.1% SDS, which was able to remove any non-specifically bound probe. Only strain *C. jejuni* HF5-4A-4, which contains the prophage sequence, revealed a positive signal and the intensity of this signal was proportional to the

concentration of spotted genomic DNA (Figure 3.6). No signal was observed for all other stains, which was consistent with PCR results.

3.2.5 Colony blot

Since the specificity of all the probes was confirmed and the stringency wash conditions determined, a series of colony blots were carried out to determine the subpopulation of *C. jejuni* isolates containing the prophage sequence among all *Campylobacter.* Plating with a dilution factor of 5 $\log_{10}$ of bacterial suspension were found to reveal countable *Campylobacter* colony numbers on mCCDA plates (between 30-300 colonies per plate), and these colonies were subsequently lifted onto 6 to 8 membranes for each flock time point as described in section 2.7.3.1. During the analysis, blots were first hybridised with prophage probe to avoid any false positive signals caused by incompletely stripping the DNA and colour. After visualization of the signal with alkaline phosphatase coupled antibody and the NBT/BCIP mix, the number of hybridisation positive colonies for each plate lifted was recorded. The probes were then stripped and new probes were re-hybridised until all probes had been tested.

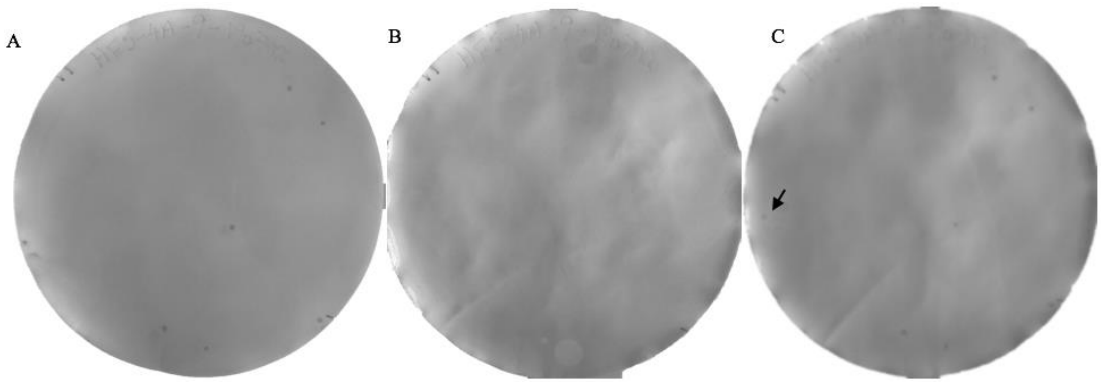


Figure 3.7 Colony blots representatives from HF5-4A-9-190712 at 30 days post hatching. A. Colony blot hybridised with prophage probe, B. Colony blot hybridised with *C. coli* probe, C. Colony blot hybridised with *Campylobacter* probe. Arrow head indicates a colony that is not observed when hybridising with the prophage probe suggesting the colony belongs to a subpopulation without prophage integration.

As shown in the example presented in Figure 3.7, after hybridisation with the prophage probe, 6 positive signals were detected on the blot and these were considered as the number of *Campylobacter* colonies containing prophage sequences on this blot (Figure 3.7-A). After stripping the blot and re-hybridising with the *C. coli* probe, no signal was observed suggesting there are less than the limit of detection (<2 $\log_{10}$ cfu g^{-1}) *C. coli* present in the caecal contents (Figure 3.7-B), and therefore all *Campylobacter* signals present on the blot are likely to represent *C. jejuni*. For the blot hybridised with the *Campylobacter* probe, despite the 6 positive signal previously detected with the prophage probe, one extra signal was present (arrowhead in Figure 3.7-C), suggesting this is a *Campylobacter* colony that does not contain any prophage sequence. In summary, no *C. coli* samples were observed in this blot, whereas in total 7

C. jejuni colonies were detected with six among them were identified as containing prophage sequence.

3.2.6 Data analysis

The colonisation of total *Campylobacter, C. coli* and *C. jejuni* per gram of caecal content were calculated based on the colony counts on the blot in multiplied by plating dilution factor standardises for a 10% by weight suspension of intestinal content. For each flock, 30-32 blots were analysed and the mean values for flocks examined with different ages were plotted (Figure 3.8).

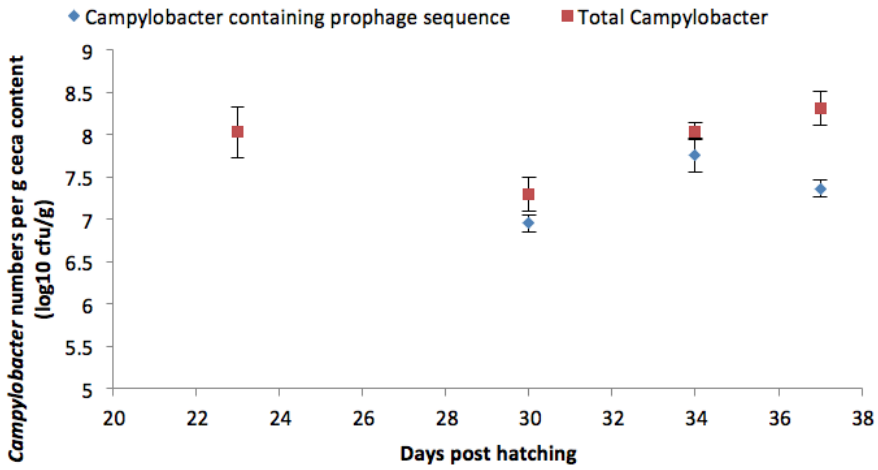


Figure 3.8 Colonisation of *C. jejuni* and *C. jejuni* subpopulation containing prophage sequence in caecal content of broiler flocks with different age. In total 30-32 blots were examined for each flock and the mean values were plotted. Error bars indicated the standard deviations within tested blots.

Since no *C. coli* signals were observed for any of the tested blots, the total *Campylobacter* signals were all identified as *C. jejuni* in this chapter. As shown in Figure 3.8, the mean count of *C. jejuni* started at 8.0 (±0.3) $\log_{10}$

cfu g^{-1} of the caecal contents on day 23 and then declined to a mean count of 7.3 (±0.2) log_{10} cfu g^{-1} on day 30. However, by day 34, the mean count of *C. jejuni* had recovered to 8.0 (±0.1) log_{10} cfu g^{-1} and increased until the end of the experiment at 37 days post hatch, with a mean value of 8.3 (±0.2) log_{10} cfu g^{-1}. The prophage sequence was first observed within the *C. jejuni* genomes on day 30 with a prophage positive mean count of 6.9 (±0.1) log_{10} cfu g^{-1}, and then 4 days later, the count increased to 7.7 (±0.2) log_{10} cfu g^{-1}. Eventually, after 37 days post hatch the mean count of *C. jejuni* with prophage sequence integrated dropped to 7.3 (±0.1) log_{10} cfu g^{-1} in the chicken caecal contents.

3.3 Discussion

Prior to the colony blot, it is important to ensure the probes were able to bind to the target sequence and be detectable using the methods available. Using a short pre-labelled commercial oligonucleotide probe designed against a Mu-like prophage sequence in *C. jejuni*, we failed to detect any positive signals when testing using a dot blot. We thought the problem could be due to the relative short length of the probe. Typically when hybridising the blot with these short probes at high concentration, the target sequence is less likely to be bound completely by one probe, but instead multiple probe molecules will bind with limited base pairing (Jayaraman et al., 2007). This leads to reduced affinity for the probes and further results in probes that can be too easily washed off during stringency washes. We therefore modified the labelling technique to generate a longer PCR fragment with the 5'-end labelled with DIG with the

help of DIG pre-labelled primers. After hybridising the blot with PCR-labelled probes, positive signals were observed. However, the binding affinity was not significantly affected by the length of the sequences for the total *Campylobacter* oligonucleotide probe or the *C. coli* oligonucleotide probe, since the expected positive signals were successfully detected using the test dot blots hybridised with the commercial DIG-labelled oligonucleotide probes. As these probes all share a similar probe length and a GC content of approximately 50%, the reason for the binding affinity difference between prophage probe and the rest remains unknown but could be a consequence of strong regional secondary DNA structures. As stripping and re-probing could permanently damage the blot, all blots used in this thesis were stripped for a maximum of three times to avoid unreliable results. Therefore, probes that were used to detect prophage sequence, *C. coli* and total *Campylobacter* were applied. By assuming that other *Campylobacter* species are minor in number compared with the population of *C. jejuni* and *C. coli* in chicken caecal content (Parker et al., 2013), the number of *C. jejuni* colonies on the blot was estimated as the variation between total *Campylobacter* and *C. coli* counts. No positive signals were observed for the colony blots hybridised with the *C. coli* probe and we assume that all the colonies lifted on the blot were *C. jejuni.* This conclusion was tested by hybridising test blots with the *C. jejuni* probe.

We observed that on day 23, the total mean *C. jejuni* count was measured at 8.0 (±0.3) $\log_{10}$ cfu g^{-1} when 5 days earlier all birds collected were

culture negative. This observation is typical of the lag phase encountered on farms in the UK and other countries, where flocks remain negative for the first 2 weeks then rapidly colonise across the whole flock (Newell and Fearnley, 2003; Newell et al., 2011). However, by day 30 the mean count had fallen to 7.3 (±0.2) $\log_{10}$ cfu g^{-1}. We hypothesised that this decline in *C. jejuni* count in chicken caecal content could be caused by the acquisition of the phage or competition with Mu-containing campylobacters. Notably, on day 30, *C. jejuni* harboring the prophage sequence were first detected, with a 45% share of the population of *C. jejuni*. By day 34, the mean count of *C. jejuni* returned to 8.0 (±0.1) $\log_{10}$ cfu g^{-1}, suggesting that members of the intestinal *Campylobacter* population had overcome the stress of phage infection to become resistant or had been succeeded by a phage insensitive population. The competitor campylobacters did not contain phage Mu-like sequences so were not a subset of lysogens that were resistant to further Mu phage infection. The recovery of the *C. jejuni* count altered the proportion of Mu-containing campylobacters but did not significantly alter their standing population 6.9 to 7.7 $\log_{10}$ cfu g^{-1}. At day 37 before depopulation of the barn the viable count of the prophage containing campylobacters was 7.3 (±0.1) $\log_{10}$ cfu g^{-1}, which represented 11% of the total *C. jejuni.* Incursion at day 30 of prophage containing campylobacters or active phage Mu resulted in a rapid increase in the fraction of the population but this situation did not persist as non-prophage containing campylobacters established dominance but did not extinguish the prophage containing population in the time frame of the experiment.

In this chapter, the presence of *Campylobacter* with Mu-like prophage sequence was detected in campylobacters colonising broiler chickens with a southern blot derived colony blot method. The data provided evidence of interactions between *Campylobacter* and phage, and the succession and modification of *Campylobacter* strains under different levels of competitive stress with respect to the age of the chicken flock.

CHAPTER 4

Characterization of bacteriophage CP30C

4.1 Introduction

Campylobacter bacteriophages have been isolated from a number of environmental sources including chicken meat and chicken excreta (Atterbury et al., 2003; Atterbury et al., 2005; El-Shibiny et al., 2005; Loc Carrillo et al., 2007). The application of *Campylobacter* bacteriophages has been reported to be an effective way of reducing the intestinal colonisation of chickens by the foodborne pathogens *C. jejuni* and *C. coli* (Loc Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009; Kittler et al., 2013). The efficacy of the treatment varies depending on the nature of phage, the titre of the phage administered, the phage-sensitivity of the host, the time interval post treatment and the route of administration (Loc Carrillo et al., 2005; Carvalho et al., 2010; El-Shibiny et al., 2009; Hammerl et al., 2014).

Early research placed the *Campylobacter* bacteriophages into three groups based on their genome size and head diameter (Sails et al., 1998). Group I included two phages with head diameters of 140.6 and 143.8 nm and large genomes, 320 kb in size. Group II had mean head diameters of 99 nm and genome sizes of approximately 180 kb, whilst Group III had a mean head diameter of 100 nm and genome sizes of approximately 140 kb. To date the *Campylobacter* bacteriophage genome sequences reported represent Groups II and III, and fall within the T4-like group. These include the Group II phages CP220, Cpt10 (Timms et al., 2010), vB_CcoM-IBB_35 (Carvalho et al., 2012) and CP21 (Hammerl et al., 2012); and the

Group III phages CP81 (Hammerl et al., 2011), NCTC12673 (Kropinski et al., 2011). Recently these phages and those present in nucleotide sequence databases have been proposed as a new subfamily of the T4-like phage superfamily, the *Eucampyvirinae*, based on their genomic DNA sequences/sizes and particle morphologies (Javed et al., 2014).

Bacteriophage CP30 was reported along with several other phages as able to discriminate *Campylobacter* isolates from a single broiler house (Scott et al., 2007b), within which campylobacters and phage were found to carry over from one flock to the next (Connerton et al., 2004). Strain succession of one genotype over another appeared to occur due to incursion of new genotypes with innate phage-resistance, rather than the development of resistance. Subsequent strain competition experiments in *Campylobacter* colonised chickens revealed bacteriophage sensitive strains to have a competitive advantage but this was reversed in the presence of bacteriophage CP30 (Scott et al., 2007b). Serial passage of CP30 in the laboratory and subsequent genome sequencing revealed that CP30 had become a mixed phage population containing identifiably different sequence types. This chapter reports the purification and DNA sequence differences between the CP30 phage derivatives that resemble the diversity first observed in the broiler flock environment.

4.2 Results

Group III phage CP30 was previously isolated from poultry excreta in UK (Connerton et al., 2004). The genome of this was sequenced and *de novo*

assembled by Prof. I. Connerton. During assembly, multiple sequence configurations were observed. The phage CP30 stock was then propagated on universal phage host strain *Campylobacter jejuni* PT14 and single plaques were recovered as a plaque purified sample (2.4.3). To date, two configurations were individually isolated and sequenced. First recovered phage CP30 derivative was named as CP30A, which was deposited in GenBank with accession number JX569801.1, and later another configuration, CP30B, was obtained. When isolating phage CP30B, we noticed that there is still another configuration, CP30C, within original stock of CP30.

4.2.1 PCR characterization of phage CP30B and C

In order to isolate phage CP30C, phage CP30 stock was propagated on *C. jejuni* PT14 using the whole plate lysis method and after lysis, ten clear plaques were randomly selected and recovered. These ten phage CP30 isolates were PCR-analysed with primers CP30A/BC (Figure 4.1). Primers CP30A/BC would allow PCR amplification of a fragment of the D302_gp005 gene, which encodes a hypothetical protein, with an amplicon size of 881bp for CP30A and an amplicon size of 948bp for both CP30B and CP30C. These amplicon size differences were caused by many insertion, deletions and substitutions between amplified regions of phage CP30A and CP30B, C (appendix 4).

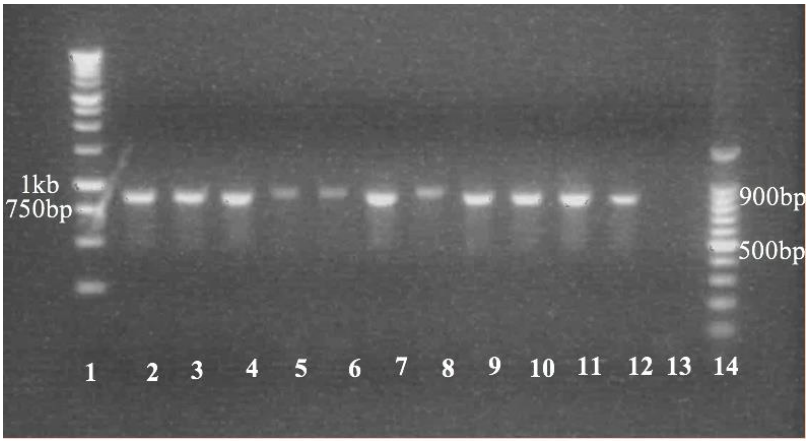


Figure 4.1 Agarose gel showing PCR samples of phage CP30 isolates amplified with CP30A/BC primers. Lane 1. 1kb DNA ladder, 2. CP30-1, 3. CP30-2, 4. CP30-3, 5. CP30-4, 6. CP30-5, 7. CP30-6, 8. CP30-7, 9. CP30-8, 10. CP30-9, 11. CP30-10, 12. Positive control-CP30A, 13. Negative control, 14. 100bp DNA ladder. Isolates 1, 2, 3, 6, 8, 9 and 10 revealed same pattern with positive control suggesting they are all CP30A. Isolates 4, 5, 7 showed larger bands than control indicates they are either CP30B or CP30C.

As shown in Figure 4.1, the fragment sizes of all the PCR amplified products were in the range between 750bp and 1kb according to the ladder, where the fragments of CP30 isolates 4, 5 and 7 migrate slightly slower than other samples, indicating these three samples have a larger amplicon size. As samples with a faster migration rate revealed a same fragment size compared with CP30A amplicon, they were identified as CP30A. Therefore, phage isolates CP30-4, 5, 7 were considered as either CP30B or CP30C.

4.2.2 PCR characterization of phage CP30C

Since phage sample CP30 isolates 4, 5, 7 were considered as either CP30B or CP30C, another pair of primers CP30AB/C were applied to

discriminate CP30C samples from CP30B. After PCR, the amplified fragments were analysed on an agarose gel (Figure 4.2). Primers CP30AB/C would specifically bind to the *D302_gp005* gene, which encodes a hypothetical protein, with an amplicon size of 1493bp for both phage CP30A and CP30B, and an amplicon size of 717bp for phage CP30C. The amplified region in CP30C has a 776bp deletion compared to the amplicon of CP30A and CP30B (appendix 5).

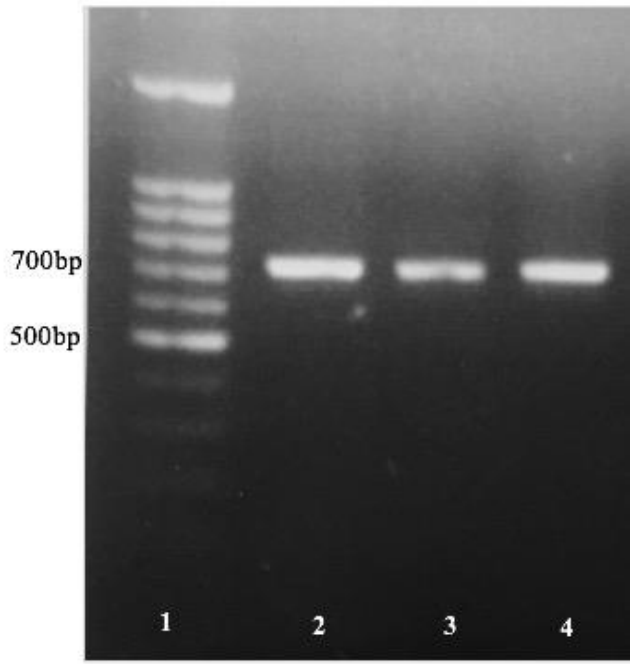


Figure 4.2 PCR samples amplified using CP30AB/C primers. Lane 1. 100bp DNA ladder, 2. CP30-4, 3. CP30-5, 4. CP30-7

Figure 4.2 shows all the loaded PCR products have fragment sizes of approximately 700bp according to ladder. Bacteriophage CP30-4, 5 and 7 were therefore all identified as CP30C.

4.2.3 Phage susceptibility

Phage susceptibility tests were carried out to investigate if phage CP30C would have a different ability to lyse different *Campylobacter* host strains (Table 4.1) compared to phage CP30A.

Table 4.1 Bacteriophage susceptibility against different host strains. Bacteriophage 1-10 correspond to phages CP30-1 to CP30-10, 11. CPX, 12. CP8, 13. Ø13, 14. CP30. Their phenotypes were recorded as confluent lysis (CL), semi-confluent lysis (SCL), opaque lysis (OL) and no plaques formed (-).

C .jejuni	**Bacteriophage**													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
BIII C3	CL	CL	CL	CL	CL	CL	CL	SCL	SCL	SCL	-	-	-	SCL
TIF 2	SCL	SCL	SCL	SCL	SCL	SCL	SCL	SCL	SCL	SCL	-	-	-	SCL
OK VIII C3	OL	OL	OL	OL	OL	OL	OL	OL	OL	OL	OL	OL	OL	OL
CII C3	SCL	SCL	SCL	-	SCL	SCL	-	SCL	SCL	SCL	SCL	SCL	-	SCL
OKIX C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OC I F3	CL	CL	CL	CL	CL	CL	CL	SCL	SCL	CL	-	-	-	CL
DV IV C1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OK X C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FDIC2	SCL	SCL	SCL	-	SCL	SCL	SCL	SCL	SCL	SCL	-	SCL	SCL	SCL
HP I F9	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	SCL	CL

Among the host strains tested, no plaques were observed for all phage isolates when propagating on *C. jejuni* OKIX C3, DV IV C1 and OK X C2. Ten phage isolates revealed the same phenotype on *C. jejuni* TIF2, OK VIII C3 and HP I F9 as semi-confluent lysis, opaque lysis and confluent lysis respectively. Phage sample CP30-8, 9 led to semi-confluent lysis on *C. jejuni* BIII C3 and OC I F3 and these were different with respect to the confluent lysis phenotype observed for other CP30 isolates. For the host strain *C. jejuni* BIII C3, a confluent lysis phenotype was observed for most of the CP30 isolates, however, semi-confluent lysis was present when the

host strain was infected by phage CP30-8, 9 and 10. Notably, phage CP30-4 could not form any plaques on either *C. jejuni* CII C3 or FDIC2. No plaques were observed on *C. jejuni* CII C3 after infection by phage CP30-7. As CP30-4 revealed a different PCR pattern with the CP30A/BC primers, and showed a different susceptibility to the host strain panel compared with the other CP30 isolates, it was selected as a representative of CP30C. This phage was propagated from which genomic DNA was isolated and subsequently sequenced using the MiSeq.

4.2.4 Genome alignment

In this thesis, whole genome alignment was carried out for phage CP30A, B and C using gpVISTA program with CP30A sequence as a reference, and the results are shown in appendices 1, 2 and 3. GpVista is a set of programs to compare between DNA sequences from microbial species up to 10 megabases long, find rearrangement in a global alignment framework and visualise the alignment with a curve (Frazer et al., 2004). The curve was calculated using a windowed mean value of identity score for the whole genome alignment. In this case, a 100bp calculation window was applied indicating that the percentage score of each X point was calculated based on the exact matches between analysed genomes in a 100bp wide window with selected X point as the centre. This analysis indicates that the major variations between phage CP30A and CP30B were present between the coordinates from 3kb to 4.5kb and from 121kb to121.5kb. Whilst the region from 3kb to 4.5kb and from 124kb to 125kb revealed a significant difference between phage CP30A and CP30C. We

also aligned the genome sequences between phage CP30B and CP30C, and as a result, major variation fell into the region with a coordinates between 124kb to 125kb. Except for these regions, the genomes alignments of the CP30 phage derivatives revealed consistent scores between 75% and 100% identity.

Genes within the major variation including the region from 3kb to 4.5kb, which encodes three hypothetical proteins (D302_gp004, D302_gp005, D302_gp006), between phage CP30A and CP30B / C, and from 124kb to 125kb, which encodes another two hypothetical proteins (D302_gp148, D302_gp149), between phage CP30A/ B, and CP30C were individually aligned with the *Campylobacter* phage NCTC12673 present in the NCBI database.

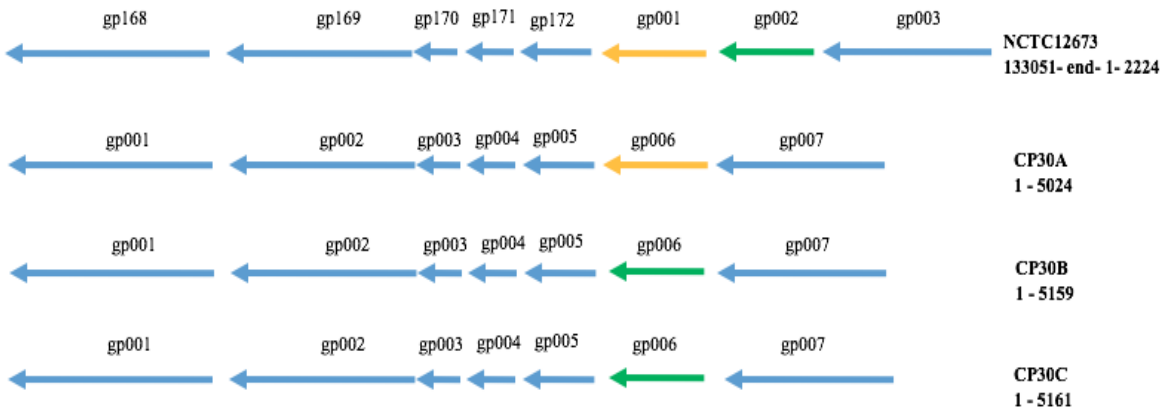


Figure 4.3 Gene alignment between CP30A, B and C in the region showing variation between 3kb to 4.5kb which observed in whole genome alignment. Gene *gp006* in CP30A shares homology with *gp001* in NCTC12673 genome, and gene *gp006* in CP30B and CP30C is homologue for *gp002* in phage NCTC12673.

As shown in Figure 4.3, in this particular region, most of the genes in phage CP30A, B and C were identical with NCTC12673. However, gene *NCTC12673_gp001*, which encodes a hypothetical protein, was present in CP30A as *D302_gp006* but absent in both phage CP30B and CP30C. Instead, the *NCTC12673_gp002* gene, which also encodes a hypothetical protein, was observed in both phage CP30B and CP30C as homologue *gp006,* but not in CP30A.

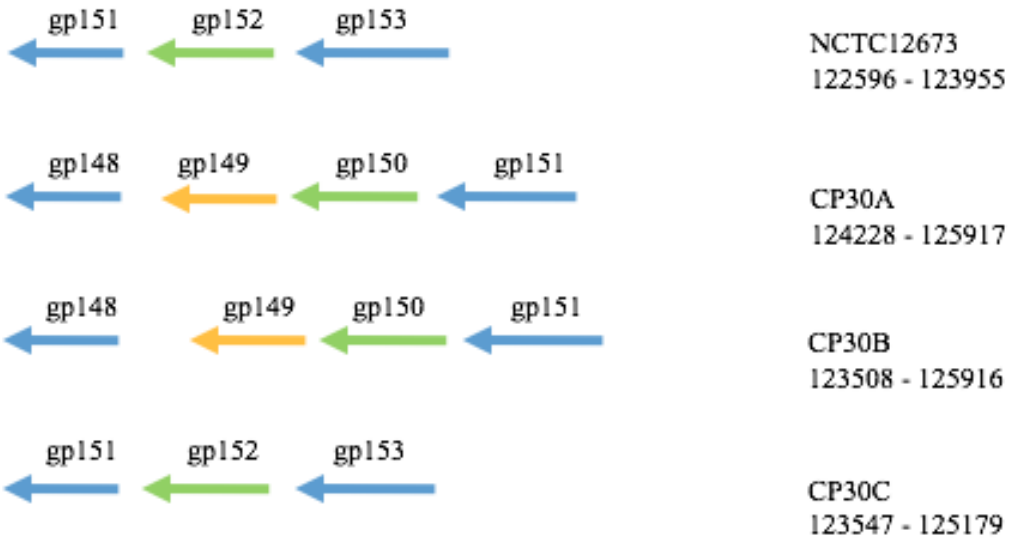


Figure 4.4 Gene alignment between CP30A, B and C in the variation region between 124kb and 125kb which was observed in the whole genome alignment. Gene *gp149* in CP30A shares homology with *gp149* in CP30B, and gene *gp150* in CP30A and CP30B is homologue for *gp152* in phage NCTC12673 and CP30C.

We then analysed the genes that fall within the genome region between 124kb and 125kb, where major variation was observed in the CP30 phage derivatives. The genes *NCTC12673_gp151, NCTC12673_gp152* and *NCTC12673_gp153*, were all identical between the CP30 phage derivatives

and NCTC12673 (Figure 4.4). However, in both phage CP30A and CP30B, a 407 base pair gene *D302_149* was present between *D302_148* (homologue of *NCTC12673_gp151*) and *D302_150* (homologue of *NCTC12673_gp152*), but was not observed in phage NCTC12673 and CP30C.

Another large variation was observed in the phage CP30B genome at coordinates between 121kb to 121.5kb compared to CP30A. The genes within this region include *D302_gp142* and *D302_gp143*, which respectively encode a hypothetical protein and a putative sugar-phosphate nucleotidyltransferase. After alignment, no gene insertion or deletions were present, instead a number of point mutations were observed.

4.2.5 Alignment of specific genes

Followed by whole genome alignment, we focused on several specific genes to predict the possible variation between phage CP30A and CP30C in host adaption and their morphology. The aligned genes and observed mutations are listed in Table 4.2.

The first gene we focused on was *D302_gp053*, that encodes a putative ssDNA binding protein, and after gene alignment of phage CP30C with CP30A, the gene sequences were completely identical. In addition, we analysed several other structural genes that constitute the basis for the morphology of the phage. As presented in Table 4.2, *D302_gp150,*

Table 4.2 List of several gene variations in phage CP30C compared to CP30A

Gene ID	Product	Synonymous substitution	Nonsynonymous substitution
D302_gp053	Putative ssDNA binding protein	N/A	N/A
D302_gp075	Major capsid protein	S49S, E152E, E168E, A185A, G213G, T217T, A218A, A219A, G224G, I232I, I252I, D272D, D351D, L419L and N437N	A122G, D125N, N159D, I160V, M181I, T186S and K222A
D302_gp121	Major head protein II	L33L, G48G, I144I, K158K, K218K, D222D, N246N and G309G	T102A, I244S and Q310R
D302_gp150	Head completion protein	N/A	N/A
D302_gp081	Neck protein	R4R, S6S, T25T, N34N, D67D, F114F, S115S, N118N, V163V, Y165Y, Y176Y, Y184Y, N187N and L230L	P68S, K69R, I119V, K149E and T168I
D302_gp040	putative tail tube protein	F50F, E52E, F61F, V62V and R65R	N/A
D302_gp041	putative tail tube protein	T153T, K156K, L157L, C158C, F161F, E169E, L170L, S171S, E176E, V182V, T183T, A185A and T187T	T151I, T160A, A166G and V168I
D302_gp084	putative tail tube protein III	R18R, K49K, T52T, S129S, F137F, I145I, P146P, I147I, K149K, N150N, K157K, T166T, T177T, K183K, N193N, P195P, T200T, E214E, S216S, V241V and K242K	V12I, R23K, N27T, A30T, V63I, T69N, N71S, T85I, T102H, V109I, I127V, V148I, V199L, T211I and I240L

D302_gp062	tail sheath protein	A68A, L112L, K121K, Q122Q, S139S, K163K, E208E, K212K, I234I, N296N, N317N, L323L, N374N, E412E, L483L, Y496Y, A581A, F582F, I676I, T677T, L678L and V683V	T111A, A113T, A130T, H168Y, I174T, N247K and K252S
D302_gp108	tail completion and sheath stabiliser	N9N, T50T, T64T, L65L, E69E, N108N, K109K, L111L, Y117Y, S119S, K120K and I136I	N/A
D302_gp124	tail sheath stabiliser and completion protein	G98G, K99K, N265N, D284D and E341E	V252I
D302_gp044	putative baseplate wedge subunit	N/A	N/A
D302_gp045	putative baseplate wedge subunit	N/A	N/A
D302_gp050	putative tail fibre protein	N/A	Single base pair deletion lead to frameshift mutation

D302_gp044 and *D203_gp045* that respectively encode the putative head completion protein, and two putative baseplate wedge subunits, are completely identical. Synonymous substitutions were observed in genes *D302_gp040* and *D302_gp108*, which respectively encode a putative tail tube protein and the tail completion/sheath stabiliser. For the genes *D302_gp075* (major capsid protein), *D302_gp121* (major head protein II), *D302_gp081* (neck protein), *D302_gp041* (putative tail tube protein), *D302_gp084* (putative tail tube protein III), *D302_gp062* (tail sheath protein) and *D302_gp124* (tail sheath stabiliser and completion protein), a number of single base pair substitutions were observed. Notably, among these substitutions, the majority of them are synonymous substitutions, and no frameshift mutations were observed for any of the genes stated above. However, for the gene *D302_gp050*, which encodes putative tail fibre protein, a single cytosine deletion was observed in residue 10 of the gene that led to a frameshift (appendix 19)**.** An alternative start codon is positioned downstream of the frameshift, translation initiation from which would produce a protein missing 44 amino acids from the N-terminal end (appendix 20). The predicted protein structures of these alternatives are presented in Figure 4.5.

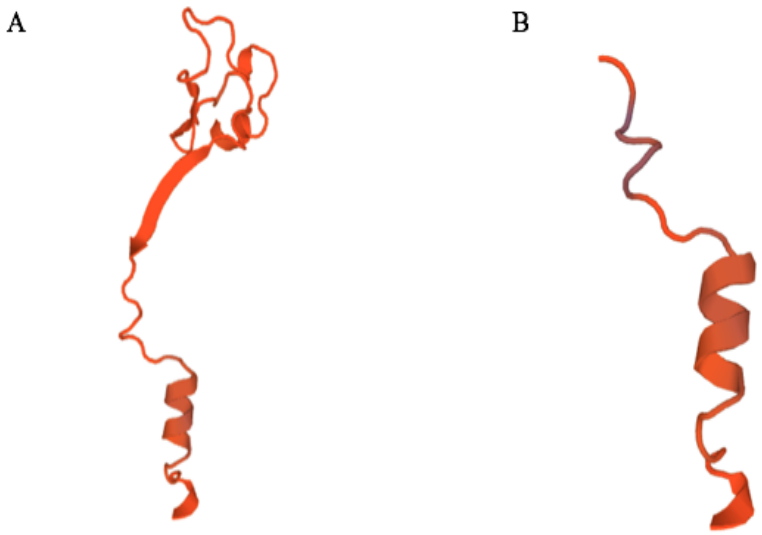


Figure 4.5 Protein structure prediction of gene *D302_gp050* based on template protein hyaluronidase, phage associated (2c3f.1.A), that covers the N terminal end of the protein, using Swiss Prot system. A. Phage CP30A, B. Phage CP30C.

By comparing the N terminal protein structure, we can suggest that the missing amino acid sequence would form one alpha helix and three beta sheets as shown at the top of the protein structure in Figure 4.5-A.

4.2.6 Phage susceptibility test on *C. jejuni* with different flagellin glycosylation modification

As the encoding protein of *gp050,* Gp050 functions as a binding protein with a specific target of acetamidino-modified pseudaminic acid on the host flagella (Javed et al., 2013). In order to find out if phage CP30C with a defective Gp050 would have a different infection ability than CP30A, *C. jejuni* host strains with varied flagellin glycosylation modification were required. Such modification was previously characterised by Scott (2006) in *C. jejuni* OCII F1, OCII F3 and OCII C10 with the addition of different

moieties on one typical flagellin peptide, and the strains were all isolated from a single flock. Among them, strain OCII F3 was identified as the product of horizontal transfer of genomic DNA from strain OCII F1 to OCII C10 with the length of at least 112kb (Scott et al., 2007a). In this case, differences in phage susceptibility between CP30A and CP30C were examined for *C. jejuni* OCII F1, OCII F3 and OCII C10 as host strains.

Table 4.3 Bacteriophage susceptibility against host strains with different flagellin glycosylation modification. Their phenotypes were recorded as confluent lysis (CL) and no plaques formed (-).

Phage name	OCII FI	OCII F3	OCII C10
CP30A	-	CL	CL
CP30C	-	CL	CL

Table 4.3 shows that phage CP30A and CP30C have similar abilities to infect *C. jejuni* OCII F3 and OCII C10, and are similarly unable to infect OCII FI. Notably both CP30A and CP30C phages produced clear plaques on *C. jejuni* OCII F3 and OCII C10, which further lead to almost complete cell clearance of the bacterial lawn.

4.3 Discussion

The double stranded DNA virus CP30 is a *Campylobacter* specific type III phage, which was previously isolated from poultry excreta (Loc Carrillo et al., 2005). As revealed by initial genome sequencing data, after multiple rounds of propagation in the laboratory CP30 had become a mixed phage population containing identifiably different sequence types. In order to

purify the phage for further experiments, ten phage CP30 isolates were recovered from single plaques and characterised using PCR with specific primers designed to discriminate the sequence types. As a result, isolates 4, 5 and 7 were considered as phage CP30C. These phage isolates were further examined for any differences in their ability to replicate in different host strains. Different plaque phenotypes after infecting *C. jejuni* CII C3 and FDIC2 were observed for isolate 4 compared with other CP30 isolates. This isolate was selected to definitively represent phage CP30C since it was demonstrated to be genotypically and phenotypically different to CP30A and CP30B. This phage was propagated and the genome sequence determined. After whole genome sequencing and data assembly, an alignment was made for genome sequence of phage CP30C, with the available genome sequences for CP30A and CP30B. Most genome regions for the CP30 phage derivatives retained identity, whereas major variation was observed in the region from 3kb to 4.5kb, which encodes three hypothetical proteins (D302_gp004, D302_gp005, D302_gp006), between phage CP30A and CP30B / C, and from 124kb to 125kb, which encodes another two hypothetical proteins (D302_gp148, D302_gp149), between phage CP30A/ B, and CP30C. These genes were individually aligned with the *Campylobacter* phage NCTC12673 present in the NCBI database.

In the region from 3kb to 4.5kb, majority of the genes in phage CP30A, B and C were conserved with NCTC12673. However, gene *NCTC12673_gp001*, was present in CP30A as a homologue *D302_gp006*

but absent in both phage CP30B and CP30C. Instead, *NCTC12673_gp002* gene was observed in both phage CP30B and CP30C but not in CP30A. This was considered as the reason for the genome variation observed between phage CP30A and CP30B/ C in this particular region. In the genome region between 124kb and 125kb of phage CP30A derivatives. Most of the genes were also identical between the CP30 phage derivatives and NCTC12673. However, gene *D302_149* was only present in both phage CP30A and CP30B, but not in phage NCTC12673 and CP30C. This inserted gene in phage CP30A and B was thought to be the cause for the variation observed between phage CP30A/ B and CP30C. Besides the regions stated above, another large variation was also observed in the phage CP30B genome at coordinates between 121kb to 121.5kb compared to CP30A based on whole genome alignment. However, no gene insertion or deletions were observed after alignment, instead a number of point mutations were present.

We have examined several genes for which the products are likely to affect phage morphology, including the major capsid protein, major head protein II, head completion protein, neck protein, putative tail tube protein, tail sheath protein, tail completion and sheath completion protein and a putative baseplate wedge subunit, and most of the genes are generally well conserved between phage CP30A and CP30C but with several single base substitutions. Based on this, we predict the morphology and host interactions are likely to be similar. However, in gene *D302_gp050*, that encodes putative tail fibre protein, a single

cytosine deletion was observed in residue 10 of the gene that leads to a potential frameshift. An alternative start codon is positioned downstream of the frameshift, translation initiation from which would produce a protein missing 44 amino acids from the N-terminal end. The Swiss-prot protein modelling for the N terminal of protein D302_gp050 suggesting that the missing 44 amino acid sequence would form one alpha helix and three beta sheets.

Gene *gp047* of *Campylobacter* phage NCTC12673, is the homologue of *gp050* in phage CP30C. The product of *gp047* has been identified as a putative tail fibre protein, of which the C-terminal quarter has been proposed as a putative receptor binding protein (Javed et al., 2013). However, the failure of an anti-gp047 antibody to bind to the NCTC12673 phage particle (Javed et al., 2015b), and the absence of Gp047 upon proteomic analysis by mass spectrometry of purified phage virions (Kropinski et al., 2011), suggests that Gp047 is not a structural component of the phage particle. Although evidence suggests that phage NCTC12673 binds to capsular polysaccharides of host cells (Coward et al., 2006), Gp047 was found to specifically bind acetamidino-modified pseudaminic acid on the host flagella, which results in host cell agglutination and motility reduction upon binding (Javed et al., 2015b). It is reported that the Gp047 protein has a much broader host recognition and binding range than the phage from which it was derived. Therefore, instead of a receptor binding protein, Javed et al. (2015a) hypothesised Gp047 of phage NCTC12673 functions as an extracellular effector protein,

which is released after cell lysis followed by binding nearby bacterial cells to reduce their motility and further allow an increase in the binding frequency of released phage progeny.

In the phage susceptibility tests presented within this chapter, we observed that instead of forming semi-confluent lysis like CP30A, phage CP30C was unable to form plaques on host strains *C. jejuni* CII C3 and FDIC2. This could potentially be caused by a defect in the ability of phage CP30C to bind bacterial acetamidino-modified pseudaminic acid decorated flagellar filaments, owing to the mutation present in *gp050.*

Flagellins from *Campylobacter* spp. are decorated with as many as 19 O-linked glycans and can contribute to approximately 10% of the flagellin molecular weight (Thibault et al., 2001). Most *Campylobacter* spp. are able to synthesise two distinct nine carbon sugars to decorate flagellin: pseudaminic acid and legionaminic acid (Logan et al., 2008). Previously, Dr. Andy Scott used flagellin peptide mass fragment (PMF) spectra with tryptic digested FlaA protein as a precursor molecule to determine the types of modification present on flagella of *C. jejuni* OCII F1, OCII F3 and OCII C10 isolates from broiler flocks (Scott, 2006). These strains originate from the broiler house study of Connerton et al. (2004) and can be discriminated on the basis of their sensitivity to CP30A infection (Scott et al., 2007a). The chemical decoration of the flagellin subunit was first predicted on the basis of the best characterised *C. jejuni* strain 81-176, which was known to lack the legionaminic acid (Leg) synthesis pathway,

only synthesising pesudaminic acid (Pse), with acetamidino-modified Pse as a major modification, which is bound by the recombinant protein of Gp047 fused to glutathione S-transferase (Stephenson et al., 2014; Javed et al., 2015a). The peptide fragments identified by Scott (Scott, 2006) as glycosylated are located at positions 464-480 TTAFGVK/DETAGVTTLK within flagellin. In *C. jejuni* OCII F1, the peptide of DETAGVTTLK is modified by the addition of three moieties predicted as 5-acetamidino,7-acetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (Pse5Am7Ac). Whilst, for *C. jejuni* OCII F3 and OCII C10, the peptide of TTAFGVK / DETAGVTTLK is modified with the addition of a single moiety, which is predicted as 5,7-diacetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (Pse5Ac7Ac). Both predictions are based on similarities with the work reported by Thibault et al. (2001). Scott also suggested that the differential modifications would be significant for phage binding, and demonstrated that the modification with the three moieties (Pse5Am7Ac) was associated with non-binding by phage CP30A, whilst the addition of single moieties (Pse5Ac7Ac) modification was conducive for phage CP30A binding.

