Dairy Farm *Campylobacteraceae*

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ABSTRACT

Infection with *Campylobacter* is considered to be the most common bacterial cause of human gastroenteritis worldwide. In light of the dramatic increase of antibiotic resistant bacteria, alternative solutions including biological controls such as bacteriophage therapy and bacteriophage biosanitization are being considered. One way in which campylobacters enter the human food chain is through consumption of contaminated raw milk. An updated study of the ability of campylobacters to survive in milk, including species other than *C. jejuni,* was carried out. Isolation of bacteriophages from bovine slurry, with potential for biocontrol and therapeutic purposes was attempted using conventional methods. *Campylobacter* and *Arcobacter* hosts were isolated and characterised, including genome sequencing, from the same environment. The method used for this purpose was proven efficacious for porcine slurry; however, no lytic phage were isolated from bovine samples. During the isolation experiments unusual plaques were formed on the lawn of the *C. hyointestinalis* S12 host strain. The causative agent of this lytic activity was found to be due to a new predatory bacterium, which was characterised with respect host range and genome sequence. Phylogenetic analysis placed the new bacterium in the family *Oceanospirillaceae* and the name *Venatorbacter cucullus* gen. nov. sp. nov proposed.

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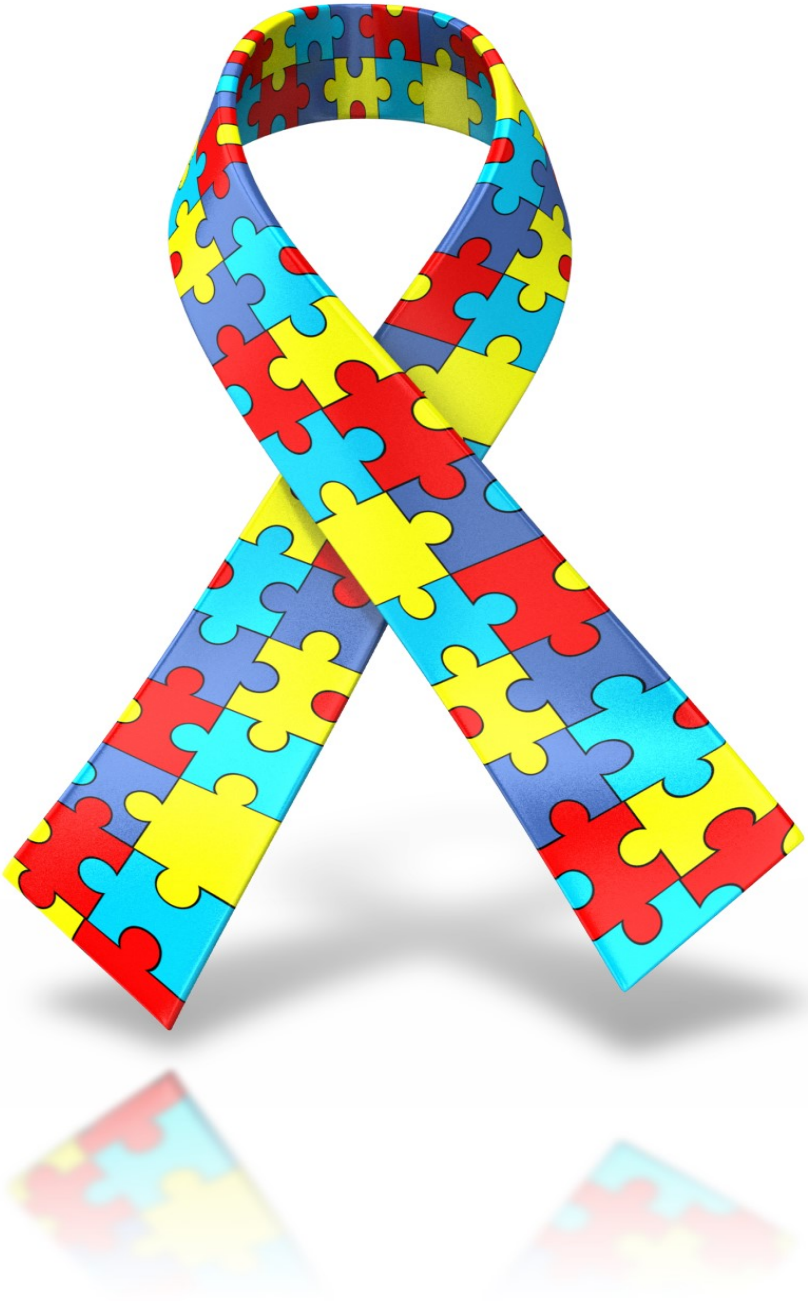
For Adam . . .



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ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BA	Blood agar
BHI	Brain heart infusion agar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
$CaCl_2$	Calcium chloride
CAT	Cefoperazone, Amphotericin B, and Teicoplanin
CDC	Centers for Disease Control and Prevention
CDT	Cytolethal distending toxin
CFU	Colony forming unit
CIP	Ciprofloxacin
CME	*Campylobacter* multidrug efflux pump
CO2	Carbon dioxide
CP	*Campylobacter* phage
CPS	Capsular polysaccharide
CPS	Capsular polysaccharide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
Da	Daltons
DNA	Deoxyribonucleic acid
ED	Entner-Doudoroff
EDTA	Ethylene Diamine Tetra acetic Acid
EFSA	European Food Safety Authority
EOP	Efficiency of plating
EtOH	Ethanol
EU	European Union
Fe^{+3}	Iron(III) ferric
FSA	Food Safety Authority
g	Gramm
h	Hour
HCl	Hydrochloric acid
ICOMSFF	International Commission on Microbiological Specifications for Foods
Kb	Kilo base
KCl	Potassium chloride
kDa	Kilo dalton
LOS	Lipooligosaccharides sialylation
MACS	Modular Atmospheric Controlled System
mCCDA	Modified *Campylobacter* blood-free selective agar
MCPs	Methyl-accepting chemotaxis proteins
MCPs	methyl-accepting chemotaxis proteins
MgSO4	Magnesium sulphate
MH	Mueller-Hinton
ml	Millilitre
MLST	Multi-locus sequence typing
MRD	Maximal Recovery Diluents
mRNA	Messenger ribonucleic acid
N_2	Nitrogen
NA	Nutrient Agar
NaCl	Sodium chloride
NaOH	Sodium Hydroxide

NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
nm	Nanometre
NZCYM	New Zealand Casamino Yeast Medium
O2	Oxygen
OD	Optical density
P	probability value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming unit
psi	Pound per square inch
PT14	*Campylobacter jejuni* NCTC 12662
QRDR	Quinolone Resistance Determining Region
RDM	Raw Drinking Milk
RNA	Ribonucleic acid
RO	Reverse osmosis
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
SM	Salt Magnesium
T3SS	Type three secretion system
TAE	Tris-acetate ethylene diaminetetraacetic acid
TAE	Tris-Acetated EDTA
TCS	Two-component regulator system
TCS	Two-component regulator system
TEM	Transmission electron microscopy
Tris	(hydroxymethyl)aminomethane
UHT	Ultra-high temperature processing
UK	United Kingdom
UV	Ultraviolet
V	Voltage
V3-V4 region	semi-conserved hypervariable16S ribosomal RNA regions
WGS	Whole genome sequencing
x g (RCF)	Relative centrifugal force
µl	Microliter
µm	Micrometre
% v/v	Percentage volume per volume
% w/v	Percentage weight per volume
°C	Degree Celsius

Chapter 1

INTRODUCTION

1.1 *Campylobacteraceae* family

1.1.1 Campylobacteraceae family taxonomy

The *Campylobacteraceae* and *Helicobacteraceae* families lie within the order Camylobacterales within the epsilon subdivision of the phylum Proteobacteria, the *Campylobacteraceae* family contains three closely related genera, *Campylobacter*, *Arcobacter*, and *Sulfurospirillum* (On et al., 2017; Lastovica et al., 2014). A number of species that at one time were included the genus *Campylobacter*, have been reclassified into other genera and families within the *Epsilonproteobacteria*, before the genus was first described in 1963, what we now know as *Campylobacter* were classified within the genus *Vibrio* (Moore and Matsuda, 2002; Moore et al., 2005). The move to a new genus coincided with recognition of their clinical, economical, and ecological importance. The family *Campylobacteraceae* phylogenetic reconstruction based on 16S rRNA is illustrated by On et al.(2017) (Figure 1.1).