Due to the variation in Pse modification of flagellin, these host strains provide a possibility to characterise the consequences of the defective Gp050 in the absence of the N-terminal region present in phage CP30C. A susceptibility test of phage CP30A and CP30C was carried out on *C. jejuni* host strains OCII F1, OCII F3 and OCII C10 to characterise their infection abilities. Phages CP30A and CP30C were able to infect and lyse *C. jejuni* CII

F3 and OCII C10 cells, which resulted in confluent lysis of the bacterial lawns. However, neither phage could infect *C. jejuni* OCII F1. Therefore, we conclude that the N-terminal 44 amino acids do not influence the ability of Gp050 to differentially promote infection of the susceptible *C. jejuni* exhibiting differential flagellin glycosylation patterns. This result is consistent with the findings reported by Javed et al. (2015a), as only C-terminal quarter of Gp047 (residues 1040-1365) in phage NCTC12573 is responsible for its binding activity. The different susceptibility of phage CP30C than CP30A on host strains *C. jejuni* CII C3 or FDIC2 needs to be investigated further.

CHAPTER 5

Genome variation of *Campylobacter jejuni* in the bacteriophage carrier state

5.1 Introduction

Bacteriophage life cycles are generally described as either lytic or lysogenic. During the lytic cycle, after initial infection, phage DNA redirects the host metabolism to express and replicate phage DNA, assembles the phage progeny, before eventual host cell lysis. Whilst, lysogenic phage can integrate their DNA into the host genome and replicate with the host, these phages retain the ability to enter the lytic cycle, often at times of stress on the host. However, there are other alternative life cycles (reviewed by Abendon, 2009). Although the functional classification and terminology applied to alternative phage life cycles can be confused due to their inherent instability, pseudolysogeny and carrier state are the terms used most frequently to describe these associations (Adams, 1959; Lwoff, 1953).

Ripp and Miller (1997) defined the life cycle of pseudolysogeny as a host phage interaction that phage nucleic acid neither entered in to a lytic cycle to redirect host metabolism, nor functioned as lysogen to enter a long-term relatively stable relationship, but remained as a non-active state within the host cell. The pesudolysogeny life cycle is commonly related with starvation conditions, and is able to re-enter either the lytic or lysogenic life cycle when nutrients are replenished (Ripp and Miller, 1998; Los et al., 2003).

The carrier state life cycle (CSLC) describes a situation where the host bacteria and bacteriophage persist in an equilibrium, where some of the bacteria are resistant to phage infection whilst others are sensitive and support phage replication, with the result that bacteria and phage maintain similar population levels in serial culture (Siringan *et al.,* 2014). CSLC conforming to this definition have been identified within various host strains including *Shigella dysenteriae, Brucella abortus, Proteus mirabilis, Salmonella Typhimurium* (Li et al., 1961; Jones et al., 1962; Coetzee and Hawtree, 1962; Cenens et al., 2013).

The study of CSLC in *C. jejuni* PT14 was initiated by Siringan et al. (2011) when determining the dispersive ability of Class III *Campylobacter* specific phage on *Campylobacter* biofilms. Siringan et al. (2011) found that bacteria recovered from biofilms were capable of spontaneously generating phage by cell lysis. With a combination of PFGE and southern blot approaches, they confirmed that the phage DNA had not integrated into the host chromosome, and concluded that phage genomes were in the form of non-integrated elements within cell cultures. After examination of the *Campylobacter* levels from the caecal contents of chickens experimentally colonised with CSLC bacteria, they were able to confirm that CSLC were unable to colonise (Siringan et al., 2014). Later Brathwaite et al. (2015) found that CSLC bacteria during early-logarithmic phase were non-motile and further resulted in a reduced ability to adhere and infect HCA-7 colonic epithelial cells. A significant downregulation of the *flaA* gene encoding major flagellin of *C. jejuni* was

observed in CSLC in transcriptional studies, and is thought to be responsible for the non-motile phenotype (Brathwaite et al., 2015). On the other hand, the *flaB,* that encodes minor flagellin, revealed an increase in expression.

This chapter describes genetic variation in *Campylobacter* CSLC as compared with their parental strains using WGS. The universal phage host strain *C. jejuni* PT14 and the broiler chicken isolate *C. jejuni* HPC5 were used as host strains.

5.2 Results

The *Campylobacter jejuni* PT14CP8, PT14CP30A, HPC5CP8 and HPC5CP30A CSLC strains were previously isolated from biofilms by Siringan et al., (2014). Selected *Campylobacter* strains were first cultured on glass coverslips inside Petri dishes flooded with Mueller-Hinton broth at 42˚C under microaerobic conditions for 120 hours to allow biofilm formation, before applying phages to the biofilm and incubating at 42˚C under microaerobic conditions for a further 24 hours. Bacterial cells were detached from the glass coverslip and subsequently resuspended in MRD before measuring the respective count and titre of the *Campylobacter* and phage in the suspensions Picking 100 *Campylobacter* colonies from the enumeration plates and determining their phage sensitivity and ability to produce phage capable of infecting naïve host bacteria enabled estimates of the frequency of phage resistance amongst other surviving bacteria. Isolates that were continuously able to produce plaques after a minimum

of five generations were considered as having entered the carrier state life cycle.

5.2.1 Motility test

After isolation, the motility of *Campylobacter* carrier state cultures were individually determined (Figure 5.1).

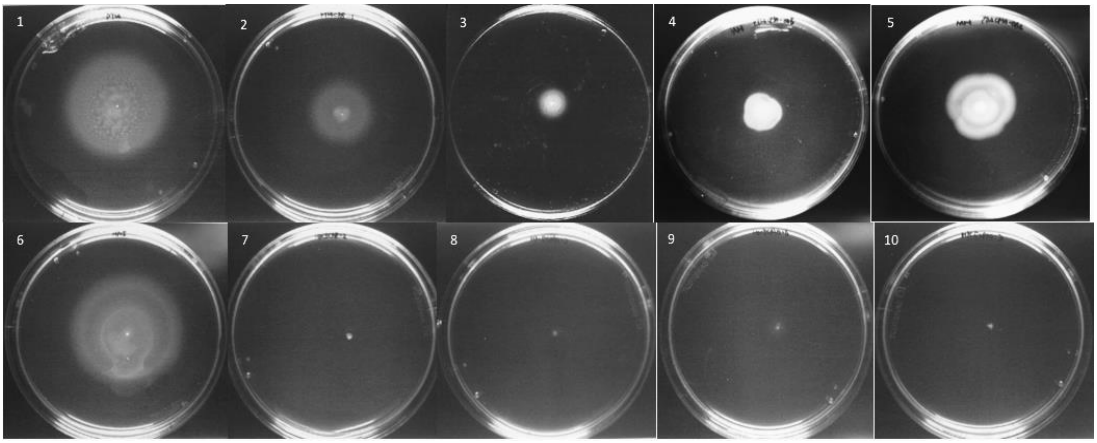


Figure 5.1 Motility test on *C. jejuni* PT14, HPC5 and their carrier state isolates. 1. *C. jejuni* PT14 wild type, 2. *C. jejuni* PT14CP8CS, 3. *C. jejuni* PT14CP30ACS, 4. *C. jejuni* PT14CP30A-035CS, 5. *C. jejuni* PT14CP30A-064CS, 6. *C. jejuni* HPC5 wild type, 7. *C. jejuni* HPC5CP8CS, 8. *C. jejuni* HPC5CP30ACS, 9. *C. jejuni* HPC5CP30A-6CS, 10. *C. jejuni* HPC5CP30A-7CS.

As shown in Figure 5.1 both *C. jejuni* PT14 and HPC5 were fully motile as expected. The *C. jejuni* PT14CP8CS was motile but with significantly reduced motility than wild type (Figure 5.1-2). Severely impaired motility was observed in *C. jejuni* PT14CP30ACS with aggregation observed at the centre of the motility plate. Similar phenotypes were observed for the independent isolates PT14CP30A-035 and PT14CP30A-064 but with pronounced irregular edges to the growth fronts. All the HPC5 carrier state variants were profoundly non-motile. In order to investigate the

genetic variations present in CSLC *Campylobacter*, genomic DNA was isolated from *C. jejuni* PT14CP8CS, PT14CP30ACS and PT14CP30A-035CS from which libraries were prepared and sequenced using the MiSeq platform.

5.2.2. Genome variation between *C. jejuni* carrier state strains

Whole genome sequencing was carried out for *C. jejuni* PT14CP8, PT14CP30A, PT14CP30-035, and the sequences subsequently aligned with their parental genomes. Several genetic modifications were identified using the variant detection algorithm available within CLC Genomics workbench (version 9.0). In *C. jejuni* PT14CP30ACS, an adenine to guanine substitution at nucleotide 1102 of the *flhF* gene was observed, which results in T368A substitution that could be linked with the non-motile phenotype of the carrier state strain. This substitution was present in all sequences from PT14CP30ACS (Figure 5.2).

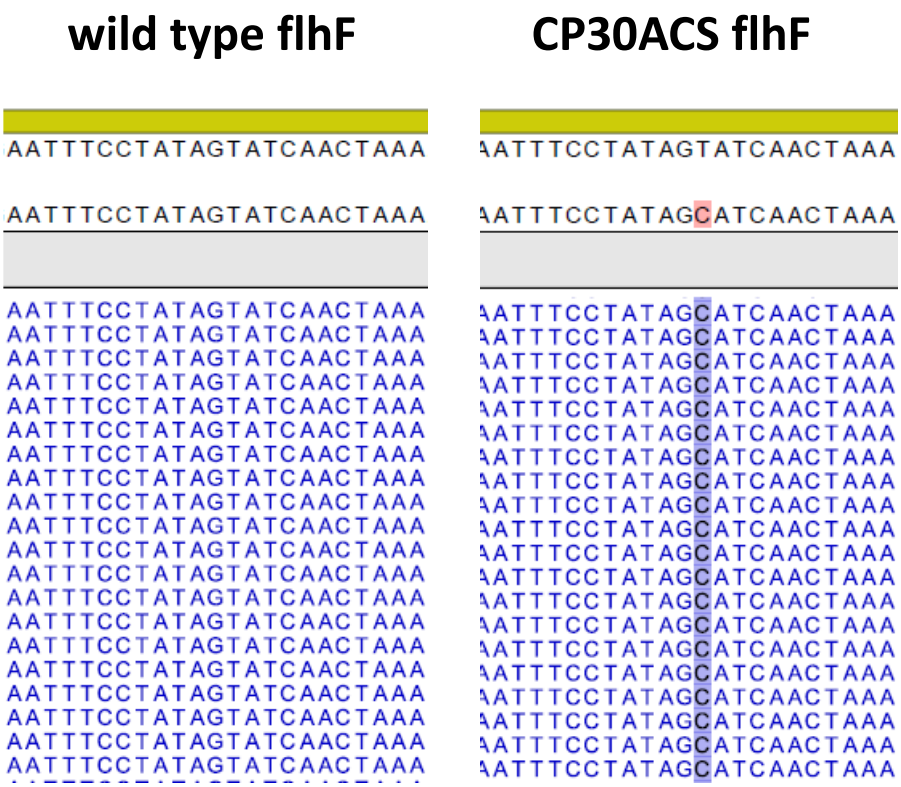


Figure 5.2 Whole genome sequencing output from CLC Genomics workbench after mapping to the PT14 genome showing part of complementary strand of the *flhF* gene from wild type *C. jejuni* PT14 and PT14CP30ACS. Highlighted is the nucleotide location showing the thymine was changed into cytosine in *C. jejuni* PT14CP30ACS. The multiple sequences at the bottom of the figure illustrated different paired-end sequence reads obtained from whole genome sequencing are identical within this region.

Two further independent isolates were available from contemporary laboratory stocks. Genomic DNAs were prepared and the *flhF* gene PCR amplified and subject to Sanger sequencing. These data confirm independent selection of the *flhF* mutation compared to wild type *C. jejuni* PT14 (Figure 5.3). The initial sequencing result from *C. jejuni* PT14CP30ACS-3 revealed a mixed population indicated by the chromatogram containing wild type flhF and the mutant. However, the phenotypes could be separated on the basis of motility. *C. jejuni* PT14 wild type was easily recovered from the leading edge of growth on a motility plate and confirmed by PCR sequencing.

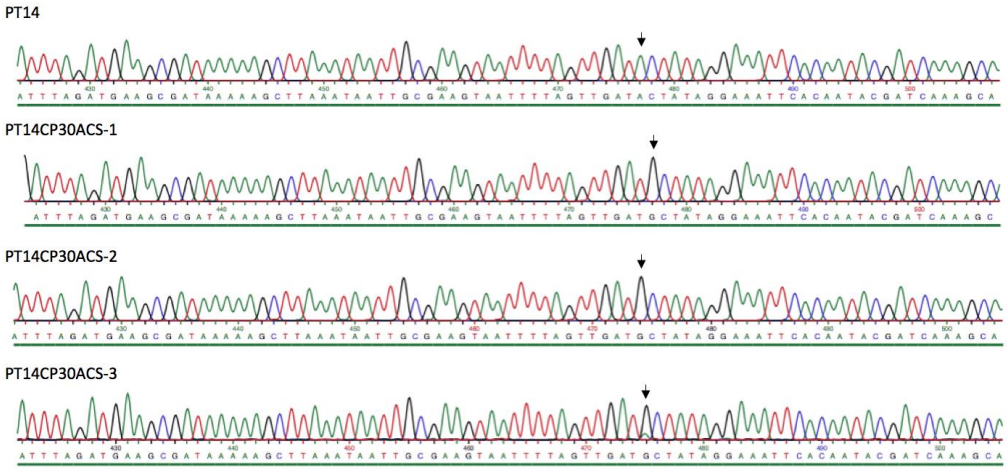


Figure 5.3 Chromatograms of Sanger sequencing for *flhF* gene from *C. jejuni* PT14 and three independent isolates of *C. jejuni* PT14CP30ACS. Arrowheads indicated the substitution from adenine in *C. jejuni* PT14 to a guanine in *C. jejuni* PT14CP30ACS.

A single adenine deletion was detected in the *C. jejuni* PT14CP30A-035 carrier state strain at nucleotide 325 of the gene A911_00360 that encodes L-lactate permease. The nucleotide deletion results in frameshift that disrupts the reading frame of A911_00360 (appendix 21). The frameshift would prevent translation from the predicted N-terminus but would leave an internal initiation codon intact to translate a protein missing the N-terminal 110 amino acids (appendix 22). The predicted protein structures of the *C. jejuni* PT14CP30A-035CS L-lactate permease reading frame and the wild type are shown in Figure 5.4. As illustrated by the protein structure modelling, deletion of the N- terminal 110 amino acid leads to the absence of several alpha-helices in the protein structure.

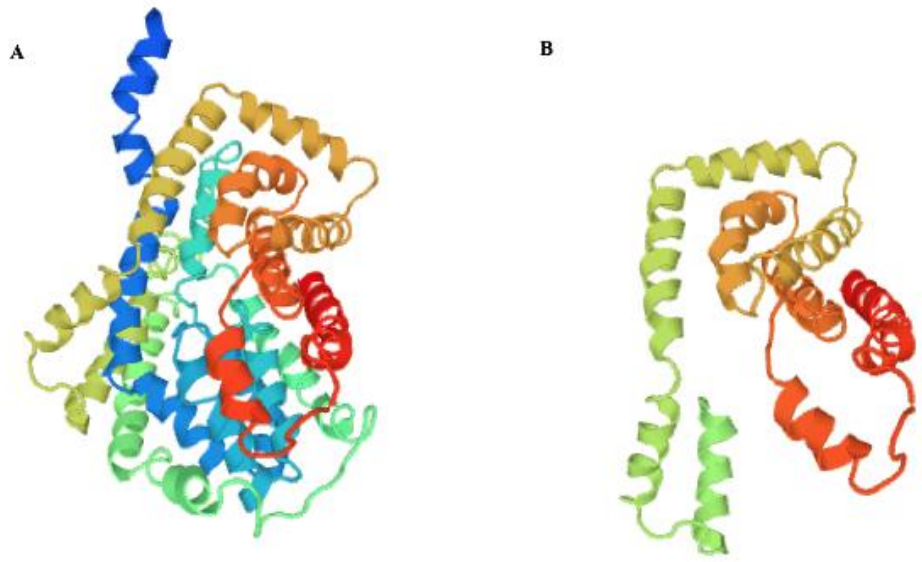


Figure 5.4 Protein structure modelling of gene A911_00360 using Swiss prot modelling system based on template AbgT putative transporter family (4r0c.1.A). A. *C. jejuni* PT14, B. *C. jejuni* PT14CP30A-035,

For *C. jejuni* PT14CP8CS, a single thymine deletion at nucleotide 297 to create a frameshift in the gene A911_05040 that encodes a putative transcriptional regulatory protein containing an AraC-type DNA-binding domain (appendix 23).

5.3 Discussion

Based on the motility plate assays, *C. jejuni* PT14CP8, PT14CP30A and PT14CP30A-035 carrier state strains revealed impaired motility phenotypes compared to wild type *C. jejuni* PT14. Whole genome sequence studies were undertaken to investigate any genetic variations responsible for these phenotypes. After genome alignment, *C. jejuni* PT14CP30A-035 revealed a single adenine deletion was observed in gene A911_00360 that encodes L-lactate permease. This adenine deletion causes a frameshift mutation and the translated protein would have N-

terminal 110 amino acids deleted and further leads to the absence of several alpha-helices in the predicted protein structure.

Since L-lactate permease functions as a transporter for L-lactate across the membrane and plays a significant role in *C. jejuni* metabolism. The frameshift mutation of *A911_00360* could impair L-lactate uptake and catabolism in the carrier state strain *C. jejuni* PT14CP30A-035 and could even potentially affect the swarming motility. However, Thomas et al. (2011) reported that *C. jejuni* are likely to possess an alternative route for L-lactate transport, since inactivation of the *C. jejuni* NCTC11168 homologue *cj0076c* did not eliminate the transport of L-14C-lactate but resulted in a reduction of approximately 30%. In this case, the impaired motility of *C. jejuni* PT14CP30A-035 is not likely to be caused by frameshift mutation in L-lactate permease encoding gene *A911_00360*, but could be epigenetic modifications present in this strain.

Whole genome sequencing of the carrier state *C. jejuni* PT14CP30ACS identified a point mutation showing the substitution of adenine to guanine within the *flhF* gene (A911_00305) that corresponds to the amino acid substitution of T368A in FlhF as compared to the wild type sequence from *C. jejuni* PT14. The *flhF* genes of three further independent isolates of *C. jejuni* PT14CP30ACS were PCR amplified and sequenced to reveal the same mutation (appendix 26). Deletion of the *flhF* gene of *C. jejuni* 81-176 results in a non-motile phenotype, and the FlhF protein has been reported to function in the control of flagella biosynthesis (Balaban

& Hendrixson, 2011). It may be hypothesised that the FlhF substitution present in PT14CP30ACS carrier state cultures could contribute to their non-motile phenotype.

CHAPTER 6

FlhF mutation is responsible for the non-motile phenotype of carrier state PT14CP30ACS

6.1 Introduction

Flagella biosynthesis is a complex process that requires the coordinate expression of over fifty genes to produce a functional flagella (Chevance & Hughes, 2008). Flagellar export in *C. jejuni* is controlled by a two-component regulator FlgSR, where the FlgS histidine kinase autophosphorylates to activate the response regulator FlgR. Phosphorylated FlgR in conjunction with σ^{54} –RNA polymerase activates the transcription of genes encoding flagellar components including the flagellar hook and hook-associated proteins, and the minor flagellin FlaB. FlgSR and σ^{54} are also required for the full expression of σ^{28} –dependent genes involved in flagellar biosynthesis including the expression of *flaA* encoding the major flagellin protein (Lertsethtakarn et al., 2011; Gilbreath et al., 2011). Presumably at the front of the transcriptional cascade, several specific flagellar genes are required for σ^{54} transcription, including *flhA, flhB, flip, fliR* as well as *flhF* (Hendrixson & Dirita, 2003). FlhF is reported to impart spatial and/ or numerical control of flagella biosynthesis in *Campylobacter jejuni* (Balaban & Hendrixson, 2011) and to function at an early stage of flagella biosynthesis (Green *et al*., 2009).

FlhF together with the signal sequence-binding protein SRP54 (or Ffh in bacteria) and signal recognition particle (SRP) receptor FtsY, form a unique subfamily within the SIMIBI-type nucleoside triphosphate-binding protein class referred to as SRP GTPase proteins that are universally conserved (Leipe et al., 2002; Zanen et al., 2004). Unlike SRP54 and FtsY that function as a SRP-SRP Receptor (SRP-SR) heterodimer in the

presence of GTP, FlhF proteins form a stable homodimer structure through interaction of their GTP binding domains (G domain) (Bange et al., 2007). The G domain is located in the C-terminus of FlhF, which is conserved with SRP54 and FtsY. FlhF also contains conserved B and N domains. The B domain functions to regulate dimerization in the presence of GTP (Bange et al., 2007), whilst the N domain is thought to stabilise the GTP bound state by rearranging the SRP conformation upon ribosome binding and further prime it for the formation of subsequent homodimer or heterodimer complex (Halic et al., 2006). The G domain is where the GTP molecules bind, and within which it contains five conserved nucleotide-binding elements (G1-G5) (Eichler and Moll, 2001). The functions of the SRP GTPases, including regulation of translation, protein translocation, cell polarity, signal transduction and possibly cell division, are established (Bulyha *et al.*, 2011).

Carrier state *C. jejuni* PT14CP30ACS show impaired motility and the development of a growth phase dependent sub-population in broth culture that are associated with phage resistance (Siringan *et al.,* 2014). Transmission electron microscope (TEM) images show the non-motile bacteria to have truncated flagella (Siringan *et al.,* 2014). However, the reason for this defect in flagellar biosynthesis remains unknown. The previous chapter identified a point mutation within the *flhF* gene of *C. jejuni* carrier state cultures harboring bacteriophage CP30A. This chapter examines the effect of the *flhF(T368A)* allele on flagella function and phage infection of *C. jejuni* PT14.

6.2 Results

6.2.1 The *flhF(T368A)* mutation is a feature of carrier state PT14CP30ACS

Whole genome sequencing of the carrier state *C. jejuni* PT14CP30ACS identified the point mutation T368A in FlhF as compared to the wild type sequence of *C. jejuni* PT14. The NCBI database currently contains 604 protein sequences of FlhF from *Campylobacter* and *Helicobacter* species, which are well conserved and all of which feature a threonine residue at the equivalent sequence position. The substitution is therefore unique and has the potential to modify the function of FlhF.

The observed T368A mutation is identified in the third GTP binding element of the G domain within FlhF, where the threonine residue is universally conserved within different *Campylobacter* species (Table 6.1).

Table 6.1 Claustal Omega alignment of G3 domains in FlhF between several *Campylobacter* species, the highlighted amino acid shows the location of the conserved threonine changed to alanine in *C. jejuni* PT14CP30CS .

Species name	G1 domain	G2 domain	G3 domain
C. jejuni	288 GPTGVGKT 295	321 DTYR 324	367 DTIGN 371
C. coli	280 GPTGVGKT 287	313 DTYR 316	359 DTIGN 363
C. iguaniorum	238 GPTGVGKT 245	272 DTYR 275	318 DTMGS 322
C. fetus	235 GPTGVGKT 242	269 DTYR 272	315 DTMGS 319
C. volucris	266 GPTGVGKT 273	299 DTYR 302	345 DTTGN 349
C. subantracticus	264 GPTGVGKT 271	297 DTYR 300	343 DTTGN 347
C. peloridis	264 GPTGVGKT 271	297 DTYR 300	343 DTTGN 347
C. lari	264 GPTGVGKT 271	297 DTYR 300	343 DTTGN 347

We therefore hypothesise that the *flhF(T368A)* mutation contributes to the impaired motility phenotype exhibited by *C. jejuni* PT14CP30ACS isolates.

6.2.1 Construction of non-functional *flhF* mutants

Non-functional *flhF* mutant*s* were first constructed to determine the role of *flhF* in *Campylobacter* motility (Figure 7.1). Briefly, the wild type *flhF* gene was first PCR amplified from *C. jejuni* PT14 and cloned into TOPO pCR2.1 vector. The *flhF* clone sequence was confirmed by DNA sequencing (appendix 27, 28). The plasmid construct pCR2.1::*flhF* was then digested with the restriction enzyme EcoRI to obtain an EcoRI- *flhF*-EcoRI fragment. This fragment was ligated into pUC4K vector, which was previously digested with EcoRI to produce an EcoRI- pUC4kΔ*kan*- EcoRI backbone. The construct was linearised by digestion with the restriction enzyme XbaI followed by ligation with a kanamycin cassette into the XbaI site to form the plasmid construct pUC4KΔ*kan*::*flhF::kan* (Figure 6.1, 6.2A). This construct was examined for the presence of the kanamycin resistance cassette within *flhF* gene by PCR amplification using flanking *flhF* primers.

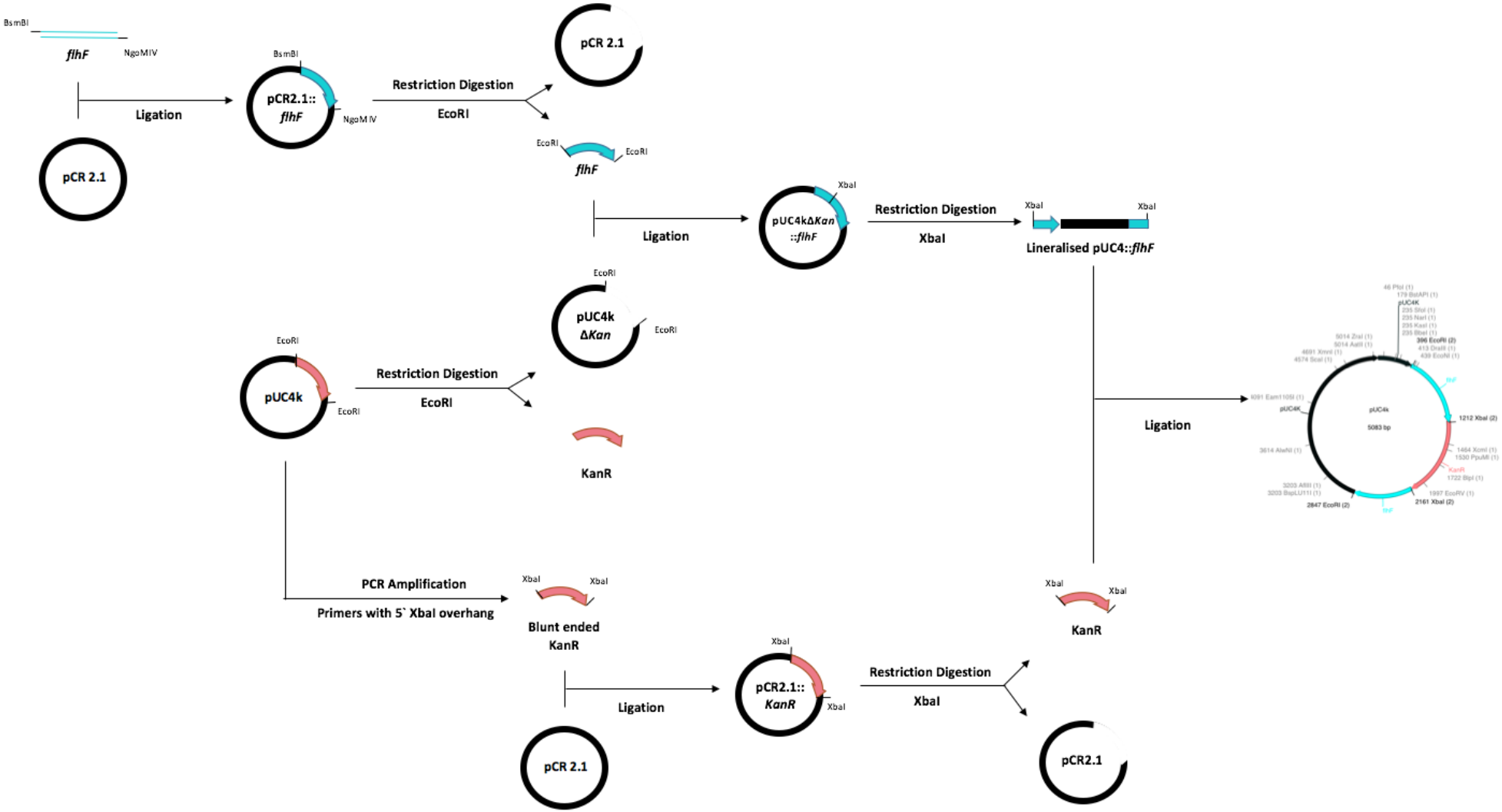


Figure 6.1 Diagram illustrates the cloning strategy to construct pUC4KΔ*kan*::*flhF*::*kan*. The final construct pUC4KΔ*kan*::*flhF*::*kan* was naturally transformed into *C. jejuni* to form non-functional *flhF* mutants.

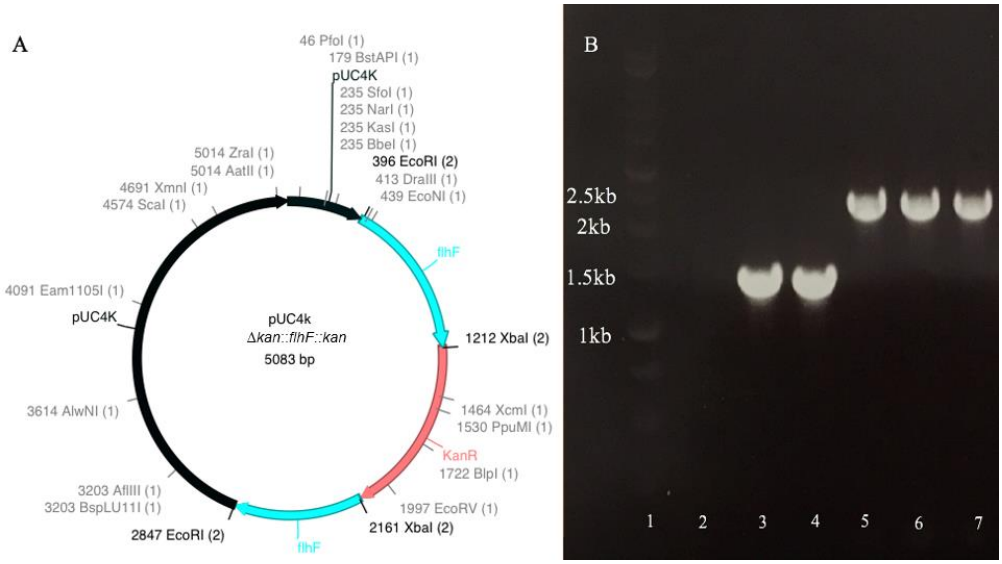


Figure 6.2 *flhF* inactivation plasmid construction A. Map of the construct pUC4KΔ*kan*::*flhF::kan* B. PCR products of the *flhF* gene. lane 1. 1kb DNA ladder; 2. negative control; 3. positive control of *C. jejuni* PT14 genomic DNA; 4. pUC4KΔ*kan*::*flhF*; 5. sample 1 PT14*flhF::kan* genomic DNA; 6. sample 2 PT14*kan*::*flhF::kan* genomic DNA; 7. sample 3 PT14*flhF::kan* genomic DNA. PCR products in lane 3, 4 revealed bands with approximately 1.5kb size indicating wild type *flhF* whilst the bands in lane 5, 6, 7 with a size of approximately 2.5kb suggesting the presence of kanamycin cassette within *flhF.*

The plasmid construct was then naturally transformed into *C. jejuni* PT14 wild type with the aim of targeting cross-over of the *flhF* sequences flanking the kanamycin cassette. However, no colonies were observed on kanamycin selective blood agar plates after incubation. As *C. jejuni* NCTC 11168 is widely used strain for the construction of mutants by natural transformation, and the *flhF* sequences are completely conserved, the construct pUC4KΔ*kan*::*flhF::kan* was transformed into *C. jejuni* NCTC 11168. After five days' incubation, putative transformant colonies were obtained and PCR analysed for insertion into the *flhF* gene. Genomic DNA from one of several correct *flhF* inactivated mutants was isolated and used for natural transformation of *C. jejuni* PT14 wild type. After

transformation and kanamycin selection, successful transformants were again confirmed by colony PCR examining the presence of the kanamycin cassette within *flhF* (Figure 6.2B). As controls, *C. jejuni* PT14 and pUC4KΔ*kan*::*flhF* amplicons revealed a fragment size of approximately 1.4kb, which is consistent with the length of the native amplified *flhF* gene. Compared with the *flhF* amplicon from *C. jejuni* PT14 wild type and the *flhF* gene containing plasmid, the putative transformant genomic DNAs were approximately 1kb larger, which this is equal to the fragment size of the kanamycin resistant cassette (949bp) inserted in the *flhF* gene. These three transformants were then considered as representing *C. jejuni* PT14::*flhF::kan.*

6.2.2 Construction of *flhF* complementation mutants

In order to determine the role of the *flhF* gene product on the formation of a functional flagellum in *Campylobacter,* and how *flhF(T368A)* would affect the motility of *Campylobacter, flhF* complementation mutants were constructed with either *flhF* or *flhF(T368A)* alleles (figure 6.3). The ectopic complementation of *flhF* and *flhF(T368A)* was carried out in pseudogene *Cj0046* (*A911_00230*) to prevent adversely affecting any functional genes. Wild type *flhF* and *flhF(T368A)* were PCR amplified from *C. jejuni* PT14 wild type and PT14CP30ACS respectively, and cloned into TOPO pCR2.1 vector. The sequences of the *flhF* and *flhF(T368A)* insertions were respectively confirmed in the constructs of pCR2.1::*flhF* and pCR2.1::*flhF(T368A)* by PCR sequencing with internal *flhF* primers and

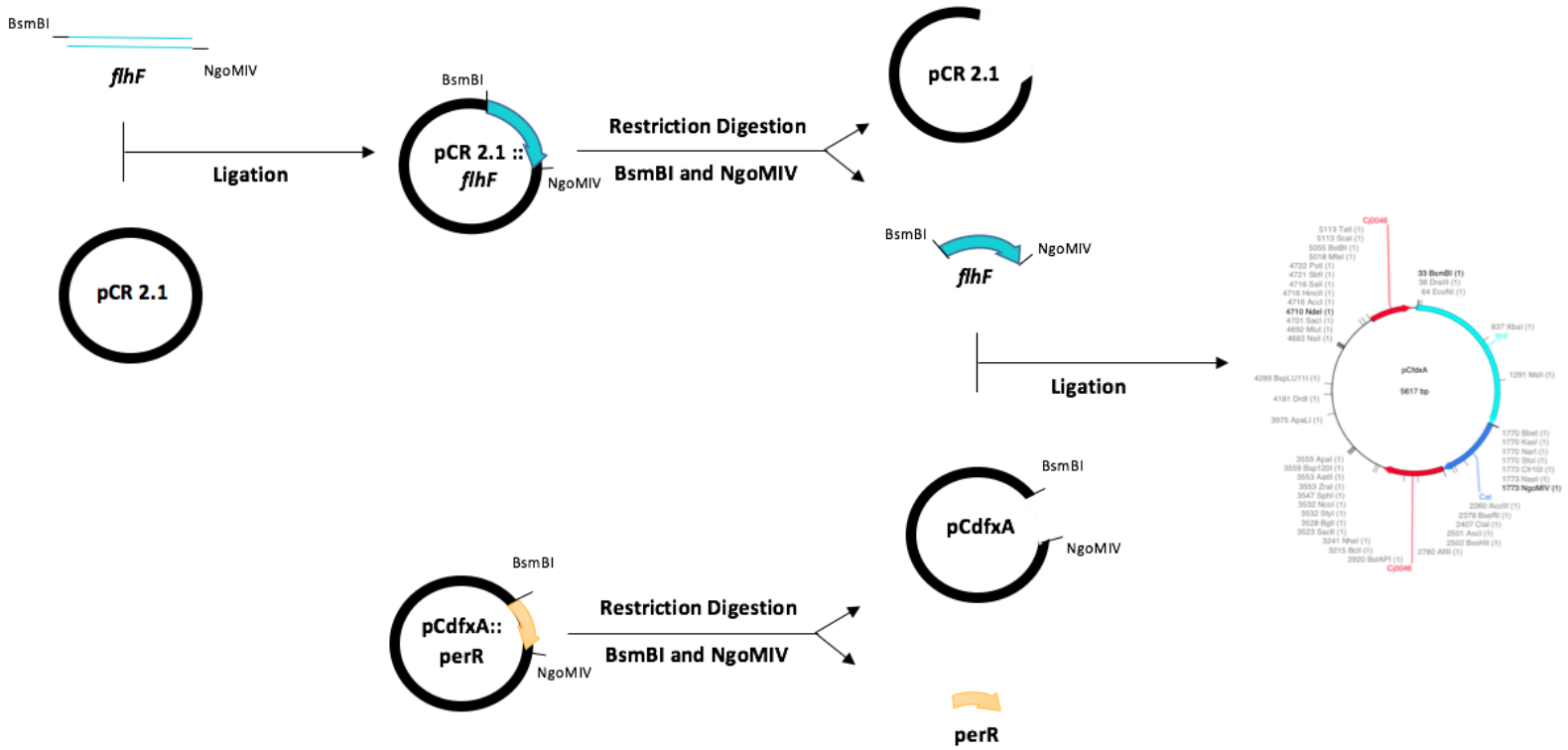


Figure 6.3 Diagram illustrates the cloning strategy to construct pCfdxA *00230*::*flhF-cat*. The final construct pCfdxA *00230*::*flhF-cat* was naturally transformed in *C. jejuni* to form ectopic *flhF* complementation. The *flhF(T368A)* ectopic complementation was constructed using the same cloning strategy.

M13 commercial primers. The sequencing results were aligned with *flhF* sequence in *C. jejuni* PT14 from the database (http://www.ncbi.nlm.nih.gov/nuccore/CP003871.4) to demonstrate the clones were intact and contained the anticipated DNA sequences (appendix 27, 28). PCfdxA::perR vector was digested with BsmBI and NgoMIV to produce the pCfdxA backbone, which contains the flanking *Cj0046 (A911_00230)* pseudogene. DNA fragments containing *flhF* and *flhF(T368A)* were then individually cloned into the pCfdxA vector to form constructs of pCfdxA *00230*::*flhF-cat* and pCfdxA *00230*::*flhF(T368A)-cat* (Figure 6.3, 6.4A). These constructs were then respectively examined for the presence of *flhF* within the vector *Cj0046* pesudogene by PCR (data not shown).

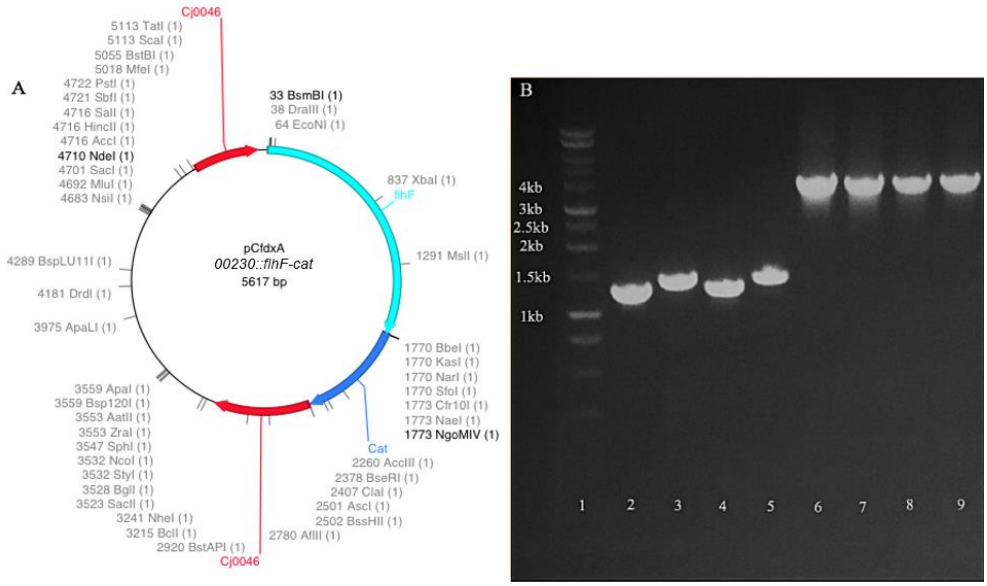


Figure 6.4 *flhF* complementation plasmid construction A. Map of the construct pCfdxA *00230*::*flhF-cat* B. Colony PCR amplification of the Cj0046 pseudogene, lane 1, 1kb DNA ladder; 2, *C. jejuni* PT14; 3, *C. jejuni* NCTC11168; 4, *C. jejuni* PT14*flhF::kan*; 5, *C. jejuni* NCTC11168*flhF::kan*; 6, *C. jejuni* PT14*flhF::kan 00230::flhF-cat*; 7, *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat*; 8. *C. jejuni* NCTC11168*flhF::kan 00230::flhF-cat*; 9. *C. jejuni*

NCTC11168*flhF::kan 00230::flhF(T368A)-cat.* The band size in lane 2, 4 and 3, 5 matches the PCR amplicon size of *Cj0046* in *C. jejuni* PT14 and NCTC11168 respectively indicating the presence of *Cj0046.* The sizes of bands in lane 6, 7, 8, 9 are approximately 2.4kb larger than wild type amplicon suggesting the presence of *flhF-cat* within *Cj0046* gene.