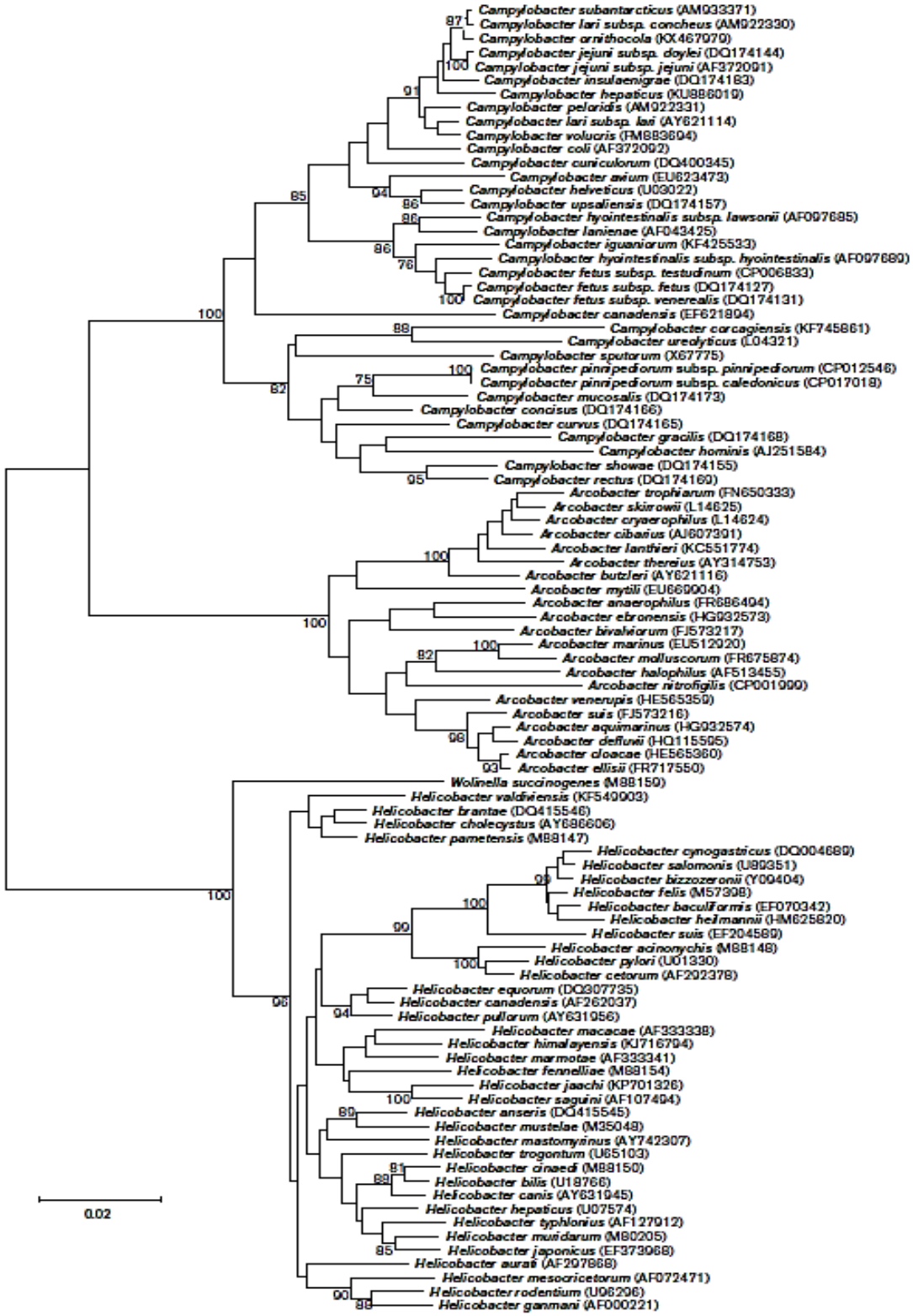


Figure 1.1 Phylogenetic relationships based on 16S rRNA genes of type strains belonging to the families *Campylobacteraceae* and *Helicobacteraceae* which include *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* .spp genus,

presented by neighbour-joining tree using the Kimura 2-parameter distance estimation method, with bootstrapping based on 500 replicates

Spiral shape microbes have been observed in clinical samples from patients with diarrhoeal, gastric problems, and abortion cases since the late 1800s (Escherich, 1886; Solomon, 1896). However, the difficulty in culturing these bacteria was an important issue that prevented further progress. Taxonomy methods in the early part of the twentieth century were primitive and bacterial classification was limited to the microscopic study of cell morphology, growth requirements, biochemical and immunological tests. It was established that the genus *Vibrio* contained species with varied growth requirements and hosts. For example, aerobic species like *V. cholera* had been isolated from human hosts. Microaerobic and anaerobic species that caused abortions and fertility problems like *V. fetus* and *V. bubulus* had bovine and ovine hosts (Smith and Taylor, 1919; Florent, 1953; Costas et al., 1987). Some species had been isolated from human oral specimens such as *V. sputorum* (Prévot, 1940) and finally the intestinal species *V. coli* and *V. jejuni,* from porcine and bovine hosts (Doyle, 1948; Jones et al., 1931). All these bacteria showed profound differences from *V. cholera* and were reclassified in a new genus called *Campylobacter*, which means curved bacillus (Sebald and Véron 1963). The combination of the guanine-cytosine content (G+C content) of the genomic DNA, together with fermentation experiments by Hugh and Leifson (1953) that revealed that *V. fetus* and *V. bubulus* have different metabolic pathways, both confirmed a difference to other *Vibrio* species (Sebald and Veron, 1963). Ten years later, other microaerobic and anaerobic species previously classified as *Vibrio,* were reclassified as *Campylobacter* species by a study based on their different biochemical and serological properties and the G+C content of deoxyribonucleic acid (DNA) (Véron and Chatelain, 1973). The importance of the *Campylobacter* genus was highlighted after it was discovered that *Campylobacter spp*. were often linked with human diarrhoea (Butzler et al., 1973; Skirrow, 1977). This discovery

was only possible once the first selective media had been developed by Skirrow in 1977 together with incubation at 43°C in a microaerobic atmosphere and following this significant step, many new taxa were described, which included: *C. mucosalis* and *C. hyointestinalis* from pig intestine (Lawson et al., 1975), *C. concisus* from human oral cavity (Tanner et al., 1981), *C. nitrofigilis* (now reclassified in the *Arcobacter* genus) from plant roots (McClung et al., 1983), *C. lari* from gulls (Benjamin et al., 1983), *C. pylori* (now reclassified in the *Helicobacter* genus) from human gastric mucosa (Skirrow, 1983), *C. cryaerophilus* from pig and cattle abortions cases (Neill et al., 1985) and *C. cinaedi* and *C. fennelliae* (now reclassified in the *Helicobacter* genus) from human intestine (Totten et al., 1985). Modifications of selective culture media allowed other new species to be described (Karmali et al., 1986). For example *C. mustelae* (now reclassified in the *Helicobacter* genus) was isolated from ferret gastric mucosa (Fox et al., 1988), *C. jejuni* subsp. *doylei* from human enteritis and gastritis (Steele and Owen, 1988), *C. intracellularae* from porcine proliferative enteritis (McOrist et al., 1990), *C. upsaliensis* from dog faeces (Sandstedt and Ursing, 1991) and *C. butzleri* (now reclassified in the *Arcobacter* genus) from human diarrhoea (Kiehlbauch et al., 1991). More novel *Campylobacter* species were described as a variety of different animal hosts species were investigated (Priest and Austin, 1993; Gupta, 1998; Vandamme et al., 1992b). New species were distinguished by chemotaxonomic based methods which included: cellular fatty acid and isoprenoid quinone analysis, protein profile study and, DNA-DNA hybridization, which was considered as one of the most important tools to discriminate a new bacterial species (Wayne et al., 1987). Notably the DNA sequence of the 16S subunit of bacterial rRNA gene was demonstrated to be one of the powerful tools to defining the phylogenetic diversity of *Campylobacter* species (Romaniuk et al., 1987; Paster and Dewhirst, 1988). A new genus called *Helicobacter* (Goodwin et al., 1989a) was proposed for "*C. pylori"* and "*C. mustelae"* after analysis of their 16S rRNA gene sequence, electron microscopic studies of their flagella (Han et al., 1989), and their variation in

cellular fatty acid composition (Goodwin et al., 1989b). A further significant change in *Campylobacter* species classification occurred after the use of rRNA-DNA hybridisation resulted in the reclassification of the aerotolerant campylobacters into a new genus named *Arcobacter* to give, *A. nitrofigilis*, *A. cryaerophilus*, and *A. butzleri* (Vandamme et al., 1992b). *Sulfurospirillum*, as a novel genus was then created, which included species from environmental backgrounds only (Schumacher et al., 1992). *Bacteroides ureolyticus* was reclassified to the *Campylobacter* genus (Vandamme et al., 2005).

1.1.2 Campylobacteraceae Identification

A wide review by Lastovica et al. (2014) described the identification, diagnostic methods, genetic studies and epidemiology of the taxa of *Campylobacteraceae* and it was noted that identification of isolates to species or subspecies level, was required for epidemiological subtyping studies.

1.1.3 *Campylobacter* Phenotypical Features

The requirement for a microaerobic atmosphere is an important distinguishing feature for most *Campylobacter* species. A microaerobic gas mixture containing approximately from 3 to 8 % O_2, 10% CO_2, and 85% N_2 is required for optimal growth (Jorgensen and PFaller, 2015; Agnetti et al., 2019; Khan et al., 2018). Some *Campylobacter* strain such as *C. coli* OR12 and probably others, show a more aerotolerant behaviour under certain circumstances (O'Kane and Connerton, 2017). The optimal growth temperature for most *Campylobacter* species is 37 to 42°C. No growth occurs below 30°C or above 45°C for most *Campylobacter* species. The group known as thermophilic campylobacters including *C. jejuni*, *C. coli* and *C. lari* grow at temperatures above 37°C and form an abundant lawn at 42°C (Bhunia, 2018a; Firlieyanti et al., 2016). *Campylobacter upsaliensis* is

thermotolerant rather than thermophilic, as it does not grow at 42°C (Debruyne et al., 2008; Vandamme, 2000).

Analysis of G+C content of campylobacters indicates that genus members have a low ratio of approximately 29 to 47 mol% (On et al., 2017; Lastovica et al., 2014). In general, *Campylobacter* spp. are less robust toward environmental stress compared to other food-borne pathogens. Environmentally stressed or old *Campylobacter* cultures cells, may change their morphology to spherical or coccal forms (Klančnik et al., 2013; Griffiths, 1993). The cells size varies from 0.2–0.8 μm wide and 0.5–5.0 μm in length (Vandamme, 2000). Some members of the genus such as *C. showae* and occasionally *C. jejuni* isolates, may form straight rod-shaped cells rather than curved ones (Wassenaar and Newell, 2006). The majority of *Campylobacter* species are motile with a characteristic corkscrew-like motion by a single unsheathed polar flagellum at one end, for example *C. hyointestinalis* (Gebhart et al., 1985) or at both ends of the cell for example *C. jejuni* (Vandamme, 2000; Nachamkin et al., 1993). Another species, C*. showae* has multiple unipolar flagella. In contrast *C. gracilis* and *C. hominis* are non-motile due to their lack of flagella (Vandamme and De Ley, 1991; Lawson et al., 2001). Most *Campylobacter* spp. will die below pH 4.9 and above pH 9.0, while the optimum pH is 6.5–7.5 (Silva et al., 2011). *Campylobacter* colonies are beige grey, have a domed shape and are 1-2 mm diameter when grow on blood agar in a microaerobic atmosphere (On and Zhang, 2014). Dry conditions are not well tolerated (Fernandez et al., 1985). Campylobacters are sensitive to unfavourable osmotic conditions and will be inhibited at concentrations of ≥2% sodium chloride (Doyle and Roman, 1982b). Until relatively recently it was believed that carbohydrates were neither fermented nor oxidised with amino acids or tricarboxylic acid cycle intermediates being used to generate energy (Debruyne et al., 2008). However, it has now been demonstrated that the genomes of 1.7% of *C. jejuni* and *C. coli* strains encode Entner-Doudoroff (ED) pathway genes and that

incubation of such strains with glucose enhanced stationary-phase survival and biofilm formation (Vegge et al., 2016).