The constructs pCfdxA::*flhF* and pCfdxA::*flhF(T368A)* were naturally transformed into *C. jejuni* PT14*flhF::kan* and *C. jejuni* NCTC11168*flhF::kan* genomes by cross-over of flanking *Cj0046 (A911_00230)* pseudogene sequences. However, no colonies were observed on chloramphenicol selective blood agar plates after incubation in either case. To promote recombination the constructs were linearised with the restriction enzyme NdeI before natural transformation of *C. jejuni* PT14*flhF::kan* and *C. jejuni* NCTC11168*flhF::kan*. Using this approach transformants were obtained, which were then confirmed by examining the presence of *flhF* gene within the *Cj0046* or *A911_00230* pseudogenes via colony PCR with *Cj*0046 primers. As shown in Figure 6.4B, PCR products amplified from wild type *C. jejuni* PT14 and *C. jejuni* PT14*flhF::kan* showed a fragment size of approximately 1.2kb whilst fragments of 1.4kb in size were observed respectively for the *C. jejuni* NCTC11168 and *C. jejuni* NCTC11168*flhF::kan* amplicons. These results met the expected amplicon size of the *Cj0046/A911_00230* pseudogenes present in *C. jejuni* PT14 (1289bp) and *C. jejuni* NCTC11168 (1407bp). Notably, wild type *C. jejuni* NCTC11168 and *C. jejuni* NCTC11168*flhF::kan* are approximately 100bp larger than *C. jejuni* PT14 and *C. jejuni* PT14*flhF::kan*, which is due to a 118bp deletion present in *A911_00230* pseudogene of wild type *C. jejuni* PT14 compared to *cj0046* of *C. jejuni* NCTC11168. PCR products amplified from *C. jejuni* PT14(*flhF::kan 00230::flhF-cat)*, *C. jejuni* PT14(*flhF::kan*

00230::flhF(T368A)-cat), *C. jejuni* NCTC11168(*flhF::kan 0046::flhF-cat)* and *C. jejuni* NCTC11168(*flhF::kan 0046::flhF(T368A)-cat)* revealed fragment sizes of 3.8kb, which were roughly 2.4kb larger than the wild type amplicon. This is consistent with the size of *flhF-cat* insertion (2502 bp).

6.2.3 *FlhF* is required for motility and *flhF(T368A)* has a negative impact on motility.

In order to determine the role of *flhF(T368A)* on the motility of *C. jejuni* PT14CP30ACS, three *flhF* constructs were prepared in *C. jejuni* PT14. *C. jejuni* PT14*flhF::kan* was created by inserting a kanamycin resistance gene to inactive *flhF.* Complements of the *flhF::kan* mutant with either *flhF* wild type or *flhF(T368A)* alleles were prepared by insertion into the *A911_00230* pseudogene. As the *flhF* gene sequence is identical between *C. jejuni* PT14 and *C. jejuni* NCTC11168, similar constructs were prepared in *C. jejuni* NCTC11168 in parallel experiments.

The swarming motility of wild type *C. jejuni* PT14 and its *flhF* mutants were assessed on 0.4% agar plates. As expected, the wild type *C. jejuni* strain was fully motile (Figure 6.5A) and over 95% of cells were observed as having typical bipolar flagella when observed using transmission electron microscopy (TEM; Figure 6.5F). Inactivation of the *flhF* gene resulted in a non-motile phenotype (PT14*flhF::kan*) where flagella structures were absent from all the cells visualised by TEM (Figure 6.5B and G). The *flhF* mutant cells also appeared somewhat straighter than

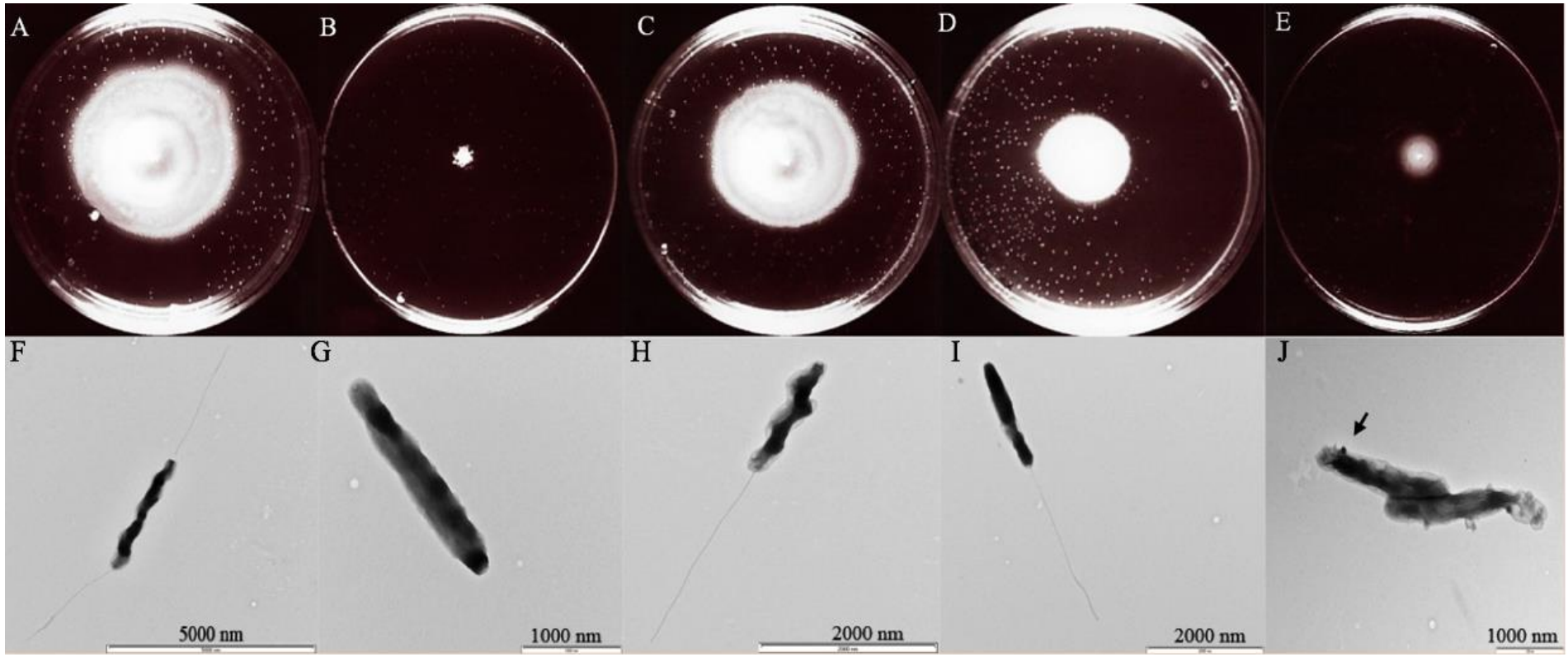


Figure 6.5 Motility plates and TEM images. A and F Wild type *C. jejuni* PT14; B and G *C. jejuni PT14flhF::kan;* C and H *C. jejuni* PT14*flhF::kan 00230::flhF-cat;* D and I *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat;* E and J *C. jejuni* PT14CP30ACS. The arrowhead in panel J indicates a bacteriophage binding to the capsule of a *C. jejuni* PT14CP30ACS cell.

wild type, which exhibited the spiral form that typifies campylobacters. Complementation of the *flhF* mutation in *C. jejuni* PT14(*flhF::kan 00230::FlhF-cat)* restored 90% motility compared to wild type *C. jejuni* PT14 with 88% cells exhibiting single polar flagella structures by TEM (Figure 6.5C and H). These cells had also notably regained spiral morphology. Alternatively complementation with *FlhF(T368A)* produced strains (*flhF::kan 00230::FlhF(T368A)-cat*) with approximately 50% of the swarming motility of the wild type with 14% of cells exhibiting single polar flagella structures by TEM but the majority did not regain the spiral morphology. (Figure 6.5D and I). By comparison the carrier state strain *C. jejuni* PT14CP30ACS was non-motile under these conditions and TEM revealed the cells to possess a truncated flagella phenotype (Figure 6.5E and J) with around 10% of the cells showing the flagella to be positioned aberrantly at a non-polar location. Phage CP30A was also observed adsorbed to the cell surface (Figure 6.5J).

The swarming motility of *C. jejuni* NCTC11168 and its *flhF* mutants were also determined. Similar to *C. jejuni* PT14, wild type *C. jejuni* NCTC11168 was fully motile whilst its *flhF::kan* mutant was non-motile. Complementation the *flhF::kan* mutant with either *flhF* or *flhF(T368A)* successfully restored its motility, and *flhF* complementation revealed a higher motility recovery than *flhF(T368A)* complementation (data not shown).

6.2.5 *FlhF(T368A)* negatively regulates the expression of both σ^{28} and σ^{54} dependent genes

To test if *flhF(T368A)* mutation would influence flagellar gene expression, quantitative reverse transcription PCR (RT-qPCR) was performed in this thesis. Insertion of the kanamycin gene within *flhF* resulted in 3-fold less expression of the native promoter of *flhF* as compared to wild type *C. jejuni* PT14 (Figure 6.6). The *flhF* complement (*flhF::kan 00230::flhF-cat)* restored the expression of *flhF* transcription to that of wild type *C. jejuni* PT14 (Figure 6.6). However, complementation with *flhF(T368A)* produced transformants that achieved maximally 80% of the *flhF* transcript level of wild type (Figure 6.6). The gene for *flhG* is located directly downstream of *flhF*, and is reported to encode an ATPase that represents a member of the ParA superfamily of proteins that regulate cell division, and in the case of *C. jejuni* mediates the site and numerical control of flagellar synthesis (Gulbronson et al., 2015). Inactivation of *flhF* reduced the relative transcription of *flhG* to 39% of wild type. Transformants carrying ectopic copies of either *flhF* or *flhF(T368A)* did not restore *flhG* transcription to wild type levels but significantly increased the transcription of *flhG* compared to the *flhF* mutant. The increase in transcription of *flhG* as a consequence of second site *flhF* expression would militate against a polar effect of the kanamycin gene insertion and suggests either activation of the promoter of *flhF* and *flhG* or an increase in transcript stability.

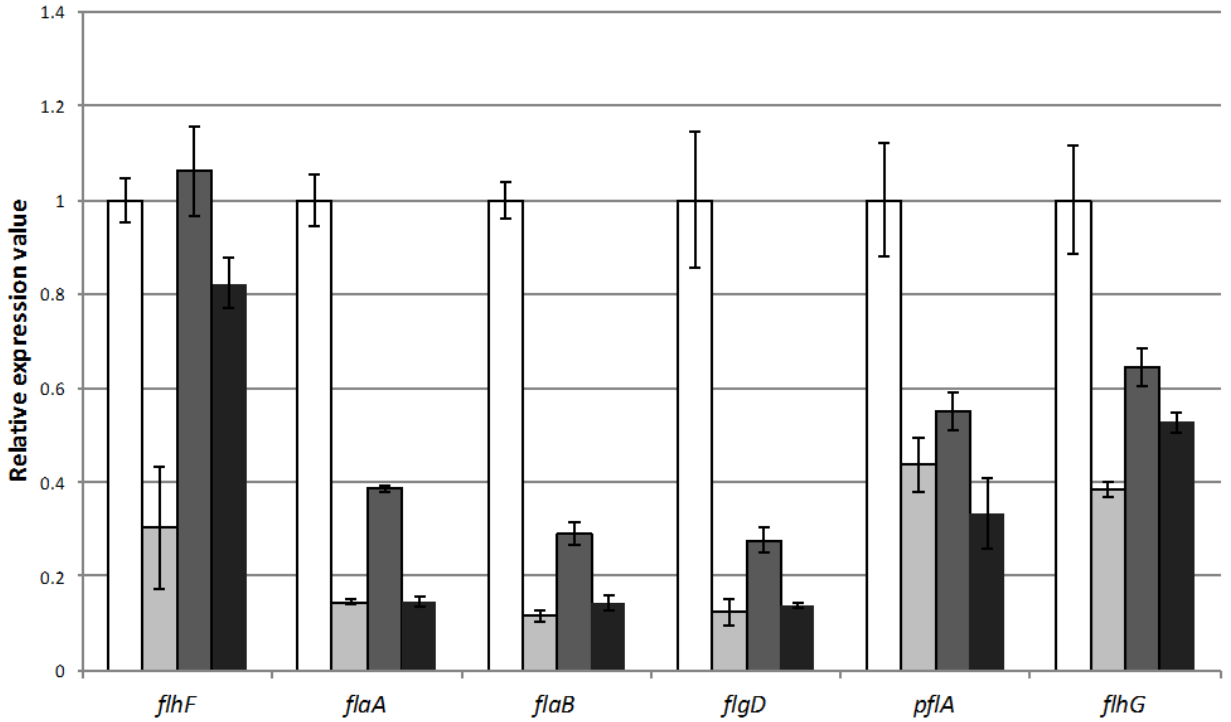


Figure 6.6 qPCR measurements of the relative expression levels of flagellar genes *flhF*, *flaA, flaB, flgD pflA* and *flhG* from *C. jejuni* PT14, PT14*flhF::kan*, PT14*flhF::kan 00230::flhF-cat* and PT14*flhF::kan 00230::flhF(T368A)-cat*. Values reported for each gene represent determinations from triplicate cultures of a verified transformant. The gene encoding phosphoglycerokinase (*pgk*) was used as housekeeping gene to normalise the data. □, *C. jejuni* PT14 wild type; ■, *C. jejuni* PT14*flhF::kan;* ■, *C. jejuni* PT14 *flhF::kan 00230::flhF-cat ;* ■, *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat*.

We next determined if *flhF* was essential for the expression of σ^{28} and σ^{54} –dependent flagellar associated genes, and whether the expression of *flhF(T368A)* affected the regulation of such genes (Figure 6.6). The *flaA* gene encoding the major flagellin FlaA was down-regulated 6-fold in the *flhF* mutant. Ectopic expression of *flhF* restored *flaA* expression to approximately 40% of wild type, whereas ectopic expression of *flhF(T368A)* did not produce a significant increase in *flaA* expression over the *flhF* mutant. In contrast to *flaA*, which has a σ^{28}-dependent promoter the minor flagellin encoding gene, *flaB*, has a σ^{54}-dependent promoter (Guerry et al., 1991; Wassenaar et al., 1993). The *flaB* gene was also down-regulated 6-fold in the *flhF* mutant, and ectopic expression of *flhF* also increased *flaB* expression over the *flhF* mutant but this increase was not evident upon ectopic expression of the *flhF(T368A)* allele. Similarly, *flgD* (encoding a hook assembly protein) uses a σ^{54}-dependent promoter (Balaban et al., 2009), and is reduced in the *flhF* mutant but increases upon ectopic expression of wild type *flhF* but not *flhF(T368A)*. Expression of the *pflA* gene encoding the paralysed flagella protein was also significantly reduced in the *flhF* mutant. In the absence of pflA expression affected cells produce intact but paralysed flagella (Gao et al., 2014). In summary ectopic complementation of *flhF* inactivated mutants of *C. jejuni* PT14 (*flhF::kan 00230::flhF-cat*) resulting in a 2.3 to 2.6-fold increase in σ^{28} and σ^{54} dependent genes compared to the *flhF* mutant, implying that *flhF* either directly or indirectly acts to positively regulate the expression of these genes, whereas *flhF(T368A)* appears to have lost this function.

6.2.6 *C. jejuni* PT14 *flhF* mutants are less sensitive to phage infection

Bacteriophage CP30A, CPX, Φ3, Φ15, CLP6 and CLP47 at a test dilution of $\log_{10}$ 7 pfu ml^{-1} were applied to bacterial lawns formed by either *C. jejuni* PT14, or PT14*flhF::kan* or PT14*flhF*::*kan* 00230::*flhF-cat* or PT14*flhF*::*kan* 00230::*flhF(T368A)-cat* in order to investigate if *flhF(T368A)* has any impact on *C. jejuni* acting as a host for bacteriophage infection. Data are presented as the efficiency of plating (EOP) for each phage relative to wild type *C. jejuni* PT14 for each of the host strains (Figure 6.7).

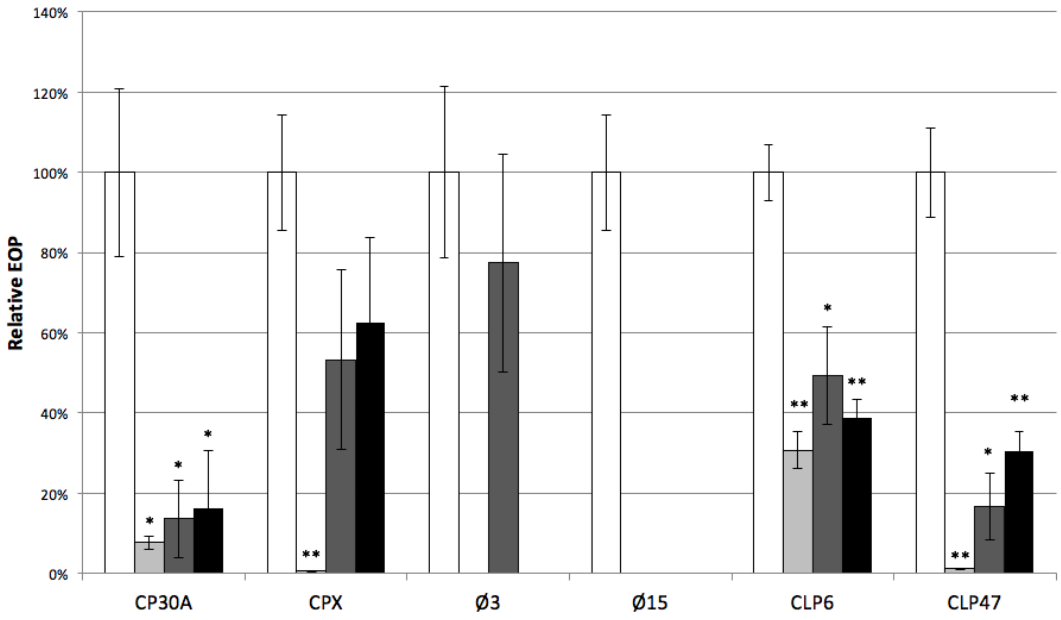


Figure 6.7 EOP for different phages replicate on different host strains. Approximately 7 log10 PFU of each phage type was applied onto triplicate bacterial lawns in decimal dilutions. Plaques were scored after 24 h incubation and the relative EOPs were calculated against wild type *C. jejuni* PT14, PT14*flhF*::*kan*, PT14*flhF*::*kan* 00230::*flhF*-cat and PT14*flhF*::*kan* 00230::*flhF(T368A)*-cat. □, *C. jejuni* PT14 wild type; ■, *C. jejuni* PT14*flhF*::*kan*; ■, *C. jejuni* PT14*flhF*::*kan* 00230::*flhF*-cat ■, PT14*flhF*::*kan* 00230::*flhF(T368A)*-cat. The error bars represent standard deviations. * indicates EOP in tested group is significantly different to the EOP of the control wild type *C. jejuni* PT14 group (p<0.05), ** indicates p<0.01.

The *flhF* mutant strains showed reductions in the EOP for all bacteriophage compared to wild type ($p < 0.05$), and for phage Φ3 and

Φ15 no replication at all was observed. Complementation with wild type *flhF* restored susceptibility to phage CPX and Φ3 with an EOP not significantly different to wild type *C. jejuni* PT14 and similar restoration was observed for *flhF(T368A)* complementation when being infected by phage CPX but not Φ3. Whilst phage Φ15 could only replicate on wild type *C. jejuni* PT14 with no plaque formation detected on any other *flhF* mutant derivative (Figure 6.7). The EOPs of phages CP30A and CLP6 revealed no significant difference between *flhF* mutant and the complementation either carried out with *flhF* or *flhF(T368A),* but all were significantly decreased compared to wild type *C. jejuni* PT14. Notably, the EOP of most of the phages tested showed no significant difference between *flhF* complementation and *flhF(T368A)* complementation ($p>0.05$), with the exception of phage Φ3. Moreover, a 100 fold less plaque forming ability was observed for phage CPX and CLP47 when infecting *C. jejuni* PT14*flhF*::*kan* compared with either *flhF* or *flhF(T368A)* complemented strains. All the phages have tailed morphologies and are members of the *Myoviridae.* However, phages ϕ3 and ϕ15 are classified as group II *Campylobacter*-specific bacteriophages based on their genome sizes (180 and 190 kb) and head diameters (Sails et al., 1998), whereas the remaining phages are all group III with genomes sizes of approximately 140 kb (Connerton et al., 2004; Siringan et al., 2011; Firlieyanti et al., 2016).

Since the group III phages revealed higher EOPs on wild type *C. jejuni* PT14 than the *flhF(T368A)* complement, we further investigated if this

would result in different host responses and phage replication. Phage CP30A was used to infect *C. jejuni* PT14, PT14*flhF::kan*, and PT14*flhF::kan 00230::flhF(T368A)-cat* in broth cultures. Rapid increases in phage titre were observed when the density of bacteria was >7 $\log_{10}$ CFU ml^{-1} - the phage proliferation threshold. The phage proliferation threshold is that the bacterial cell density above which would lead to the probability of single phage progeny replicating greater than the chance of it being lost (Robert and Vincent, 2002; Cairns et al., 2009), and presented as rapid increases in phage titre as shown in figure 6.8. In the wild type *C. jejuni* PT14 cultures an increase in phage titre was accompanied by a 3 $\log_{10}$ CFU ml^{-1} fall in the bacterial count at 10 h due to bacteriophage mediated cell lysis. As observed previously in laboratory culture the bacterial growth recommenced with the development of phage resistance (Scott et al., 2007). The *flhF* mutant did not experience the fall in viable count but remained static once the bacterial density had reached 7 $\log_{10}$ CFU ml^{-1}. The phage titre of CP30 increased from 6 h to reach a similar level to that produced by wild type PT14. The *flhF(T368A)* complement of the *flhF* mutant restored the sensitivity to phage mediated lysis and the characteristic fall in viable count observed in wild type at densities >7 $\log_{10}$ CFU ml^{-1} .

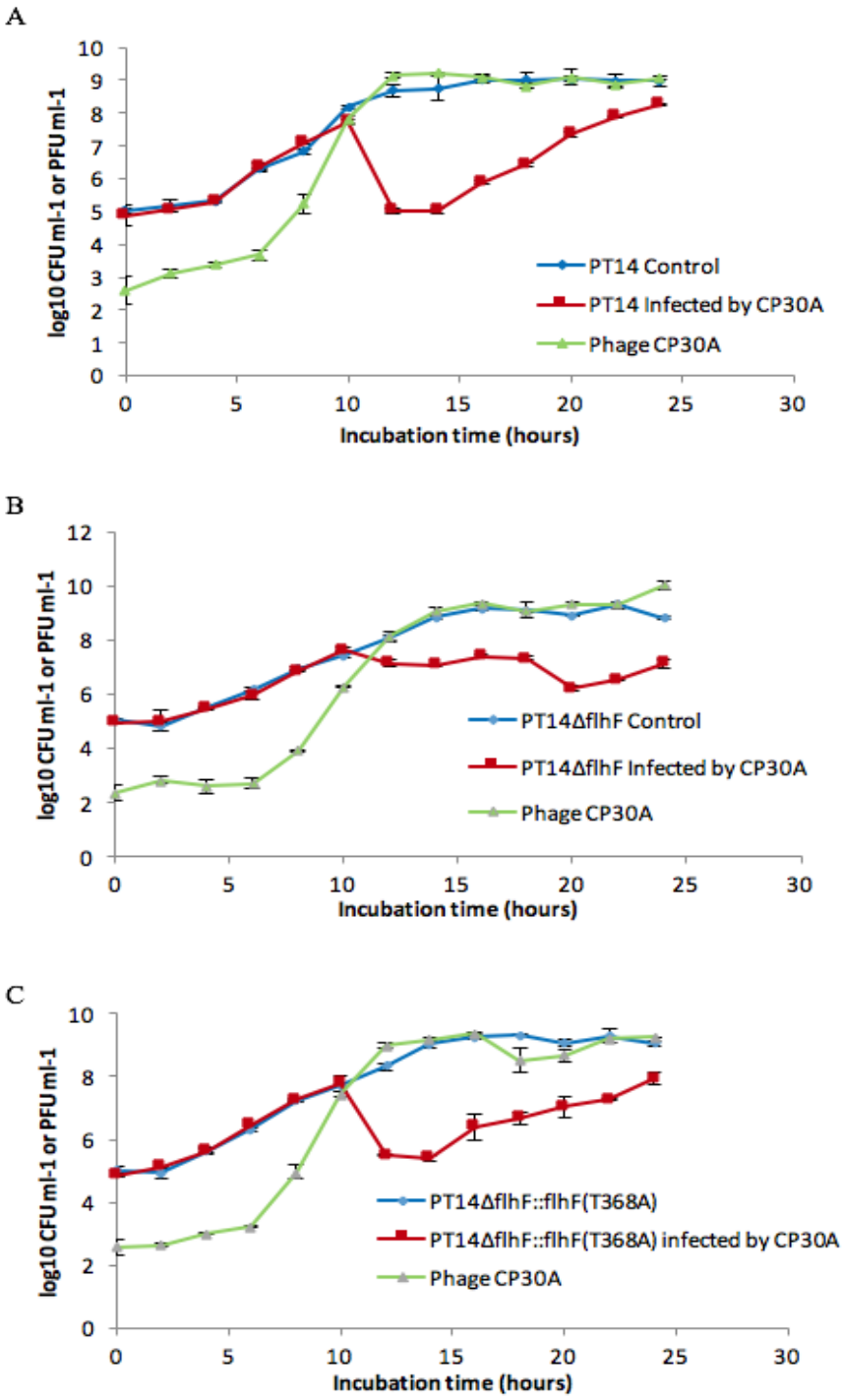


Figure 6.8 Growth characteristics of *C. jejuni* PT14 wild type and its *flhF* mutants against infection by bacteriophage CP30A. Broth cultures containing 4 $\log_{10}$ CFU *C. jejuni* were pre-incubated for two hours and 2 $\log_{10}$ PFU bacteriophage CP30A was then added. The time when phage sample was added counted as time zero and the suspension was further cultured for 24 hours. Sample aliquots were removed every two hours to determine the count and titre of bacteria and phage. A. the *C. jejuni* PT14, B. the *C. jejuni* PT14*flhF::kan* , C. the *C. jejuni* PT14*flhF::kan 00230:: flhF(T368A)-cat.*

6.2.8 His-tagged FlhF protein purification

FlhF protein is reported to have GTPase activity that is required for proper biosynthesis of functional flagella (Balaban et al., 2009). Wild type FlhF and FlhF(T368A) proteins were expressed as N-terminal His6-tagged fusions in *E. coli* using a pET28b vector. After purification with Ni-NTA spin columns (Qiagen), both FlhF and FlhF(T368A) proteins were examined using a SDS-PAGE gel (Figure 6.9).

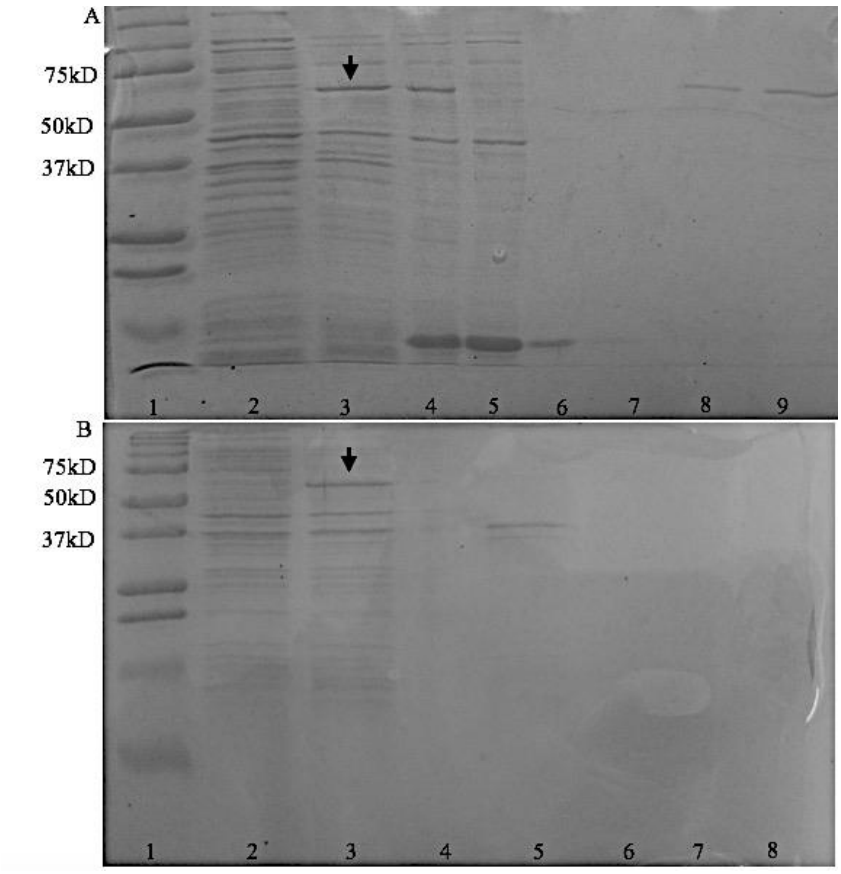


Figure 6.9 SDS-PAGE gel of FlhF purification from recombinant *E. coli* BL21DE3 star cells. A. FlhF wild type protein, lane 1. Protein ladder, 2. Pre-induction culture, 3. Post- induction culture, 4. Clear lysate, 5. Flow through, 6. Wash-1, 7. Wash-2, 8. Elution-1, 9. Elution-2; B. FlhF(T368A) protein purification, lane 1. Protein ladder, 2. Pre-induction, 3. Post-induction, 4. Clear lysate, 5. Flow through, 6. Wash-1, 7. Wash-2, 8. Elution. Arrow-heads indicate the expressed FlhF(T368A) protein product after inducing with IPTG.

After inducing with ITPG to trigger transcription of the *lac* promoter, both FlhF and FlhF(T368A) proteins show expression in post-induction cells but not in pre-induction cultures, where the arrowheads in Figure 6.9 indicate the corresponding protein bands. The molecular weight of this band was estimated as 55kD and this is consistent with the molecular weight for FlhF (55.36kD) and FlhF(T368A) (55.33 kD), and therefore, was considered as the target protein. After lysing *E. coli* host cells, FlhF wild type protein was released to the cleared lysate whereas no bands were observed for the FlhF(T368A) protein sample. The absence of FlhF(T368A) protein in the cell lysate could be due to the protein being expressed as an inclusion body and required further investigation. After applying the lysate containing the total soluble cell proteins to the column support, those that cannot bind to immobilized nickel ions on the affinity chromatography support matrix due to the lack of coordinating amino acid (histidine) residues, were observed in the flow through. These samples contained no FlhF protein. The support was then washed with washing buffer containing a low concentration of imidazole to completely remove the total cell protein as well as any non-specific binding proteins. After approximately 3-5 column washes, the his-tagged protein sample was eluted with elution buffer containing high concentration of imidazole, and a sharp single band representing the FlhF protein was observed upon SDS-PAGE (Figure 6.9-A). FlhF(T368A) protein was not observed in any step after cell lysis (Figure 6.9-B).

4.2.9 FlhF(T368A) inclusion body determination

Since FlhF(T368A) was not present in the clear lysate it was suspected that it may have been expressed as an inclusion body. To assess this, the location of expressed FlhF(T368A) protein was determined as described in 2.8.9.

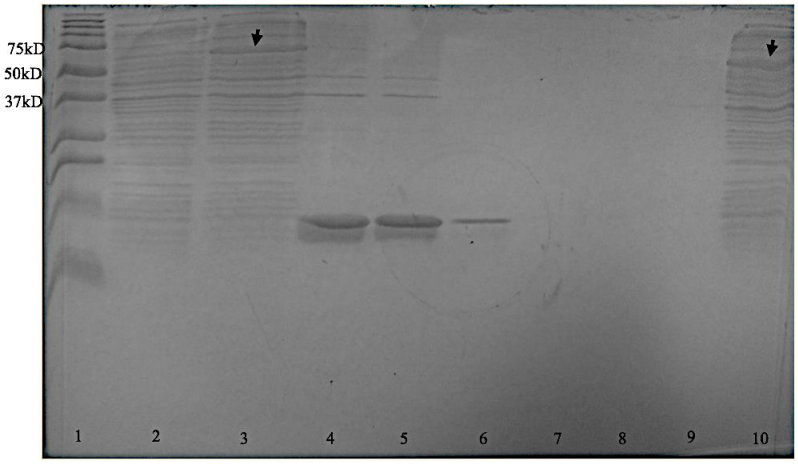


Figure 6.10 SDS-PAGE gel to determine the location of FlhF(T368A) expressed in *E. coli* BL21DE3 star cells. Lane 1, Protein ladder, 2. Pre-induction culture, 3. Post induction culture, 4. Periplasm-1, 5. Periplasm-2, 6. Periplasm-3, 7. Cytoplasm-1, 8. Cytoplasm-2, 9. Cytoplasm-3, 10. Inclusion body. Arrow heads indicate the bands of correct molecular mass for the FlhF(T368A) protein.

As shown in Figure 6.10, the target band was not present in either the periplasmic space or the cytoplasm but instead was located within inclusion body material as had been hypothesised. In order to avoid the formation of inclusion bodies and to promote proper protein folding, the expression conditions were modified by lowering the inducing quantity of IPTG to give a final concentration of 0.1mM and reducing the culture temperature to 25˚C after IPTG induction. Under these modified conditions FlhF(T368A) protein was still expressed within an inclusion

body (data not shown). It was noted that there were three cysteine residues in the FlhF and FlhF(T368A) proteins, and we hypothesised that the likelihood of a free cysteine being present may have an influence on protein folding ability during expression. Therefore, the expression host was changed from *E. coli* BL21DE3 Star to *E. coli* Origami 2 DE3 pLysS in order to stabilise any free cysteine.

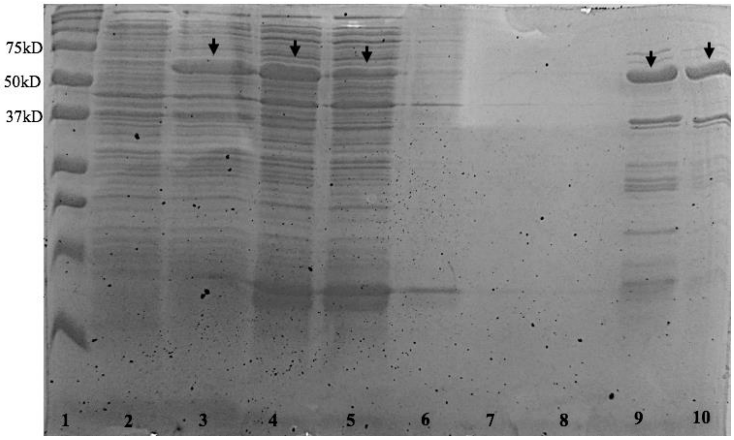


Figure 6.11 SDS-PAGE gel of FlhF(T368A) purification with host *E. coli* Origami 2 DE3 pLysS. Lane 1. Protein Ladder, 2. Pre-induction culture, 3. Post-induction culture, 4. Clear lysate, 5. Flow through, 6. Wash-1, 7. Wash-2, 8. Wash-3, 9. Elution-1, 10. Elution-2. Arrow heads indicate FlhF(T368A) representative bands.

Using the expression host Origami 2 DE3 pLysS, FlhF(T368A) was eventually expressed as soluble protein (Figure 6.11). After lysing *E. coli* cells, FlhF(T368A) was released to the clear lysate as indicated by the arrow head in lane 4 of Figure 6.11. Expressed protein was also observed in the flow through sample which was considered as the his-tagged target protein exceeding the capacity of the purification column. After three washes containing low concentrations of imidazole, non-binding proteins were completely removed from the support as shown in lane 8, and

immobilised proteins were then eluted. However, it was noted that multiple bands were present in the elution sample, suggesting that the eluted sample contained a mixed population of proteins.

6.2.10 FlhF(T368A) purification with imidazole gradient wash

In order to obtain pure FlhF(T368A) protein, an imidazole gradient wash was carried out during protein purification. Wash solutions with imidazole concentration of 50mM, 75mM, 100mM, 150mM and 200mM were prepared and individually applied in the protein purification protocol as wash solutions. Elution from each purification was analysed by SDS-PAGE.

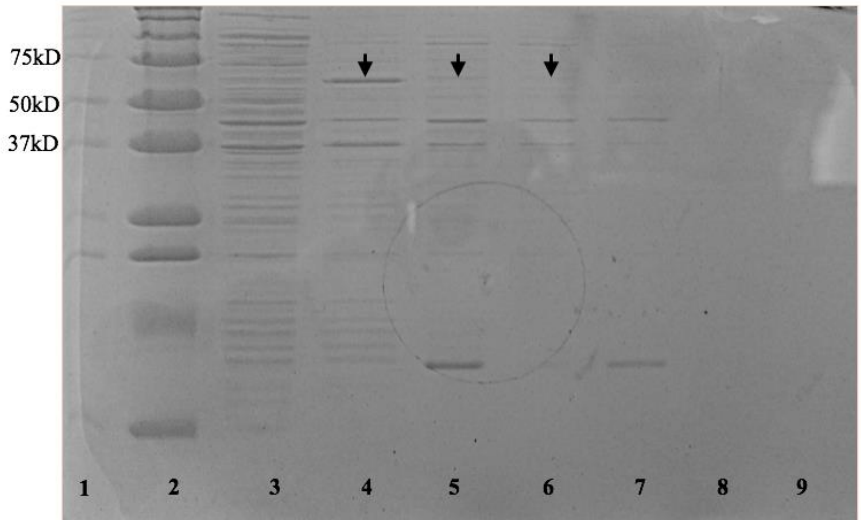


Figure 6.12 SDS-PAGE gel of elution samples obtained with different imidazole concentrations contained within the wash solutions. Lane 1, Protein ladder, 2. Pre-induction culture, 3. Post-induction culture, 4. Elution from 50mM imidazole wash, 5. Elution from 75mM imidazole wash, 6. Elution from 100mM imidazole wash, 7. Elution from 150mM imidazole wash, 8. Elution from 200mM imidazole wash. Arrow heads indicate the FlhF(T368A) protein band.

As Figure 6.12 illustrates, the target protein was present in the elution samples obtained with 50mM and 75mM imidazole washes and was absent in those washed with higher imidazole concentrations, suggesting 75mM imidazole is the upper criterion for immobilization of target protein on the support and wash buffer with imidazole over than this would result in complete loss of target protein. We also noted that even though the affinity support was washed with higher concentrations of imidazole, non-specifically bound proteins were not removed as multiple bands were still observed in lane 5, 6 and 7.

6.2.11 FlhF(T368A) purification with increased elution times

As increasing imidazole concentration in the wash solution failed to remove non-specific binding proteins from the target FlhF(T368A), we then planned to increase the number of elution samples in order to elute protein contaminates first and obtain pure FlhF(T368A) when all other proteins have been eluted.



Figure 6.13 SDS-PAGE of FlhF(T368A) purification with 5 elution steps. Lane 1. Protein ladder, 2. Pre-induction culture, 3. Post- induction culture, 4. Wash-1, 5. Wash-2, 6, Elution-1, 7. Elution-2, 8. Elution-3, 9. Elution-4, 10. Elution-5. Arrow heads indicate the band representing FlhF(T368A) protein.

As we expected, multiple bands were present in the first two elution samples (Figure 6.13). After the third elution, two bands were observed in the elution sample including target FlhF(T368A) protein and an extra band, that has a molecular weight of approximately 37kD. The extra band was considered as either the product of partial degraded FlhF(T368A), or host cell protein non-specifically bound to the support.

6.2.12 FlhF(T368A) purification and fractionation with protease inhibitor

Since protein degradation was considered as one of the potential causes for the contaminated band, phenylmethylsulfonyl fluoride (PMSF) was applied as protease inhibitor at a final concentration of 0.5mM in the cell growth medium, purification buffers and fractionation buffers. In addition

to including protease inhibitor, all steps were carried out on ice or at 4 ˚C to avoid degradation of the protein sample. The purified protein was then analysed on SDS-PAGE gel.

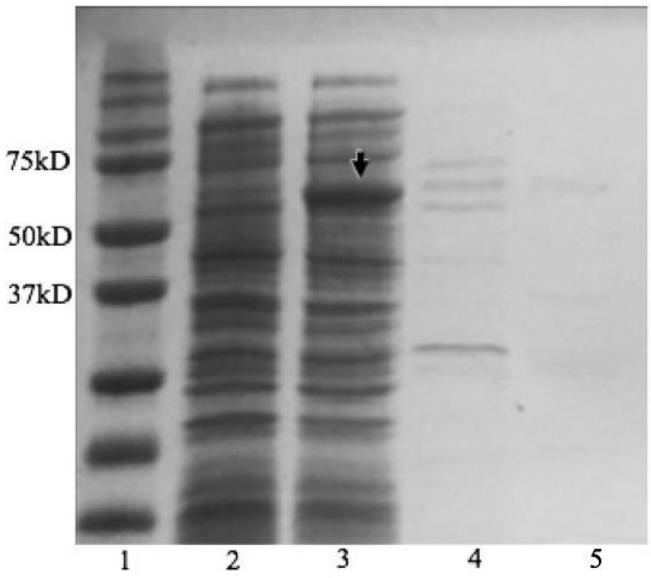


Figure 6.14 SDS-PAGE for FlhF(T368A) purification in the presence of PMSF. Lane 1. Protein ladder, 2. Pre-induction culture, 3. Post-induction culture, 4. Elution-1, 5. Elution-2. The arrow head indicates the band representing for FlhF(T368A).

Even with the presence of protease inhibitor and affinity chromatography performed in a cold environment to avoid protein degradation, the extra band with a molecular weight of 37kD was still observed in the elution sample as illustrated in lane 5 of Figure 6.14. Therefore, this protein was considered as cell protein from the expression host strain.

6.2.13 FlhF(T368A) fractionation

To separate FlhF(T368A) from non-specific binding proteins the affinity column eluate was fractionated using a Superose 12 high performance gel filtration column. To retain the protein quantity required the elution-2

sample from section 6.2.11 was applied to the column. The protein fractions are shown as an elution chromatogram as individual peaks with absorbance at 280nm (Figure 6.15-A).

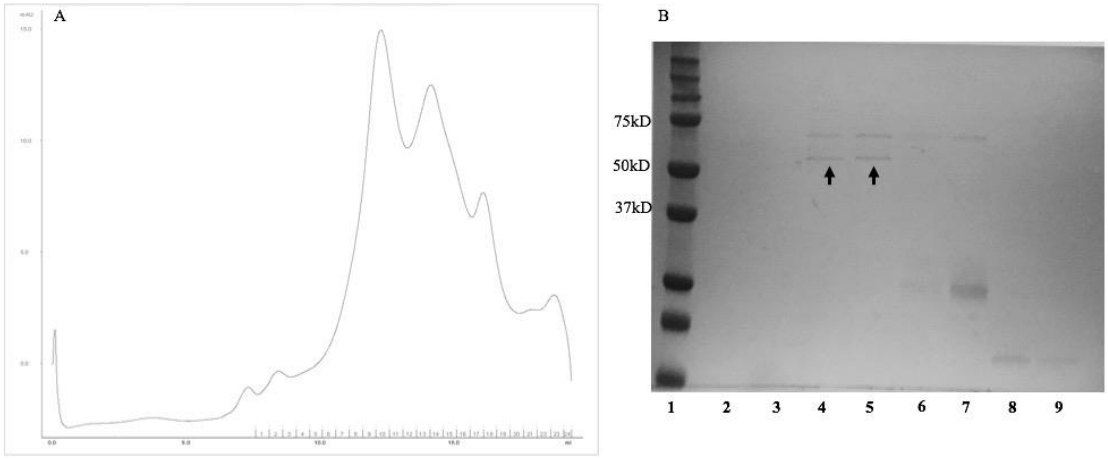


Figure 6.15 FlhF(T368A) fractionation superose size exclusion chromatography. A. chromatogram of FlhF(T368A) protein fractionation using superose 12 column, B. SDS-PAGE gel analysing protein fractions obtained from fractionation, lane 1. Protein ladder, 2. Fraction-2, 3. Fraction-3. 4. Fraction-10, 5. Fraction-11, 6, Fraction-14, 7. Fraction-18, 8. Fraction-22, 9. Fraction-23. Arrow heads indicate band representing FlhF(T368A).

From the gel image, we can demonstrate that protein samples with larger molecular weight were fractionated from the column first followed by smaller proteins (Figure 6.15-B). Most of the proteins were well separated in this run. However, in the fraction containing the target FlhF(T368A) protein, there was still one extra band present with a molecular weight of approximately 67kD. We thought this could be a contaminating protein that has a similar molecular weight to FlhF(T368A) and cannot be separated using Superose 12 high performance gel filtration column.

6.2.14 FlhF(T368A) protein separation with a centrifugal filter

As protein fractionation failed to isolate the target protein from a contaminated protein of molecular of 67kD, fraction-10 sample from section 6.2.13 was then ultrafiltered through a centrifugal filter with a 30 kDa cut off size. The retentate was discarded and the filtrate collected for analysis by SDS-PAGE gel.

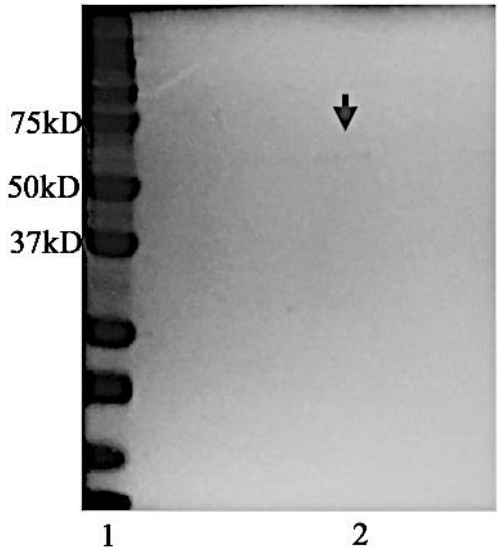


Figure 6.16 SDS-PAGE gel of FlhF(T368A) after separation by ultrafiltration, lane 1. Protein ladder, 2. Flow through sample. Arrow head indicates the band representing FlhF(T368A).

As shown in Figure 6.16, a single band was present in the filtrate with a molecular weight of approximately 55kD, which is consistent with the molecular weight of FlhF(T368A). This sample was then considered as pure FlhF(T368A) protein.

6.2.15 Bradford assay standard curve

Prior to carry out any protein related work, the concentrations of isolated FlhF and FlhF(T368A) protein samples were determined by Bradford assay. A standard curve was prepared by plotting the absorbance at 595nm of a series of BSA with known concentration, as illustrated in Figure 6.17.

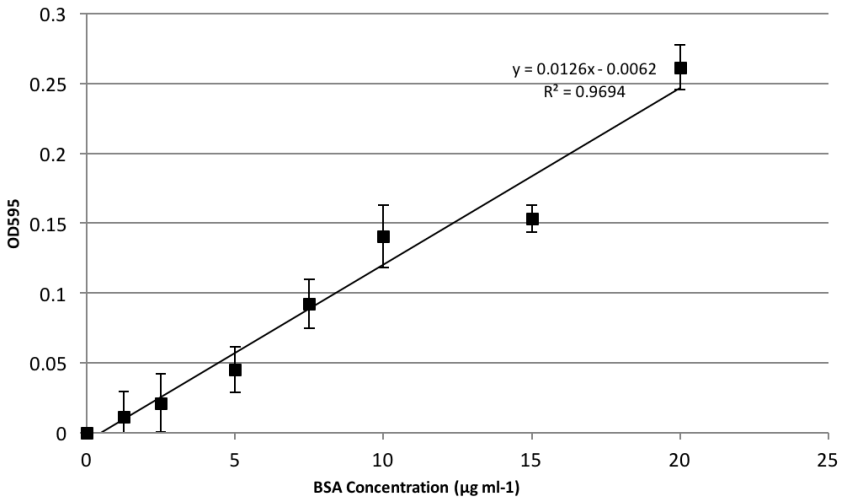


Figure 6.17 Bradford assay standard curve. BSA with concentration of 1.25, 2.5, 5, 7.5 10, 15 and 20 µg/ml was applied as standard sample.

FlhF and FlhF(T368A) proteins were assayed under standard conditions. Absorbance at 595nm for FlhF and FlhF(T368A) were 0.1226±0.027 and 0.222±0.005 corresponding to 102.2µg ml^{-1} and 181.1µg ml^{-1}.

6.2.16 Free phosphate standard

In order to convert the measured absorbance to the level of free phosphate, a standard Pi curve was prepared before carrying out the GTP

hydrolysis assay by plotting the absorbance at 612nm of a series of known concentration Pi samples (Figure 6.18).

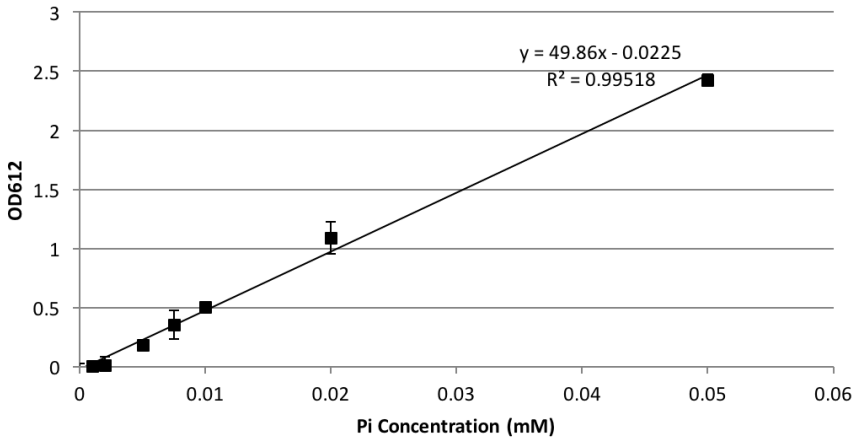


Figure 6.18 Free phosphate standard curve using buffers containing 1, 2, 5, 7.5, 10, 20, 50 μM of free phosphate as standard samples.

6.2.17 FlhF(T368A) has a less thermal stability and GTPase activity than wild type FlhF

As FlhF is reported to have GTPase activity and play a significant role in flagella biosynthesis GTPase activities were measured, by detecting the release of free phosphate (P_i) as a product of GTP hydrolysis. The specific activity of the wild type flhF GTPase was 2.5-fold greater than that of FlhF(T368A). The thermal tolerance of the wild type FlhF and FlhF(T368A) proteins were assessed by pre-incubating the proteins for 5 minutes at different temperatures before addition of the substrate.

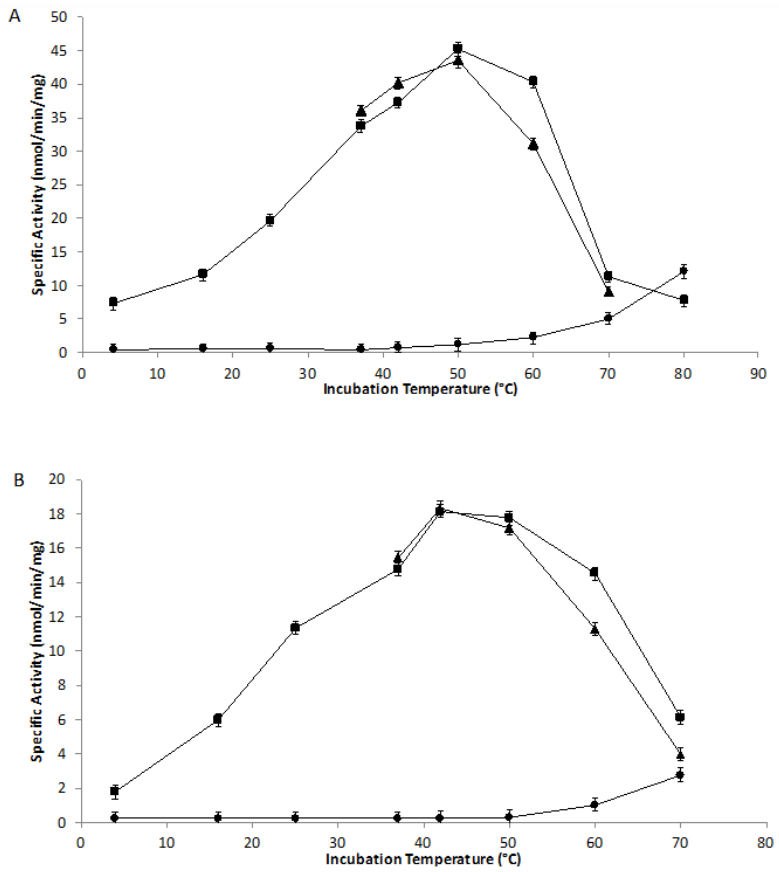


Figure 6.19 Thermal stability of FlhF proteins. A. FlhF wild type protein, reached its highest activity at 50°C; B. FlhF(T368A) protein attained the highest specific activity present at 42°C. ■, FlhF GTPase activity temperature profile; ▲, FlhF protein preheated at the desired temperature for 5 minutes before commencing the reaction; ●, GTP self-hydrolysis control.

The contribution of GTP self-hydrolysis was monitored by control incubations without protein (Figure 6.19- A and B), and corrections made to the corresponding enzymatic activities. The temperature optimum of FlhF GTPase was 50°C with a specific activity of 45 nmol min^{-1} mg^{-1}. Pre-heated FlhF retains 96% specific activity at 50°C compared to control (Figure 6.19- A). Pre-heated FlhF samples pre-incubated at 37°C and 42°C produced a 7% increase in specific activity, implying that wild type FlhF protein remains stable up to 42°C. The GTPase activity of FlhF(T368A)

has a reduced temperature optimum of 42°C (Figure 6.19B). Pre-heated FlhF(T368A) at 37°C increased the specific activity by 4% but pre-incubation at 42°C resulted in a 4% decrease in specific activity, suggesting FlhF(T368A) GTPase activity is subject to thermal instability at the growth temperature of *C. jejuni*.

6.2.18 GTPase kinetics

The enzyme kinetic properties of wild type FlhF and FlhF(T368A) were determined. Protein samples were separately assayed at 42°C across a range of substrate concentrations. GTP concentrations of 0.05mM, 0.1mM, 0.25mM, 0.5mM, 1mM were used for wild type FlhF. However, since the 0.05mM substrate concentration was too low to give any detectable activity for FlhF(T368A), 0.1mM, 0.175mM, 0.25mM, 0.5mM, 1mM were used as substrate concentrations.

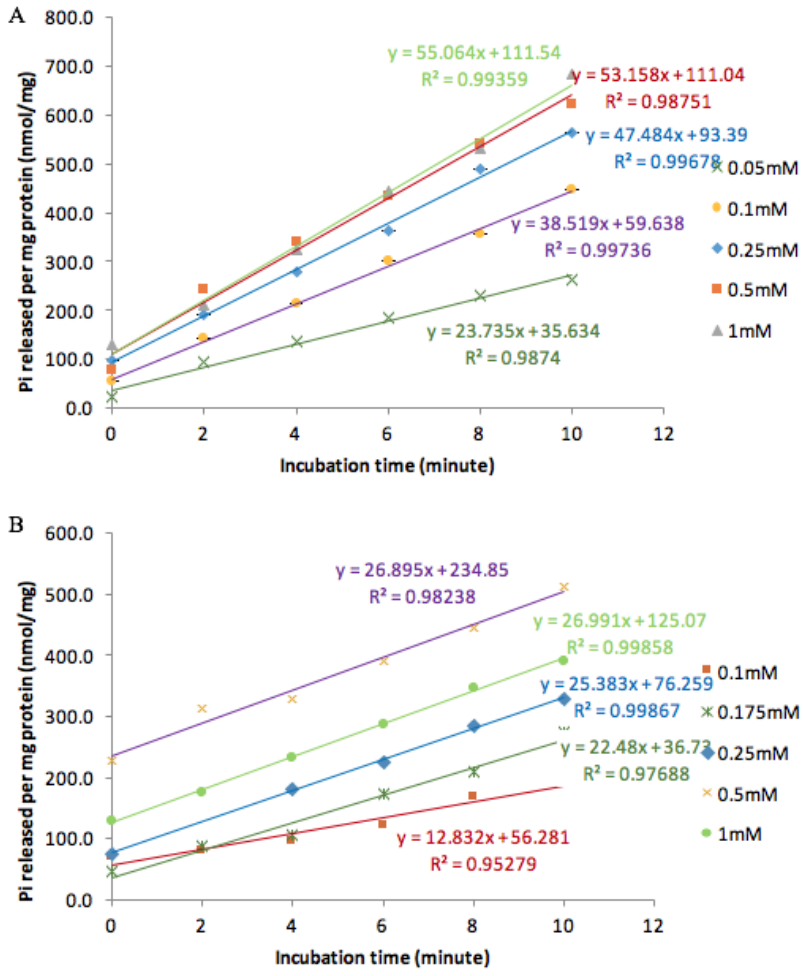


Figure 6.20 GTPase reaction rates of FlhF and FlhF(T368A) proteins. 42°C was selected as assay temperature.

We observed that with different substrate concentration, the initial reaction velocities (V_0) showed a linear increase with time (Figure 6.20). After rapid mixing GTP hydrolysis proceeds at a constant reaction rate. This is typical when substrate concentration [S] is greater than the Michaelis constant of the enzyme (K_m), and the reaction velocity at this time period is constant, and equivalent to its maximal velocity for the substrate concentration applied. For wild type FlhF the GTPase initial rate velocities were 23.735, 38.519, 47.484, 53.158 and 55.064 nmol min^{-1} with substrate GTP concentrations of 0.05, 0.1, 0.25, 0.5 and 1mM respectively. For FlhF(T368A), V_0 of 12.832, 22.48, 25.383, 26.895 and 26.991 nmol min^{-1} were observed respectively with 0.1, 0.175, 0.25, 0.5 and 1mM of substrate concentrations. As substrate concentration kept increasing, an increase in initial velocity was observed for both FlhF wild type and FlhF(T368A) protein. These data were plotted as a reciprocal Lineweaver-Burke plot and the raw data as Michaelis-Menten plot (Figure 6.21). The Km for FlhF at 42°C was 0.075mM and the V_{max} 62.5 nmol min^{-1} mg^{-1}, and for FlhF(T368A) the K_m was 0.1736mM and V_{max} is 37.74 nmol min^{-1} mg^{-1}. The k_{cat} for FlhF and FlhF(T368A) were calculated as 0.0115 $second^{-1}$ and 0.007 $second^{-1}$ respectively.

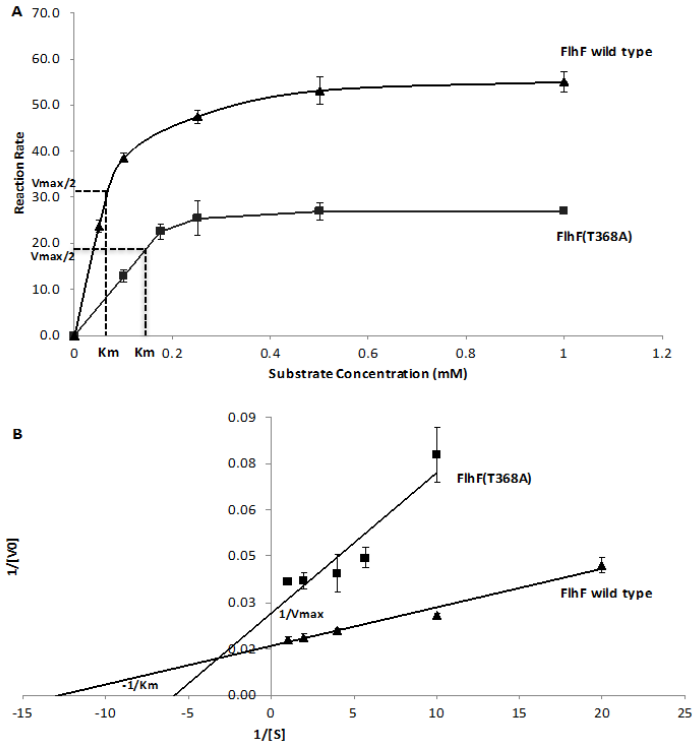


Figure 6.21 Lineweaver-Burke plots and Michaelis-Menten for GTPase activities of FlhF and FlhF(T368A).

6.2.19 FlhF protein structure modelling

In order to investigate the cause for the reduced thermal stability and GTPase activity of FlhF(T368A), protein structure of FlhF and FlhF(T368A) was predicted using Swiss prot system (Figure 6.22).

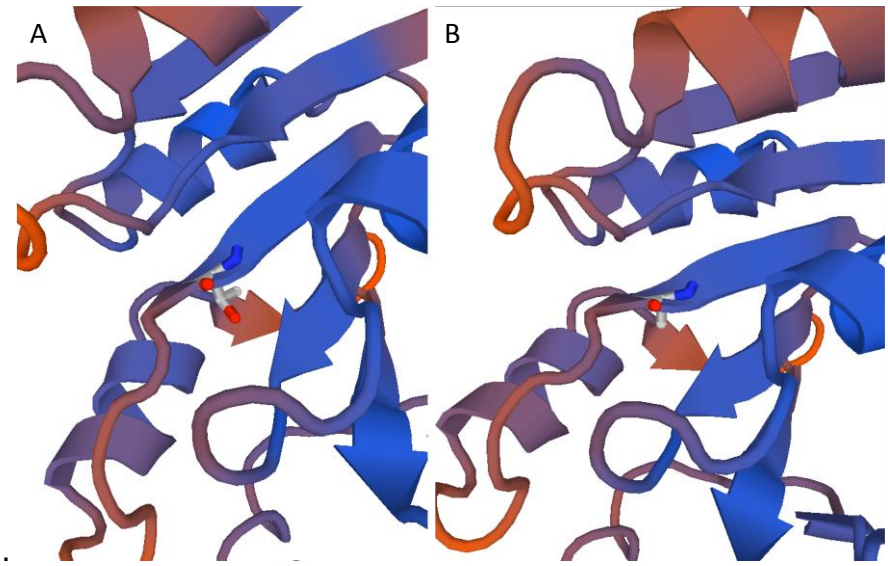


Figure 6.22 Predicated location of T368A mutation based on the template flagellar biosynthesis protein FlhF -2px0.1.A (Swiss-Prot, 2014). A, FlhF wild type, B. FlhF(T368A).

As shown in the figure above, changing a threonine into an alanine in FlhF would cause the loss of a -HCOH- group, and this mutation is located in a β-sheet on the basis of the model structure.

6.3 Discussion

The *flhF* knock-out mutant of *C. jejuni* PT14 showed a non-motile phenotype, and after complementing the *flhF::kan* mutant with wild type *flhF* motlitity was restored. Although the *flhF* is still expressed under its native promoter, due to the translocation from its original location to the *cj0046* psuedogene gene the expression of flhF may be reduced but nevertheless regains motility. Therefore, the non-motile phenotype observed for the *flhF* knockout and its *flhF* dependent restoration is evidence that *flhF* is essential for the motility of *Campylobacter jejuni* PT14. However, when the *flhF::kan* mutant was complemented by

flhF(T368A), the resulting strain exhibited significantly reduced motility compared to wild type and the wild type complement. The *flhF(T368A)* mutation confers impaired motility for *C. jejuni*. No motility was observed for *C. jejuni* PT14CP30ACS, which carries the mutation in association with chronic CP30 phage infection, a finding that is consistent with a previous study (Siringan et al., 2014). Critically these studies show the *flhF(T368A)* mutation to confer impaired motility and flagella synthesis independent of the phage infection associated with carrier state culture from where the mutation was identified.

Transmission electron microscopy (TEM) was employed to examine cell and flagellar structures of *C. jejuni* PT14 and the *flhF* mutant derivatives. *C. jejuni* PT14 has typical bi-polar flagella but the *flhF::kan* mutant was unable to synthesise any flagella structure confirming the importance of the flhF gene product in the development of the flagella (Balaban et al., 2009). Most of the *C. jejuni* PT14*flhF::kan 00230::flhF-cat* cells possessed single flagella. Whereas the majority of the *C. jejuni* PT14*flhF::kan00230::flhF(T368A)-cat* cells had no flagella, this did not apply to all cells, which was consistent with the reduced swarming motility observed on 0.4% agar motility plates. Notably, when using the standard bacteria fixing protocol, even low speed centrifugation for the recovery of cell pellets can lead to damage and the absence of flagella. It was noted that complemented *C. jejuni* PT14*flhF::kan 00230::flhF-cat* and *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* were susceptible to damage and specific care was required for these preparations that was

not required for wild type *C. jejuni* PT14. The flagella of *C. jejuni* PT14*flhF::kan 00230::flhF-cat* and *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* are apparently more delicate than the flagella of wild type *C. jejuni* PT14. In order to test this hypothesis the complementation strains were fixed in same fixative, but without any centrifugation, and from these preparations it was possible to successfully observe flagella by TEM.

We then investigated if the non-flagella phenotype of the majority of *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* cells was due to downregulation of flagellar synthesis associated genes by focusing on the role of *flhF(T368A)* in expression of σ^{28} and σ^{54} dependent genes. We demonstrated that inactivating *flhF* by inserting the kanamycin resistance gene significantly reduced the expression of *flhF* itself. The reduced expression of *flhF* could be restored to wild type levels if the *flhF::kan* mutant was complemented with wild type *flhF.* However, if the complementing allele was *flhF(T368A),* the expressed *flhF* was approximately 20% less, suggesting that *flhF(T368A)* influences *flhF* expression.

In the absence of *flhF,* the expression level of *flaA* (σ^{28} transcription dependent); *flaB* and *flgD* (σ^{54} transcription dependent) were all significantly reduced, and a less severe but still significant downregulation was observed for *pflA* and *flhG*. These results indicate that *flhF* is required for proper expression of both σ^{28} and σ^{54} transcribed

genes. Hendrixson and DiRita (2004) hypothesised that *flhF* exerted it's influence at the start of the σ^{54} transcriptional cascade, and therefore deletion of *flhF* would result in improper transcription of the downstream genes and lead to reduced levels of σ^{54} dependent gene expression, which would account for the observations that *flaB* and *flgD* showed downregulated transcription in this study. Correa et al. (2005) reported that in *Vibrio cholera*e, *flhF* is also essential for the expression of some σ^{28} dependent flagellar genes including the major flagellin homologue, whereas Hendrixson and DiRita (2003) found that in *Campylobacter jejuni*, σ^{28} dependent *flaA* was largely unaffected by deletion of *flhF*. However, σ^{54} dependent gene expression was affected by *flhF* deletion and similarly by the deletion of the regulatory genes *flhA, flhB, fliP* and *fliR* (Hendrixson and DiRita, 2003). In this study, it has been demonstrated that *flhF* is also essential for proper expression of σ^{28} dependent *flaA*, and deletion of *flhF* would lead to a significant reduction of expression level of *flaA*. We presume that the difference between *flhF* and other putative regulatory genes affecting σ^{28} dependent *flaA* could be related with the GTPase activity of FlhF.

After complementation of *C. jejuni* PT14*flhF::kan* with wild type *flhF,* 1.8 to 2.6 fold upregulation of σ^{28} and σ^{54} dependent genes was observed that was not observed for the *flhF(T368A)* complement. Instead, expression of *flaA, flaB* and *flgD* genes in *flhF(T368A)* complement were similar to the level they expressed in *flhF::kan* mutant (1.16-fold increase for *flaB* and *flgD* but not significant). The significant upregulation of wild type

complementation confirms that *flhF* is required for the proper expression of *flaA, flaB* and *flgD* genes. We can also demonstrate that unlike wild type FlhF, the *flhF(T368A)* encoded protein FlhF(T368A) may not be able to function as an indirect positive regulator of the *flaA, flaB* and *flgD* genes. This could be the reason why *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* has few cells with flagella compared to wild type and the wild type complement.

Inactivation of *flhF* reduced transcription of *flhF* and the downstream gene *flhG*. Transformants carrying either *flhF* or *flhF(T368A)* at the *A911_00230* psuedogene locus added to the expression of *flhF* to achieve levels not significantly different to wild type but this was not so for *flhG* transcription. However, the expression of *flhF* did lead to a significant increase in *flhG* transcription compared to the *flhF* knock-out mutation suggesting that *flhF* expression affects *flhF* and *flhG* transcription. Surface structures are candidates for adhesion and receptors for bacteriophage infection. In *C. jejuni* capsular polysaccharide and flagella have been proposed to constitute receptors for the infection of certain phage (Rakhuba et al., 2010; Sørensen et al., 2015). After binding to the flagella, phages could be drawn toward the host receptors via flagella rotation, and therefore, increase the chance of infection (Gurrero-Ferreira et al., 2011). Here it is observed that phages generally had a higher EOP on wild type *C. jejuni* PT14 than *flhF* mutants that are largely non-flagellated, suggesting that although flagella may not be essential, the presence of a functional flagella increases infection efficiency. For phage CPX and

CLP47, *C. jejuni* PT14 *flhF::kan* mutant revealed an EOP of 0.01 than other complementation strains, which may indirectly emphasise the important role of flagella in infection for these two particular phages. Phages Φ3 can only infect *C. jejuni* PT14 and PT14*flhF::kan 00230::flhF-cat,* that are identified as either having proper flagellar at one or both ends, suggesting that Φ3 may potentially belongs to a flagellatropic phage class, and that flagella is essential for its infection. In summary, a higher EOP was generally observed for those host strains with proper synthesised flagella, and no significant difference was observed in EOP between *C. jejuni* PT14*flhF::kan 00230::flhF-cat* and PT14*flhF::kan 00230::flhF(T368A)-cat* with the exception of phages Φ3 indicating that for certain phages, the *flhF(T368A)* would not prevent infection ability but reduce efficiency and that *flhF(T368A)* contributes to a resistant phenotype for the host strain.

Broth cultures of wild type and mutant PT14 *C. jejuni* were examined for growth dynamics and ability to propagate phage. CP30 phage was selected as it had provoked the *flhF(T368A)* mutation in the carrier state. Infection of wild type *C. jejuni* PT14 at densities >7 $\log_{10}$ CFU ml^{-1} resulted in a crash in the bacterial population due to phage lysis and a rise in phage titre. The crash was not evident in the *flhF* mutation but a cessation in growth was observed. The cessation in growth was reminescent of the behaviour of the carrier state bacteria, which in the case of the CSLC bacteria is coincident with an increase in phage titre (Siringan *et al.,* 2014). An increase in phage titre was also evident for the CP30 infected *flhF* knock-out mutant culture demonstrating that the impaired flagella

biosynthesis may affect the efficency but not halt phage replication. Complemetation of the *flhF* mutation with the *flhF(T368A)* allele all but restored wild type behaviour with culture experiencing a reduced but significant population crash of 3 $\log_{10}$ CFU ml^{-1}. The greater drop in cell number for wild type *C. jejuni* PT14 over *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat*, suggests that wild type *C. jejuni* PT14 is more sensitive to phage infection than *flhF(T368A)* complementation. As *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* cultures are likely to contain low populations of cells that have flagella which if required for cellular engagement or as a possible receptor for phage binding, this would mean the culture would be less sensitive towards phage infection. The absence of phage mediated lysis and bacterial population crash is likely due to non-flagella phenotype of the *flhF* knock-out mutation. Notably, we observed that within the first six hours of incubation, phage CP30A with *C. jejuni* PT14*flhF::kan* and PT14*flhF::kan 00230::flhF(T368A)-cat* mutants revealed a slower growth rate than wild type *C. jejuni* PT14, which may also be influenced by the defective flagella phenotype.

The predicted the protein structures of FlhF and FlhF(T368A) are presented based on the Swiss-Model algorithm to investigate the structural difference the mutation may impart. Changing a threonine into an alanine in FlhF would cause the loss of a -HCOH- group, and this mutation is located in a β-sheet on the basis of the model structure. Since the β-sheet is connected by hydrogen bonds between oxygen and hydrogen atoms (Nowick, 2008) , the missing oxygen atom in FlhF(T368A)

could break the hydrogen bond network between this beta sheet and another. Therefore, the structure of FlhF(T368A) would be less stable compared to wild type FlhF. This hypothesis was confirmed by FlhF thermal stability experiment as FlhF(T368A) revealed less stability against heat treatment than wild type FlhF protein.

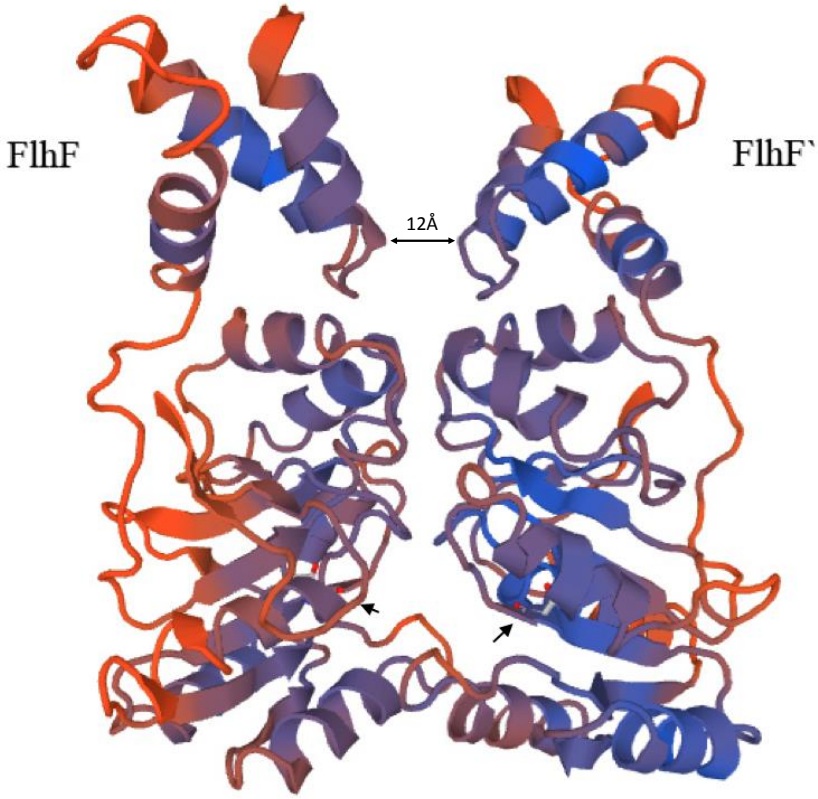


Figure 6.23 FlhF protein homodimer structure with N domains on top and G domains at the bottom. Arrow heads in G domain indicate the location of T368A substitution. (Swiss-prot, 2014)

Bange et al. (2007) reported that FlhF of *B subtilis* forms a stable homodimer structure in the presence of GTP (Figure 6.23), and that the G domains form a composite active site harbouring two GTP molecules. The N domains are not identified as the interface of the dimer structure and

are separated by a distance of 12Å. Since FlhF(T368A) has a mutation in the G3 domain, it is likely to have a reduced affinity or residency time for GTP nucleotides. As a result of reduced GTP binding ability, we would expect this mutation to show a decrease in GTPase activity. After comparison, FlhF wild type had a 2.3-fold less K_m than FlhF(T368A), suggesting that FlhF wild type required 2.3-fold less substrate to achieve its half V_{max} than FlhF(T368A), and therefore had a higher substrate affinity. Comparison of the V_{max} per milligram protein sample, FlhF wild type showed a maximum reaction rate of 62.5 nmol min^{-1} mg^{-1}, which was also higher than FlhF(T368A) protein with a V_{max} of 37.74 nmol min^{-1} mg^{-1}. Therefore, we demonstrate that when [S] was much greater than K_m, the maximum velocity FlhF protein could reach was 1.65-fold higher than FlhF(T368A). The turnover number k_{cat} was also a signicicant parameter for enzyme kinetics, and as a result, k_{cat} of 0.0115 $second^{-1}$ for FlhF wild type was also higher than k_{cat} of 0.007 $second^{-1}$ for FlhF(T368A), demonstrates within one minute, FlhF wild type has a 1.62-fold faster ability to hydrolyze GTP than FlhF(T368A). In summary, FlhF wild type protein is more stable against thermal stress and more active than FlhF(T368A) as a GTPase.

The instability of FlhF(T368A) was also evident in attempts to express the protein in *E. coli*. We found that the BL21DE3 Star *E. coli* expression host strain could efficiently express active wild type FlhF but FlhF(T368A) was expressed as an inclusion body, where reducing the incubation temperature to 25°C did not improve the solubility. Betz (1993) and

(Pantoliano et al., 1987) state that the disulphide bonds, formed by oxidation of a pair of cysteines, can significantly stabilize the protein structure. FlhF and FlhF(T368A) proteins contain three cysteine residues, and the possibility of free cysteine could lead to mis-pairing of the cysteine residues, and further cause protein misfolding and aggregation (Ke and Berkmen, 2012). To overcome this potential problem we used the expression host strain into Origami 2 DE3 pLysS, which contained mutations in thioredoxin reductase and glutathione reductase, to improve the formation of the correct disulphide bonds. Using this host it was possible to produce and purify FlhF(T368A) protein.

In this chapter, evidence that FlhF(T368A) has reduced GTPase activity and stability than wild type FlhF, and that the *flhF(T368A)* mutation results in a downregulation of $\sigma^{28}$and σ^{54} dependent flagellar genes that collectively cause defective flagella biosynthesis and impaired motility. Therefore, it may be concluded that *flhF(T368A)* is responsible for the non-motile phenotype of *C. jejuni* PT14CP30ACS.

Chapter 7

Discussion

7.1 General discussion

Campylobacter jejuni is the major cause of gastroenteritis worldwide and is the most commonly reported foodborne disease in the EU. Consumption or handling of contaminated poultry meat is considered as the main source of infection. Conventional biosecurity measures for poultry flocks have not reduced the incidence of *Campylobacter* on product, and as a consequence several alternative interventions have been developed to control *Campylobacter* infection including bacteriophage therapy. An increasing number of studies have successfully reduced *Campylobacter* colonization after the application of phage therapy both *in vivo* and *in vitro*.

Coevolution interactions between *Campylobacter* and phage are central to understanding the application of phage therapy as well as natural *Campylobacter* ecology. Bacteriophage are capable of driving rapid molecular modification (Paterson et al., 2010) through reciprocal higher mutation rates (Pal et al., 2007), and it is therefore important to understand how these processes can affect *Campylobacter* populations in agriculture and for the application of phage therapy how to select the correct phage and when to apply them. .

Chapter 3 of this thesis started the study with the determination of the presence of Mu-like prophage in *C. jejuni* caecal isolates from broiler flocks as they progressed over the rearing period. As Mu-like prophage are considered as transposable elements they constitute a source of

genetic diversity for *C. jejuni.* We followed the course of *Campylobacter* colonization in a broiler chicken flock. Birds selected from the flock first exhibited *C. jejuni* colonization of their intestinal contents at 23 days of age to achieve mean colonization levels of 8.0 $\log_{10}$ cfu g^{-1}, which was followed by a significant decrease in colonization at 30 days of age, which coincided with the first appearance of Mu-like prophage in the *Campylobacter* populations recovered from intestinal contents. The proportion of the *Campylobacter* population carrying Mu-like prophage was almost 45% suggesting rapid spread given that it was absent in the populations sampled 7 days earlier. The dissemination of the Mu-like prophage within the flock could occur by Mu-like carriers outcompeting resident campylobacters or infection and replication of the free phage or a combination of both. By day 34 the proportion of the campylobacters carrying Mu-like prophage had increased to become the dominant component of the population at 52% before declining to 11% at day 37. If the prophage carrying campylobacters had a competitive advantage compared to the resident campylobacters when first colonizing the flock this advantage was eroded over 7 days at the same time the total *Campylobacter* population increased to 8.3 $\log_{10}$ cfu g^{-1}. It was not the case that a favourable prophage configuration had been selected amongst the population that conferred fitness in the intestine and resisted phage super-infection rather the resident bacteria had become resistant to Mu-like infection or a new insensitive *C. jejuni* had been introduced. Representative strains of these time points are available and could add to

our understanding if the whole genome sequences were determined and analysed.

Coevolution interactions between *Campylobacter* and phage are a constant source for genetic mutation and variation in phage and host bacteria in the environment. A T4-like *Campylobacter* specific phage CP30 within *Eucampyvirinae* subfamily was found become a mixed phage population containing identifiably different sequence types after serial passage in the laboratory. Chapter 4 analysed the genetic variation arising to identify and characterise a new sequence type, phage CP30C. Following repeated plaque purification, phage CP30C was purified from CP30A and B. After whole genome sequencing and alignment, the genome variations were determined to reside within two regions (3-4.5kb, 121-125kb) and all genes involved in these regions were identified as hypothetical protein encoding genes. Several point mutations were also observed in genes that encode proteins responsible for phage morphogenesis and assembly. Of significance, a single cytosine deletion caused a frameshift mutation in gene *gp050* that encodes a putative tail fibre protein. The sequence of *gp050* shares homology with *gp047* in phage NCTC12673, for which the C-terminal region of the protein product was proposed to the receptor binding protein. The frameshift mutation in phage CP30C resulted in 44 amino acids from N-terminal to be lost and an alternative initiation codon employed. We hypothesized this could potentially affect the adsorption ability to host cells of such phage. As the Gp050 binding protein specifically targets acetamidino-modified pseudaminic acid on flagellin,

we used three *C. jejuni* strains isolated from the same flock, which were previously identified as having different flagellin glycosylation modification, to determine if the defective Gp050 in phage CP30C would lead to a reduction in infection ability compared with CP30A. However, no significant difference was observed between two types of phages, potentially suggesting that N-terminal 44 amino acid deletion was not responsible for differences in host susceptibility. The cause for the variation in susceptibility of phage CP30C to *C. jejuni* CII C3 and FDIC2 to CP30A therefore needs to be further investigated.