1.1.4 *Arcobacter* Phenotypical Features

The species of the *Arcobacter* genus can be discriminated from those of the *Campylobacter* genus by two characteristics. Firstly the ability of arcobacters to grow aerobically and secondly their ability to grow at 15-30°C which is a lower temperature range than campylobacters can tolerate (Vandamme et al., 1992a). *Arcobacter* cell morphology, having curved, S-shaped, or spiral rods, closely resembles that of the species of the genus *Campylobacter*. In general arcobacters like campylobacters, are motile with a corkscrew-like motion both having a single polar unsheathed flagellum at one or both ends of the cell (Vandamme et al., 1992a; Vandamme, 2000). Frequently, both *Arcobacter* species and *Campylobacter* species can be isolated from the same primary isolation plate (Serraino et al., 2013; Vilar et al., 2010b; Lastovica, 2006) and the discrimination of the two genera can be difficult using phenotypic or biochemical methods in routine isolation laboratories (Yan et al., 2000; Bhunia, 2018b). Misidentification can be avoided by simply incubating the isolates at 25°C and 37°C under aerobic conditions *Arcobacter* are not known to ferment or oxidize carbohydrates, with energy obtained from amino acids or tricarboxylic acid cycle intermediates (On et al., 2017). On blood agar, *Arcobacter* appear as smooth domed colonies that are off-white or cream in colour and approximately 1 mm in diameter (On et al., 2017; Lastovica et al., 2014).

1.2 *Campylobacter* and *Arcobacter* typing methods

Bacterial strain typing is important because it allows epidemiological tracking of strains with antibiotic resistance, pathogens with increased virulence or transmissibility. Genetic heterogeneity among *Campylobacter* and *Arcobacter* species has meant that until recently there was a lack of effective rapid typing methods available (Wassenaar and Newell, 2000). Modern genome sequencing technology has superseded most of the methods that had previously been useful but are now obsolete and examples of some of these methods include: Serotyping (Penner et al., 1980; Lior et al., 1982); Amplified fragment length polymorphism (AFLP) (Vos et al., 1995; Duim et al., 1999); Restriction fragment length polymorphism (RFLP; Meinersmann et al., 1997); Pulsed field gel electrophoresis (PFGE; Nielsen et al., 1998); phage typing (Grajewski et al., 1985) and ribotyping (Maidak et al., 1997; Kiehlbauch et al., 1994). There are two methods, multi-locus sequence typing (MLST) and whole genome sequencing (WGS), that give sensitive, reproducible, and standardised isolate typing results are described in more detail below (section 1.2.1 and 1.2.2).

1.2.1 Multi-locus sequence typing

The principle of the MLST method is based on sequencing the nucleotide sequence of specific regions of seven housekeeping genes, which are amplified by Polymerase chain reaction **(PCR)**. The nucleotide polymorphisms generated by natural genetic variation in multiple chromosomal locations provide a type of "fingerprint" than can be used to group strains. The combination of each these alleles give the sequence type that may be identified by reference to a database. This method has been used for the typing *Campylobacter* species such as *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* (Miller et al., 2005; Mossong et al., 2016; Kiatsomphob et al., 2019). It has also been used for typing *Arcobacter* species like *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*, one

advantage of using the MLST technique that is highly reproducible, providing a standardised typing scheme for *Arcobacter* and *Campylobacter* species. (Miller et al., 2009; Pérez-Cataluña et al., 2017; Parisi et al., 2019). Internet-based MLST databases assist nomenclature and exchange of MLST results between laboratories. This database is stored at Oxford University (http://mlst.zoo.ox.ac.uk; Clark et al., 2005; Merga et al., 2013).

1.2.2 Whole genome sequencing

The whole genome sequence (WGS) technique is one of the most effective methods for typing *Campylobacter* isolates, the use of WSG for *Campylobacter* isolates has also enhanced understanding in many areas of research particularly in epidemiology, source attribution, evolution and ecology (Llarena et al., 2017). The first whole *Campylobacter* genome was published in 2000 (Parkhill et al., 2000). Since then many hundreds of *Campylobacter* genomes have deposited in the NCBI database. Obtaining the WGS of bacteria has become more accessible due to a advances in technology allowing this technique to be used for routine surveillance and other different studies (Anjum et al., 2016; O'Kane and Connerton, 2017; Liang and Connerton, 2018; Köser et al., 2012). Molecular epidemiology of *Campylobacter* is challenging because of the huge phenotypical diversity with extensive genomic structure changes in isolates within the same species (Ridley et al., 2008, Wilson et al., 2009). An important application of WGS is in surveillance and outbreak detection, establishing the pathogen source, transmission pathway and risk factors (Mullner et al., 2009). For example, WSG was used in an investigation into an outbreak of 69 cases of campylobacteriosis caused by consumption of contaminated raw milk, showing that the *Campylobacter* genomic DNA sequences were identical in both patients and milk samples (Kenyon et al., 2020). Moreover, several studies have highlighted the application of WGS to detect and understand mechanisms of antimicrobial

resistance of *Campylobacter* isolated from different sources (Collineau et al., 2019; DiDonato et al., 2020; Elhadidy et al., 2019). Also the WGS can be used to study the interactions of *Campylobacter* with bacteriophage and the effect of phage on their epidemiology (Hooton and Connerton, 2015; Llarena et al., 2017).

1.3 *Campylobacter* pathogenicity

1.3.1 Virulence factors

The exact pathogenesis mechanisms of *Campylobacter* spp. are still not fully understood, due to the unique characteristics of campylobacters compared to other pathogens (Al-Banna et al., 2018; Costa and Iraola, 2019; Guerry, 2007; Dasti et al., 2010). Various requirements for pathogenicity, known as virulence factors, have been identified (Epps et al., 2013), which include flagella for motility, adherence and invasion factors and the ability to excrete toxins (Van Vliet and Ketley, 2001; Asakura et al., 2007; Dasti et al., 2010). Flagella are also important in the colonisation of the small intestine and colon in humans (Poly and Guerry, 2008; O'Hara et al., 2012). Cellular inflammation in the intestine is caused by the invasion stage, which is followed by a reduction in the intestine's absorptive capacity for the nutrients (Van Deun et al., 2007). The ability of *Campylobacter* to colonise and live in the intestinal tract is a source of discussion for many reasons. Firstly, bile salts impact on the bacterial cell survival, secondly, gastric acids impact the ability to survive through the stomach (Van Deun et al., 2007), and finally the host involved for instance colonisation of poultry caeca has little if any detrimental effect whilst colonisation of the human intestine causes severe enteritis. In poultry, the caeca is the main location for colonisation by *Campylobacter*, with the viable count reaching 10^6 to 10^8 cfu/g (Meade et al., 2009). The first colonisation location in humans is the small intestine and it appears that the individual strain virulence and the host immunity both have an important part in determining the disease severity (Havelaar et al., 2009; Zibauer et al., 2008) and the suggested mechanism for *C. jejuni* infection was characterised by Backert and Hofreuter, (2013)(Figure 1.2).

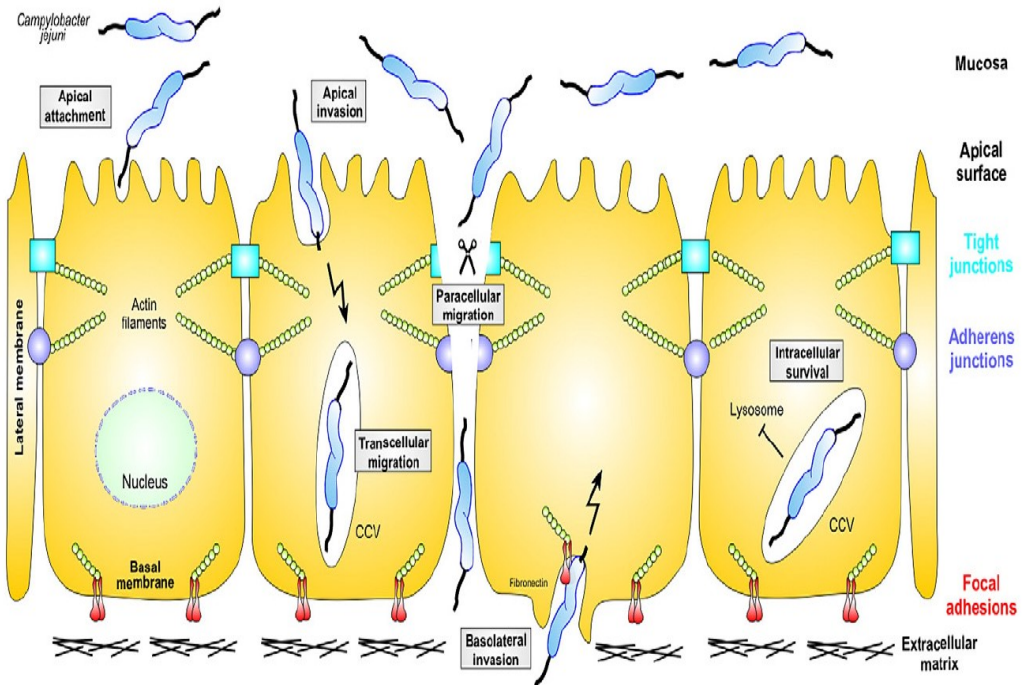


Figure 1.2 Proposed mechanisms of *C. jejuni* of infection (Backert and Hofreuter, 2013)

To gain a better understanding of pathogenicity, a different study focused on genes that participate in producing proteins that are responsible for virulence of *Campylobacter* (Bolton, 2015).