Phage therapy in broiler flocks is considered as a therapeutic usage of lytic bacteriophage to control *C. jejuni*. However, when Patcharin Siringan was using lytic phage to reduce *Campylobacter* biofilms, bacteria recovered post phage treatment were found to produce phage spontaneously and were investigated to establish they had entered the carrier state life cycle. Carrier state bacteria are impaired in motility and limit the propagation of virulent bacteriophage with which they are associated. The association has benefits to the host in that it improves survival and therefore transit of extra-intestinal environments but with the drawback is they do not colonize chickens efficiently (Siringan *et al.,* 2014). Carrier state bacteria are therefore repositories for virulent phage to remain with their host and vehicles for the dissemination of virulent bacteriophage. Chapter 5 focused on the genome variation between *C. jejuni* in CSLC with their parental strain. Based on motility tests, *C. jejuni* PT14CP30-035CS and PT14CP30-064CS revealed an uncommon

phenotype with irregular shaped bacterial halos, whilst *C. jejuni* PT14CP8CS showed a significantly reduced motility than wild type *C. jejuni* PT14. *C. jejuni* PT14CP30CS is non-motile but not profoundly so with aggregation observed extending from centre inocula on motility assay plates. Based on whole genome sequencing, several mutations were determined in *C. jejuni* CSLC over their parental strain. These include a substitution of adenine to guanine in *flhF* in *C. jejuni* PT14CP30ACS; an adenine deletion in *A991_00360* encoding L-Lactate permease in *C. jejuni* PT14CP30A-035CS; a thymine deletion in *A911_05040* encoding an AraC-type DNA-binding protein in *C. jejuni* PT14CP8CS; and a substitution of thymine to adenine in *A911_04139* that encodes a hypothetical protein.

It was hypothesised that the point mutation in *flhF* present in carrier state bacteria carrying the bacteriophage CP30A caused impaired motility and could be a key adaption to limit phage replication to maintain a balanced host and phage association. To test this hypothesis, it was necessary to create isogenic strains carrying the *flhF(T368A)* mutation independent of the influence of phage infection and expression. We first constructed several variants of *C. jejuni* PT14, including non-functional *flhF* (*C. jejuni* PT14*flhF::kan*), wild type *flhF* complementation (*C. jejuni* PT14*flhF::kan 00230::flhF-cat*) and *flhF(T368A)* complementation (*C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat*).

Based on a combination of motility test and TEM, *flhF* is identified as essential for proper flagella biosynthesis and *flhF(T368A)* as significantly

reduced swarming motility due to most of *C. jejuni* cells with the *flhF(T368A)* mutation having lost their ability to synthesise flagella. The *flhF* may also correlated with the morphology of *C. jejuni* cells as both *flhF* mutant and *flhF(T368A)* complementation revealed a straight shape cells, whilst wild type *C. jejuni* and the *flhF* complementation cells were normal spiral shaped. We then analyzed whether the reduced swarming motility was due to the downregulation of certain flagellar genes through qPCR. We identified that *flhF* affects the expression of both σ^{28}-dependent (*flaA*) and σ^{54}-dependent (*flaB*, *flgD, pflA*) genes, whilst *flhF(T368A)* has lost this function. Hendrixson and DiRita (2003) suggested that *flhF* transcription was presumably at the start of the σ^{54} transcriptional cascade, and the *flhF* mutant would cause improper transcription of the downstream genes in the transcriptional cascade. Correa et al. (2005) reported that *flhF* was essential for proper expression of σ^{28} dependent flagellar genes including *flaA* in *Vibrio cholerae,* that is identical with our observations in *C. jejuni.* The reduced motility in *C. jejuni* PT14*flhF::kan 00230::flhF(T368A)-cat* further generally resulted in a less sensitive phenotype than its parental strain against phage infection using EOP test comparisons and growth characterization in broth cultures with phage CP30A infection. Notably, phage ϕ3 can only infect *C. jejuni* PT14 and *C. jejuni* PT14*flhF::kan 00230::flhF-cat,* that are identified as capable of synthesizing flagella properly. We hypothesized that phage ϕ3 might be a flagellatropic phage and the presence of flagella on its host strains plays a significant role for its infection. Moreover, with the exception of phage ϕ3, the EOP for all other tested phage between *C. jejuni* PT14*flhF::kan 00230::flhF-cat* and *C.*

jejuni PT14*flhF::kan 00230::flhF(T368A)-cat* revealed no significant difference, suggesting that *flhF(T368A)* potentially would not prevent infection ability but reduce efficiency and potentially contribute to a resistant phenotype for the host strain. The GTPase activity of FlhF protein was confirmed and FlhF(T368A) exhibited significantly less GTP hydrolysis ability and impaired folding compared to wild type FlhF. This was considered as due to the loss of a -HCOH- group in G3 domain of FlhF with possibility of reduced affinity or residency time for GTP nucleotides. In summary, we can also conclude that *flhF(T368A)* enabled *C. jejuni* to be less sensitive against phage infection but the fitness costs are severe.

In conclusion, this thesis identified genetic modifications present in either bacteria or phage during coevolution interactions occurring under *in vivo* and *in vitro* conditions, and illustrated the potential consequences of such modifications and mutations. This study also proved that the occurrence of phage resistance in *C. jejuni* is associated with great fitness costs as a consequence of losing flagella synthesis. Interestingly, as a product of genetic diversity, a newly characterized phage CP30C revealed a defect in tail fibre protein that had been implicated in infection efficiency. Whether this mutation is spontaneously produced or caused by certain environmental stress needs further investigation.

7.2 Future work

Due to time constraint, there are certain subjects this study could not cover. In future work, the infection ability of phage CP30C could be

investigated to confirm the role of putative tail fibre protein in the adsorption of phage to host cells. The CRISPR-Cas system is possibly another area that could be analyzed in *C. jejuni* CSLC. As part of this study but not presented, a northern blot based CRISPR-Cas detection was carried out with DIG-labelled probes in order to efficiently determine the presence of foreign insertions within the CRISPR-array and the relative expression value of the CRISPR-array with incorporation of proto-spacer in *C. jejuni* CSLC compared with the array in their parental strain at the same time. Next generation sequencing was employed by Hooton and Connerton (2015) to identify new host-derived proto-spacers in carrier state cultures. The development of techniques identifies when and in which bacteria these events occur would be of significant benefit to understanding if they are subject to self-destruction by CRISPR mediated autoimmunity or represent an evolving system. However, even with relatively high amounts of RNA, no signal was observed except the well-expressed positive control 5s RNA. This was considered as due to a relatively low expression from CRISPR-array; RNA loss during blot transfer techniques or natural degradation; and the low labelling efficiency of DIG compared with radioactive labelling techniques. In the future, it may worth to try another labelling method to analysis the CRISPR-Cas system in *C. jejuni* CSLC or utilize PCR and RT-PCR to separately determine the presence of foreign incorporation elements and its relative expression level.

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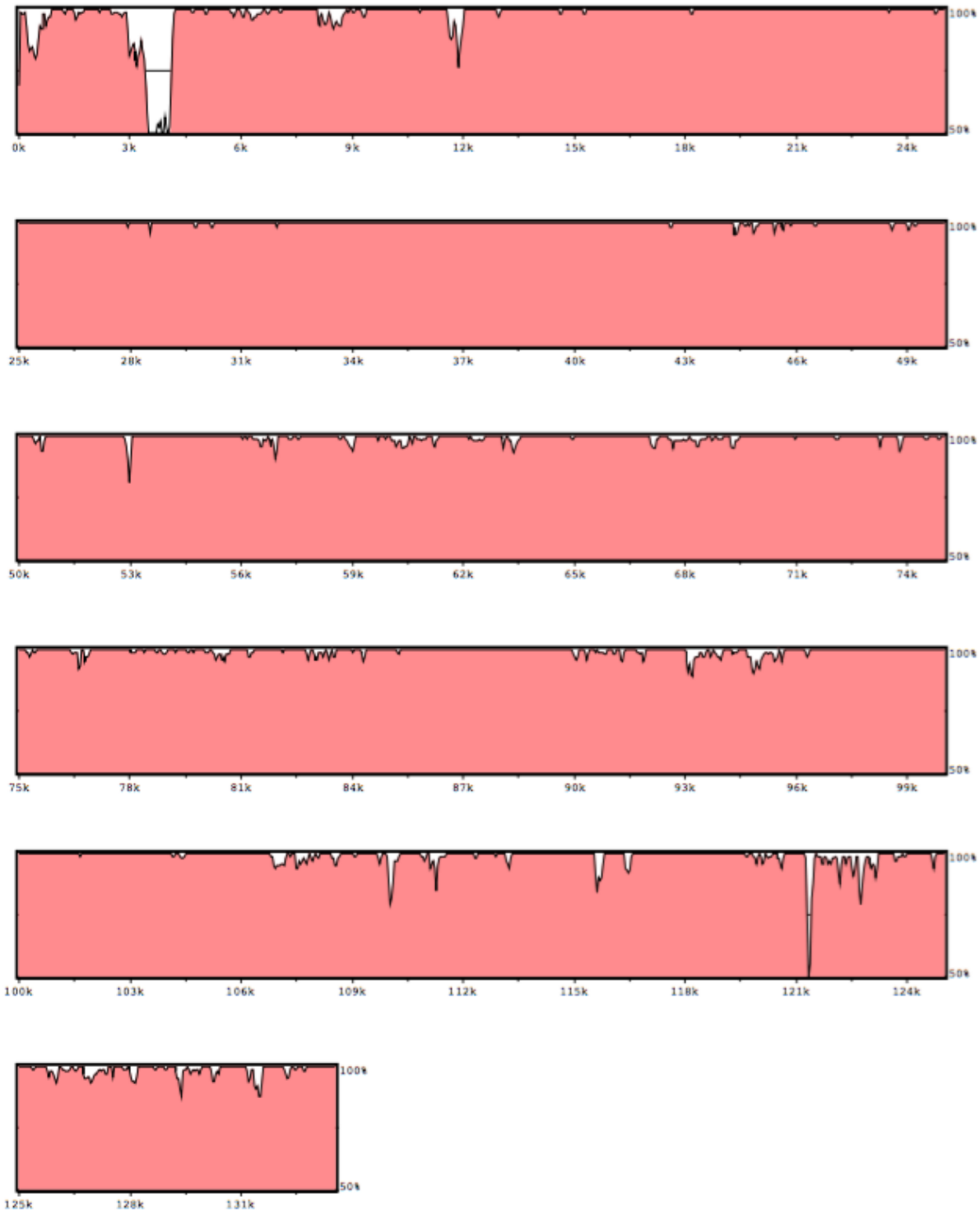
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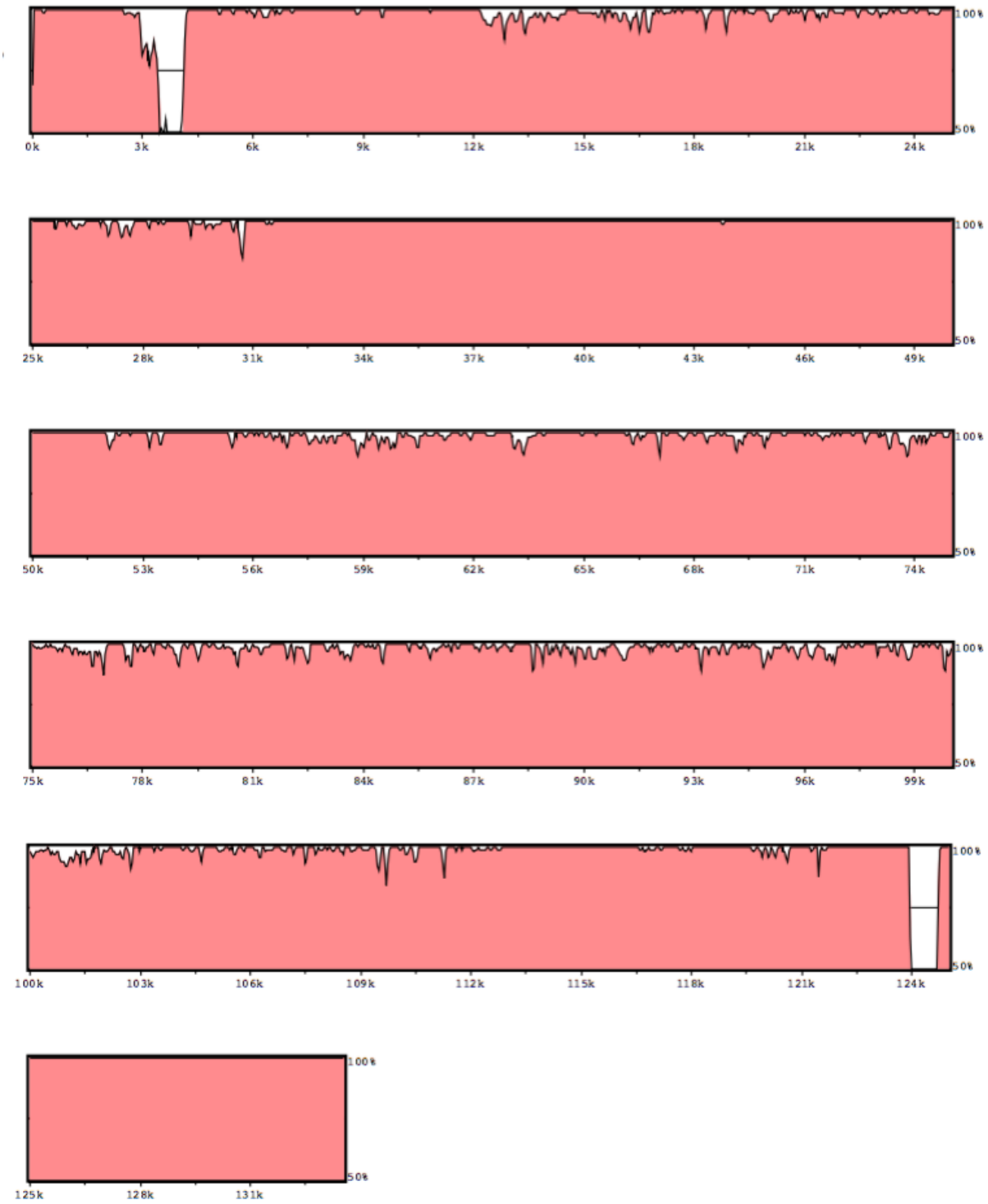
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Appendix

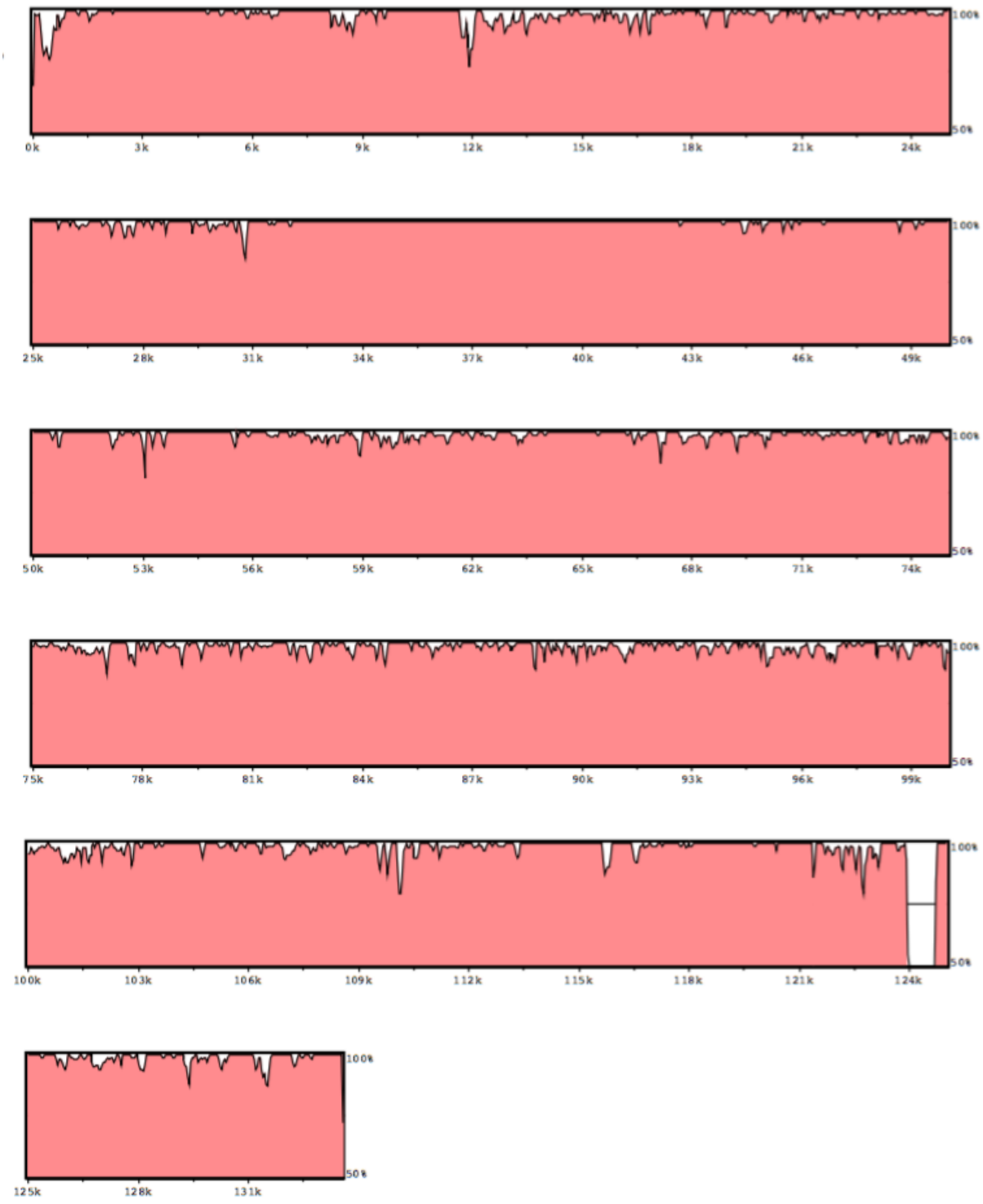
Appendix 1. Genome alignment between phage CP30A and CP30B using gpVISTA alignment. Window size of 100bp is applied. CP30A sequence is used as the reference.



Appendix 2. Genome alignment between phage CP30A and CP30C using gpVISTA alignment. Window size of 100bp is applied. CP30A sequence is used as the reference.



Appendix 3. Genome alignment between phage CP30B and CP30C using gpVISTA alignment. Window size of 100bp is applied. CP30A sequence is used as the reference.



Appendix 4. DNA sequence of amplified fragment using primer CP30AB/C in phage CP30A, B and C. Primer binding locations are indicated in red.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTGTTAATTG TTTTTCATTT TCAATCAATA TAGCATTTGA TTTTTCTAAT GAAATATATC
CP30B  GTGTTAATTG TTTTTCATTT TCAATTAACA CAGCGTTTGA TTTTTCCAAA GAAATATATC
CP30C  GTGTTAATTG TTTTTCATTT TCAATTAACA CAGCGTTTGA TTTTTCCAAA GAAATATATC

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATAAGCAGT AAAAGCTAAC AAAGATCCTA ACAAAATATA AATTATGTTT GACTTTATAA
CP30B  TATAAGTTAT AGCAACTAAT AAACAACCAA ATATAATATA AACTATGTTT GATTTTATAA
CP30C  TATAAGTTAT AGCAACTAAT AAACAACCAA ATATAATATA AACTATGTTT GATTTTATAA

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGAAAATAA AAAATTAAAC AT-----TTG CATCCTTTAA ATGT------ --TTGATACT
CP30B  CTGATAGTAT AATATTAAAC ATGTTATCTA TTTTCCTAGA ATATAGTATA ATTTTCCATC
CP30C  CTGATAGTAT AATATTAAAC ATGTTATCTA TTTTCCTAGA ATATAGTATA ATTTTCCATC

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CGAAAAAAAC G-AGTATCAA ACATGTTTAT TT-------- -TTTAGGTTT TACATATAAA
CP30B  TGATGCAAAT GTAGCACCAA ATCAACTGAT TCACATAAAT ATTTAGAATA CATATTTTGC
CP30C  TGATGCAAAT GTAGCACCAA ATCAACTGAT TCACATAAAT ATTTAGAATA CATATTTTGC

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCAGAGTCTT CTAAATCA-- ---AATTCAG CACCAAAGTT ATCAATAACA ATTTCCCACT
CP30B  TTACCTCTTT TTAAATTATT TATAATTAAG T-TAAATCTT TTGAATAATT AGTGATGAGT
CP30C  TTACCTCTTT TTAAATTATT TATAATTAAG T-TAAATCTT TTGAATAATT AGTGATGAGT

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CACCTTCATA ATTTTTTATT TTCA-TAGCT GAGTTATCAT CATAACCAAC TCTTACTTTT
CP30B  ACAAATAATT ATATTTTGTA CCAACTTGAC CATTTACTTT AATAACTTTA TCGTTTTTTA
CP30C  ACAAATAATT ATATTTTGTA CCAACTTGAC CATTTACTTT AATAACTTTA TCGTTTTTTA

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAACCAATAT CACCTTCATA ATATTCCCAA TCATATAGCT CATCTTTGAT GATATTATCT
CP30B  AAGTAAATAT CATACTT--- -TCTCCATAA TCATTAAATA ATTCTACAAC ACTTTCACCT
CP30C  AAGTAAATAT CATACTT--- -TCTCCATAA TCATTAAATA ATTCTACAAC ACTTTCACCT

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATATCATCAA CTTTTGATAC TATAACGCTT GCTAATTTTT GTAGTGATTT TTCATCAAAA
CP30B  ATATTACC-- -TTCTACTAT TTTAAATTGT TTTGATTTAA GTTCATTTTG TATAATATCA
CP30C  ATATTACC-- -TTCTACTAT TTTAAATTGT TTTGATTTAA GTTCATTTTG TATAATATCA

              490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAATTATAA TAGGAGGGTA TTTTTCTCCA TCAAAACTTA TG----TTTG CTACTTTTTC
CP30B  GTAAAATTAT TTATATAATA TTCTTTTTCG AGTAATCTTG CAACATTATA TTCTGTTATC
CP30C  GTAAAATTAT TTATATAATA TTCTTTTTCG AGTAATCTTG CAACATTATA TTCTGTTATC

              550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTAT---ATT TCATTTAAAA T----GTCTT CTTTTATACC AGTAACTCTC ACTGTTATAC
CP30B  ATATCCAATT TAAGTGATAA TACAGGTTCA TTTTTACTCT TGCAATTTTT AAGTTTATCT
CP30C  ATATCCAATT TAAGTGATAA TACAGGTTCA TTTTTACTCT TGCAATTTTT AAGTTTATCT

              610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAGGTTT-TA TTAAGTTTAG TCTTGTTCGA GCAGTTCGTT CTTTAACAAA GAATGA----
CP30B  TCTATTGATT TTAAAGTATC TTTTTTTAAA TCTGTTACAT TAACAACTAT GGATGGCTCA
CP30C  TCTATTGATT TTAAAGTATC TTTTTTTAAA TCTGTTACAT TAACAACTAT GGATGGCTCA

              670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ----TATCAG AATTTGTA-T ATCAGAGTCA TTAACTAT-- ---AGAACTT AATTCATAAC
```

```
CP30B  ATTTTCTTAT AATTTCCAAT ATTATAATGC TCAAATACTC CAAATATTTC AATGTCTAAA
CP30C  ATTTTCTTAT AATTTCCAAT ATTATAATGC TCAAATACTC CAAATATTTC AATGTCTAAA

               730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TGTCTAA-TA TAGTTTTACC ATAACTAAC- -ATTTACCTT TAAATTAGCT TTAGCTATTG
CP30B  CATATATCTT TCAACTTGCC TTCTTCAATA GATCTATCAT TTAAA-AACA TCACATATTC
CP30C  CATATATCTT TCAACTTGCC TTCTTCAATA GATCTATCAT TTAAA-AACA TCACATATTC

               790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAGTA---T TAAACTA--- GTAATATTAT TGCTAATATT TTTATCGTTG TTTTTTAAAT
CP30B  TATATTATCT TGTATTACAG GTAATATTAG TTTGCGTAAT TTTATATCAT TTTTTGCAAA
CP30C  TATATTATCT TGTATTACAG GTAATATTAG TTTGCGTAAT TTTATATCAT TTTTTGCAAA

               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CATTTATA-- ---------G ATTCAACTAA AAATTTTGAG TATATCATAC TTATATCCTT
CP30B  TACCCATAAG TTTTCAGATG ATTCATTTAA AAATTTTGAA TACATTATAT TTAGATCCTT
CP30C  TACCCATAAG TTTTCAGATG ATTCATTTAA AAATTTTGAA TACATTATAT TTAGATCCTT

               910        920        930        940        950
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|..
CP30A  TTTCAAT-TA TTTATATTTT ATGTCGTATT TTATAAGAGC AGCTACTAAA GCACTTA
CP30B  TTCAACTATT TTTATATTTT ATATCATATT TTATAAGAGC AGCTACTAAA GCACTTA
CP30C  TTCAACTATT TTTATATTTT ATATCATATT TTATAAGAGC AGCTACTAAA GCACTTA
```

Appendix 5. DNA sequence of amplified fragment using primer CP30A/BC in phage CP30A, B and C. Primer binding locations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTGTCTTTAT GTTCTTTCGG AGTATCAGTA TGTGCATAAC GGAATATATG GGTACCTAGT
CP30B  GTGTCTTTAT GTTCTTTCGG AGTATCAGTA TGTGCATAAC GGAATATATG GCTACCTAAT
CP30C  GTGTCTTTAT GTTCTTTCGG AGTATCAGTA TGTGCATAAC GGAATATATG GGTACCTAGT

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTGTTAATTA ATGTTTCAAT ATCCACATTT TTATCATTTA TTTCTAAGTA TTTTATATTA
CP30B  ATGTTAATTA ATGTTTCAAT ATCCACATTT TTATCATTTA TTTCTAAGTA TTTTATATTA
CP30C  CTGTTAATTA ATGTTTCAAT ATCCACATTT TTATCATTTA ATTTCTAAGT TTTTATATTA

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTTTCATAC AGCTTATGAA GTTGGTAATG CTTTTAAATT CATCATCTGA AAGATTACAC
CP30B  TTTTTCATAC AGCTTATGAA GTTGGTAATT CTTTTAAATT CATCATCTGA AAGATTACAC
CP30C  TTTTTCATAC AGCTTATGGA GTTGGTAATG CTTTTAAATT CATCATCTGA AAGATTACAC

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATATATTTAA CATTGTAGAA CTCATCATCA TAATAACATA TGCAATTATT TAAAAATGCT
CP30B  ATATATTTAA TATTGTAGAA CTCATCATCA TAATAACATA TGCAATTATT TAAAAATGCT
CP30C  ATATATTTAA CATTGTAGAA CTCATCATCA TAATAACATA TGCAATTATT TAAAAATGCT

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATTGCTCAG TTTCAAATTT AAAAGATATC ACAGTTGCGT TATTCATATT ATTCCTTTCA
CP30B  AATTGCTCAG TTTCAAATTT AAAAGATATC ACAGTTGCGT TATTCATATT ATTCCTTTCC
CP30C  AATTGCTCAG TTTCAAATTT AAAAGATATC ACAGTTGCGT TATTCATATT ATTCCTTTCA

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATGTTTATG GTATTATACC ACAAATAATC TTAAAGATTA CTTAAATTAA TTCATTCTAT
CP30B  AATGTTTATG GTATTATACC ACAAATAATC TTAAAGATTA CTTAAATTAA TTCATTCTAT
CP30C  AATATTTGTA GTATTATATC ACAAACAATC TTAAAGATTA CTTAAAT--- ----------

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTATAAAAT TCTCACTCTT TATCTTTCAA CAGCTCTAAA CTTTTGGTGG ATACACAATA
CP30B  ATTATAAAAT TCTCACTCTT TATCTTTCAA CAGCTCTAAA CTTTTGGTGG ATACACAATA
CP30C  ---------- ---------- ---------- ---------- ---------- ----------

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTCATTTATT TAGTTTTATT TTTGATTCTT TTAACTTCTC TTGTTGCTTA AATTTCCTGT
CP30B  CTCATTTATT TAGTTTTATT TTTGATTCTT TTAACTTCTC TTGTTGCTTA AATTTCCTGT
CP30C  ---------- ---------- ---------- ---------- ---------- ----------

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTAATTTATG ATTAAGATAT AAATAAGGCG GGTCAGTTAT TATACAGTCT ATCGTCTCAT
CP30B  CTAATTTATG ATTAAGATAT AAATAAGGCG GGTCAGTTAT TATACAGTCT ATCGTCTCAT
CP30C  ---------- ---------- ---------- ---------- ---------- ----------

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTTTATTAAT CATATCCTTG TATAAACTCT ATACAGTCAC AATTGTATAC TTTATAATTA
CP30B  CTTTATTAAT CATATCCTTG TATAAACTCT ATACAGTCAC AATTGTATAC TTTATAATTA
CP30C  ---------- ---------- ---------- ---------- ---------- ----------

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTTATTCACA TCCCTTGAGT TAAATAATTC AAAATTATGA TTATTTATAT ATTTAGTTAA
CP30B  CTTATTCACA TCCCTTGAGT TAAATAATTC AAAATTATGA TTATTTATAT ATTTAGTTAA
CP30C  ---------- ---------- ---------- ---------- ---------- ----------

               670        680        690        700        710        720
```

```
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TGATATATCA TTCTTTATAC AATAATTTTT ACATTCTTCC GAGTTTAAAA TATCTAGTAG
CP30B   TGATATATCA TTCTTTATAC AATAATTTTT ACATTCTTCC GAGTTTAAAA TATCTAGTAG
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                720        740        750        760        770        780
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TTCTTTTTCA ACTTCTGATA TTTTTTTAAT TTTACCTATA ACTCCATGTT TGCTGCTTAT
CP30B   TTCTTTTTCA ACTTCTGATA TTTTTTTAAT TTTACCTATA ACTCCATGTT TGCTGCTTAT
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                790        800        810        820        830        840
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GTATTCTTCA AGTTTTGAAG GAGATATA-- -ATGCTTAGG TCTAAAGAAA ACATTTTTAT
CP30B   GTATTCTTCA AGTTTTGAAG GAGATATA-- -ATGCTTAGG TCTAAAGAAA ACATTTTTAT
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                850        860        870        880        890        900
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GGTTTTACAT TTAGGACATT CAGAGATATA CCATTTTAAG TTATTTTCTG TCAATTTTAA
CP30B   GGTTTTACAT TTAGGACATT CAGAGATATA CCATTTTAAG TTATTTTCTG TCAATTTTAA
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                910        920        930        940        950        960
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   ATAAACCACT TTATCTGTAA ATAATTTTTT AAATTCTCCA AAATTACAAG TTTTACATTT
CP30B   ATAAACCACT TTATCTGTAA ATAATTTTTT AAATTCTCCA AAATTACAAG TTTTACATTT
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                970        980        990        1000       1010       1020
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GCAATTTTTA GAATCTTTAT GAAAAATACT TGTTATATGT TTTAACATTT TACTTTTATC
CP30B   GCAATTTTTA GAATCTTTAT GAAAAATACT TGTTATATGT TTTAACATTT TACTTTTATC
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                1030       1040       1050       1060       1070       1080
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   CTTTAATTAA TTTTAAAACT TATTCTTTGG TTTATAAAAT CTTCCATGTT -TTATTCTCC
CP30B   CTTTAATTAA TTTTAAAACT TATTCTTTGG TTTATAAAAT CTTCCATGTT -TTATTCTCA
CP30C   ---------- ---------- ---------- ---------- ---------- ----------

                1090       1100       1110       1120       1130       1140
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   AATGTTTATG GTATTATACC ACAAATAATC TTAAAGATTA CTTAAATAAT TCATTTTCAG
CP30B   AATATTTGTA GTATTATATC ACAAACAATC TTAAAGATTA CTTAAATAAT TCATTTTCAG
CP30C   AATATTTGTA GTATTATATC ACAAACAATC TTAAAGATTA ----AATAAT TCATTTTCAG

                1150       1160       1170       1180       1190       1200
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TTAATACCAT AAATTCCATA TTATTTATTC TACAGAACTC TCTTGCAGCT TCCCATTTTG
CP30B   TTAATACCAT AAATTCCATA TTATTTATTC TACAGAACTC TCTTGCAGCT TCCCATTTTG
CP30C   TTAATACCAT AAATTCCATA TTATTTATTC TACAGAACTC TCTTGCAGCT TCCCATTTTG

                1210       1220       1230       1240       1250       1260
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   CTTGATTTTT TGAAACTGTT AATGCTTGTT CTACTACTCT TTTTTTATTT TTTTCTGTGA
CP30B   CTTGATTTTT TGAAACTGTT AATGCTTGTT CTACTACTCT TTTTTTATTT TTTTCTGTGA
CP30C   CTTGATTTTT TGAAACTGTT AATGCTTGTT CTACTACTCT TTTTTTATTT TTTTCTGTGA

                1270       1280       1290       1300       1310       1320
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TTATTTTTGG TTTTTTAAAT ATAGCGTCTT TTTGTGGTTT AATTTCAATA AGATATTTTT
CP30B   TTATTTTTGG TTTTTTAAAT ATAGCGTCTT TTTGTGGTTT AATTTCAATA AGATATTTTT
CP30C   TTATTTTTGG TTTTTTAAAT ATAGCGTCTT TTTGTGGTTT AATTTCAATA AGATATTTTT

                1330       1340       1350       1360       1370       1380
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TTATGTTATT GTCTTTATCT TTTACTTCAA TATAAAAATC AGGGAAATAT CTATGTTTTT
CP30B   TTATGTTATT GTCTTTATCT TTTACTTCAA TATAAAAATC AGGGAAATAT CTATGTTTTT
CP30C   TTATGTTATT GTCTTTATCT TTTACTTCAA TATAAAAATC AGGGAAATAT CTATGTTTTT

                1390       1400       1410       1420       1430       1440
```

```
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TACCTAGGAA CTCATAAGGT ATAATTATTG ATTCACTAGC CCATTTAAGA ACACCAGCAT
CP30B  TACCTAGGAA CTCATAAGGT ATAATTATTG ATTCACTAGC CCATTTAAGA ACACCAGCAT
CP30C  TACCTAGGAA CTCATAAGGT ATAATTATTG ATTCACTAGC CCATTTAAGA ACACCAGCAT

               1450       1460       1470       1480       1490
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|..
CP30A  TATTATCGCA CCATAACATA AATTTATATT CCCATGAGCT ACGATAGATG ATGTTTG
CP30B  TATTATCGCA CCATAACATA AATTTATATT CCCATGAGCT ACGATAGATG ATGTTTG
CP30C  TATTATCGCA CCATAACATA AATTTATATT CCCATGAGCT ACGATAGATG ATGTTTG
```

Appendix 6. DNA sequence of D302_gp053 gene in phage CP30A and C, which encode putative ssDNA binding protein.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGAATGAAT TTGACATTTT AACAGGATTT TCAGGTGCAG ATTTAATGCA AAAAATGCCA
CP30C  ATGAATGAAT TTGACATTTT AACAGGATTT TCAGGTGCAG ATTTAATGCA AAAAATGCCA

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAAAATATTG GCCAAAAAAG TTATGTTGAT AACAGATTTT GGAAGCTGTC AAAAAACAAA
CP30C  CAAAATATTG GCCAAAAAAG TTATGTTGAT AACAGATTTT GGAAGCTGTC AAAAAACAAA

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAGGGCAGTG GAGCAGCTGT TATTAGATTA ATAACAGATA AACATAAAAC ACCATTCGTT
CP30C  GAGGGCAGTG GAGCAGCTGT TATTAGATTA ATAACAGATA AACATAAAAC ACCATTCGTT

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CACATATATC ACTATAACTC TAAAAAGAAT GTAGGTGGCA AAGATCGCTG GTTAATAGCA
CP30C  CACATATATC ACTATAACTC TAAAAAGAAT GTAGGTGGCA AAGATCGCTG GTTAATAGCA

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGTCCAA GTACAATTGG ATTACCTTGT CCTATTCAAG AAGAGTATTT TGAAGTATTA
CP30C  AATAGTCCAA GTACAATTGG ATTACCTTGT CCTATTCAAG AAGAGTATTT TGAAGTATTA

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGTGGTG ATGAAAAACT GGCAAGATCA CTATATGGTA GAAAGGTAAA ATACTACACT
CP30C  AATAGTGGTG ATGAAAAACT GGCAAGATCA CTATATGGTA GAAAGGTAAA ATACTACACT

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AACATTTTAG TTGTAAAAGA TCCAGCTAAT CCTGAAAATG AAGGTAAAGT GTTTCTATTT
CP30C  AACATTTTAG TTGTAAAAGA TCCAGCTAAT CCTGAAAATG AAGGTAAAGT GTTTCTATTT

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAATTTGGAA GTAAGTTAAA AGAAAAGTTC CTAGCTTGGA TGAATCCAGA TGAAACACAA
CP30C  GAATTTGGAA GTAAGTTAAA AGAAAAGTTC CTAGCTTGGA TGAATCCAGA TGAAACACAA

              490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGGTCTCTAG GACATACAGA AAAAGAACTA TATAACCCTA TAAATGGTTA TAATATAGAG
CP30C  AGGTCTCTAG GACATACAGA AAAAGAACTA TATAACCCTA TAAATGGTTA TAATATAGAG

              550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTAACTATTA AAAAAGATCC ACAATCAGGT TTCTTTAACT ATGATAACAC AAGTTTAGCA
CP30C  CTAACTATTA AAAAAGATCC ACAATCAGGT TTCTTTAACT ATGATAACAC AAGTTTAGCA

              610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CCATCACCTT CAAAGTTAGG CGGGTTAGAA AAAAATGAAG ATATTATAGA CATAATTCTT
CP30C  CCATCACCTT CAAAGTTAGG CGGGTTAGAA AAAAATGAAG ATATTATAGA CATAATTCTT

              670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAAAACTT ACGATTTAAG TGAATTTACA AAGCCTGAGT ATTTTCCTTC TTATGAAGAA
CP30C  AATAAAACTT ACGATTTAAG TGAATTTACA AAGCCTGAGT ATTTTCCTTC TTATGAAGAA

              730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAAAAGAAA AACTAGAAAG GTTTAAAAAT CCTTTTGGCA CTAAAACTTC AAGTGTTCCA
CP30C  TTAAAAGAAA AACTAGAAAG GTTTAAAAAT CCTTTTGGCA CTAAAACTTC AAGTGTTCCA

              790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCAGTGGTTG AAAAAACAAA TGATAATCCA CCATTTGAAA CACAAGAATC AAAACCACAA
```

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CP30C  TCAGTGGTTG AAAAAACAAA TGATAATCCA CCATTTGAAA CACAAGAATC AAAACCACAA

               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CCTCAACAAC AAGTGGTTCA ACAACAAAAA CCTAAACAAG AAAACAGTCA GGATGATGAT
CP30C  CCTCAACAAC AAGTGGTTCA ACAACAAAAA CCTAAACAAG AAAACAGTCA GGATGATGAT

               910
       ....|....| ....|...
CP30A  TGGTTAAATA ATCTTTAA
CP30C  TGGTTAAATA ATCTTTAA
```