1.3.1.1 Motility

Motility is essential for any pathogen to move towards the host target attachment sites, which then allows the organism to penetrate the mucus lining of the intestine, and finally colonise the intestine (Szymanski et al., 1995). Motility and chemotaxis are also vital for campylobacters to survive and multiply in the gastrointestinal tract by controlling flagellar movement according to the environmental conditions around the bacteria (Bolton, 2015) and *Campylobacter* genes responsible for motility have been outlined in (Table 1.1).

Table 1.1 *Campylobacter* motility factors

Gene (s)	Product
*fla*A	FlaA, the major flagellin protein
*fla*B	FlaB, the major flagellin protein
*fli*F	FliF, hook basal body protein
*fli*M and *fli*Y	FliM & FliY, flagellar motor proteins
*flg*I	FlgI, P-ring in the peptidoglycan
*flg*H	FlgH, L ring in the outer membrane
*flg*E and *fli*K	FlgE & FliK, minor hook components
*fli*A	σ^{28} promoter regulates *fla*A gene expression
*rpo*N	σ^{54} promoter regulates *fla*B gene expression
cj1321_ cj1325/6	Proteins involved in flagellin O-linked glycosylation

Adapted from (Bolton, 2015)

1.3.1.1.1 Flagella

On encountering the highly viscous conditions in the intestinal mucosa, *Campylobacter* will show an unusual increase in motility, which is thought to be important for colonisation of the small intestine (Ketley and Konkel, 2005; Guerry, 2007). A key role of the flagellum was to enable persistence in the difficult conditions encountered in the gastrointestinal tract (Guerry, 2007) the one or two polar flagella and the helical cell shape of cells both contributed to motility of campylobacters (Ferrero and Lee, 1988). The flagellum of *Campylobacter* is composed of the flagellar basal body and the extracellular filament structural components, the flagellar basal body is comprised of a series of discs that link it to the cytoplasm through the inner membrane (Burnham and Hendrixson, 2018). Different ring structures with a central rod within the periplasm form the mechanistic components, the hook protein structure anchors the flagellum filament to the outer membrane (Lertsethtakarn et al., 2011). The *Campylobacter* flagellum structure and genes were characterised by Burnham and Hendrixson (2018)(Figure 1.3).

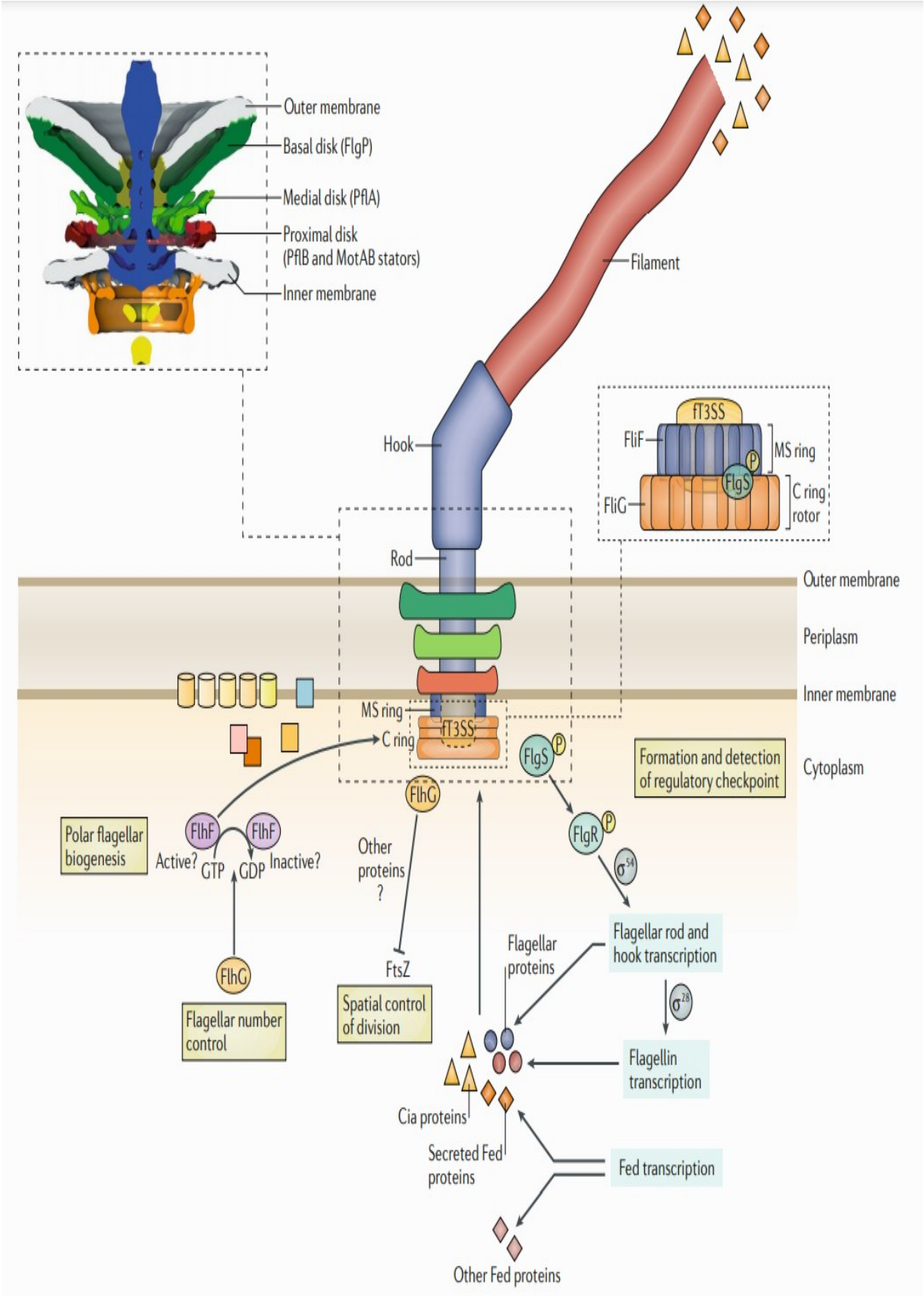


Figure 1.3 *Campylobacter* flagella structure and their genes (Burnham and Hendrixson, 2018)

The flagellar basal body is a complex structure with many different components, The flagellar M-ring protein **(FliF)** protein is linked to a multimer of flagellar motor switch protein FliF **(MS ring)** in the inner membrane that attaches the hook assembly to the cell membrane, Flagellar biosynthesis protein required for formation of the rod structure of the flagellar apparatus, together with FliI and FliH, may constitute the export apparatus of flagellin Flagellar biosynthesis protein **(FlhA and FlhB)**, Flagellar biosynthetic protein which have role in flagellar biosynthesis protine **(FliO)**, Flagellar biosynthetic protein which plays a role in the flagellum-specific transport system **(FliP)**, Flagellar biosynthetic protein which have role in flagellar biosynthesis **(FliQ)** and **(FliR)**proteins are responsible for the type III secretion system **(T3SS)**. The flagellar basal-body rod protein **(FliG)**, Flagellar motor switch protein **(FliM)**, **(FliN)** and **(FliY)** proteins are all parts that form the mechanistic core of flagella is the cytoplasmic C ring **(C ring)** with FliM and FliY proteins serving as flagellar motor switch proteins (Carrillo et al., 2004). Moreover, putative flagellar motor proton channel **(MotA)** and Flagellar motor protein **(MotB)** proteins are the motor components, while Flagellar P-ring protein **(FlgI)** makes up the P ring in the peptidoglycan, Flagellar L-ring protein **(FlgH)** protein is embed in the L ring in the outer membrane, and proteins are minor hook components **(FlgE** and **FliK)**. The main flagellin protein FlaA makes up the majority of extracellular filament structure while the FlaB is a minor component (Nachamkin et al., 1993; Wassenaar et al., 1993; Sommerlad and Hendrixson, 2007; Lertsethtakarn et al., 2011). The Flagellin A **(FlaA)** is encoded by the *fla*A gene and transcription is regulated by the σ^{28} promoter which is highly conserved among different *Campylobacter* species (Guerry, 2007; Ketley and Konkel, 2005; Bolton, 2015) and the transcription of the Flagellin B gene ***(fla*B)** gene is controlled by the σ^{54} promoter which responsible for encoding the hook basal body filament structure (Guerry, 2007; Ketley and Konkel, 2005; Bolton, 2015). The is transcriptional step of these genes is regulated by two elements comprising the sensor kinase Histidine kinase **(FlgS)** protein and the response regulator Sigma-

54 associated transcriptional activator (FlgR)(Hendrixson, 2006). The mutation studies reported that the *fla*A gene is essential for the invasion of epithelial cells and mutations in this gene make a significant change in flagella filament structure leading to a reduction in bacterial motility (Guerry, 2007). However, no significant differences were observed in flagella structures after mutations were created in the *flaB* gene (Guerry, 2007). The mutation of the RNA polymerase sigma-54 factor gene ***(rpoN)*** or RNA polymerase sigma factor for flagellar operon gene ***(fliA)*** genes which are regulated by σ^{54} and σ^{28} also leads to a reduction in colonisation, as does mutation of motility accessory factor gene **(*maf5)*** genes, which encode mobility accessory element but mutations in sigma-54 associated transcriptional activator gene ***(flgR)*** genes resulted in extended colonisation times (Hendrixson and DiRita, 2004; Wösten et al., 2004; Fernando et al., 2007). Other studies have shown the FlaA protein is vital in colonisation of chickens (Wassenaar et al., 1993, Jones et al., 2004). This means that, transcription of the *flaA* gene is key to the control of adherence, colonisation of the gastrointestinal tract and invasion of the host cells and avoiding the immune system response (Jain et al., 2008). In addition, most of the flagella genes have been reported to have roles in adherence or invasion of intestine 407 cell in human intestine epithelial (Yao et al., 1994) or in the chick intestine colonisation stage (Hendrixson and DiRita, 2004). Another characteristic of the flagella proposed by Poly and Guerry (2008) is the capability of secretion of non-flagellar proteins which are vital to virulence of *Campylobacter*. The flagella of *Campylobacter* are O-linked glycosylated which is central for successful flagellin assembly, motility and chick colonisation (Bolton, 2015). Importantly, a few studies have highlighted that the flagellar can functions as a T3SS, transporting *Campylobacter* invasion antigens **(Cia)** into the host cell (Ziprin et al., 2001; Konkel et al., 2004; Biswas et al., 2007; Fernando et al., 2007).