Appendix 7. DNA sequence of D302_gp075 gene in phage CP30A and C, which encode major capsid protein. Point mutations are indicated in red.

```
                10         20         30         40         50         60
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   ATGGCTGACA AATATTTGTT AGATGAATCA ACAAAAGAAA AATTTATTAC AAGCAACTTA
CP30C   ATGGCTGACA AATATTTGTT AGATGAATCA ACAAAAGAAA AATTTATTAC AAGCAACTTA

                70         80         90        100        110        120
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TATCCTAATC TCAATGAATC TGAGAAAAAT ATAATGAGAA CTGTGCTTGA AAACCAAGGA
CP30C   TATCCTAATC TCAATGAATC TGAGAAAAAT ATAATGAGAA CTGTGCTTGA AAACCAAGGA

               130        140        150        160        170        180
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   AACGAAGTAA AAATGCTTAT GGAAAGTACT GTAACTGGTG ATATTGCAAA ATTTACACCA
CP30C   AACGAAGTAA AAATGCTTAT GGAAAGCACT GTAACTGGTG ATATTGCAAA ATTTACACCA

               190        200        210        220        230        240
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   ATCTTAGTTC CAGTAATTCG TAGAGCATTA CCTAGTCTGA TTGGTACTGA AATAGCTGGT
CP30C   ATCTTAGTTC CAGTAATTCG TAGAGCATTA CCTAGTCTGA TTGGTACTGA AATAGCTGGT

               250        260        270        280        290        300
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GTTCAAGCAC TTAAAACTCC AACTGCTTAT TTGTATGCAA TGGTTCCACA CTATGTAGGT
CP30C   GTTCAAGCAC TTAAAACTCC AACTGCTTAT TTGTATGCAA TGGTTCCACA CTATGTAGGT

               310        320        330        340        350        360
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GATGGTAATA ATAGTGTTAG TCCAACAAAA AATGCAATTG TTTTAAAACT TAAAACAGAA
CP30C   GATGGTAATA ATAGTGTTAG TCCAACAAAA AATGCAATTG TTTTAAAACT TAAAACAGAA

               370        380        390        400        410        420
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   TCTGCAAATA AAGATGACTT TAATTATACT GGAACTCCTA TTGAAGTTTC ATTTAAGACA
CP30C   TCTGGAAATA AAAATGACTT TAATTATACT GGAACTCCTA TTGAAGTTTC ATTTAAGACA

               430        440        450        460        470        480
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GCTACAACTG TTAAAGGTAA AATAGTATAT AGTGAAAAAC AAGCAGGTAC AGATAATATA
CP30C   GCTACAACTG TTAAAGGTAA AATAGTATAT AGTGAGAAAC AAGCAGGTAC AGATGATGTG

               490        500        510        520        530        540
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GTAAATGTAT TACTTCGCTT AGAATCTAAC TCAACTGGTT CTGTTGCTAT TGGTGATGAA
CP30C   GTAAATGTAT TACTTCGCTT AGAGTCTAAC TCAACTGGTT CTGTTGCTAT TGGTGATGAA

               550        560        570        580        590        600
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   ATGGATAAAG CAGCCACATT TGCTACTAAA AAAGCAACTG TTGAGGCTGT ATATACAAAT
CP30C   ATTGATAAAG CAGCTTCATT TGCTACTAAA AAAGCAACTG TTGAGGCTGT ATATACAAAT

               610        620        630        640        650        660
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GAAGCTTTAT GGTTAAAAGT TCTTAAAAAC TATACTGGTC CTTATGCAAC TGCTGCAGGT
CP30C   GAAGCTTTAT GGTTAAAAGT TCTTAAAAAC TATACTGGAC CTTATGCAAC AGCAGCTGGT

               670        680        690        700        710        720
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GAAAAACTTG GAAAAGACAT GAAAGAAATG GGTATCAGTG TTCAAAGAGT GTTAGCTGAA
CP30C   GAAGCTCTTG GTAAAGACAT GAAAGAAATG GGTATTAGTG TTCAAAGAGT GTTAGCTGAA

               730        740        750        760        770        780
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   GCTAAAACTA GAAAAGTAAA AGGTACTTAT ACTATCGAAA TGTTACAAGA CTTAAAAGCA
CP30C   GCTAAAACTA GAAAAGTAAA AGGTACTTAT ACTATTGAAA TGTTACAAGA CTTAAAAGCA

               790        800        810        820        830        840
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A   CAACACGGAA TAAATGCTGA AAAAGAACTA GCTGACATTT TAAGTGCAGA AGTTGCTCTT
```

```
CP30C  CAACACGGAA TAAATGCTGA AAAAGAACTA GCTGATATTT TAAGTGCAGA AGTTGCTCTT

               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAATTGACA GAACTATTAT TGAAAAAGCA AATGAAGTTG CTACTGTATG TACTGATTTT
CP30C  GAAATTGACA GAACTATTAT TGAAAAAGCA AATGAAGTTG CTACTGTATG TACTGATTTC

               910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGTTAATA GTGCAGATGG TAGATGGTTT ATTGAAAAAG CAAGAGGTTT AAGTATGAGA
CP30C  GATGTTAATA GTGCAGATGG TAGATGGTTT ATTGAAAAAG CAAGAGGTTT AAGTATGAGA

               970        980        990        1000       1010       1020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTAGCAATG AAGCTAGAGA AATCGGTCGC CAAACTAGAA AAGGTGGTGG TAATAAATTA
CP30C  ATTAGCAATG AAGCTAGAGA AATCGGTCGC CAAACTAGAA AAGGTGGTGG TAATAAATTA

               1030       1040       1050       1060       1070       1080
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATCGTTTCTC CAAAAGTTGC TACAATTCTT GATGAAATTG GATCATTTGT TTTATCTCCA
CP30C  ATCGTTTCTC CAAAAGTTGC TACAATTCTT GACGAAATTG GATCATTTGT TTTATCTCCA

               1090       1100       1110       1120       1130       1140
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAGGAAGTA AGATTGATGC TATTAACAGT GGTATTAAAC CAAATGTTGG CAAATTCGAT
CP30C  GCAGGAAGTA AGATTGATGC TATTAACAGT GGTATTAAAC CAAATGTTGG CAAATTCGAT

               1150       1160       1170       1180       1190       1200
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATCGTTACG ATGTAATTGT TGATAACTTT GCTGAATTTG ATTATTGTAC TGTTGCTTAC
CP30C  AATCGTTACG ATGTAATTGT TGATAACTTT GCTGAATTTG ATTATTGTAC TGTTGCTTAC

               1210       1220       1230       1240       1250       1260
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGGAGCAT CAAACTTTGA TGCTGGTATT TTCTTTGCAC CATATAACAT TACATTGCAA
CP30C  AAAGGAGCAT CAAACTTTGA TGCTGGTATT TTCTTTGCAC CATATAACAT TACATTACAA

               1270       1280       1290       1300       1310       1320
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAAAATTTAA CAGATCCAGT AAGTGGGCAA CCTGCTATGA TTTTAAATAA CAGATATGAT
CP30C  CAAAATTTAA CAGATCCAGT AAGTGGGCAA CCTGCTATGA TTTTAAATAA TAGATATGAT

               1330       1340       1350       1360       1370       1380
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTGTTGCTA CTCCATTACA CCCAGAAGCA TTCATCAGAA CATTTGCTGT TAATTTAAAT
CP30C  GTTGTTGCTA CTCCATTACA CCCAGAAGCA TTCATCAGAA CATTTGCTGT TAATTTAAAT

               1390
       ....|....| ....|...
CP30A  AACTACATAA TCTCTTAG
CP30C  AACTACATAA TCTCTTAG
```

Appendix 8. DNA sequence of D302_gp121 gene in phage CP30A and C, which encode major head protein II. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGAGTAAGG TTATAAATGA ATCAACCACA ACAGTTGATA TTGCTGGTGT TGAATTAAAA
CP30C  ATGAGTAAGG TTATAAATGA ATCAACCACA ACAGTTGATA TTGCTGGTGT TGAATTAAAA

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTTGCAAAAT TAATATATCG TATATACACC GAATCTTTGT TAAATAGAAT AGGAGCAAGA
CP30C  CTTGCAAAAT TAATATATCG TATATACACC GAATCTTTAT TAAATAGAAT AGGAGCAAGA

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAAATGTAG CAGTTCCTAA TGGTAGTATT TTTGCTTTTA AGGGAAAATA TTTAACAGAT
CP30C  ATAAATGTAG CAGTTCCTAA TGGCAGTATT TTTGCTTTTA AGGGAAAATA TTTAACAGAT

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATACTGGAA CTGATAAAAG CAGCACCCCT TATGCTACTA TTTTACCTGA TTTTGCTGGA
CP30C  TATACTGGAA CTGATAAAAG CAGCACCCCT TATGCTACTA TTTTACCTGA TTTTGCTGGA

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGAGATA ATAATCAGGA AACTGATGTA AAAGCTGAAA TGAATTATAA AATTGTAAAA
CP30C  AATAGAGATA ATAATCAGGA AACTGATGTA AAAGCTGAAA TGAATTATAA AATTGTAAAA

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGAACAATAA ACTGTCAAAC TAAAAAAATA AGATCAAAAT GGTCAATAGA AGCTATTACC
CP30C  AGAGCAATAA ACTGTCAAAC TAAAAAAATA AGATCAAAAT GGTCAATAGA AGCTATTACC

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATTTAGTTG CTCTTACTGG TAAAACAACT GTTGAAGATA TATTAGAAAA AGAACTTTTA
CP30C  GATTTAGTTG CTCTTACTGG TAAAACAACT GTTGAAGATA TATTAGAAAA AGAACTTTTA

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACAGAAATTA TTCAAGAAAT TGACTTTTCA GCTTTAAAAA TGATGACAAC TAAAGCAACA
CP30C  ACAGAAATTA TCCAAGAAAT TGACTTTTCA GCTTTAAAAA TGATGACAAC TAAGGCAACA

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAACACAAT TAACATTAAA AGCACCTAAT GATCCATTAG TTGGTATAGA ATTATTTAAT
CP30C  AAAACACAAT TAACATTAAA AGCACCTAAT GATCCATTAG TTGGTATAGA ATTATTTAAT

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAGCTCAGA AAAAAATATT GGAAATGGCT GCTTCAACCA AAAGAGCTAT AACTATGTGT
CP30C  GCAGCTCAGA AAAAAATATT GGAAATGGCT GCTTCAACCA AAAGAGCTAT AACTATGTGT

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTACAGCAC CATATGAAAC TTGCGCTAAA TTGATGTCAC ATCCTAATTT TAAGGCAAAT
CP30C  ATTACAGCAC CATATGAAAC TTGCGCTAAA TTGATGTCAC ATCCTAATTT TAAAGCAAAT

               670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAGATTTCA CAAACTCATA TTTCATGGGA TCCATAGGTG CAACAGAAAT ATATTGTGAT
CP30C  GAAGACTTCA CAAACTCATA TTTCATGGGA TCCATAGGTG CAACAGAAAT ATATTGTGAT

               730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATTATAATA TTTTAAACAA AGAATATATG TTAATCTCTT ATAAGCATAG AAATAAAGAA
CP30C  TATTATAATT CTTTAAATAA AGAATATATG TTAATCTCTT ATAAGCATAG AAATAAAGAA

               790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTGAAATAG CTGATGGTTC TACTTGCTTT GCATTTTATA GTTATAACAT AACAAAAGCT
```

```
CP30C  ATTGAAATAG CTGATGGTTC TACTTGCTTT GCATTTTATA GTTATAACAT AACAAAAGCT

               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTGACGCTA CAAGTGGTGC TGAATCATAT TTTCATTTTT TAAGGTATGA TGTAGTTCAA
CP30C  TTTGACGCTA CAAGTGGTGC TGAATCATAT TTTCATTTTT TAAGGTATGA TGTAGTTCAA

               910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CATCCACTAG ATAATACTAA TGATGGACAA TCTATTTTCT TACATTGTAT TGAAATACAA
CP30C  CATCCACTAG ATAATACTAA TGATGGGCGA TCTATTTTCT TACATTGTAT TGAAATACAA


       ...
CP30A  TAG
CP30C  TAG
```

Appendix 9. DNA sequence of D302_gp150 gene in phage CP30A and C, which encode major head completion protein.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGCCAGAGT TCAAACAAGG TTTTTATAAA CCTATTAACC CAGAAAAATA TATAGATGAT
CP30C  ATGCCAGAGT TCAAACAAGG TTTTTATAAA CCTATTAACC CAGAAAAATA TATAGATGAT

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTAGCAAACA TCATCTATCG TAGCTCATGG GAATATAAAT TTATGTTATG GTGCGATAAT
CP30C  GTAGCAAACA TCATCTATCG TAGCTCATGG GAATATAAAT TTATGTTATG GTGCGATAAT

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATGCTGGTG TTCTTAAATG GGCTAGTGAA TCAATAATTA TACCTTATGA GTTCCTAGGT
CP30C  AATGCTGGTG TTCTTAAATG GGCTAGTGAA TCAATAATTA TACCTTATGA GTTCCTAGGT

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAAAACATA GATATTTCCC TGATTTTTAT ATTGAAGTAA AAGATAAAGA CAATAACATA
CP30C  AAAAAACATA GATATTTCCC TGATTTTTAT ATTGAAGTAA AAGATAAAGA CAATAACATA

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAAAATATC TTATTGAAAT TAAACCACAA AAAGACGCTA TATTTAAAAA ACCAAAAATA
CP30C  AAAAAATATC TTATTGAAAT TAAACCACAA AAAGACGCTA TATTTAAAAA ACCAAAAATA

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATCACAGAAA AAAATAAAAA AAGAGTAGTA GAACAAGCAT TAACAGTTTC AAAAAATCAA
CP30C  ATCACAGAAA AAAATAAAAA AAGAGTAGTA GAACAAGCAT TAACAGTTTC AAAAAATCAA

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAAAATGGG AAGCTGCAAG AGAGTTCTGT AGAATAAATA ATATGGAATT TATGGTATTA
CP30C  GCAAAATGGG AAGCTGCAAG AGAGTTCTGT AGAATAAATA ATATGGAATT TATGGTATTA

              430        440
       ....|....| ....|....| ....
CP30A  ACTGAAAATG AATTATTTAA GTAA
CP30C  ACTGAAAATG AATTATTTAA GTAA
```

Appendix 10. DNA sequence of D302_gp081 gene in phage CP30A and C, which encode neck protein. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGGCTACTA GGCAAAGTCT CAAAGATTAT ATTTTTGGAA TGTTAGGTTC CCCAGTTATT
CP30C  ATGGCTACTA GACAAAGCCT CAAAGATTAT ATTTTTGGAA TGTTAGGTTC CCCAGTTATT

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTGTGGAAC TTACTGATTT CCAAATAGAT GAGAATATTA ATTTTACTAT ACAAAAGTTT
CP30C  ACTGTGGAAC TTACCGATTT CCAAATAGAT GAGAATATTA ACTTTACTAT ACAAAAGTTT

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTGAGTTTG CTATGTATGG TAAATTAAAA GGCACACTAT TAATTGACCT ACCTAAAGGT
CP30C  TCTGAGTTTG CTATGTATGG TAAATTAAAA GGCACACTAT TAATTGACCT ACCTAAAGGT

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTGAGAAAAA TTAAATTAGA TCCTAAAATT TCTGAGGTTA TAACATTACG AATATATCCA
CP30C  GTGAGAAAAA TTAAATTAGA CTCTAGAATT TCTGAGGTTA TAACATTACG AATATATCCA

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTGGCGGAG GTTTTTTAGG GTTAAGTATT CCAGGTGGTT TAGTAATAAC ACCAACTGAA
CP30C  AGTGGTGGAG GTTTTTTAGG GTTAAGTATT CCAGGTGGTT TAGTAATAAC ACCAACTGAA

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGCAAGCAA TGCTATTTGG TGGAACAGTA CAAGGTAACT TTAGTATGCA AAACATATAT
CP30C  ATGCAAGCAA TGCTATTTGG TGGAACAGTA CAAGGTAACT TCAGCATGCA AAATGTATAT

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTGTATTAG CAAATATGTC TATACTTGAT ACATATTTTA CAATAATACC AAATTATGCT
CP30C  TCTGTATTAG CAAATATGTC TATACTTGAT ACATATTTTA CAATAATACC AAATTATGCT

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTAATCCAT TTACAAATAT GTTAGAGTTT TTTGAAGATA TAACTTCTGA AAAGGTTTTA
CP30C  TTTAATCCAT TTACAAATAT GTTAGAATTT TTTGAAGATA TAACTTCTGA AAAGGTTTTA

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAGAAGTCA GATATAAGTA TACACCAGAA GAAGAAGATG GGATATACGA GCAACCATGG
CP30C  TTAGAAGTTA GATACAAGTA TATACCAGAA GAAGAAGATG GGATATATGA GCAACCATGG

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTAAAGAAT ACGCTTTAAA CTTATGCAAA AGAACTTGGG GATCAAATAT AGGAAAATAT
CP30C  GTTAAAGAAT ATGCTTTAAA TTTATGCAAA AGAACTTGGG GATCAAATAT AGGAAAATAT

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGCACCGT TAATAGGTGG TATTAAAGCT AATTATGAAA GAATTATACA AGAAGCAAAT
CP30C  GATGCACCGT TAATAGGTGG TATTAAAGCT AATTATGAAA GAATTATACA AGAAGCAAAT

               670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTGAATTAG AAAGATTAGA AACAGTACTA CTAGAAAACT ATTGTGAACC ACTTCCATTA
CP30C  ACTGAATTAG AAAGATTAGA AACAGTATTA CTAGAAAACT ATTGTGAACC ACTTCCATTA

               730
       ....|....| ..
CP30A  TTAAGAGGTT AA
CP30C  TTAAGAGGTT AA
```

Appendix 11. DNA sequence of D302_gp040 gene in phage CP30A and C, which encode putative tail tube protein. Point mutations are indicated in red.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGCCTTTAT ATACAGTTGA TAAATTAGCA AATGCTCTTA AAGGTGGAGC AAAAAGTGAT
CP30C  ATGCCTTTAT ATACAGTTGA TAAATTAGCA AATGCTCTTA AAGGTGGAGC AAAAAGTGAT

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAATATTTTA TAGAAATAGG TACTCCGTTA GGTGCTCCAG AAGTAGCATT TACAGAAGAG
CP30C  AAATATTTTA TAGAAATAGG TACTCCGTTA GGTGCTCCAG AAGTAGCATT TACAGAAGAG

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATATTATAT TATGTAAAAC TGCTAGTTTT CCAGAAAGAA CTCTTGGAGA AGTTGAAGCA
CP30C  GATATTATAT TATGTAAAAC TGCTAGTTTC CCAGAGAGAA CTCTTGGAGA AGTTGAAGCA

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTCGTTCAAG GTAGAAAATT AAAATTACCA GGTGATTCAA CATTTGATGC AGCTTGGAGC
CP30C  TTTGTCCAAG GTAGGAAATT AAAATTACCA GGTGATTCAA CATTTGATGC AGCTTGGAGC

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CCAGTATTTT ATCAAACACC TGATCATAAT ATTAGAGCGA AATTTTTAAC ATGGATTGAT
CP30C  CCAGTATTTT ATCAAACACC TGATCATAAT ATTAGAGCGA AATTTTTAAC ATGGATTGAT

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAATTGATG TGTACAAAAA CAATTATCAT ACTTGTGATC CATATAGTTT AATGGTTACA
CP30C  AAAATTGATG TGTACAAAAA CAATTATCAT ACTTGTGATC CATATAGTTT AATGGTTACA

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTAAGGTTC ATCAAGTTAA TTGTAATGGT GAGCCTGTTG CAACTTATGA GTTTTTTAAT
CP30C  GCTAAGGTTC ATCAAGTTAA TTGTAATGGT GAGCCTGTTG CAACTTATGA GTTTTTTAAT

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTATGGCCAT CAAAAGTCGG TGAAATAGAA GTGGCAGCAG ATAAAACAAA TTCTATACAA
CP30C  GTATGGCCAT CAAAAGTCGG TGAAATAGAA GTGGCAGCAG ATAAAACAAA TTCTATACAA

              490        500        510        520
       ....|....| ....|....| ....|....| ....|....| ....|...
CP30A  GAATTTACAG TTGATTTTAC ATACTCACAT TGGGAAAAGA TAGCTTAA
CP30C  GAATTTACAG TTGATTTTAC ATACTCACAT TGGGAAAAGA TAGCTTAA
```

Appendix 12. DNA sequence of D302_gp041 gene in phage CP30A and C, which encode putative tail tube protein. Point mutations are indicated in red.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGTCTTTAT ATAATATTGA TAGACTAAGA AGCTCCCTTA AACAAGGGGG TGCCATCAAT
CP30C  ATGTCTTTAT ATAATATTGA TAGACTAAGA AGCTCCCTTA AACAAGGGGG TGCCATCAAT

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCAAAATATA AAATTGACAT AAAAATACCT ACCCTATTGA GGTCTCTACC GTTTTTTAAA
CP30C  TCAAAATATA AAATTGACAT AAAAATACCT ACCCTATTGA GGTCTCTACC GTTTTTTAAA

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACAGTAAATA TATCAGGTGA ATATTTAAGT ATAATGGCTA ATAGAACATC TATTCCAGGT
CP30C  ACAGTAAATA TATCAGGTGA ATATTTAAGT ATAATGGCTA ATAGAACATC TATTCCAGGT

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAGTCTATGA GTACTGTAAA AGTGTATCAT CGTGGGCAAC CATTTGTTAT AAGAGGTGCA
CP30C  AAGTCTATGA GTACTGTAAA AGTGTATCAT CGTGGGCAAC CATTTGTTAT AAGAGGTGCA

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCACAATTTA ATAATACACA TAAAATAACA TTTTATAATA CACCTGATAT GGATATTCAT
CP30C  GCACAATTTA ATAATACACA TAAAATAACA TTTTATAATA CACCTGATAT GGATATTCAT

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAATTATTTA GTGATTGGAT TTATAGAATT GATAGTTTTG ATAGTACAAT TACACAATCA
CP30C  CAATTATTTA GTGATTGGAT TTATAGAATT GATAGTTTTG ATAGTACAAT TACACAATCA

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATATTCTTAG GTAACTATGT TGGTTTTAAT AGTGTAGGTG CTGGTTATAT GAGTGATATA
CP30C  ATATTCTTAG GTAACTATGT TGGTTTTAAT AGTGTAGGTG CTGGTTATAT GAGTGATATA

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAGTATCAC AATTATCATC TGATGGTAAA ACTGAAACTA AATTTAAACT ATGTTATACT
CP30C  ATAGTATCAC AATTATCATC TGATGGTAAA ATTGAAACAA AATTTAAGTT GTGCTATGCT

              490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTCCCTATTG ATATAGCTGA AGTTGAATTA AGTGCTTCTG GAAAAGAAAT ATCATCAACA
CP30C  TTTCCTATTG ATATAGGTGA AATTGAGTTG TCAGCTTCTG GAAAAGAGAT ATCATCAACA

              550        560        570
       ....|....| ....|....| ....|....| ....|....
CP30A  GAAGTAACAT TTGCCTATAC ATATTGGGAA AGAGTATAA
CP30C  GAAGTTACTT TTGCTTATAC TTATTGGGAA AGAGTATAA
```

Appendix 13. DNA sequence of D302_gp084 gene in phage CP30A and C, which encode putative tail tube protein III. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGGGTTTAA ACAAGTTTGA TTCTGTTGAT TATGTTTTAA GTTCTGGACA AAGGCCTTTT
CP30C  ATGGGTTTAA ACAAGTTTGA TTCTGTTGAT TATATTTTAA GTTCTGGACA AAGACCTTTT

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGATATAGAG TATCATTAAA TTTACCTGCT AAAATAGCAA AAATATCAGG TGCTTTATAT
CP30C  AGATATAAAG TATCATTAAC TTTACCTACT AAAATAGCAA AAATATCAGG TGCTTTATAT

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GACAATGCAG TTAATATATT GTGCAAGGGT GCTACTTTAC CAGCACCTTC TATATTAACT
CP30C  GACAATGCAG TTAATATATT GTGCAAAGGT GCTACCTTAC CAGCACCTTC TATATTAACT

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACACCTGTTG GTTTAGATGG TAGAACTATA AACATACCAA CATTAATGAA ACTTGATAAT
CP30C  ACACCTATTG GTTTAGATGG TAGAAATATA AGCATACCAA CATTAATGAA ACTTGATAAT

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTACAAATA TGACATTTTT TATAGATGAA AAATCAAGTG TAAGGCGTAT ATTAGAATAC
CP30C  ACTACAAATA TGATATTTTT TATAGATGAA AAATCAAGTG TAAGGCGTAT ATTAGAATAT

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TGGACTTTTT GTATTGATTC AGGTGTAACC GCAAATGAAG AAACACCATC AGTTCCTGGT
CP30C  TGGCATTTTT GTATTGATTC AGGTATAACC GCAAATGAAG AAACACCATC AGTTCCTGGT

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTGGTGTTG CTAATATTAT AGGATCAGTT GCTAATATTG GAGCAGGTTT TATATCTGAT
CP30C  GCTGGTGTTG CTAATATTGT AGGATCTGTT GCTAATATTG GAGCAGGTTT CATATCTGAT

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTACAAGTG ATATACCAAT TGTTGGTAAC GCTGTTAATA GTTTCCTAGG TATAAATAAA
CP30C  ATTACAAGTG ATATTCCTAT CATAGGAAAT GCTGTTAATA GTTTCCTAGG CATAAATAAA

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTGTAAGTG GTAATACTGA TATTAATATG ACTGGTGAAT TGAAATTAAC CTTATTAAAT
CP30C  GGTGTAAGTG GTAATACCGA TATTAATATG ACTGGTGAAT TGAAATTAAC ATTATTAAAT

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATAGTGGCA ATGCTGTTGG AAGTTATACA TATAAAAATA TATTTCCTAT TGATGTGACC
CP30C  TATAGTGGTA ATGCTGTTGG AAGTTATACA TATAAAAACA TATTCCCTAT TGATTTGACT

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGAAGTGATA TGCAAGATGA TCAAACAGAA ACAATTAATG AATTTAGCGT AACATTTGGA
CP30C  GGAAGTGATA TGCAAGATGA TCAAACAGAA ATAATTAATG AGTTTAGTGT AACATTTGGA

               670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATACACATT ATGTATATAA AAAAGAAACA GAATCTATTA TAGATGCTGT TACAGGAATA
CP30C  TATACACATT ATGTATATAA AAAAGAAACA GAATCTATTA TAGATGCTGT TACAGGACTA

               730
       ....|....| ..
CP30A  GTTGGGTTAT GA
CP30C  GTGGGATTAT GA
```

Appendix 14. DNA sequence of D302_gp062 gene in phage CP30A and C, which encode tail sheath protein. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGGCTTTAC TAAGTCCAGG TGTAGAAGTT AAAGAAATTG ACTTATCTCT TACGGTTTCA
CP30C  ATGGCTTTAC TAAGTCCAGG TGTAGAAGTT AAAGAAATTG ACTTATCTCT TACGGTTTCA

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTGCTTCAT CTAGTTTTGG AGCGTTTTGT GGTATTTTTC CAAAAGGTCC ATGTGATGGT
CP30C  AGTGCTTCAT CTAGTTTTGG AGCGTTTTGT GGTATTTTTC CAAAAGGTCC ATGTGATGGT

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAGTTTTCA TAAATGATAT TCCAACACTT GAAAGTGTAT TTGGAAAACC TACAAATTCA
CP30C  GCAGTTTTCA TAAATGATAT TCCAACACTT GAAAGTGTAT TTGGAAAACC TACAAATTCA

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATTATAATG ATTTTTTTCA AGCGTATTGT TTTTTAAGAA GAGCAGGTAG TTTATATGTT
CP30C  AATTATAATG ATTTTTTTCA AGCATATTGT TTTTTAAGAA GAGCAGGTAG TTTATATGTT

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTAAGAGCTA TTGATAAACT TGGAAAATCT ACAAGAAAAG ATTCAGGTTT AACAATAAAT
CP30C  GTAAGAGCTA TTGATAAACT TGGAAAATCT ACAAGAAAAG ATTCAGGTTT AACAATAAAT

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTGTATTAA GTGAAAAAGC AACAGAAATA ACCTTGGCAG ATACAACAGG TTTATATGTT
CP30C  GCTGTATTAA GTGAAAAAGC AACAGAAATA GCTTTAACAG ATACAACAGG TTTATATGTT

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGACAGCAAA TTATGTTTGG TGAAAAAGCT GATGCTAATG TTTATACTAT TGCTAGCATT
CP30C  GGCCAACAAA TTATGTTTGG TGAAAAAACT GATGCTAATG TTTATACTAT TGCTAGTATT

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAAGCTAATA CTAAAATAAC TTTCACTCCA GAAATACAAA CAGGTGATGG TACTGGTAAC
CP30C  CAAGCTAATA CTAAAATAAC TTTCACTCCA GAAATACAAA CAGGTGATGG TACTGGTAAC

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTTCAAAAA TTTATATTTG TCACCCATCA ATGAATGCAA TAGGTGAAGT TTTAAAAACT
CP30C  TCTTCAAAGA TTTATATTTG TTATCCATCA ATGAATGCAA CAGGTGAAGT TTTAAAAACT

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTTCAAGTA ATACTATAAC AGATGCTAAA TTAAAAGAAA CGCTTAAAAT AATTCCTAAT
CP30C  GGTTCAAGTA ATACTATAAC AGATGCTAAA TTAAAAGAAA CGCTTAAAAT AATTCCTAAT

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATGATGTTT ATGAAACTTT AGAGCCTTCT ATTAAATTTA GTGATACTGA AACAAAATTA
CP30C  AATGATGTTT ATGAAACTTT AGAACCTTCT ATTAAGTTTA GTGATACTGA AACAAAATTA

               670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAATTTATTG CTAAATCAGT TGGTTCTTGG GGAAATAATA TTAAAGTAGC AGTTGCAACA
CP30C  AAATTTATTG CTAAATCAGT TGGTTCTTGG GGAAATAATA TCAAAGTAGC AGTTGCAACA

               730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGCTGATT TTGGAGCAAA TAAAAATATT ATTAAAGGTA TTCCATTAGA TGATAATTTT
CP30C  AAAGCTGATT TTGGAGCAAA GAAAAATATT ATTAGTGGTA TTCCATTAGA TGATAATTTT

               790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAATATATTC CAGATACAGA TCAAGTTGCT GTAGTTGTAT TAGAAAACAA CGAAATAAAA
CP30C  GAATATATTC CAGATACAGA TCAAGTTGCT GTAGTTGTAT TAGAAAACAA CGAAATAAAA
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               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAACATATA TGGTTTCAAT TAAAGAAGGT GCTAAAGATT ATAATAATAA ATCTAATTAT
CP30C  GAAACATATA TGGTTTCAAT TAAAGAAGGT GCTAAAGATT ATAATAACAA ATCTAATTAT

               910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTGAAGATG TTATTAATAG AAAATCATCT TATGTTTATT GTAAAAATAA CACTACAATA
CP30C  ATTGAAGATG TTATTAATAG AAAATCATCT TATGTTTATT GTAAAAATAA TACTACAATA

               970        980        990        1000       1010       1020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTGATCTAC CAAAATCTGC TTTGGATTCT GAAATCATAA CACTTAAATT TGGTGAAGAT
CP30C  ACTGATTTAC CAAAATCTGC TTTGGATTCT GAAATCATAA CACTTAAATT TGGTGAAGAT

               1030       1040       1050       1060       1070       1080
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGAGCTCCAA CAAAAGCTGA TATTATTAGT GGTTATACAG ATAATTTTAG TTCAAAAGAA
CP30C  GGAGCTCCAA CAAAAGCTGA TATTATTAGT GGTTATACAG ATAATTTTAG TTCAAAAGAA

               1090       1100       1110       1120       1130       1140
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAATAGATA TTGATATTGT TATTGCAAAT GAAATGGCTA ATAAAGAATG TGCTGATTTT
CP30C  GAAATAGATA TTGATATTGT TATTGCAAAT GAAATGGCTA ACAAAGAATG TGCTGATTTT

               1150       1160       1170       1180       1190       1200
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TGTGTAACTC GTGGTGATGT TATAGGTTAT GGTGGTGTTC CATTTAGTGA AGTTGTTGGT
CP30C  TGTGTAACTC GTGGTGATGT TATAGGTTAT GGTGGTGTTC CATTTAGTGA AGTTGTTGGT

               1210       1220       1230       1240       1250       1260
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAAAAGCTG AAGATTGTGT TAAAAATCTT TTAGAATATA GAAGCACTGG TGAAATGAAT
CP30C  TTAAAAGCTG AAGATTGTGT TAAAAATCTT TTAGAGTATA GAAGCACTGG TGAAATGAAT

               1270       1280       1290       1300       1310       1320
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTGATAATA AGTATTTTTC ATTTATAGGA AATTATGGTT ATATATATGA TAAGTATAAT
CP30C  ATTGATAATA AGTATTTTTC ATTTATAGGA AATTATGGTT ATATATATGA TAAGTATAAT

               1330       1340       1350       1360       1370       1380
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GACAAATATA GATGGATCAA CTTAGCTGGT GCAACAGCAG GATTAAGAGC TTATACAAAT
CP30C  GACAAATATA GATGGATCAA CTTAGCTGGT GCAACAGCAG GATTAAGAGC TTATACAAAT

               1390       1400       1410       1420       1430       1440
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAAGCAAGAC AGCCATGGTT TGCTGCAGCT GGTTTAAATC AAGGACAATA TTTAGATATT
CP30C  CAAGCAAGAC AGCCATGGTT TGCTGCAGCT GGTTTAAATC AAGGACAATA TTTAGATATT

               1450       1460       1470       1480       1490       1500
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATCAAGCTGG CATTCAATCC TAATAATGGA CAAAGAGATC TTTTGTATAA GAGTGCTATA
CP30C  ATCAAGCTTG CATTCAATCC TAATAATGGA CAAAGAGATC TTTTGTACAA GAGTGCTATA

               1510       1520       1530       1540       1550       1560
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATCCTGTTG TTAGCTTTCC AAGTTTAGGA ATTTGTTTAT GGGGACAAAA AACTTGTACT
CP30C  AATCCTGTTG TTAGCTTTCC AAGTTTAGGA ATTTGTTTAT GGGGACAAAA AACTTGTACT

               1570       1580       1590       1600       1610       1620
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAAAAACCTA GTGCATTTGA TAGAGTAAAT GTTAGAATGT TGTTTAATTA TCTTGAAAGA
CP30C  CAAAAACCTA GTGCATTTGA TAGAGTAAAT GTTAGAATGT TGTTTAATTA TCTTGAAAGA

               1630       1640       1650       1660       1670       1680
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AACATAGCTA ACTCAGCTAG ATATGTTGTA TTTGAGCAAA ATGACACTCA TACTCAAAAT
CP30C  AACATAGCTA ACTCAGCTAG ATATGTTGTA TTTGAGCAAA ATGACACTCA TACTCAAAAT

               1690       1700       1710       1720       1730       1740
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       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGTTTGTAA GTATGTGTTC TCCGTTATTA ACTCAAGTTC AAGCGGGTCG CGGTATAGAT
CP30C  ATGTTTGTAA GTATGTGTTC TCCGTTATTA ACTCAAGTTC AAGCGGGTCG CGGTATAGAT

               1750       1760       1770       1780       1790       1800
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCATTTAAAA TAGTATGTGA TGATAGCAAT AACACGCCTT TGGTTAAATC CAATAATCAG
CP30C  GCGTTCAAAA TAGTATGTGA TGATAGCAAT AACACGCCTT TGGTTAAATC CAATAATCAG

               1810       1820       1830       1840       1850       1860
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTGTTGCGT CTTTCTTAAT TAAACCGACT TATGCAATAG AATTTATAAC TTTGAATTTT
CP30C  TTTGTTGCGT CTTTCTTAAT TAAACCGACT TATGCAATAG AATTTATCAC ACTTAATTTC

               1870       1880       1890       1900       1910
       ....|....| ....|....| ....|....| ....|....| ....|....| .
CP30A  GTCGCTGTAG GTGCTACAAT TAGTTTTGAA GAAGCAATAG GCTCAATTTA A
CP30C  GTCGCTGTTG GTGCTACAAT TAGTTTTGAA GAAGCAATAG GCTCAATTTA A
```

Appendix 15. DNA sequence of D302_gp108 gene in phage CP30A and C, which encode tail completion and sheath stabiliser protein. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGAACTTTA GAAATATTGC TTTAAATTCA AATATAGTTT TTAGGACATT ATTATTTTCT
CP30C  ATGAACTTTA GAAATATTGC TTTAAACTCA AATATAGTTT TTAGGACATT ATTATTTTCT

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGATACTC AATATTATTG TCAAAAAGTT AAATTGCCTA GAATATCTTT AGAAGGACAA
CP30C  GATGATACTC AATATTATTG TCAAAAAGTT AAATTGCCTA GAATATCTTT AGAAGGACAA

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGTAGGGC ATTCAACTGG TACTTTAACG TTAGGTGGTG AAGTAGCAAA ATTTGATTCA
CP30C  AAAGTAGGGC ATTCAACTGG TACTTTAACA TTAGGTGGTG AAGTAGCAAA ATTTGATTCA

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAACATTGA CACTATTAGT AGATGAAAAT TTAGAAGTGT GGAAAAATTT TGTCAATTTA
CP30C  ATAACATTGA CGCTGTTAGT AGATGAGAAT TTAGAAGTGT GGAAAAATTT TGTCAATTTA

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTAATAAAT ATAACAAAAT TTCTACAAAT ACTGGATGTG GCATTGAAGC TACATCGTGG
CP30C  ATTAATAAAT ATAACAAAAT TTCTACAAAT ACTGGATGTG GCATTGAAGC TACATCGTGG

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAGAAATAT ATGATTCTAA AAACAAATAT TTATTTAAAG TTGAATTTTA TAAAAGTAAA
CP30C  TTAGAAATAT ATGATTCTAA AAATAAGTAT TTGTTTAAAG TTGAATTTTA CAAAAGCAAG

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAGATGAGG TAAGTGAATT GGAATATTCT ACAACTGATA ATAATATCAT AACCTTAGAT
CP30C  TTAGATGAGG TAAGTGAATT GGAATATTCT ACAACTGATA ATAATATTAT AACCTTAGAT

               430        440        450
       ....|....| ....|....| ....|....| ....|.
CP30A  ATAACACTTA ATTTTGATTA TATGAAAATC ATATAA
CP30C  ATAACACTTA ATTTTGATTA TATGAAAATC ATATAA
```

Appendix 16. DNA sequence of D302_gp124 gene in phage CP30A and C, which encode tail sheath stabiliser and completion protein. Point mutations are indicated in red.

```
                10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGGAGTTTT TCTTTTTCGA AACAACTAAG AAGTATTGTA AAGGGTTACT CGATATATTC
CP30C  ATGGAGTTTT TCTTTTTCGA AACAACTAAG AAGTATTGTA AAGGGTTACT CGATATATTC

                70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGTATAC AAGTTAAAAA GAAAGTAGAT GAAAAAACAG ATAAGTATGT CACAGTACCT
CP30C  AATAGTATAC AAGTTAAAAA GAAAGTAGAT GAAAAAACAG ATAAGTATGT CACAGTACCT

               130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTAGTTTTG GAAGTAAAGA TGCTGCTTCT GTTTTTAGTG ATACAGAATT AGATCAATTA
CP30C  ATTAGTTTTG GAAGTAAAGA TGCTGCTTCT GTTTTTAGTG ATACAGAATT AGATCAATTA

               190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAAATGGTA ATTTTAATAT ATTACCTAGA ATGTCTTTAG CTTTAATGAG TATGGAAAGA
CP30C  TTAAATGGTA ATTTTAATAT ATTACCTAGA ATGTCTTTAG CTTTAATGAG TATGGAAAGA

               250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGATCAAA GAGCTACAAG TAGATTTCAA ATACCTATAA AAGATATTGA CGGAAAGAAT
CP30C  GATGATCAAA GAGCTACAAG TAGATTTCAA ATACCTATAA AAGATATTGA CGGGAAAAAT

               310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAACTTTCC AACATAACTG TGTTCCTTAT TCTTTTGATT TTGTATTAAG TATAGCTACA
CP30C  ATAACTTTCC AACATAACTG TGTTCCTTAT TCTTTTGATT TTGTATTAAG TATAGCTACA

               370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGATCTTTAA CTGACTTAAC TTCTATATTA GAGCAAATTT TACCTTTCTT TAACCCAAAC
CP30C  AGATCTTTAA CTGACTTAAC TTCTATATTA GAGCAAATTT TACCTTTCTT TAACCCAAAC

               430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTAATTTAA GGGTTAGAGA GTTAGAATGG TTAACAGAAC CTACAACTAT ACAAGTTGAA
CP30C  ATTAATTTAA GGGTTAGAGA GTTAGAATGG TTAACAGAAC CTACAACTAT ACAAGTTGAA

               490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTAATTAGTG TAGATTATGA ATTACCTGAT GAAAATGATG GAGCTGATAT AAGAGTTTGT
CP30C  TTAATTAGTG TAGATTATGA ATTACCTGAT GAAAATGATG GAGCTGATAT AAGAGTTTGT

               550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTGCTAATG TTACAATGAG ACTTCACGGG AATATATATC CCCCTATTAA AAATGGTGCT
CP30C  AGTGCTAATG TTACAATGAG ACTTCACGGG AATATATATC CCCCTATTAA AAATGGTGCT

               610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTATCCAAC AAGTTAAATT ATACTTATCA CCAGTTGTAG ATTTCTCTGA AGATAGTAAA
CP30C  GTTATCCAAC AAGTTAAATT ATACTTATCA CCAGTTGTAG ATTTCTCTGA AGATAGTAAA

               670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAATAGTGC ATAAGTTTAA TATCGATGAA AATACACATA TGATGGATAT AGATTCATTT
CP30C  GAAATAGTGC ATAAGTTTAA TATCGATGAA AATACACATA TGATGGATAT AGATTCATTT

               730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTAGAATAG ATTATGGTGA AGAATGGAAT AAAGTAAAGC CAGTTATAGA TGGTGTAAAA
CP30C  GTTAGAATAG ATTATGGTGA AGAATGGAAT AAAATAAAGC CAGTTATAGA TGGTGTAAAA

               790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTGAAGTTA AAAATTTACC TATTCAAGAA AACATAAAAT ATAGGATATT ATACACAGAT
CP30C  GGTGAAGTTA AAAACTTACC TATTCAAGAA AACATAAAAT ATAGGATATT ATACACAGAT
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```
               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATACTGATG ACAATATTAA GTTTATTATA AATGTATTAG AAGATAATGG TGTTAATCCT
CP30C  GATACTGATG ATAATATTAA GTTTATTATA AATGTATTAG AAGATAATGG TGTTAATCCT

               910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTATATCAA AACAATTAAA TTATTTTACT GTGTTTGCTA AAAATAAAGG TACCTTAAAG
CP30C  ATTATATCAA AACAATTAAA TTATTTTACT GTGTTTGCTA AAAATAAAGG TACCTTAAAA

               970        980        990        1000       1010       1020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTAAGTATTC AAGCAGTAAA TTCATTTGAT TTACAAAGTA ATATTTATGA AATGGAGTTA
CP30C  TTAAGTATTC AAGCAGTAAA TTCATTTGAT TTACAAAGTA ATATTTATGA AATGGAGTTA

               1030
       ....|....| ..
CP30A  GAATTCTCAT GA
CP30C  GAGTTCTCAT GA
```

Appendix 17. DNA sequence of D302_gp044 gene in phage CP30A and C, which encode putative baseplate wedge subunit.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGGCGGATA ATATTTTAAT TCCATATAAT TATGATGATA TAAAAGATGA AGTAATAAAA
CP30C  ATGGCGGATA ATATTTTAAT TCCATATAAT TATGATGATA TAAAAGATGA AGTAATAAAA

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTACTTAAAA ATAAAGGGTA TAATGCTGAT GTAAAAAGTT CAAATGCAAA CCTATTAGCA
CP30C  TTACTTAAAA ATAAAGGGTA TAATGCTGAT GTAAAAAGTT CAAATGCAAA CCTATTAGCA

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATATATTAT CTTATTTAGC TTATAGTATA AATGTAAATA CCTCTTTTCA AGCAGGTGAA
CP30C  GATATATTAT CTTATTTAGC TTATAGTATA AATGTAAATA CCTCTTTTCA AGCAGGTGAA

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGCTATTAT CAACTGCTCA ATATAGGAAA AATATACTTA TGGGTGCTAG ACAATTAGGA
CP30C  ATGCTATTAT CAACTGCTCA ATATAGGAAA AATATACTTA TGGGTGCTAG ACAATTAGGA