1.3.1.1.2 Chemotaxis

Chemotaxis is the ability of bacterial cells to interpret signals from chemical stimuli allowing them to move towards or away from specific conditions. Some pathogenic bacteria like *Campylobacter* rely on this mechanism to invade their hosts cells (Bolton, 2015; Korolik, 2019). Chemotaxis has been extensively studied in *Escherichia coli* as an example of an enteric pathogen. These studies provide a model for analysis of chemotaxis in campylobacters (Yao et al., 1997; Hendrixson and DiRita, 2004; Hartley - Tassell et al., 2010; Kanungpean et al., 2011). The chemotaxis mechanism is achieved by the actions of two-component regulator system **(TCS)** which includes a membrane-associated histidine autokinase sensor and a cytoplasmic response regulator protein. The fact that TCS assist to recognising and responding to stimulus in the environment means they are essential in the pathobiology of enteric pathogens (Howitt et al., 2011). There are six chemotaxis proteins comprising the TCS, Che A, B, R, W, Y and Z with methyl-accepting chemotaxis proteins **(MCPs)** (Hamer et al., 2010) and the *Campylobacter* chemotaxis genes are outlined by Bolton (2015) in (Table 1.2).

Table 1.2 *Campylobacter* chemotaxis factors

Gene (s)	Product
cheA, cheB, cheR, cheV, cheW and *cheZ*	Chemotaxis proteins; Che A, B, R, V, W, & Z.
tlp1, tlp4 and *tlp10*	Methyl-accepting chemotaxis proteins (MCPs) also called transducer-like proteins
cheY	CheY, response regulator controlling flagellar rotation
cetA and *cetB*	*Campylobacter* energy taxis system proteins CetA (Tlp9) and CetB (Aer2)
luxS	AI-2 biosynthesis enzyme
acfB	AfcB, MCP protein required for persistence in the cecum

Adapted from (Bolton, 2015).

The role of the CheW protein is to bind the MCPs to the CheA protein that triggers the transfer of a phosphoryl group to either CheY or CheB (Bolton, 2015). In many *Campylobacter* strains the CheW protein is replaced by CheV, which is also enables phosphorylation of CheY protein, the phosphorylated protein is joined the to the FliM component of the flagellar motor which leads to a change in rotation direction from counterclockwise to clockwise(Bolton, 2015). This results in a change in motility mode, from smooth forward to sideways tumbling motility and a change in direction, the CheY protein is then dephosphorylated by CheZ which will reverses the effect and returns to the smooth motility (Lertsethtakarn et al., 2011). The mechanisms are controlled by CheR, which allocates methyl groups to the MCPs enhancing their ability to activate CheA protein (Guccione et al., 2008). These steps are reversed by phosphorylated CheB, which removes the methyl groups from MCPs decreasing the ability to activate CheA, which resets the system to pre-stimulation position (Hamer et al., 2010; Lertsethtakarn et al., 2011; Chandrashekhar et al., 2017). Many metabolic substrates including: alpha-ketoglutarate, cysteine, L-glutamate, L-aspartate, L-asparagine, L- L-serine and pyruvate may be considered as chemotaxis activators (Westfall et al., 1986; Mohammed et al., 2004; Velayudhan et al., 2004; Guccione et al., 2008). Other metabolites have a role in the chemotaxis system such as L-malate, D-lactate and succinate, electron donors including formate and electron acceptors including fumarate, dimethyl sulfoxide, nitrite, nitrate and hydrogen peroxide (Myers and Kelly, 2004; Weingarten et al., 2008). The components of bile components have a significant negative impact to *C. jejuni* chemotaxis (Hugdahl et al., 1988). The *Campylobacter* genome includes several genes encoding important elements called methyl accepting chemotaxis proteins also called transducer like proteins **(Tlps)** (Hugdahl et al., 1988). These work as extracellular signals, sensors or stimulators in the form of ligands and they transmit signals to the cytoplasmic core chemotaxis signal transduction Che proteins network (Lertsethtakarn et al., 2011). Variation in the components of the chemotaxis system allows flexibility in

adaptation to different environments and may contribute to their success as foodborne pathogens (Bolton, 2015; Chandrashekhar et al., 2017).

1.3.1.2 Adhesion and binding factors

Adherence of *Campylobacter* to epithelium cells in the intestine is an important step in the colonisation process (Krause-Gruszczynska et al., 2007; Chlebicz and Śliżewska, 2018). In vitro experiments using human and non-human cell lines, have identified a number of adhesion proteins in *C. jejuni* including, fibronectin-binding outer membrane protein CadF (Konkel et al., 2001; Konkel et al., 1997). The *cadF* gene, is one of the most highly conserved regions of the chromosome and encodes the 37 kDa CadF outer membrane protein (Hofreuter et al., 2006). Mutation of the *cad*F gene was shown to result in a significant reduction in *Campylobacter* internalisation in INT 407 human intestinal epithelial cells (Krause-Gruszczynska et al., 2007). Interaction with fibronectin, results in activation of the GTPases Rac1 and Cdc42 proteins which stimulate internalisation of *Campylobacter* cells (Bolton, 2015). Interestingly, Ziprin et al. (1999) confirmed that the absence of cadF proteins renders *C. jejuni* unable to colonise chickens. Other genes associated with *C. jejuni* adhesion include: *cap*A, *pld*A, *jlp*A, the lipoprotein peb genes, *flp*A and *vir*B11.

1.3.1.3 Invasion

In addition to motility, *Campylobacter* flagella have an important role in invasion of host cells acting as a type three secretion system (T3SS) secreting non-flagellar proteins (Poly and Guerry, 2008). Various different experimental animal models have shown that invasion of *C. jejuni* into the colonic epithelial cells results in early mucosal damage (Poly and Guerry, 2008; Field et al., 1986; Humphrey et al., 1985; Welkos, 1984). The ability of *Campylobacter* to invade host cells is strain dependent (Newell et al., 1985). The invasion mechanism, involves actin- and

microtubule-independent mechanisms (Bouwman et al., 2013). The adhesion factor CadF is also involved in invasion via fibronectin signalling leading to internalisation (Dasti et al., 2010). *Campylobacter* has been shown to enter into INT-407 epithelial cells with its tip first, followed by the flagella (Krause-Gruszczynska et al., 2007). The contribution of various other factors in the invasion process is still unclear, for example lipooligosaccharides sialylation (LOS; Louwen et al., 2008), outer core or the capsular polysaccharide (CPS; Karlyshev and Wren, 2001), and invasive antigens (Cia) which are produced in the presence of bile (Rivera-Amill et al., 2001). The genes that contribute to invasion in *Campylobacter* were detailed by Bolton (2015) (**Table 1.3**). Several studies have highlighted the role of different flagellar export proteins like CiaB, FlaA, FlaB and their homolog FlaC that is secreted via the T3SS into host cell. Homologues of a type IV secretion system have identified in a plasmid of *C. jejuni* 81–176. Mutation experiments have shown that the absence of the genes encoding these proteins could lead to a decrease in invasion (Bacon et al., 2000; Bacon et al., 2002).

Table 1.3 *Campylobacter* invasion genes

Gene (s)	Product
flhA, flhB, fliQ, fliP, fliO and fliR	FlhA, FlhB, FliO, FliP, FliQ & FliR, components of the flagellar T3SS
flaC	FlaC protein secreted into the host cells and essential for colonisation and invasion
ciaB	CiaB, 73-kDa protein involved in adhesion
ciaC	CiaC, protein required for full invasion of INT-407 cells
ciaI	CiaI, reported role in intracellular survival
iamA	IamA, invasion associated protein
htrA	HtrA, chaperone involved in the proper folding of adhesions
virK	VirK, may have a role in protection against antimicrobial proteins
fspA	FspA, protein with a role in apoptosis

Adapted from (Bolton, 2015)

1.3.1.4 Iron acquisition

In the light of dramatic increase in multidrug resistant bacteria and their negative impact on human life, the synergistic relationships among microbial communities need to be better understand (Jiricny et al., 2010; Griffin et al., 2004). Pathogenic bacteria such as *Campylobacter* require iron III **(Fe^{+3})** for survival and growth, the accessibility of the beneficial form of iron is restricted for various reasons such as it is bound in a complex such as haemoglobin or lactoferrin (Andrews et al., 2003), there is limited water solubility in certain conditions and pHs, more importantly competition from other microbes within the host (Schalk and Guillon, 2013; Raymond et al., 2015). Moreover, iron deficiency may be a non-specific host defence mechanism to eliminate pathogenic bacteria activity (Andrews et al., 2003). The pathogenic bacteria have evolved efficient Fe^{+3} uptake mechanisms in order to overcome this limited iron abundance, they secrete high-affinity Fe^{+3} chelating agents, called siderophores (Hider and Kong, 2010) for example enterobactin (Raines et al., 2016). Sharing of siderophores is an example of cooperation between cells which supports bacterial virulence (West and Buckling, 2003). A study of the *C. jejuni* NCTC 11168 genome revealed a lack of the genes encoding enterobactin from the annotated sequence, which suggested that *C. jejuni* 11168 may utilise enterobactin hydrolysis products for the uptake of Fe^{+3} from other microflora in their environment (Raines et al., 2016; Palyada et al., 2004). It has been suggested that Fe^{+3} uptake across the outer membrane is controlled by two receptors CfrA and CfrB (Xu et al., 2010). Other genes important in iron uptake are *chuA* and *chuD* which encode outer membrane receptors for hemin and haemoglobin uptake (Johnson et al., 2016; Palyada et al., 2004). The genes *cfrA*, *cfrB* encode outer membrane ferric enterobactin (FeEnt) receptors and the *fur* gene which controls ferric uptake regulator all of which are essential for colonisation (Hoang et al., 2012, Woodall et al., 2005, Hermans et al., 2011).