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATGAAGCAT CAAGAAAAGT ATCTTATGTT TATTCTTTGG AAATAAAACC TTTGAAAGAT
CP30C  TATGAAGCAT CAAGAAAAGT ATCTTATGTT TATTCTTTGG AAATAAAACC TTTGAAAGAT

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATACAAAAG ATAATGATAA CGAAGATAAA AGAATTTATT CAATACCCAA ATATACAATG
CP30C  GATACAAAAG ATAATGATAA CGAAGATAAA AGAATTTATT CAATACCCAA ATATACAATG

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTAACAGTG GTTCAAATAC CTACTATTAT ATGGGTAGTG ATATTGAAGT TGAGTTATCT
CP30C  TTTAACAGTG GTTCAAATAC CTACTATTAT ATGGGTAGTG ATATTGAAGT TGAGTTATCT

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AACAAAGATA TAACCACTGG AAAAGCATCT ACTATAAAAA TAGATGTAAA GGAAGGAATT
CP30C  AACAAAGATA TAACCACTGG AAAAGCATCT ACTATAAAAA TAGATGTAAA GGAAGGAATT

              490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTACATAAAT GGGACAAAAA TAAAGACACA CAAGTTTTTA CTATTAAAGC TATTGAACAA
CP30C  TTACATAAAT GGGACAAAAA TAAAGACACA CAAGTTTTTA CTATTAAAGC TATTGAACAA

              550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGAAGTA TAAAATCTTC AAATAAAATA TCATTATATC AGGACAACAT AGAAGAAAAT
CP30C  AATAGAAGTA TAAAATCTTC AAATAAAATA TCATTATATC AGGACAACAT AGAAGAAAAT

              610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGGCTTGAGG TTTTTGTAAC TTATATTGAT ATCGAAACTG GAGATAGTAA AGTTGATGAG
CP30C  GGGCTTGAGG TTTTTGTAAC TTATATTGAT ATCGAAACTG GAGATAGTAA AGTTGATGAG

              670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATTGGGAAA AGTCAGATCA ATTTATGATA GATGCAGATA GTGATACAAA TAAAAAATAT
CP30C  TATTGGGAAA AGTCAGATCA ATTTATGATA GATGCAGATA GTGATACAAA TAAAAAATAT

              730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTGTTTTAA ATAACATAGA TTATAGTGGT GTTGATATCT ATTTTTCAAT ATCAGGTATA
CP30C  TTTGTTTTAA ATAACATAGA TTATAGTGGT GTTGATATCT ATTTTTCAAT ATCAGGTATA

              790        800        810        820        830        840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTACAAATT TACTACCAGG AAGTACCGTA AAAGTTACAT ATTTAGAATC TAAAGGAAGT
CP30C  GGTACAAATT TACTACCAGG AAGTACCGTA AAAGTTACAT ATTTAGAATC TAAAGGAAGT
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              850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTGGTAAAT GTGGGGACAA TTTTGCATTT TCACAAAATA CATATCCTTT TAATCTCATG
CP30C  AGTGGTAAAT GTGGGGACAA TTTTGCATTT TCACAAAATA CATATCCTTT TAATCTCATG

              910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAATTGATA AATTTGAAAC AAAAATAGTT GGCACTGATG AAGAAACCAA TAGTTCAATA
CP30C  GAAATTGATA AATTTGAAAC AAAAATAGTT GGCACTGATG AAGAAACCAA TAGTTCAATA

              970        980        990        1000       1010       1020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGAAAATG CTCCTATATT TCATAATTCA GCAAACAGGG CAGTTACAGT AAGAGATTAT
CP30C  AAAGAAAATG CTCCTATATT TCATAATTCA GCAAACAGGG CAGTTACAGT AAGAGATTAT

              1030       1040       1050       1060       1070       1080
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTGCAATCT GTAATAGATA TACAAATATA TATCAAACCC AAGTGTGGGG TGGGGATGAA
CP30C  ATTGCAATCT GTAATAGATA TACAAATATA TATCAAACCC AAGTGTGGGG TGGGGATGAA

              1090       1100       1110       1120       1130       1140
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAGCAAGTTG TACAACTAGG ACACATTTGG TTTTCATTTA TCCCTGAATA CCGTAATCAA
CP30C  GAGCAAGTTG TACAACTAGG ACACATTTGG TTTTCATTTA TCCCTGAATA CCGTAATCAA

              1150       1160       1170       1180       1190       1200
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATTTTTCAT TAGATGAAAC AACACAAACA TATTCATTAG TAAACAAAAA TGATTCATAT
CP30C  GATTTTTCAT TAGATGAAAC AACACAAACA TATTCATTAG TAAACAAAAA TGATTCATAT

              1210       1220       1230       1240       1250       1260
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TACTTAAAAC AAAGTGAGTT AAGATCTAAT ACATTAGATA AAAATGGATA TTTAGTAAAT
CP30C  TACTTAAAAC AAAGTGAGTT AAGATCTAAT ACATTAGATA AAAATGGATA TTTAGTAAAT

              1270       1280       1290       1300       1310       1320
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGGTATAT TTGATGAGTT AGATTCTTAT AAAATAATGA CTATGGAATT ACACAATAGA
CP30C  AAAGGTATAT TTGATGAGTT AGATTCTTAT AAAATAATGA CTATGGAATT ACACAATAGA

              1330       1340       1350       1360       1370       1380
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATCCTATAT ATATGGATTT TGATTATGAA ATAAGGATAA TCAAACAAAA TATTGTAGTT
CP30C  TATCCTATAT ATATGGATTT TGATTATGAA ATAAGGATAA TCAAACAAAA TATTGTAGTT

              1390       1400       1410       1420       1430       1440
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCGAAAAATG AAACACAGGA TAAACTATTT AATATTTTAA AAGATTATTT TAAATCAGAT
CP30C  TCGAAAAATG AAACACAGGA TAAACTATTT AATATTTTAA AAGATTATTT TAAATCAGAT

              1450       1460       1470       1480       1490       1500
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAGAAAGTT TTGAATCTTC TTATTTTCAT TCAAGTGTTA TAAAAAGGTT AGGAACAGAA
CP30C  ATAGAAAGTT TTGAATCTTC TTATTTTCAT TCAAGTGTTA TAAAAAGGTT AGGAACAGAA

              1510       1520       1530       1540       1550       1560
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTATATGATT TATCAGGTAT ACAAGTAGAT GTTAGTATGA ATATACCTTT GTATTTAAGA
CP30C  TTATATGATT TATCAGGTAT ACAAGTAGAT GTTAGTATGA ATATACCTTT GTATTTAAGA

              1570       1580       1590       1600       1610       1620
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAAAGAAC CAAACAAAGA TATTTTATAT ATTTATCTGG CTATACCTTT TGAGCAAATA
CP30C  AATAAAGAAC CAAACAAAGA TATTTTATAT ATTTATCTGG CTATACCTTT TGAGCAAATA

              1630       1640       1650       1660       1670       1680
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAACTAAAA CACAGGATGA TCAAAATGAA TTACATGTTA ACTTACTGCC ACAAATAAGT
CP30C  ATAACTAAAA CACAGGATGA TCAAAATGAA TTACATGTTA ACTTACTGCC ACAAATAAGT

              1690       1700       1710       1720       1730       1740
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       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCGGATGACT TTGGTGGAAA ATTAGAAGTG GATTTTAAAA ATCCTATAAA AGGATTTACA
CP30C  TCGGATGACT TTGGTGGAAA ATTAGAAGTG GATTTTAAAA ATCCTATAAA AGGATTTACA

               1750       1760       1770       1780       1790       1800
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTCATAGGAA GTTCTAATGC TATAATAGGT ACTTTTGATG TAGGAAATGG TCAATCAGCA
CP30C  GTCATAGGAA GTTCTAATGC TATAATAGGT ACTTTTGATG TAGGAAATGG TCAATCAGCA

               1810       1820       1830       1840       1850       1860
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTTTCAATG GTAAAAATGT TGTCACTTCA GATGGATTAA TTATAAAGAA TACTAATATA
CP30C  GTTTTCAATG GTAAAAATGT TGTCACTTCA GATGGATTAA TTATAAAGAA TACTAATATA

               1870       1880       1890       1900       1910       1920
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTTTTAATA TATTTGCAAA TGGAACAATA ATAGGAACTT ATAAAATAAT ATATGATAAT
CP30C  AGTTTTAATA TATTTGCAAA TGGAACAATA ATAGGAACTT ATAAAATAAT ATATGATAAT

               1930       1940       1950       1960       1970       1980
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGAAGAAGGT TTATAGTTAT AGAAATAACA GATAGTATAG TTTTAAGCAG TCTTGATAAT
CP30C  AGAAGAAGGT TTATAGTTAT AGAAATAACA GATAGTATAG TTTTAAGCAG TCTTGATAAT

               1990       2000       2010       2020       2030       2040
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAACTCCAA AATATATAAG GGTAAAATAT TCTGATGATA ACCTATCATT TTATAAAAAT
CP30C  ATAACTCCAA AATATATAAG GGTAAAATAT TCTGATGATA ACCTATCATT TTATAAAAAT

               2050       2060       2070       2080       2090
       ....|....| ....|....| ....|....| ....|....| ....|....| ....
CP30A  ACAATAGCTA GATTATCTTC AGTAAAATTT GTAAGTGAAA GTGATGTTAT ATGA
CP30C  ACAATAGCTA GATTATCTTC AGTAAAATTT GTAAGTGAAA GTGATGTTAT ATGA
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Appendix 18. DNA sequence of D302_gp045 gene in phage CP30A and C, which encode putative baseplate wedge subunit.

```
               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTGATACAAT ACAAAGATAT AAATCCAAAA AACATTGAAA AAGATATAAT AAATGTTGAT
CP30C  GTGATACAAT ACAAAGATAT AAATCCAAAA AACATTGAAA AAGATATAAT AAATGTTGAT

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTTTTTATG TGTCATTAAA AAATATTGTA AGCACTACTA TTGGGGATAT AGCTGGATTT
CP30C  ACTTTTTATG TGTCATTAAA AAATATTGTA AGCACTACTA TTGGGGATAT AGCTGGATTT

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CCAGAGTTTT CAAATAATGC TCAGCTATTA TTTGATCAAT ATAGCTCAGT TGCATTAGAT
CP30C  CCAGAGTTTT CAAATAATGC TCAGCTATTA TTTGATCAAT ATAGCTCAGT TGCATTAGAT

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTTATAAGA CATCTTTAAA AACATCAATT CAAAAATTTG ATTATCGTAT TATAGTTGAT
CP30C  GCTTATAAGA CATCTTTAAA AACATCAATT CAAAAATTTG ATTATCGTAT TATAGTTGAT

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATATAAATA TTTCAAAAGG AGACGCTGAT AATAGTGTAT ATATTGAAAT AAAATATAGA
CP30C  AATATAAATA TTTCAAAAGG AGACGCTGAT AATAGTGTAT ATATTGAAAT AAAATATAGA

              310        320        330        340
       ....|....| ....|....| ....|....| ....|....| ....|...
CP30A  GTTAGAGATA CAACTATATC AGATACAGCT AGTATAAAGG TTGGATAA
CP30C  GTTAGAGATA CAACTATATC AGATACAGCT AGTATAAAGG TTGGATAA
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Appendix 19. DNA sequence of D302_gp050 gene in phage CP30A and C, which encode putative tail fibre protein. Mutation is indicated in red. Highlighted TAA would be the stop codon for gene D302_gp050 in phage CP30C after mutation.

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               10         20         30         40         50         60
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGATAGAAC CAAAAAGAGA GCCTACACAA GACTTTTTTG TATGTTTATT AAAAGAACCT
CP30C  ATGATAGAAC -AAAAAGAGA GCCTACACAA GACTTTTTTG TATGTTTATT AAAAGAACCT

               70         80         90        100        110        120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGATGGATTA GTACTGATGA CTTATATCCC ATTTTATTAA TACCAGGAGT CAATTATCCT
CP30C  AGATGGATTA GTACTGATGA CTTATATCCC ATTTTATTAA TACCAGGAGT CAATTATCCT

              130        140        150        160        170        180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTGAAATAG CTATGATGCA TCCTGATTTT TTCGGTGGCG ATGATGTTGT ATTTAAGCCT
CP30C  GCTGAAATAG CTATGATGCA TCCTGATTTT TTCGGTGGCG ATGATGTTGT ATTTAAGCCT

              190        200        210        220        230        240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAACCTCCAA TTGATCCAGA TAATCCTGAT TTATCTAACT ATTATACAAA ACCAGAAACA
CP30C  GAACCTCCAA TTGATCCAGA TAATCCTGAT TTATCTAACT ATTATACAAA ACCAGAAACA

              250        260        270        280        290        300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATAGTTTAT TAGATAAGAA AGCAGATAAA GTTCATACAC ATGTTGTAGC TGATATAACA
CP30C  AATAGTTTAT TAGATAAGAA AGCAGATAAA GTTCATACAC ATGTTGTAGC TGATATAACA

              310        320        330        340        350        360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATTTGAATT TAAATAAATT TGCTACAAAA GAAGAAACAT ATACTAAACA AGAGATAGAT
CP30C  GATTTGAATT TAAATAAATT TGCTACAAAA GAAGAAACAT ATACTAAACA AGAGATAGAT

              370        380        390        400        410        420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATAAAATAG ATGAAATAGT ACCACCTGAA ATTGATTTAA CTAATTATGC AAAGAAAGAT
CP30C  GATAAAATAG ATGAAATAGT ACCACCTGAA ATTGATTTAA CTAATTATGC AAAGAAAGAT

              430        440        450        460        470        480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAGCTAATA TTTTTACAAA AGCTAATATT TTTACAGAAG CACCTTCGGT AGAAGTAGAT
CP30C  GCAGCTAATA TTTTTACAAA AGCTAATATT TTTACAGAAG CACCTTCGGT AGAAGTAGAT

              490        500        510        520        530        540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAACACTAG ATAATCATGT TATTAGAAAG AAACAGTTTG ACAATAGCAT AAAAGAAGTT
CP30C  GCAACACTAG ATAATCATGT TATTAGAAAG AAACAGTTTG ACAATAGCAT AAAAGAAGTT

              550        560        570        580        590        600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGATTTAC TATCTAATGT ATTTTCATAT AAAGGATCAA AACCTACATA TACAGAAATA
CP30C  AAAGATTTAC TATCTAATGT ATTTTCATAT AAAGGATCAA AACCTACATA TACAGAAATA

              610        620        630        640        650        660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GAAGCCATTG TTGATAAAAA GATAGGTGAT GTATGGTATG CTGAAGATAC TGGATATATG
CP30C  GAAGCCATTG TTGATAAAAA GATAGGTGAT GTATGGTATG CTGAAGATAC TGGATATATG

              670        680        690        700        710        720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATATATGGA ATGGTAAAAC TTGGTATGAT TTAGGTAAAT CTTTTGATGC TAGTAAATTT
CP30C  TATATATGGA ATGGTAAAAC TTGGTATGAT TTAGGTAAAT CTTTTGATGC TAGTAAATTT

              730        740        750        760        770        780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTGATATAA CTTCAGACCA AATAACAATA AATGGTATTA AAAAATTCAC AGGAAAATTA
CP30C  GTTGATATAA CTTCAGACCA AATAACAATA AATGGTATTA AAAAATTCAC AGGAAAATTA

              790        800        810        820        830        840
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       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGCATTAA CACCTGTTGA TTCTGATGAT GTGGCTATTT TGAGCTGGAC AACAAAACAA
CP30C  AAAGCATTAA CACCTGTTGA TTCTGATGAT GTGGCTATTT TGAGCTGGAC AACAAAACAA

               850        860        870        880        890        900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAAATGACA AAGTTGAATC TGTTATTGGT GATTTAAATT CATTAAATAA TGAAGTTTCT
CP30C  ATAAATGACA AAGTTGAATC TGTTATTGGT GATTTAAATT CATTAAATAA TGAAGTTTCT

               910        920        930        940        950        960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAGATAATT TAGTTAATGC TATAAATAGT GTAGATGATA AGTTTAAAAC AACAGCTAAA
CP30C  AAAGATAATT TAGTTAATGC TATAAATAGT GTAGATGATA AGTTTAAAAC AACAGCTAAA

               970        980        990        1000       1010       1020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATCAATAAAT CAAATACATT TACAGGTGAT CAAACTTATG TAGATCATAT TTTACTGGAA
CP30C  ATCAATAAAT CAAATACATT TACAGGTGAT CAAACTTATG TAGATCATAT TTTACTGGAA

               1030       1040       1050       1060       1070       1080
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTGTTCCTT CTGAAAGAAA TCATGCAGTT AATTTGGGAT ACATTTTAGA TAATCCTGGA
CP30C  TCTGTTCCTT CTGAAAGAAA TCATGCAGTT AATTTGGGAT ACATTTTAGA TAATCCTGGA

               1090       1100       1110       1120       1130       1140
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTATAAAAC TTCCTGATCA TACAGCACTT ACACAAAATT CTGTTACAGA AATAACTTTT
CP30C  GGTATAAAAC TTCCTGATCA TACAGCACTT ACACAAAATT CTGTTACAGA AATAACTTTT

               1150       1160       1170       1180       1190       1200
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGATATGCAA ATCCAGTATC ATATTCTGCA CAACAATTAA AAAATGTATT CCTAAAAGAT
CP30C  GGATATGCAA ATCCAGTATC ATATTCTGCA CAACAATTAA AAAATGTATT CCTAAAAGAT

               1210       1220       1230       1240       1250       1260
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAGTAGGTA ATGAATATAA AGCTATAATG GCAGATAAAA CATCATTTAC AGAAAATCCT
CP30C  ATAGTAGGTA ATGAATATAA AGCTATAATG GCAGATAAAA CATCATTTAC AGAAAATCCT

               1270       1280       1290       1300       1310       1320
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCAAAGGAAA TGGTTGTTAT ACTTTCTAGA ACTGATTATA CAAAAAATAT AGATGTTAAG
CP30C  TCAAAGGAAA TGGTTGTTAT ACTTTCTAGA ACTGATTATA CAAAAAATAT AGATGTTAAG

               1330       1340       1350       1360       1370       1380
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTTGATATAA CTAAAACAGT TGATGAGCTT AAACAATATG AGCTTAAAGA AGGAGAAGTT
CP30C  TTTGATATAA CTAAAACAGT TGATGAGCTT AAACAATATG AGCTTAAAGA AGGAGAAGTT

               1390       1400       1410       1420       1430       1440
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGAGTTATAC TATCTTATGA TACTGTATCT GTTTATTCTA GTGGGTATGG TTATGGAGCT
CP30C  AGAGTTATAC TATCTTATGA TACTGTATCT GTTTATTCTA GTGGGTATGG TTATGGAGCT

               1450       1460       1470       1480       1490       1500
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATGTTTGCTA GAAACGCTAA TAAAAAAGAC GGAGATTTAA TATATGATTA TTATTCTGGA
CP30C  ATGTTTGCTA GAAACGCTAA TAAAAAAGAC GGAGATTTAA TATATGATTA TTATTCTGGA

               1510       1520       1530       1540       1550       1560
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTCAAAATG ATATAACAAA TAATAGAAAA GTATCTATAA AAATAGATAA ACTTGGTATT
CP30C  AGTCAAAATG ATATAACAAA TAATAGAAAA GTATCTATAA AAATAGATAA ACTTGGTATT

               1570       1580       1590       1600       1610       1620
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATACTCCAG ATATTGTAAG TATATCTATG ACTACAAATG GTTCTGAAAA ATTAACAGTA
CP30C  AATACTCCAG ATATTGTAAG TATATCTATG ACTACAAATG GTTCTGAAAA ATTAACAGTA

               1630       1640       1650       1660       1670       1680
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAACAGATA CACTAGATCC TGTAGAAAAT ACATACGAAT CTGCGGATAT GACTTATATT
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CP30C  AAAACAGATA CACTAGATCC TGTAGAAAAT ACATACGAAT CTGCGGATAT GACTTATATT

              1690       1700       1710       1720       1730       1740
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CATACTCCTG TCAGCAAAAT TGCAGGAGAT ATTCTGTATA GTAATATATC TCAAGCAATT
CP30C  CATACTCCTG TCAGCAAAAT TGCAGGAGAT ATTCTGTATA GTAATATATC TCAAGCAATT

              1750       1760       1770       1780       1790       1800
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAATCAATAC ATGTGTTAGA AAATAATATA TGTTCTTTAA AGCCTGCAAG TATGGAATTA
CP30C  AAATCAATAC ATGTGTTAGA AAATAATATA TGTTCTTTAA AGCCTGCAAG TATGGAATTA

              1810       1820       1830       1840       1850       1860
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAACTTGTAA GGCTCAAAGA ACTTAATCAA ACAATAAATA ACATGTTGCA ATCTATGTTT
CP30C  CAACTTGTAA GGCTCAAAGA ACTTAATCAA ACAATAAATA ACATGTTGCA ATCTATGTTT

              1870       1880       1890       1900       1910       1920
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGAGAGTC CTGTAGCACT AAAAAATGGA GATTACATAG ATGTTTCATT TAGTGGTAGT
CP30C  GATGAGAGTC CTGTAGCACT AAAAAATGGA GATTACATAG ATGTTTCATT TAGTGGTAGT

              1930       1940       1950       1960       1970       1980
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCAAGCTATG GAACAGGATA TTGTGGGTAT GTTAATATAA AAGATACTAT AAGAGATATT
CP30C  GCAAGCTATG GAACAGGATA TTGTGGGTAT GTTAATATAA AAGATACTAT AAGAGATATT

              1990       2000       2010       2020       2030       2040
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACATATAAAT CTTATAAAGT ATCTTCAAAT GCTTTTGATA CAACTAGTGG AACTAAAGTT
CP30C  ACATATAAAT CTTATAAAGT ATCTTCAAAT GCTTTTGATA CAACTAGTGG AACTAAAGTT

              2050       2060       2070       2080       2090       2100
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATTGCAGTTC TCACTTCTGA TAACAGTAAA ACAAATGTAA CTTATTCCGA TAGTGTATCA
CP30C  ATTGCAGTTC TCACTTCTGA TAACAGTAAA ACAAATGTAA CTTATTCCGA TAGTGTATCA

              2110       2120       2130       2140       2150       2160
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACATTAGAAT ATTATGAAGT AGCAGAAAAT GAGATATTAT TAGAAATATC ATTTTCAACT
CP30C  ACATTAGAAT ATTATGAAGT AGCAGAAAAT GAGATATTAT TAGAAATATC ATTTTCAACT

              2170       2180       2190       2200       2210       2220
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCTAAGCAAT ATTCTGCTAA ATACGGGTAT GGAGCTATGT TAGAGTATTG GGGTTCTGTA
CP30C  GCTAAGCAAT ATTCTGCTAA ATACGGGTAT GGAGCTATGT TAGAGTATTG GGGTTCTGTA

              2230       2240       2250       2260       2270       2280
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTGATCTAT GTTATGATTA TTATATGGGT TCAAACCTAG ATATGAACTG TCCATTTAAA
CP30C  TCTGATCTAT GTTATGATTA TTATATGGGT TCAAACCTAG ATATGAACTG TCCATTTAAA

              2290       2300       2310       2320       2330       2340
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAACAATAC TAAAACTAGG AAGTTCTGTT AAAGCAGATA CTATACAAAT AGGTGCTCCT
CP30C  ATAACAATAC TAAAACTAGG AAGTTCTGTT AAAGCAGATA CTATACAAAT AGGTGCTCCT

              2350       2360       2370       2380       2390       2400
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACTTTTGCAG GGTCATTAGT TATGCACCTA AGAAAAAATA CAAACGATGT TATTAATTTC
CP30C  ACTTTTGCAG GGTCATTAGT TATGCACCTA AGAAAAAATA CAAACGATGT TATTAATTTC

              2410       2420       2430       2440       2450       2460
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CTTGTATCTA CAGGTGTGAA TGTAACAGGA AGAGATGGGA CTGAAAGTGG ATCAGTATAT
CP30C  CTTGTATCTA CAGGTGTGAA TGTAACAGGA AGAGATGGGA CTGAAAGTGG ATCAGTATAT

              2470       2480       2490       2500       2510       2520
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATTTAGTAG AAAAATCATT GAAACCATTA AAAGTTCTTA CATCTGAAGC ACATCAATCT
CP30C  AATTTAGTAG AAAAATCATT GAAACCATTA AAAGTTCTTA CATCTGAAGC ACATCAATCT
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              2530       2540       2550       2560       2570       2580
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAAATGGTA TAACTAATTT CAATAATAAA GTGTATATGA ATATAGAAAA TGAAAGAATT
CP30C  ATAAATGGTA TAACTAATTT CAATAATAAA GTGTATATGA ATATAGAAAA TGAAAGAATT

              2590       2600       2610       2620       2630       2640
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACAGACAGCA AACAACTAAT ACATAAAGAA TACCTAGATA AAAATATTAC AGATAATGTA
CP30C  ACAGACAGCA AACAACTAAT ACATAAAGAA TACCTAGATA AAAATATTAC AGATAATGTA

              2650       2660       2670       2680       2690       2700
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GCATATAATA TTAGTAATAC TCCATTAGTA CCTTACAATG ATGTTAGTTC TTTAAATACA
CP30C  GCATATAATA TTAGTAATAC TCCATTAGTA CCTTACAATG ATGTTAGTTC TTTAAATACA

              2710       2720       2730       2740       2750       2760
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAAAATATTT GTGTAAAAAT ATCTGCGACA ACTGATAGCT CTAATTATTC ATCTGGTGAT
CP30C  AAAAATATTT GTGTAAAAAT ATCTGCGACA ACTGATAGCT CTAATTATTC ATCTGGTGAT

              2770       2780       2790       2800       2810       2820
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ACAGTAGTAG TTAGTGATTT CAAGATAAAA CTTAAAGGAG ATGATGAGTA CCTTAAACCT
CP30C  ACAGTAGTAG TTAGTGATTT CAAGATAAAA CTTAAAGGAG ATGATGAGTA CCTTAAACCT

              2830       2840       2850       2860       2870       2880
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATGGGGTTG AAGTTATTGA TAGAGAAAAT AATAAAATAG GTTTAAATTT AATAGGAGAT
CP30C  TATGGGGTTG AAGTTATTGA TAGAGAAAAT AATAAAATAG GTTTAAATTT AATAGGAGAT

              2890       2900       2910       2920       2930       2940
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATACAGTAT ATAATGATAA TGATGCTTTA TTATCTACAA GACCTTCTGA TTTCAACTCA
CP30C  GATACAGTAT ATAATGATAA TGATGCTTTA TTATCTACAA GACCTTCTGA TTTCAACTCA

              2950       2960       2970       2980       2990       3000
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTAATGTAG ATCTTTCTAG TGGAAGTAAC GCCTTAGTAA CTGTTAAAAC TAATGGAGCT
CP30C  TCTAATGTAG ATCTTTCTAG TGGAAGTAAC GCCTTAGTAA CTGTTAAAAC TAATGGAGCT

              3010       3020       3030       3040       3050       3060
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TATGATTATA GTGGAGTTTA TGATATACCA AATCCTTTTA GAGAATATAA TAAAAAATAC
CP30C  TATGATTATA GTGGAGTTTA TGATATACCA AATCCTTTTA GAGAATATAA TAAAAAATAC

              3070       3080       3090       3100       3110       3120
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCTTTATTCT TAAGTAATGA TGGGTTAAAA AATCCTTATT ACCAAGTTGA TATAAATAGC
CP30C  TCTTTATTCT TAAGTAATGA TGGGTTAAAA AATCCTTATT ACCAAGTTGA TATAAATAGC

              3130       3140       3150       3160       3170       3180
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TCCAAACAAG TTGATAATAT ATCTTTTCAA TTATTTGGAA CTAGTGCTAC AAATCCTTTT
CP30C  TCCAAACAAG TTGATAATAT ATCTTTTCAA TTATTTGGAA CTAGTGCTAC AAATCCTTTT

              3190       3200       3210       3220       3230       3240
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  TTATATTCTA AAGACTGTAA AATTGAATTG TTTATAGAAA ATACTATTGT AAAAACCTTC
CP30C  TTATATTCTA AAGACTGTAA AATTGAATTG TTTATAGAAA ATACTATTGT AAAAACCTTC

              3250       3260       3270       3280       3290       3300
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATATTAAAG GCAGCTCTGG AAATAATAGT CCTGTGAATA TTAATATAGA TTATAAAGAT
CP30C  AATATTAAAG GCAGCTCTGG AAATAATAGT CCTGTGAATA TTAATATAGA TTATAAAGAT

              3310       3320       3330       3340       3350       3360
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTATGTTTT TAATATCTAT TCTTGATTCT ATAAATTACA TAAATAATAG AATTAATAAA
CP30C  GGTATGTTTT TAATATCTAT TCTTGATTCT ATAAATTACA TAAATAATAG AATTAATAAA

              3370       3380       3390       3400       3410       3420
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
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CP30A  AATGCAGAAT TACTTACAGG GTCAGGCAAA CCTAATTTTT CATTAAACCC TAACAAAATA
CP30C  AATGCAGAAT TACTTACAGG GTCAGGCAAA CCTAATTTTT CATTAAACCC TAACAAAATA

               3430       3440       3450       3460       3470       3480
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTTCTCTAT ACTCTGATAC AACTAATAAA GCTGTATATA TGTGTATAGA CAATACTTCT
CP30C  GGTTCTCTAT ACTCTGATAC AACTAATAAA GCTGTATATA TGTGTATAGA CAATACTTCT

               3490       3500       3510       3520       3530       3540
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GGTGCTAATA AATGGGTAAA TATAGTAACA GGAGATGAAA TTAAACCAAA CCTTAGAAAA
CP30C  GGTGCTAATA AATGGGTAAA TATAGTAACA GGAGATGAAA TTAAACCAAA CCTTAGAAAA

               3550       3560       3570       3580       3590       3600
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  ATAGAAATTA CTTGTAATGT AAGATTAAGA AGTGGCCAAT ATGGTGGTTG TATGAGCGGT
CP30C  ATAGAAATTA CTTGTAATGT AAGATTAAGA AGTGGCCAAT ATGGTGGTTG TATGAGCGGT

               3610       3620       3630       3640       3650       3660
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GTTAAAATAG GATTTGATAA CGGATATGCT TCTACAAAAC AAATAGTTAA AGGGTTAAAT
CP30C  GTTAAAATAG GATTTGATAA CGGATATGCT TCTACAAAAC AAATAGTTAA AGGGTTAAAT

               3670       3680       3690       3700       3710       3720
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTGGTCAAA TATTGCTATC TCTAGATGGG TTGGGTAACC TTTCTGGATA TTCTGAAGTC
CP30C  AGTGGTCAAA TATTGCTATC TCTAGATGGG TTGGGTAACC TTTCTGGATA TTCTGAAGTC

               3730       3740       3750       3760       3770       3780
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AGTTCTTTAA CTCCTAGCAG TCAAGATATA AAAGTTGATG TGGATACTAC TGGGATATAT
CP30C  AGTTCTTTAA CTCCTAGCAG TCAAGATATA AAAGTTGATG TGGATACTAC TGGGATATAT

               3790       3800       3810       3820       3830       3840
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AATGACCCTT CATATCATTG CGTTACTAAT ATATTTAAAG AATACCTTGG CAATGCTGAT
CP30C  AATGACCCTT CATATCATTG CGTTACTAAT ATATTTAAAG AATACCTTGG CAATGCTGAT

               3850       3860       3870       3880       3890       3900
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  CAATGTTCGC TATGGTCTGA TGCTAGTGTT AAACAACTTA AAATAACTTT ACTATCTGAA
CP30C  CAATGTTCGC TATGGTCTGA TGCTAGTGTT AAACAACTTA AAATAACTTT ACTATCTGAA

               3910       3920       3930       3940       3950       3960
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  AAGATTCCTA CCAAAATATT ATATGTAGGA AACGGATATT ATGGTCAAAC ATCTGTTTCT
CP30C  AAGATTCCTA CCAAAATATT ATATGTAGGA AACGGATATT ATGGTCAAAC ATCTGTTTCT

               3970       3980       3990       4000       4010       4020
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATGTAAAAG CTGTATGGTA TTATGTAAAT GATAACGGAG ACAAAATAGA AGGTAATGTA
CP30C  GATGTAAAAG CTGTATGGTA TTATGTAAAT GATAACGGAG ACAAAATAGA AGGTAATGTA

               4030       4040       4050       4060       4070       4080
       ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A  GATAATGATC TAAAGGTAAG TAATAATGTT TCTGAAACAA ACGATAGTTC TTATATATAT
CP30C  GATAATGATC TAAAGGTAAG TAATAATGTT TCTGAAACAA ACGATAGTTC TTATATATAT

               4090
       ....|....| ....|...
CP30A  GCGTTCAATA TAAATTAG
CP30C  GCGTTCAATA TAAATTAG
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Appendix 20. Protein sequence of D302_gp050 gene in phage CP30A and C, start codon is indicated in red.

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                10         20         30         40         50         60
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  MIEPKREPTQ DFFVCLLKEP RWISTDDLYP ILLIPGVNYP AEIAMMHPDF FGGDDVVFKP
CP30C_gp050  -DRTKREPTQ DFFVCLLKEP RWISTDDLYP ILLIPGVNYP AEIAMMHPDF FGGDDVVFKP

                70         80         90        100        110        120
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  EPPIDPDNPD LSNYYTKPET NSLLDKKADK VHTHVVADIT DLNLNKFATK EETYTKQEID
CP30C_gp050  EPPIDPDNPD LSNYYTKPET NSLLDKKADK VHTHVVADIT DLNLNKFATK EETYTKQEID

               130        140        150        160        170        180
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  DKIDEIVPPE IDLTNYAKKD AANIFTKANI FTEAPSVEVD ATLDNHVIRK KQFDNSIKEV
CP30C_gp050  DKIDEIVPPE IDLTNYAKKD AANIFTKANI FTEAPSVEVD ATLDNHVIRK KQFDNSIKEV

               190        200        210        220        230        240
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  KDLLSNVFSY KGSKPTYTEI EAIVDKKIGD VWYAEDTGYM YIWNGKTWYD LGKSFDASKF
CP30C_gp050  KDLLSNVFSY KGSKPTYTEI EAIVDKKIGD VWYAEDTGYM YIWNGKTWYD LGKSFDASKF

               250        260        270        280        290        300
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  VDITSDQITI NGIKKFTGKL KALTPVDSDD VAILSWTTKQ INDKVESVIG DLNSLNNEVS
CP30C_gp050  VDITSDQITI NGIKKFTGKL KALTPVDSDD VAILSWTTKQ INDKVESVIG DLNSLNNEVS

               310        320        330        340        350        360
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  KDNLVNAINS VDDKFKTTAK INKSNTFTGD QTYVDHILLE SVPSERNHAV NLGYILDNPG
CP30C_gp050  KDNLVNAINS VDDKFKTTAK INKSNTFTGD QTYVDHILLE SVPSERNHAV NLGYILDNPG

               370        380        390        400        410        420
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  GIKLPDHTAL TQNSVTEITF GYANPVSYSA QQLKNVFLKD IVGNEYKAIM ADKTSFTENP
CP30C_gp050  GIKLPDHTAL TQNSVTEITF GYANPVSYSA QQLKNVFLKD IVGNEYKAIM ADKTSFTENP

               430        440        450        460        470        480
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  SKEMVVILSR TDYTKNIDVK FDITKTVDEL KQYELKEGEV RVILSYDTVS VYSSGYGYGA
CP30C_gp050  SKEMVVILSR TDYTKNIDVK FDITKTVDEL KQYELKEGEV RVILSYDTVS VYSSGYGYGA

               490        500        510        520        530        540
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  MFARNANKKD GDLIYDYYSG SQNDITNNRK VSIKIDKLGI NTPDIVSISM TTNGSEKLTV
CP30C_gp050  MFARNANKKD GDLIYDYYSG SQNDITNNRK VSIKIDKLGI NTPDIVSISM TTNGSEKLTV

               550        560        570        580        590        600
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  KTDTLDPVEN TYESADMTYI HTPVSKIAGD ILYSNISQAI KSIHVLENNI CSLKPASMEL
CP30C_gp050  KTDTLDPVEN TYESADMTYI HTPVSKIAGD ILYSNISQAI KSIHVLENNI CSLKPASMEL

               610        620        630        640        650        660
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  QLVRLKELNQ TINNMLQSMF DESPVALKNG DYIDVSFSGS ASYGTGYCGY VNIKDTIRDI
CP30C_gp050  QLVRLKELNQ TINNMLQSMF DESPVALKNG DYIDVSFSGS ASYGTGYCGY VNIKDTIRDI

               670        680        690        700        710        720
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  TYKSYKVSSN AFDTTSGTKV IAVLTSDNSK TNVTYSDSVS TLEYYEVAEN EILLEISFST
CP30C_gp050  TYKSYKVSSN AFDTTSGTKV IAVLTSDNSK TNVTYSDSVS TLEYYEVAEN EILLEISFST

               730        740        750        760        770        780
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  AKQYSAKYGY GAMLEYWGSV SDLCYDYYMG SNLDMNCPFK ITILKLGSSV KADTIQIGAP
CP30C_gp050  AKQYSAKYGY GAMLEYWGSV SDLCYDYYMG SNLDMNCPFK ITILKLGSSV KADTIQIGAP

               790        800        810        820        830        840
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  TFAGSLVMHL RKNTNDVINF LVSTGVNVTG RDGTESGSVY NLVEKSLKPL KVLTSEAHQS
CP30C_gp050  TFAGSLVMHL RKNTNDVINF LVSTGVNVTG RDGTESGSVY NLVEKSLKPL KVLTSEAHQS
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                 850        860        870        880        890        900
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  INGITNFNNK VYMNIENERI TDSKQLIHKE YLDKNITDNV AYNISNTPLV PYNDVSSLNT
CP30C_gp050  INGITNFNNK VYMNIENERI TDSKQLIHKE YLDKNITDNV AYNISNTPLV PYNDVSSLNT

                 910        920        930        940        950        960
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  KNICVKISAT TDSSNYSSGD TVVVSDFKIK LKGDDEYLKP YGVEVIDREN NKIGLNLIGD
CP30C_gp050  KNICVKISAT TDSSNYSSGD TVVVSDFKIK LKGDDEYLKP YGVEVIDREN NKIGLNLIGD

                 970        980        990        1000       1010       1020
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  DTVYNDNDAL LSTRPSDFNS SNVDLSSGSN ALVTVKTNGA YDYSGVYDIP NPFREYNKKY
CP30C_gp050  DTVYNDNDAL LSTRPSDFNS SNVDLSSGSN ALVTVKTNGA YDYSGVYDIP NPFREYNKKY

                 1030       1040       1050       1060       1070       1080
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  SLFLSNDGLK NPYYQVDINS SKQVDNISFQ LFGTSATNPF LYSKDCKIEL FIENTIVKTF
CP30C_gp050  SLFLSNDGLK NPYYQVDINS SKQVDNISFQ LFGTSATNPF LYSKDCKIEL FIENTIVKTF

                 1090       1100       1110       1120       1130       1140
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  NIKGSSGNNS PVNINIDYKD GMFLISILDS INYINNRINK NAELLTGSGK PNFSLNPNKI
CP30C_gp050  NIKGSSGNNS PVNINIDYKD GMFLISILDS INYINNRINK NAELLTGSGK PNFSLNPNKI

                 1150       1160       1170       1180       1190       1200
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  GSLYSDTTNK AVYMCIDNTS GANKWVNIVT GDEIKPNLRK IEITCNVRLR SGQYGGCMSG
CP30C_gp050  GSLYSDTTNK AVYMCIDNTS GANKWVNIVT GDEIKPNLRK IEITCNVRLR SGQYGGCMSG

                 1210       1220       1230       1240       1250       1260
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  VKIGFDNGYA STKQIVKGLN SGQILLSLDG LGNLSGYSEV SSLTPSSQDI KVDVDTTGIY
CP30C_gp050  VKIGFDNGYA STKQIVKGLN SGQILLSLDG LGNLSGYSEV SSLTPSSQDI KVDVDTTGIY

                 1270       1280       1290       1300       1310       1320
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CP30A_gp050  NDPSYHCVTN IFKEYLGNAD QCSLWSDASV KQLKITLLSE KIPTKILYVG NGYYGQTSVS
CP30C_gp050  NDPSYHCVTN IFKEYLGNAD QCSLWSDASV KQLKITLLSE KIPTKILYVG NGYYGQTSVS

                 1330       1340       1350       1360
            ....|....| ....|....| ....|....| ....|....| ....|.
CP30A_gp050  DVKAVWYYVN DNGDKIEGNV DNDLKVSNNV SETNDSSYIY AFNIN-
CP30C_gp050  DVKAVWYYVN DNGDKIEGNV DNDLKVSNNV SETNDSSYIY AFNIN-
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Appendix 21. DNA sequence of gene A911_ 00360 in *C. jejuni* PT14 and PT14CP30A-035CS, which encode l- lact permease. Mutation is indicated in red. Highlighted TGA would be the stop codon for gene A911_00360 in *C. jejuni* PT14CP30A-035CS after mutation.

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                    10         20         30         40         50
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           TTGGAACAAA TTTTAACATG GCAACAAATT TATGACCCTT TTTCAAATAT
PT14CP30A-035 TTGGAACAAA TTTTAACATG GCAACAAATT TATGACCCTT TTTCAAATAT

                    60         70         80         90        100
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           TTGGCTAAGT GCTTTAGTGG CATTTTTACC TATACTATGT TTTTTAGTTT
PT14CP30A-035 TTGGCTAAGT GCTTTAGTGG CATTTTTACC TATACTATGT TTTTTAGTTT

                   110        120        130        140        150
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GTTTGGTTGT TTTAAAACTT AAAGGCTATC AAGCAGGTTT TTTAACTGTT
PT14CP30A-035 GTTTGGTTGT TTTAAAACTT AAAGGCTATC AAGCAGGTTT TTTAACTGTT

                   160        170        180        190        200
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           ATACTTGCTA CTTTAGTAGC TTTATTTGCC TATAAAATGC CTTGGAATTT
PT14CP30A-035 ATACTTGCTA CTTTAGTAGC TTTATTTGCC TATAAAATGC CTTGGAATTT

                   210        220        230        240        250
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           AGTGGGTGCA AGTTTTATTC AAGGCTTTAC AAATGGTATG TGGCCTATAG
PT14CP30A-035 AGTGGGTGCA AGTTTTATTC AAGGCTTTAC AAATGGTATG TGGCCTATAG

                   260        270        280        290        300
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           CTTGGATTAT CATCGCGGCA ATCTTTCTTT ACAAACTTTC TATCAAGTCA
PT14CP30A-035 CTTGGATTAT CATCGCGGCA ATCTTTCTTT ACAAACTTTC TATCAAGTCA

                   310        320        330        340        350
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GGTTCTTTTG AAATCATTAA AAAAAGCGTG ATGAGCATTA CTCCAGATCA
PT14CP30A-035 GGTTCTTTTG AAATCATTAA AAAA-GCGTG ATGAGCATTA CTCCAGATCA

                   360        370        380        390        400
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           TAGAATTCAA GTGATTTTAA TAGGTTTTTG TTTTGGTTCA TTTTTAGAGG
PT14CP30A-035 TAGAATTCAA GTGATTTTAA TAGGTTTTTG TTTTGGTTCA TTTTTAGAGG

                   410        420        430        440        450
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GTGCTATAGG TTTTGGTGGC CCTGTGGCTA TAACCGCTGC ACTTTTAGTA
PT14CP30A-035 GTGCTATAGG TTTTGGTGGC CCTGTGGCTA TAACCGCTGC ACTTTTAGTA

                   460        470        480        490        500
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GGACTTGGCT TGCGTCCTTT ACAAGCTGCA GGGCTTTGTC TTATCGCAAA
PT14CP30A-035 GGACTTGGCT TGCGTCCTTT ACAAGCTGCA GGGCTTTGTC TTATCGCAAA

                   510        520        530        540        550
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           TACCGCTCCT GTTGCTTTTG GTGCGGTGGG AATTCCTATT ATTGCTATGG
PT14CP30A-035 TACCGCTCCT GTTGCTTTTG GTGCGGTGGG AATTCCTATT ATTGCTATGG

                   560        570        580        590        600
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           CAAATCTTGT CGGCATAGAA CAGCATTCAG TTTCTGCCAT GGTAGGTAGA
PT14CP30A-035 CAAATCTTGT CGGCATAGAA CAGCATTCAG TTTCTGCCAT GGTAGGTAGA

                   610        620        630        640        650
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           ATGCTAGTTC CTTTAAGCCT TACTATACCT TTTTTCATAG TTTTTTTAAT
PT14CP30A-035 ATGCTAGTTC CTTTAAGCCT TACTATACCT TTTTTCATAG TTTTTTTAAT

                   660        670        680        690        700
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                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            GGATGGATTT AAAGGTATTA AAGAAACTTT TCCAGCTATT TTAGTTGCGG
PT14CP30A-035 GGATGGATTT AAAGGTATTA AAGAAACTTT TCCAGCTATT TTAGTTGCGG

                       710        720        730        740        750
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            CGCTAAGTTT TACAACTACT CAATTTTTAA GCTCAAATCA CCTAGGAGCG
PT14CP30A-035 CGCTAAGTTT TACAACTACT CAATTTTTAA GCTCAAATCA CCTAGGAGCG

                       760        770        780        790        800
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            GAATTACCTG ATATTATTTC AGCTGTGGTT TCTTTAGCAG TTACGACTGT
PT14CP30A-035 GAATTACCTG ATATTATTTC AGCTGTGGTT TCTTTAGCAG TTACGACTGT

                       810        820        830        840        850
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            ATTTTTGAAA TTTTGGAAAC CAAAAAATAT TTTTAGATTT GACAATGAAA
PT14CP30A-035 ATTTTTGAAA TTTTGGAAAC CAAAAAATAT TTTTAGATTT GACAATGAAA

                       860        870        880        890        900
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            GCAATTTCAC TCAAGATAAC ACCTTAAGTT TTAATCAAAT TTTAAAAGCT
PT14CP30A-035 GCAATTTCAC TCAAGATAAC ACCTTAAGTT TTAATCAAAT TTTAAAAGCT

                       910        920        930        940        950
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TGGTCACCTT TTATTTTACT CATTGTTTGT ATTATCATTT GGACACAACC
PT14CP30A-035 TGGTCACCTT TTATTTTACT CATTGTTTGT ATTATCATTT GGACACAACC

                       960        970        980        990       1000
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TTGGTTTAAA GCTCTCTTTG ATAAGGATGG AATTTTAAGC TATACAAGTA
PT14CP30A-035 TTGGTTTAAA GCTCTCTTTG ATAAGGATGG AATTTTAAGC TATACAAGTA

                      1010       1020       1030       1040       1050
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TTACTTTACA ATTTAGCAAT ATCACAACTG GAATTTTAAG CCCTTCTATC
PT14CP30A-035 TTACTTTACA ATTTAGCAAT ATCACAACTG GAATTTTAAG CCCTTCTATC

                      1060       1070       1080       1090       1100
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            ACAGGCATAG GAGAAGCTAA GCCTTTATCT TTGGCCTTGG GTGTAGATTT
PT14CP30A-035 ACAGGCATAG GAGAAGCTAA GCCTTTATCT TTGGCCTTGG GTGTAGATTT

                      1110       1120       1130       1140       1150
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            AATCAATGGA AAAACCGTAG CTCAAGCAGG AACAGCGATT TTACTTGCCG
PT14CP30A-035 AATCAATGGA AAAACCGTAG CTCAAGCAGG AACAGCGATT TTACTTGCCG

                      1160       1170       1180       1190       1200
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            CTTTTTTAAC TATAGCTATT TTAAAAATCA AAGCCGAAGA TGCTGCAGAA
PT14CP30A-035 CTTTTTTAAC TATAGCTATT TTAAAAATCA AAGCCGAAGA TGCTGCAGAA

                      1210       1220       1230       1240       1250
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TGCTTTTGGG TTACCTTAAA AGAAATGGCT ATACCTTGTA TCACCATAGG
PT14CP30A-035 TGCTTTTGGG TTACCTTAAA AGAAATGGCT ATACCTTGTA TCACCATAGG

                      1260       1270       1280       1290       1300
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TTTGGTTGTG GCCTTTGCTT TTATCTCTAA AAATAGTGGT ATGAGCACTA
PT14CP30A-035 TTTGGTTGTG GCCTTTGCTT TTATCTCTAA AAATAGTGGT ATGAGCACTA

                      1310       1320       1330       1340       1350
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            CTTTAGGACT TGCTTTTGCA CACACAGGTG ACGCTTTTTC TTTCTTTTCA
PT14CP30A-035 CTTTAGGACT TGCTTTTGCA CACACAGGTG ACGCTTTTTC TTTCTTTTCA

                      1360       1370       1380       1390       1400
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            CCAATCATTG GCTGGATAGG GGTATTTTTA ACAGGCTCTG ATACAAGTGC
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PT14CP30A-035 CCAATCATTG GCTGGATAGG GGTATTTTTA ACAGGCTCTG ATACAAGTGC

                      1410       1420       1430       1440       1450
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TAATTTACTT TTTGGAACCT TGCAACAAGT AAGCGCTCAA AAACTAGGTA
PT14CP30A-035 TAATTTACTT TTTGGAACCT TGCAACAAGT AAGCGCTCAA AAACTAGGTA

                      1460       1470       1480       1490       1500
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTAGCGAAGC TTTATTTTTA GCAGCTAATT CAGTAGGTGG GGTTGTTGGG
PT14CP30A-035 TTAGCGAAGC TTTATTTTTA GCAGCTAATT CAGTAGGTGG GGTTGTTGGG

                      1510       1520       1530       1540       1550
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          AAGATGATCA GTCCACAAAG CATTGCTATA GCTTGTGCAG CGGTTGGACT
PT14CP30A-035 AAGATGATCA GTCCACAAAG CATTGCTATA GCTTGTGCAG CGGTTGGACT

                      1560       1570       1580       1590       1600
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TGTGGGAAAA GAATCTGATC TTTTCAAATT CACATTAAAA TATTCTGTAG
PT14CP30A-035 TGTGGGAAAA GAATCTGATC TTTTCAAATT CACATTAAAA TATTCTGTAG

                      1610       1620       1630       1640       1650
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          CTTTTATTAT TTTAATAGGA ATTTGGACTT GCATTATAGC TTTTTTCTTA
PT14CP30A-035 CTTTTATTAT TTTAATAGGA ATTTGGACTT GCATTATAGC TTTTTTCTTA

                      1660       1670       1680
              ....|....| ....|....| ....|....| ...
PT14          CAAGGAATCA TCCCTGAAGT TATAGTGAAA TAA
PT14CP30A-035 CAAGGAATCA TCCCTGAAGT TATAGTGAAA TAA
```