1.3.1.5 Toxin production

Cytolethal distending toxin (CDT) causes inhibition in the eukaryotic cell cycle at the G2 phase before mitosis (Smith and Bayles, 2006). It is the only toxin reported and identified in *Campylobacter* species including *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. upsaliensis* and *C. hyointestinalis* (Asakura et al., 2007; Kamei et al., 2015; Samosornsuk et al., 2015; Johnson and Lior, 1988). Over the last two decades significant progress has been achieved in terms of analysing the role of CDT in virulence (Karlyshev and Wren, 2001; Hickey et al., 2000). The CDT is composed of three active proteins: the CdtB which controls the activation step leading to cell cycle blockage, and a protein dimer consisting of both CdtA and CdtC subunits, which are associated with transfer of CdtB to the inside of the host cell (Pickett and Whitehouse, 1999). Following internalisation, the CdtB protein targets the nucleus of the cell and triggers a DNase I-like activity that results in DNA double-strand breaks. As a result, the cell cycle of the eukaryotic cell is inhibited followed by cellular distension and finally cell the death (Lara-Tejero and Galán, 2001; Smith and Bayles, 2006). The role of CDT in *C. jejuni* pathogenesis remains an active area of research. Not all *C. jejuni* isolates possess the genes that codes for CDT in their genomes, while some strains that encode the genes, do not produce CDT (Pickett et al., 1996). This is probably due to natural mutations (AbuOun et al., 2005). Mutants of *C. jejuni* lacking the CDT genes demonstrate effective colonisation of NF-kB-deficient mice similar to wild type (Fox et al., 2004).

1.4 *Arcobacter* pathogenicity

Awareness of arcobacters as important foodborne pathogens has increased dramatically due to the rising number of food-related outbreaks of illness (Ramees et al., 2017; Mottola et al., 2016; Noor and Maniha, 2019). Arcobacters are considered to be emerging, foodborne, zoonotic pathogens, worldwide (Ferreira et al., 2019; Kim et al., 2019) but their virulence and their pathogenic mechanisms

are not completely understood. The International Commission on Microbiological Specifications for Foods (ICMSF) has described *Arcobacter* as a serious hazard to human health (ICOMSFF, 2002). The transmission and pathogenesis mechanisms for arcobacters proposed by Ramees et al (2017)(Figure 1.4). Historically, *Arcobacter* was first isolated and described from aborted bovine foetal tissues (Ellis et al., 1977), and similarly isolated from porcine foetuses (Ellis et al., 1978). Later it was realised that arcobacters can cause bacteraemia, endocarditis, peritonitis, gastroenteritis and diarrhoea in humans. They cause diarrhoea, mastitis and abortion in animals (Jiang et al., 2010; Figueras et al., 2014; Ferreira et al., 2016b). The proposed mechanisms of diarrhoea include the production of a cytotoxin, affecting expression of tight junction proteins of the epithelial gut cells, release of pro-inflammatory cytokines and epithelial barrier dysfunction leading to cell death (Figueras et al., 2014). The organism is released from the intestines of animals into the environment where it contaminates water and food sources thereby entering the human food chain (Ramees et al., 2017). The most frequent species of *Arcobacter* isolated from humans or animals with clinical conditions are *A. butzleri*, *A. cryaerophilus, A. skirrowii* and *A. aremore* (Gill, 1983; Kiehlbauch et al., 1991; De Oliveira et al., 1997; Wesley, 1997; Wesley et al., 2000; Bagalakote et al., 2014; Ramees et al., 2014b). In addition, *Arcobacter* bacteria can found in healthy human stool specimens and animal faeces without any clinical signs, which means that their pathology and pathogenicity are contentious (Öngör et al., 2004; Shah et al., 2011; Houf and Stephan, 2007; Figueras et al., 2014) The confirmation of culturing is still considered as the "gold standard" method for distinguishing and diagnosing *Arcobacter* (Atabay and Corry, 1998; Rahimi, 2014; Ramees et al., 2014a).

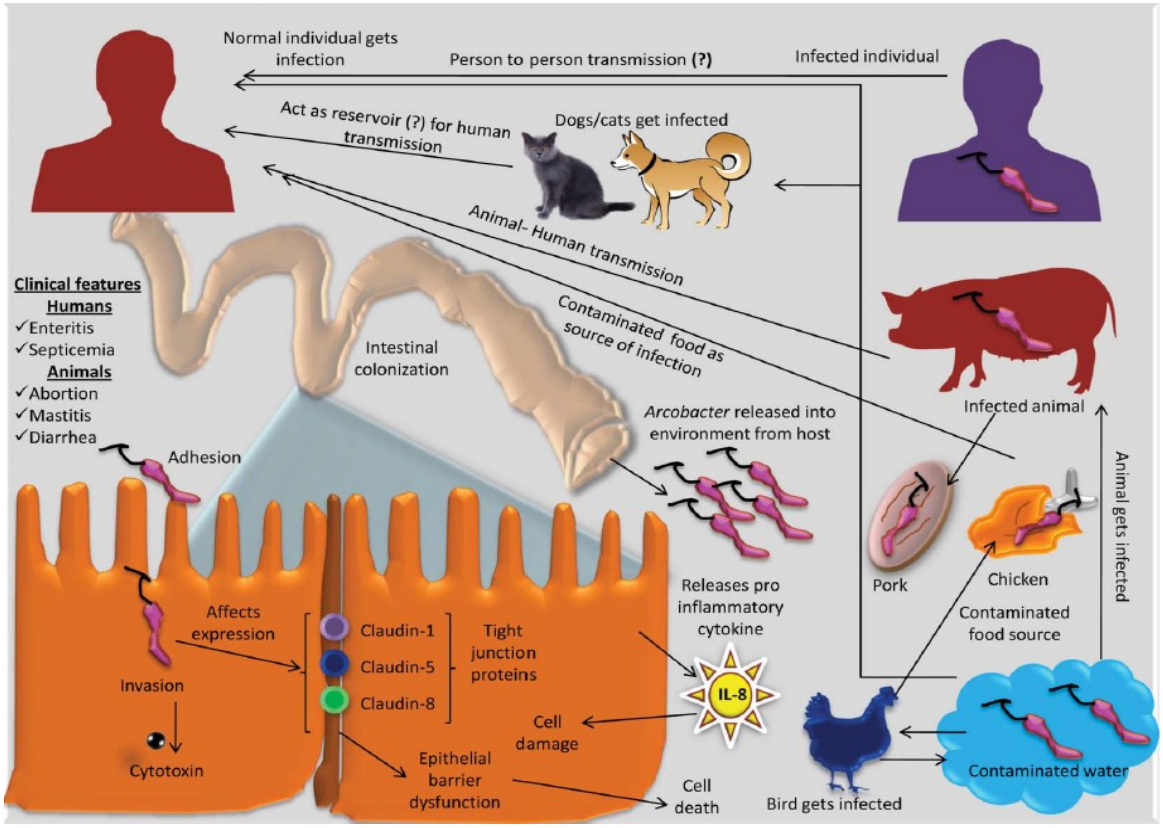


Figure 1.4 The proposed transmission and pathogenesis mechanisms for arcobacters. Adapted from Ramees et al. (2017)

1.5 *Campylobacter* and *Arcobacter* isolation, enrichment and maintenance

1.5.1 Isolation, enrichment, and maintenance of *Campylobacter*

Isolation of thermophilic campylobacters from clinical samples is achieved by using selective media supplemented with antibiotics to inhibit the competitive microflora, followed by incubation under microaerobic conditions at 42°C (Endtz et al., 1991; Jorgensen and PFaller, 2015). For food and environmental samples, which are more likely to include low numbers of campylobacters, enrichment methods are used, followed by growth on selective media supplemented with antibiotics (Gharst et al., 2013). Pre-enrichment may further increase chances of isolating injured bacteria. However, a consequence of this and other enrichment methods, is that

if mixed populations of strains and species are present only the most numerous individual strain may be isolated (Gharst et al., 2013; Silva et al., 2011). The *Campylobacter* species such as *C. hyointestinalis* and *C. concisus* have different optimum growth temperatures and atmosphere requirements for successful culture (Gebhart et al., 1985; Gebhart et al., 1983; Tanner et al., 1981; Vandamme et al., 1989). Once single colonies have been obtained, culture media such as Columbia agar, Tryptose Blood Agar, and Mueller-Hinton agar can be used to sub-culture and maintain the culture for between three to four days (Lastovica et al., 2014).

1.5.2 Isolation, Enrichment, and Maintenance of *Arcobacter*

There is no standard method for the isolation of *Arcobacter* despite many different culture media having been described for use with a variety of different sample types (Collado and Figueras, 2011). Probably the most commonly used method for isolation of *Arcobacter* is by inoculation of enrichment broth containing cefoperazone, amphotericin B, and teicoplanin (CAT) followed by microaerobic incubation at 30°C for two days. (Atabay and Corry, 1997). A study carried by Merga et al. (2011) assessed five different methods to isolate *Arcobacter* from various animal faecal samples and revealed that, using enrichment broth prior to culture on modified charcoal-cefoperazone-deoxycholate agar (mCCDA) supplemented with a combination of antibiotics was the most effective method. Many other methods to isolate *Arcobacter* have been were described (Johnson and Murano, 1999; Houf et al. 2001; Van Driessche et al. 2003). Elimination of the competitive flora by enrichment may give an advantage to some *Arcobacter* species over other species or other bacteria in the same sample (Atabay et al., 2006; Houf et al., 2001; Andersen et al., 2007). Inconsistencies outcomes between the culture and molecular detection methods for arcobacters have been reported. This could be due to reasons such as variation in isolation steps for

example enrichment periods or different culture media, which may lead to unrepresentative results (Ho et al., 2006a; Fera et al., 2004; González et al., 2006). The ability to grow under both microaerobic and aerobic conditions, has also been employed for the isolation of *Arcobacter* species but more frequently to discriminate the *Campylobacter* genus from the *Arcobacter* genus (Collado and Figueras, 2011; Vandamme et al., 1991). *Arcobacter* cultures are easy to maintain because they have can grow at a range of temperatures between 15 and 30 °C, including room temperature, under aerobic conditions. This means that *Arcobacter* may be sub-cultured and left on the laboratory bench to grow without the need for an incubator (Lastovica et al., 2014; Vandamme et al., 1992a). Due to the fastidious nature of *Arcobacter* species and their biochemical similarity with *Campylobacter* species, their phenotypic discrimination is complicated. Genetic methods such as polymerase chain reaction (PCR)-based methods are employed to successfully differentiate isolates (Brightwell et al., 2007; Douidah et al., 2010). Multiplex PCR based methods have used to target genus and species-specific sequences (Houf et al., 2000; Antoĺın et al., 2001; González et al., 2006; Ramees et al., 2014b). Conventional PCR has been used as rapid tool for the detection of *Arcobacter* spp. from food and other samples (González et al., 2010; de Boer et al., 2013).

1.6 Ecology of *Campylobacter* and *Arcobacter*

1.6.1 Ecology of campylobacters

Campylobacter species are widely distributed in the environment and have been isolated from: soil (Jäderlund et al., 2011), muddy puddles (Blaser et al., 2018), beach sand (Yamahara et al., 2012), and environmental water samples (Lévesque et al., 2008). The possible routes of transmission of *C. jejuni* into the human food chain proposed by Dasti et al.(2010)(Figure 1.5).

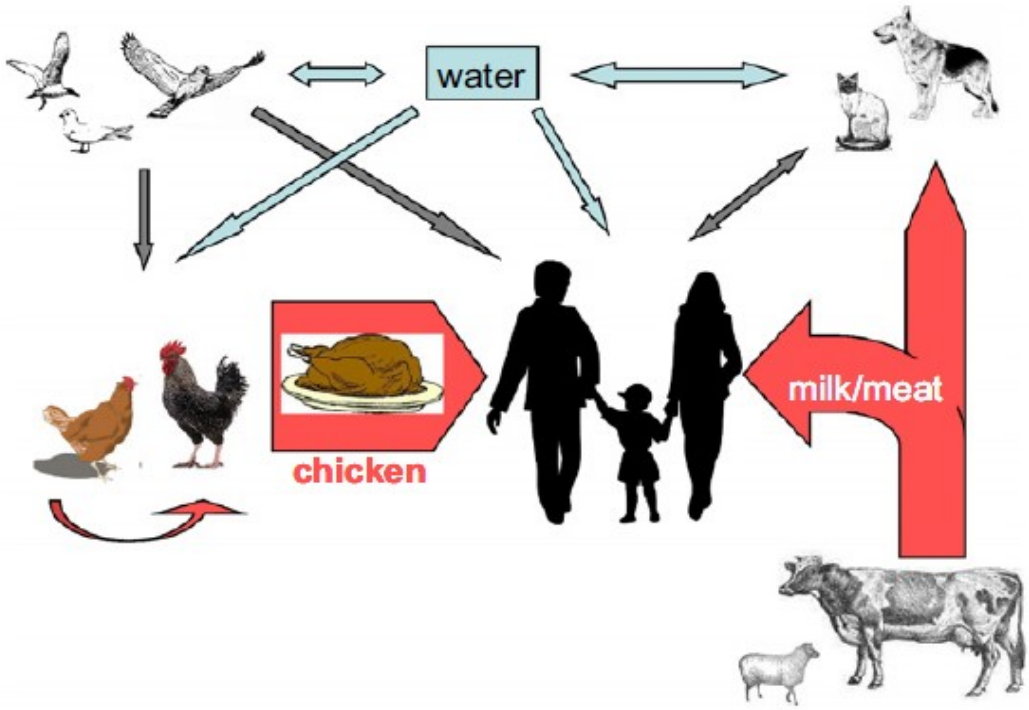


Figure 1.5 *Campylobacter jejuni* transmission routes into the human food chain Adapted from Dasti et al.(2010)

Campylobacters inhabit the intestines of birds, most mammals including humans, they are voided in faeces into the environment in large numbers where they survive until ingested by a new host (Dasti et al.2010). It has been shown that campylobacters can survive in compost made from cattle faeces for up to ten months (Inglis et al. (2010). A different *Campylobacter* species, sources and associated disease outlined by Lastovica et al.(2014)(Table 1.4). These thermophilic campylobacters have the ability to invade and colonise the intestinal mucosa and caecum of birds that have a normal body temperature of at 42°C (Pajaniappan et al., 2008; Line et al., 2010). *Campylobacter* have been isolated from the faeces of cats, dogs (Rahimi et al., 2012; Koene et al., 2009), and also from sheep, goats and cattle (Moriarty et al., 2011; Hanlon et al., 2018; Dong et al., 2016; An et al., 2018). Also, Non confirmed disease association *Campylobacter* species have been reported by Data from (Lastovica et al., 2014), from (Silva et

al., 2020), from (Boukerb et al., 2019), from (Gilbert et al., 2018), from (Cáceres et al., 2017), from (Van et al., 2016) and from (Piccirillo et al., 2016) (Table 1.5).

In the food chain, seafood, uncooked meat and raw milk are considered to serve as reservoirs for some *Campylobacter* species (Wilson and Moore, 1996; Taylor et al., 2013; Wong et al., 2007; Narvaez-Bravo et al., 2017; Davis et al., 2016; Hauri et al., 2013; Hill et al., 2012). Consumption of contaminated groundwater has led to large waterborne Campylobacteriosis outbreaks in Norway and other countries (Jakopanec et al., 2008).

Table 1.4 *Campylobacter* with known human and animal disease association

Campylobacter	**Isolation sources**	**Human disease**	**Animal disease**
C. jejuni subsp*. jejuni*	Humans, dogs, cattle, birds, poultry, cattle, sheep, milk, seafood, water	Enteritis, septicaemia, abortion, appendicitis, colitis, myocarditis, reactive arthritis, Reiter's syndrome, Guillain-Barré syndrome	Spontaneous abortion (bovine, ovine); gastroenteritis (canine, feline)
C. coli	Humans, dogs, cattle	Enteritis, septicaemia	
C. hyointestinalis subsp. *hyointestinalis*	Pigs, cattle, hamsters	Enteritis, septicaemia	
C. ureolyticus	Humans, milk, bovine faeces	Ulcerative colitis	
C. fetus subsp. *fetus*	Cattle, sheep, dogs, turtles	Septicaemia, meningitis, vascular infection, abortion	Spontaneous abortion bovine, ovine
C. concisus	Humans, dogs, cats	Inflammatory bowel disease, periodontal disease, enteritis, septicaemia, Barrett's oesophagus	Enteritis canine
C. curvus	Humans	Gastroenteritis; abscesses	
C. gracilis	Dogs, humans	Abscesses	
C. fetus subsp*. venerealis*	Cattle	Septicaemia	Infectious fertility bovine
C. insulaenigrae	Seals, porpoises	Enteritis, septicaemia	
C. jejuni subsp. *doylei*	Humans, dogs	Enteritis, septicaemia	
C. lari subsp. *lari*	Cats, dogs, chickens, seals, mussels, oysters	Enteritis, septicaemia	
C. rectus	Humans	Abscesses	
C. showae	Humans, dogs	Septicaemia, cholangitis	
C. sputorum bv. *sputorum*	Humans, cattle, pigs, sheep	Abscesses	
C. upsaliensis	Cats, dogs, ducks, monkeys	Enteritis, septicaemia, abortion, abscesses	Gastroenteritis canine, feline

Adapted from Lastovica et al.(2014)

Table 1.5 *Campylobacter* species without any confirmed disease association

Campylobacter	**Isolation sources**
C. avium [a]	Chickens, turkeys
C. canadensis [a]	Whooping and Sandhill cranes
C. cuniculorum [a]	Rabbits
C. helveticus [a]	Dogs, cats
C. hominis [a]	Humans
C. hyointestinalis subsp. *lawsonii* [a]	Pigs, poultry, birds
C. lanienae [a]	Humans, cattle
C. lari subsp. *Concheus* [a]	Unknown
C. mucosalis [a]	Pigs, dogs
C. peloridis [a]	Humans, molluscs
C. sputorum bv. *paraureolyticus* [a]	Cattle, humans
C. sputorum bv. *faecalis* [a]	Cattle
C. subantarcticus [a]	Penguins, albatrosses
C. troglodytis [a]	Chimpanzees
C. volucris [a]	Black-headed gulls
C. portucalensis [b]	Bulls
C. armoricus [c]	Surface of water and humans
C. blaseri [d]	Common seals
C. ornithocola [e]	Wild birds
C. hepaticus [f]	Chickens
C. geochelonis [g]	Hermann's tortoise

Adapted from [a]Data from Lastovica et al.(2014), [b]from Silva et al.(2020), [c]from Boukerb et al.(2019), [d]from Gilbert et al.(2018), [e]from Cáceres et al.(2017), [f]from Van et al.(2016) and [g]from Piccirillo et al.(2016).