Appendix 22. Protein sequence of gene A911_ 00360 in *C. jejuni* PT14 and PT14CP30A-035CS, which encode l- lac permease. Start codons are indicated in red.

```
                      10         20         30         40         50
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            LEQILTWQQI YDPFSNIWLS ALVAFLPILC FLVCLVVLKL KGYQAGFLTV
PT14CP30A-035 GTNFNMATNL PFFKYLAKCF SGIFTYTMFF SLFGCFKT-R LSSRFFNCYT

                      60         70         80         90        100
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            ILATLVALFA YKMPWNLVGA SFIQGFTNGM WPIAWIIIAA IFLYKLSIKS
PT14CP30A-035 CYFSSFICL- NALEFSGCKF YSRLYKWYVA YSLDYHRGNL SLQTFYQVRF

                     110        120        130        140        150
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            GSFEIIKKSV MSITPDHRIQ VILIGFCFGS FLEGAIGFGG PVAITAALLV
PT14CP30A-035 F-NH---KSV MSITPDHRIQ VILIGFCFGS FLEGAIGFGG PVAITAALLV

                     160        170        180        190        200
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            GLGLRPLQAA GLCLIANTAP VAFGAVGIPI IAMANLVGIE QHSVSAMVGR
PT14CP30A-035 GLGLRPLQAA GLCLIANTAP VAFGAVGIPI IAMANLVGIE QHSVSAMVGR

                     210        220        230        240        250
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            MLVPLSLTIP FFIVFLMDGF KGIKETFPAI LVAALSFTTT QFLSSNHLGA
PT14CP30A-035 MLVPLSLTIP FFIVFLMDGF KGIKETFPAI LVAALSFTTT QFLSSNHLGA

                     260        270        280        290        300
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            ELPDIISAVV SLAVTTVFLK FWKPKNIFRF DNESNFTQDN TLSFNQILKA
PT14CP30A-035 ELPDIISAVV SLAVTTVFLK FWKPKNIFRF DNESNFTQDN TLSFNQILKA

                     310        320        330        340        350
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            WSPFILLIVC IIIWTQPWFK ALFDKDGILS YTSITLQFSN ITTGILSPSI
PT14CP30A-035 WSPFILLIVC IIIWTQPWFK ALFDKDGILS YTSITLQFSN ITTGILSPSI

                     360        370        380        390        400
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            TGIGEAKPLS LALGVDLING KTVAQAGTAI LLAAFLTIAI LKIKAEDAAE
PT14CP30A-035 TGIGEAKPLS LALGVDLING KTVAQAGTAI LLAAFLTIAI LKIKAEDAAE

                     410        420        430        440        450
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            CFWVTLKEMA IPCITIGLVV AFAFISKNSG MSTTLGLAFA HTGDAFSFFS
PT14CP30A-035 CFWVTLKEMA IPCITIGLVV AFAFISKNSG MSTTLGLAFA HTGDAFSFFS

                     460        470        480        490        500
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            PIIGWIGVFL TGSDTSANLL FGTLQQVSAQ KLGISEALFL AANSVGGVVG
PT14CP30A-035 PIIGWIGVFL TGSDTSANLL FGTLQQVSAQ KLGISEALFL AANSVGGVVG

                     510        520        530        540        550
                ....|....| ....|....| ....|....| ....|....| ....|....|
PT14            KMISPQSIAI ACAAVGLVGK ESDLFKFTLK YSVAFIILIG IWTCIIAFFL
PT14CP30A-035 KMISPQSIAI ACAAVGLVGK ESDLFKFTLK YSVAFIILIG IWTCIIAFFL

                     560
                ....|....| .
PT14            QGIIPEVIVK -
PT14CP30A-035 QGIIPEVIVK -
```

Appendix 23. DNA sequence of gene A911_ 05040 in *C. jejuni* PT14 and PT14CP8CS, which encode AraC type DNA-binding domain-containing protein. Mutation is indicated in red. Highlighted TAA would be the stop codon for gene A911_05040 in *C. jejuni* PT14CP8CS after mutation.

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                10         20         30         40         50         60
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      ATGAATAAAA TTCTTTCTTT ACCAGAAGAT TTAAAGCAAT TAAAAGGTGT CAATTATAAA
PT14CP8CS ATGAATAAAA TTCTTTCTTT ACCAGAAGAT TTAAAGCAAT TAAAAGGTGT CAATTATAAA

                70         80         90        100        110        120
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      AAAATTAAAT CTTGCACTTT TGCTAAATAT ACACAAACGG ATACTTCGCA TTCTACTTTT
PT14CP8CS AAAATTAAAT CTTGCACTTT TGCTAAATAT ACACAAACGG ATACTTCGCA TTCTACTTTT

               130        140        150        160        170        180
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      GTTAATGTAG GAAGTCATCT TTTAACTTTT GTTCGCAAAG GATATAAAAT TTTACACACT
PT14CP8CS GTTAATGTAG GAAGTCATCT TTTAACTTTT GTTCGCAAAG GATATAAAAT TTTACACACT

               190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      GCATCAAAAG ATTATAAGAT TAACTCTTAT GAAACTTTGT TTTTAAAAGC TGGGAGCTAT
PT14CP8CS GCATCAAAAG ATTATAAGAT TAACTCTTAT GAAACTTTGT TTTTAAAAGC TGGGAGCTAT

               250        260        270        280        290        300
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      ACTTTAAGCA ATGTAGGACT TAGTAAAGGA GTTTATGAAG CTTATTTATT TTTTTTTGAC
PT14CP8CS ACTTTAAGCA ATGTAGGACT TAGTAAAGGA GTTTATGAAG CTTATTTATT TTTTTT-GAC

               310        320        330        340        350        360
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      AATGCATTTT TAATTGAGCT TATTTATAAG TATAAAGATT TTTTTAAACT AGATCAAAAG
PT14CP8CS AATGCATTTT TAATTGAGCT TATTTATAAG TATAAAGATT TTTTTAAACT AGATCAAAAG

               370        380        390        400        410        420
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      TTTCAAAACT ATGAAATTTT TTGGGTAAAA AATGATAAGA TTTTGCAAGG AATTTTGGAA
PT14CP8CS TTTCAAAACT ATGAAATTTT TTGGGTAAAA AATGATAAGA TTTTGCAAGG AATTTTGGAA

               430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      AGTTTTTCAC CGCATTTTGA AGAAAATACG CAAATTTTAG ATCCTATAGT GAGTTTGAAA
PT14CP8CS AGTTTTTCAC CGCATTTTGA AGAAAATACG CAAATTTTAG ATCCTATAGT GAGTTTGAAA

               490        500        510        520        530        540
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      TTTGAAGAGA TTTTTTTACA TTTGCTTTTA AATAAGAATA TTTATTTTAT TAGTTTTTTA
PT14CP8CS TTTGAAGAGA TTTTTTTACA TTTGCTTTTA AATAAGAATA TTTATTTTAT TAGTTTTTTA

               550        560        570        580        590        600
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      TCAGGAATTT TAAAAGAATT TCGCCTTGAT CTTTCCCAGC TTTTTGAGTA TTGTGGTAGA
PT14CP8CS TCAGGAATTT TAAAAGAATT TCGCCTTGAT CTTTCCCAGC TTTTTGAGTA TTGTGGTAGA

               610        620        630        640        650        660
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      GAATTTTTAA GCGTAAATGA AATGTCTAAT TTTGCGAAAT TAGATTTAGC AACTTTTTCT
PT14CP8CS GAATTTTTAA GCGTAAATGA AATGTCTAAT TTTGCGAAAT TAGATTTAGC AACTTTTTCT

               670        680        690        700        710        720
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      AAAGAATTTA AGAAATGTTT TGGACAAAGT CCTAAAAAAT GGTTAGATGA AAAAAGGTTG
PT14CP8CS AAAGAATTTA AGAAATGTTT TGGACAAAGT CCTAAAAAAT GGTTAGATGA AAAAAGGTTG

               730        740        750        760        770        780
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      CAAAAAGCAA AGATTTTACT TAAATTTTCT AAAAAAAATA TCAATGAAAT AGCTAATGAG
PT14CP8CS CAAAAAGCAA AGATTTTACT TAAATTTTCT AAAAAAAATA TCAATGAAAT AGCTAATGAG

               790        800        810        820        830        840
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           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       TGCGCTTTTT CTTCGGTAGC TTGGTTTATT GAAAGATTTA AAGAGAAATA TGAGCAAACT
PT14CP8CS  TGCGCTTTTT CTTCGGTAGC TTGGTTTATT GAAAGATTTA AAGAGAAATA TGAGCAAACT

                  850        860        870        880        890
           ....|....| ....|....| ....|....| ....|....| ....|....| .
PT14       CCTAAGCAAT ATCAAAAAAC AAAAAACTTG TATTTTTTAT CAAAAAACTA G
PT14CP8CS  CCTAAGCAAT ATCAAAAAAC AAAAAACTTG TATTTTTTAT CAAAAAACTA G
```

Appendix 24. Protein sequence of gene A911_ 05040 in *C. jejuni* PT14 and PT14CP8CS, which encodes AraC type DNA-binding domain-containing protein. Stop codon for gene A911_ 05040 in *C. jejuni* PT14CP8CS was highlighted as well.

```
                10         20         30         40         50         60
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      MNKILSLPED LKQLKGVNYK KIKSCTFAKY TQTDTSHSTF VNVGSHLLTF VRKGYKILHT
PT14CP8CS MNKILSLPED LKQLKGVNYK KIKSCTFAKY TQTDTSHSTF VNVGSHLLTF VRKGYKILHT

                70         80         90        100        110        120
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      ASKDYKINSY ETLFLKAGSY TLSNVGLSKG VYEAYLFFFD NAFLIELIYK YKDFFKLDQK
PT14CP8CS ASKDYKINSY ETLFLKAGSY TLSNVGLSKG VYEAYLFFLT MHF-LSLFIS IKIFLN-IKS

               130        140        150        160        170        180
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      FQNYEIFWVK NDKILQGILE SFSPHFEENT QILDPIVSLK FEEIFLHLLL NKNIYFISFL
PT14CP8CS FKTMKFFG-K MIRFCKEFWK VFHRILKKIR KF-IL--V-N LKRFFYICF- IRIFILLVFY

               190        200        210        220        230        240
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14      SGILKEFRLD LSQLFEYCGR EFLSVNEMSN FAKLDLATFS KEFKKCFGQS PKKWLDEKRL
PT14CP8CS QEF-KNFALI FPSFLSIVVE NF-A-MKCLI LRN-I-QLFL KNLRNVLDKV LKNG-MKKGC

               250        260        270        280        290
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|..
PT14      QKAKILLKFS KKNINEIANE CAFSSVAWFI ERFKEKYEQT PKQYQKTKNL YFLSKN-
PT14CP8CS KKQRFYLNFL KKISMK-LMS ALFLR-LGLL KDLKRNMSKL LSNIKKQKTC IFYQKT-
```

Appendix 25. DNA sequence of gene A911_04130 in *C. jejuni* PT14 and PT14CP8CS, which encode hypothetical protein. Mutation is indicated in red.

```
                10         20         30         40         50         60
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       ATGGAACATA AAAAAGCTCT ATTTTGTATT TTTGCCATTA TTCATTTATT TTTTTGGATC
PT14CP8CS  ATGGAACATA AAAAAGCTCT ATTTTGTATT TTTGCCATTA TTCATTTATT TTTTTGGATC

                70         80         90        100        110        120
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       ATATTTTCAT CGTTAAATCA TTTTAAATTA GATACATTTT TAAGCTATGA AATAGCTTTT
PT14CP8CS  ATATTTTCAT CGTTAAATCA TTTTAAATTA GATACATTTT TAAGCTATGA AATAGCTTTT

               130        140        150        160        170        180
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       TTTGGTGTTT TATTGATTAT TTTTACTTCT TATTTAAATT ATAAAAAAGT CATTATAAAA
PT14CP8CS  TTTAGTGTTT TATTGATTAT TTTTACTTCT TATTTAAATT ATAAAAAAGT CATTATAAAA

               190        200        210        220        230        240
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       AAATCAAAAA ATTATGAAAA AGATTTTAAT TTCATTTCAT CACTTTTTAT TAAAAAAAAA
PT14CP8CS  AAATCAAAAA ATTATGAAAA AGATTTTAAT TTCATTTCAT CACTTTTTAT TAAAAAAAAA

               250        260        270        280        290        300
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       CAAAATTTAT CAAAAATTAT TCATTTTAAA GTTCTAAAAG ACGATTTAAA GCCAAATATT
PT14CP8CS  CAAAATTTAT CAAAAATTAT TCATTTTAAA GTTCTAAAAG ACGATTTAAA GCCAAATATT

               310        320        330        340        350        360
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       AAAGAGAAAA TCCATTTTTT TACTATGTTT TTTACTCTCT TTAAATTGAT GGCTTATGTG
PT14CP8CS  AAAGAGAAAA TCCATTTTTT TACTATGTTT TTTACTCTCT TTAAATTGAT GGCTTATGTG

               370        380        390        400        410        420
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       ATATTGGTTG CTGGATTTTT ATTTTTACAT CGCCAAGATA AATTAGATAT CTTTGCTTAT
PT14CP8CS  ATATTGGTTG CTGGATTTTT ATTTTTACAT CGCCAAGATA AATTAGATAT CTTTGCTTAT

               430        440        450        460        470        480
           ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
PT14       ATTTGTGGAA TTTCATCCTT GCTAGTTTGT GTGTTTATTT TTATTTTATA TATAAAAAAG
PT14CP8CS  ATTTGTGGAA TTTCATCCTT GCTAGTTTGT GTGTTTATTT TTATTTTATA TATAAAAAAG

               490        500
           ....|....| ....|....| ....
PT14       TATGAGTCCA AAAAAAATTA TTAA
PT14CP8CS  TATGAGTCCA AAAAAAATTA TTAA
```

Appendix 26. DNA sequence of *flhF* in *C. jejuni* PT14, HPC5 and their carrier state. Point mutation is indicated in red.

```
                    10         20         30         40         50
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP8-1      ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP8-2      ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-1     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-2     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-3     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-035   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-064   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
PT14CP30-096   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5           ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP8-1      ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP8-2      ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-1     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-2     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-6     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-7     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-8     ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
HPC5CP30-3R    ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT

                    60         70         80         90        100
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP8-1      ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP8-2      ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-1     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-2     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-3     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-035   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-064   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
PT14CP30-096   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5           ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP8-1      ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP8-2      ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-1     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-2     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-6     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-7     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-8     ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
HPC5CP30-3R    ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA

                   110        120        130        140        150
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP8-1      AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP8-2      AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-1     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-2     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-3     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-035   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-064   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
PT14CP30-096   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5           AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP8-1      AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP8-2      AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-1     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-2     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-6     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-7     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-8     AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
HPC5CP30-3R    AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG

                   160        170        180        190        200
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP8-1      GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP8-2      GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP30-1     GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
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PT14CP30-2    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP30-3    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP30-035  GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP30-064  GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
PT14CP30-096  GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5          GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP8-1     GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP8-2     GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-1    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-2    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-6    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-7    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-8    GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
HPC5CP30-3R   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA

                       210        220        230        240        250
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP8-1     ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP8-2     ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-1    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-2    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-3    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-035  ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-064  ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
PT14CP30-096  ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5          ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP8-1     ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP8-2     ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-1    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-2    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-6    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-7    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-8    ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
HPC5CP30-3R   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG

                       260        270        280        290        300
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP8-1     CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP8-2     CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-1    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-2    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-3    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-035  CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-064  CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
PT14CP30-096  CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5          CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP8-1     CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP8-2     CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-1    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-2    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-6    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-7    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-8    CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
HPC5CP30-3R   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC

                       310        320        330        340        350
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP8-1     TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP8-2     TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-1    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-2    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-3    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-035  TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-064  TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
PT14CP30-096  TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5          TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP8-1     TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP8-2     TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP30-1    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
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HPC5CP30-2    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP30-6    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP30-7    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP30-8    TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
HPC5CP30-3R   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA

                       360        370        380        390        400
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP8-1     CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP8-2     CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-1    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-2    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-3    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-035  CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-064  CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
PT14CP30-096  CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5          CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP8-1     CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP8-2     CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-1    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-2    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-6    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-7    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-8    CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
HPC5CP30-3R   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA

                       410        420        430        440        450
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP8-1     ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP8-2     ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-1    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-2    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-3    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-035  ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-064  ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
PT14CP30-096  ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5          ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP8-1     ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP8-2     ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-1    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-2    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-6    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-7    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-8    ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
HPC5CP30-3R   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA

                       460        470        480        490        500
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP8-1     AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP8-2     AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-1    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-2    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-3    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-035  AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-064  AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
PT14CP30-096  AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5          AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP8-1     AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP8-2     AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-1    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-2    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-6    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-7    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-8    AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
HPC5CP30-3R   AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA

                       510        520        530        540        550
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
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PT14CP8-1     TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP8-2     TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-1    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-2    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-3    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-035  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-064  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
PT14CP30-096  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5          TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP8-1     TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP8-2     TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-1    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-2    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-6    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-7    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-8    TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
HPC5CP30-3R   TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC

                       560        570        580        590        600
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP8-1     AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP8-2     AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-1    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-2    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-3    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-035  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-064  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
PT14CP30-096  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5          AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP8-1     AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP8-2     AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-1    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-2    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-6    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-7    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-8    AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
HPC5CP30-3R   AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA

                       610        620        630        640        650
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP8-1     GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP8-2     GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-1    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-2    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-3    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-035  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-064  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
PT14CP30-096  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5          GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP8-1     GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP8-2     GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-1    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-2    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-6    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-7    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-8    GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
HPC5CP30-3R   GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC

                       660        670        680        690        700
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP8-1     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP8-2     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-1    TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-2    TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-3    TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-035  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-064  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
PT14CP30-096  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5          TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
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HPC5CP8-1      TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP8-2      TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-1     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-2     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-6     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-7     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-8     TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
HPC5CP30-3R    TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG

                      710        720        730        740        750
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP8-1      AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP8-2      AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-1     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-2     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-3     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-035   AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-064   AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
PT14CP30-096   AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5           AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP8-1      AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP8-2      AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-1     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-2     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-6     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-7     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-8     AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
HPC5CP30-3R    AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT

                      760        770        780        790        800
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP8-1      GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP8-2      GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-1     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-2     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-3     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-035   GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-064   GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
PT14CP30-096   GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5           GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP8-1      GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP8-2      GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-1     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-2     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-6     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-7     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-8     GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
HPC5CP30-3R    GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT

                      810        820        830        840        850
               ....|....| ....|....| ....|....| ....|....| ....|....|
PT14           AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP8-1      AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP8-2      AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-1     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-2     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-3     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-035   AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-064   AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
PT14CP30-096   AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5           AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP8-1      AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP8-2      AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-1     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-2     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-6     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-7     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-8     AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
HPC5CP30-3R    AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
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                  860        870        880        890        900
             ....|....| ....|....| ....|....| ....|....| ....|....|
PT14         TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP8-1    TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP8-2    TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-1   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-2   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-3   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-035 TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-064 TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
PT14CP30-096 TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5         TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP8-1    TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP8-2    TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-1   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-2   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-6   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-7   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-8   TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
HPC5CP30-3R  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT

                  910        920        930        940        950
             ....|....| ....|....| ....|....| ....|....| ....|....|
PT14         AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP8-1    AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP8-2    AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-1   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-2   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-3   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-035 AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-064 AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
PT14CP30-096 AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5         AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP8-1    AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP8-2    AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-1   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-2   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-6   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-7   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-8   AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
HPC5CP30-3R  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT

                  960        970        980        990        1000
             ....|....| ....|....| ....|....| ....|....| ....|....|
PT14         TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP8-1    TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP8-2    TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-1   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-2   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-3   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-035 TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-064 TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
PT14CP30-096 TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5         TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP8-1    TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP8-2    TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-1   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-2   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-6   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-7   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-8   TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
HPC5CP30-3R  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT

                  1010       1020       1030       1040       1050
             ....|....| ....|....| ....|....| ....|....| ....|....|
PT14         ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP8-1    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP8-2    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP30-1   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP30-2   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP30-3   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP30-035 ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
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PT14CP30-064  ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
PT14CP30-096  ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5          ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP8-1     ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP8-2     ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-1    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-2    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-6    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-7    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-8    ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
HPC5CP30-3R   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT

                      1060       1070       1080       1090       1100
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP8-1     TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP8-2     TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-1    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-2    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-3    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-035  TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-064  TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
PT14CP30-096  TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5          TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP8-1     TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP8-2     TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-1    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-2    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-6    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-7    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-8    TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
HPC5CP30-3R   TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA

                      1110       1120       1130       1140       1150
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP8-1     TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP8-2     TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-1    TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-2    TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-3    TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-035  TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-064  TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
PT14CP30-096  TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5          TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP8-1     TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP8-2     TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-1    TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-2    TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-6    TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-7    TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-8    TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
HPC5CP30-3R   TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG

                      1160       1170       1180       1190       1200
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP8-1     AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP8-2     AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-1    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-2    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-3    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-035  AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-064  AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
PT14CP30-096  AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5          AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP8-1     AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP8-2     AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP30-1    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP30-2    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP30-6    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP30-7    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
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HPC5CP30-8    AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
HPC5CP30-3R   AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT

                    1210       1220       1230       1240       1250
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP8-1     GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP8-2     GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-1    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-2    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-3    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-035  GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-064  GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
PT14CP30-096  GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5          GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP8-1     GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP8-2     GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-1    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-2    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-6    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-7    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-8    GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
HPC5CP30-3R   GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT

                    1260       1270       1280       1290       1300
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP8-1     CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP8-2     CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-1    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-2    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-3    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-035  CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-064  CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
PT14CP30-096  CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5          CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP8-1     CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP8-2     CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-1    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-2    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-6    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-7    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-8    CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
HPC5CP30-3R   CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT

                    1310       1320       1330       1340       1350
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP8-1     TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP8-2     TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-1    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-2    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-3    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-035  TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-064  TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
PT14CP30-096  TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5          TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP8-1     TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP8-2     TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-1    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-2    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-6    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-7    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-8    TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
HPC5CP30-3R   TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT

                    1360       1370       1380       1390       1400
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP8-1     TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP8-2     TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP30-1    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
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PT14CP30-2    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP30-3    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP30-035  TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP30-064  TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
PT14CP30-096  TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5          TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP8-1     TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP8-2     TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-1    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-2    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-6    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-7    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-8    TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
HPC5CP30-3R   TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA

                      1410       1420       1430       1440       1450
              ....|....| ....|....| ....|....| ....|....| ....|....|
PT14          TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP8-1     TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP8-2     TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-1    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-2    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-3    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-035  TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-064  TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
PT14CP30-096  TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5          TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP8-1     TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP8-2     TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-1    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-2    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-6    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-7    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-8    TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
HPC5CP30-3R   TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG


              ....|
PT14          AATAA
PT14CP8-1     AATAA
PT14CP8-2     AATAA
PT14CP30-1    AATAA
PT14CP30-2    AATAA
PT14CP30-3    AATAA
PT14CP30-035  AATAA
PT14CP30-064  AATAA
PT14CP30-096  AATAA
HPC5          AATAA
HPC5CP8-1     AATAA
HPC5CP8-2     AATAA
HPC5CP30-1    AATAA
HPC5CP30-2    AATAA
HPC5CP30-6    AATAA
HPC5CP30-7    AATAA
HPC5CP30-8    AATAA
HPC5CP30-3R   AATAA
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Appendix 27. DNA sequence of *flhF* isolated from *C. jejuni* PT14 and PT14CP30 in TOPO pCR2.1 vector. Sequencing was carried out with *flhF* primers and point mutations are indicated in red.

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                    10         20         30         40         50
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
flhF(T368A)-2   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
flhF            ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
flhF database   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT

                    60         70         80         90        100
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
flhF(T368A)-2   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
flhF            ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
flhF database   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA

                   110        120        130        140        150
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
flhF(T368A)-2   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
flhF            AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
flhF database   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG

                   160        170        180        190        200
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
flhF(T368A)-2   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
flhF            GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
flhF database   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA

                   210        220        230        240        250
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
flhF(T368A)-2   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
flhF            ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
flhF database   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG

                   260        270        280        290        300
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
flhF(T368A)-2   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
flhF            CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
flhF database   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC

                   310        320        330        340        350
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
flhF(T368A)-2   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
flhF            TTGGATTTCT CAAACACTCG TTTAAATACA AATTTAAACA CTGTAAAAAA
flhF database   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA

                   360        370        380        390        400
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
flhF(T368A)-2   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
flhF            CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
flhF database   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA

                   410        420        430        440        450
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
flhF(T368A)-2   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
flhF            ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
flhF database   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA

                   460        470        480        490        500
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
flhF(T368A)-2   AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
flhF            AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
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flhF database  AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA

                       510        520        530        540        550
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
flhF(T368A)-2  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
flhF           TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
flhF database  TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC

                       560        570        580        590        600
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
flhF(T368A)-2  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
flhF           AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
flhF database  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA

                       610        620        630        640        650
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
flhF(T368A)-2  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
flhF           GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
flhF database  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC

                       660        670        680        690        700
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
flhF(T368A)-2  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
flhF           TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
flhF database  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG

                       710        720        730        740        750
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
flhF(T368A)-2  AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
flhF           AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
flhF database  AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT

                       760        770        780        790        800
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
flhF(T368A)-2  GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
flhF           GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
flhF database  GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT

                       810        820        830        840        850
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
flhF(T368A)-2  AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
flhF           AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
flhF database  AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA

                       860        870        880        890        900
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
flhF(T368A)-2  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
flhF           TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
flhF database  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT

                       910        920        930        940        950
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
flhF(T368A)-2  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
flhF           AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
flhF database  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT

                       960        970        980        990        1000
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
flhF(T368A)-2  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
flhF           TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
flhF database  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
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                    1010       1020       1030       1040       1050
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
flhF(T368A)-2   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
flhF            ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
flhF database   ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT

                    1060       1070       1080       1090       1100
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
flhF(T368A)-2   TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
flhF            TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
flhF database   TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA

                    1110       1120       1130       1140       1150
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
flhF(T368A)-2   TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAGACTAAAG
flhF            TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
flhF database   TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG

                    1160       1170       1180       1190       1200
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
flhF(T368A)-2   AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
flhF            AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
flhF database   AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT

                    1210       1220       1230       1240       1250
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   GCAAACACCA AACATGGAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
flhF(T368A)-2   GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
flhF            GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
flhF database   GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT

                    1260       1270       1280       1290       1300
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
flhF(T368A)-2   CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
flhF            CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
flhF database   CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT

                    1310       1320       1330       1340       1350
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
flhF(T368A)-2   TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
flhF            TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
flhF database   TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT

                    1360       1370       1380       1390       1400
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
flhF(T368A)-2   TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
flhF            TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
flhF database   TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA

                    1410       1420       1430       1440       1450
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-1   TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
flhF(T368A)-2   TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
flhF            TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
flhF database   TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG


                ....|
flhF(T368A)-1   AATAA
flhF(T368A)-2   AATAA
flhF            AATAA
flhF database   AATAA
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Appendix 28. DNA sequence of *flhF* isolated from *C. jejuni* PT14 and PT14CP30 in TOPO pCR2.1 vector. Sequencing was carried out with M13 primers and point mutations are indicated in red.

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                    10         20         30         40         50
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
flhF            ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT
flhF database   ATGGGACAAC TTATACATAC TTTTACCGTT GAAGATACAG AACAAATTAT

                    60         70         80         90        100
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
flhF            ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA
flhF database   ACCCAAAGTA AAAGAAGATT ACGGAGATAA AGCTTTAATT ATAACAAATA

                   110        120        130        140        150
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
flhF            AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG
flhF database   AACAAATTCG TCCTAAAACC TTAAATCGCA GTGCGCTTTA TGAGGTTATG

                   160        170        180        190        200
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
flhF            GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA
flhF database   GTAGCCATAG AAGAAAGCGA CTATGAAGAA CACTTAAAAA AACAAGGAAA

                   210        220        230        240        250
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
flhF            ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG
flhF database   ATCTTTACCT GCAAAAAAAA GCTCGCCAAA ACCATCCTCT ACCTCTCTTG

                   260        270        280        290        300
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
flhF            CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC
flhF database   CAGAAGAAAA AATTAGATCT CAAATCCCTC AAGAAGATGA AGATGTAGTC

                   310        320        330        340        350
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA
flhF            TTGGATTTCT CAAACACTCT CTTAAATACA AATTTAAACA CTGTAAAAAA
flhF database   TTGGATTTCT CAAACACTCG CTTAAATACA AATTTAAACA CTGTAAAAAA

                   360        370        380        390        400
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
flhF            CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA
flhF database   CAATGACTTA GCTAAAAAAA CCTATCAAGA TTTTCCACAA AACAAAATCA

                   410        420        430        440        450
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
flhF            ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA
flhF database   ACCCTCATCA AAATAAGACT CTAGGCTTTG ATGATTTCAA AGAAAAACTA

                   460        470        480        490        500
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
flhF            AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA
flhF database   AGTGAAGTTA GCAATGAAAT TTCTAAAGTC ACAAACACAC CTTTAGAAAA

                   510        520        530        540        550
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
flhF            TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC
flhF database   TTACACCCCT AATCCAAATT ATAATAAAAA AATAGAAAAT TTTGAAAAGC

                   560        570        580        590        600
                ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2   AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
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flhF           AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA
flhF database  AATTTGAAAA ACAAATCAAC AAGCTTAATG ATAAAATAGA TCTTCTTGCA

                       610        620        630        640        650
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
flhF           GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC
flhF database  GATATGATGT GGGACGATAA AGCTGAAGCT AGAAAAAATC TTATGATACC

                       660        670        680        690        700
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
flhF           TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG
flhF database  TCCTGAATTT GCAAGTATTT ATAAACAAGC CAAAGAAAGT GGAATGCTTG

                       710        720        730        740        750
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
flhF           AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT
flhF database  AAAATCATCT AGAAGCGATT ATGAAAGCAA CTATAGAAAA TATGCCAGCT

                       760        770        780        790        800
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
flhF           GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT
flhF database  GCTATGAAAA CCAACAAAGA TGCGGTTCAA AGATATTTTC ACTCACTTTT

                       810        820        830        840        850
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
flhF           AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA
flhF database  AAGAAATATC TTGCCTTGTC GTGTTGAAAG CGATATAAAA AAACAAAAAA

                       860        870        880        890        900
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
flhF           TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT
flhF database  TCATGATGCT AGTAGGCCCA ACAGGAGTCG GAAAAACAAC AACCTTAGCT

                       910        920        930        940        950
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
flhF           AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT
flhF database  AAGTTAGCTT TTCGCTATGC TTATGGAGAT AAGCGTTATA AAACAGGTAT

                       960        970        980        990       1000
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
flhF           TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT
flhF database  TATTACGCTT GATACTTATA GAATCGGTGC AGTTGAGCAA CTTTTTCAGT

                      1010       1020       1030       1040       1050
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
flhF           ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT
flhF database  ATGCAAAAAT GATGAAACTT CCTATTATTG ATAGCATAGA ACCAAAGGAT

                      1060       1070       1080       1090       1100
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
flhF           TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA
flhF database  TTAGATGAAG CGATAAAAAG CTTAAATAAT TGCGAAGTAA TTTTAGTTGA

                      1110       1120       1130       1140       1150
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TGCTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAGACTAAAG
flhF           TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG
flhF database  TACTATAGGA AATTCACAAT ACGATCAAAG CAAACTTGCT AAAACTAAAG

                      1160       1170       1180       1190       1200
                  ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
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flhF           AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT
flhF database  AATTTTTAAT GCACTCAAAT GCAGAAATTG ATGTAAATTT AGTGGTTTCT

                      1210       1220       1230       1240       1250
               ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
flhF           GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT
flhF database  GCAAACACCA AACATGAAGA TTTAATGGAA ATTTATAAAA ATTTTTCATT

                      1260       1270       1280       1290       1300
               ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
flhF           CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT
flhF database  CTTAAATATT GATACTTTGA TTATTACTAA ATTTGATGAA ACAAAGGTTT

                      1310       1320       1330       1340       1350
               ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
flhF           TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT
flhF database  TTGGAAATAT CTTCTCTCTT GTTTATGAAA CAAATATTCC TCTAAGCTTT

                      1360       1370       1380       1390       1400
               ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
flhF           TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA
flhF database  TTCTCTATAG GACAAGAAGT GCCAGATGAT TTAGAAGTAG CCAATAGTGA

                      1410       1420       1430       1440       1450
               ....|....| ....|....| ....|....| ....|....| ....|....|
flhF(T368A)-2  TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
flhF           TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG
flhF database  TTTTCTAGTT CATTGTATTT TAGAAGGGTT TAACAAAGGA AAAAATAATG


               ....|
flhF(T368A)-2  AATAA
flhF           AATAA
flhF database  AATAA
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