1.6.2 Ecology of *Arcobacter*

Arcobacters contaminate various types of environmental water which have been associated with their transmission to humans and animals (Ho et al., 2006a; Snelling et al., 2006). These include groundwater, rivers, lakes, seawater and piggery effluent, irrigation water and even drinking water (Talay et al., 2016; Chinivasagam et al., 2007; Lee et al., 2012; Fong et al., 2007; Collado et al., 2010). The most frequently isolated species and most well studied are *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*. These are considered to be emerging pathogens

which can interact with animals and humans (Fernandez et al., 2015, Bogantes et al., 2015). Many studies have suggested that animal and human faecal contamination may play an important role in *Arcobacter* contamination of water (Van Driessche et al., 2003; Collado et al., 2008). the development of the *Arcobacter* taxonomy scheme proposed by Ramees et al.(2017)(Figure 1.6).

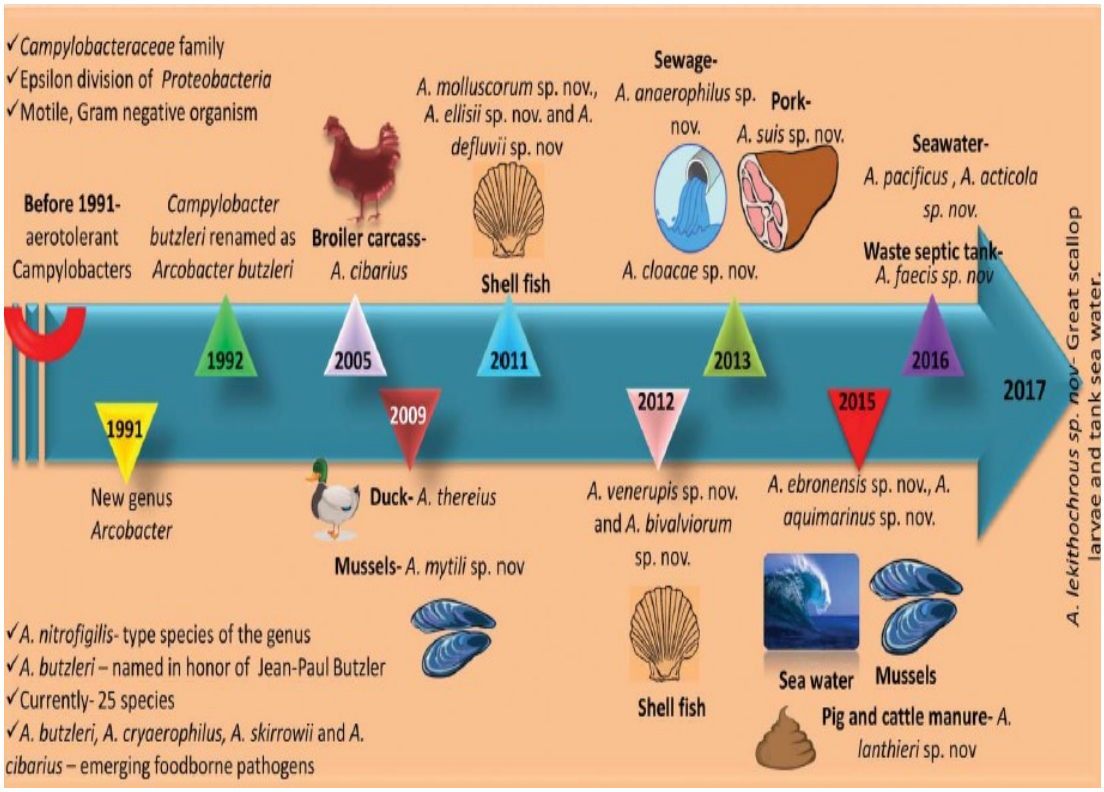


Figure 1.6 Development of *Arcobacter* taxonomy (Ramees et al., 2017)

Table 1.6 *Arcobacter* species, sources, year of isolation and pathogenicity association

***Arcobacter* species**	**Isolation source**	**Isolation year**
A. bivalviorum	Mussels, clams	2012
A. butzleri *****	Pigs, bulls, horses, cattle, chicken, dogs, cats, primates, ostriches, ducks, water, sewage	1991,2000
A. cibarius	Chicken	2005
A. cloacae	Sewage, mussels	2013
A. cryaerophilus *****	Pigs, bulls, chicken, sheep, horses, dogs, cats, sewage	1985
A. defluvii	Sewage	2011
A. ellisii	Mussels	2011
A. halophilus	Hypersaline lagoon water	2005
A. marinus	Seawater	2010
A. molluscorum	Mussels, oysters	2011
A. mytili	Mussels	2009
A. nitrofigilis *****	Estuarine plant roots	1983
A. skirrowii	Sheep, bulls, pigs, chicken, ducks	1992
A. suis	Pork meat	2013
A. thereius	Pigs, ducks	2009
A. trophiarum	Pigs	2011
A. venerupis	Mussels, clams	2012

*****Known as human and animal pathogens

Adapted from Lastovica et al.(2014)

The *Arcobacter* genus was established in the year 1991, and there are currently 27 species of *Arcobacter*. *Arcobacter* species such as *A. halophilus*, *A. marinus* and *A. defluvii* are found in sewage (Pérez-Cataluña et al., 2019, Pérez-Cataluña et al., 2018). Interestingly, some *Arcobacter* species can survive in water for 250 days at 4°C (Van Driessche and Houf, 2008). The members of *Arcobacter* species, sources of isolation and their pathogen status listed by Lastovica et al.(2014) (Table 1.6). Food from animal origin are proposed to be the source of *Arcobacter* entry in to the human food chain (Ho et al., 2006a, Ferreira et al., 2016a, On et al., 2002).

Arcobacters have been isolated from healthy cattle, sheep and pigs (Ho et al., 2006a, Ho et al., 2006b, De Smet et al., 2011, Patyal et al., 2011, Ferreira et al., 2016b). More recently, arcobacters have been isolated from raw milk in Italy (Traversa et al., 2019, Marta et al., 2020). Poultry have identified as a major

reservoir of arcobacters suggesting they have a central role in the spread of these bacteria (Collado and Figueras, 2011, Rahimi, 2014, Dekker et al., 2019). Recently, researchers have reported contaminated water and meat as important agents in the transmission of this pathogen in to the food chain (González and Ferrús, 2011, Snelling et al., 2006, Collado et al., 2010, Rahimi, 2014). Arcobacters have been frequently isolated from slaughterhouse samples, even after disinfection; therefore, slaughterhouses may be an underestimated epidemiological source of these bacteria (Collado et al., 2010, Patyal et al., 2011, Ramees et al., 2014a, Ramees et al., 2014b). A wide range of genetic diversity has reported for *Arcobacter* isolates recovered from different parts of the world with associated antibiotic resistance (Bagalakote et al., 2014, Mohan et al., 2014, Ferreira et al., 2016b). Shellfish may present an additional source of *Arcobacter* where many different species have been isolated from clams and mussels such as: *A. mytili*, *A. molluscorum*, *A. bivalviorum*, *A. venerupis*, and *A. ellisii* ,these findings have resulted in increased public health concern about consumption of seafood, which may get inadequate heat treatment before consumption (Figueras et al., 2011, Levican et al., 2012, Collado et al., 2009b, Collado et al., 2009a, Levican et al., 2013b).

1.7 Antibiotic resistance

1.7.1 *Campylobacter* antibiotic resistance

Over the decades since *Campylobacter* was first described, there has been a dramatic increase in antibiotic resistance to fluoroquinolones and macrolides. While most *Campylobacter* infections are self-limiting, severe campylobacteriosis

cases may require antibiotic therapy (Zhang and Sahin, 2020; Sproston et al., 2018). Antibiotic resistance in campylobacters has been reported in different studies since the 1980's (Flores et al., 1985; Taylor and Courvalin, 1988; Taylor et al., 1987). Studies since then have highlighted a trend of increasing resistance among *C. jejuni* isolates (Gaudreau and Gilbert, 2003). One study in the USA observed that the fluoroquinolone resistance among *C. jejuni* clinical isolates, had increased to approximately 40% in 2001 (Nachamkin et al., 2002). Moreover, fluoroquinolone resistance among *Campylobacter* isolates from human or animal background, has been detected in many European countries (Pezzotti et al., 2003; Papavasileiou et al., 2007; Avrain et al., 2003). Asian and African countries, have also observed resistance (Boonmar et al., 2005; Isenbarger et al., 2002; Nhung et al., 2016; Bester and Essack, 2008; Samuel et al., 2006). In addition, high levels of antibiotic resistance were reported in Australia and New Zealand (Sharma et al., 2003; Goodchild et al., 2001; Harrow et al., 2004). Interestingly different observations have shown that fluoroquinolone resistance is higher among *C. coli* compared to *C. jejuni* isolates (Rossi et al., 2015; EFSA, 2015; EFSA, 2014; Sproston et al., 2018) but the reasons for this were not clear. However, even distribution of fluoroquinolone resistance between the two species has also been reported (Gaudreau et al., 2014). The ciprofloxacin (CIP) resistance in *C. jejuni* and *C. coli* from different sources suggested by Sproston et al.(2018)(Table 1.6). It is believed that increasing resistance to fluoroquinolones, tetracycline, and erythromycin in *C. jejuni* and *C. coli* could be linked to the excessive usage of antibiotics in animal production (Silva et al., 2011; Sproston et al., 2018). Restrictions and limitations on the use antibiotics in animal production were applied in the EU in 2003 and 2006 and in the USA from 2005 (Sproston et al., 2018; Castanon, 2007; Nelson et al., 2007). Monitoring programs have been approved, to ensure the implementation of this legislation (Organization, 2014). The resistance to fluoroquinolone mechanism in campylobacters is based on mutation in the quinolone resistance determining region (QRDR). This results in

inhibition of DNA gyrase A (GyrA), which is responsible for initiation of DNA replication and transcription (Wieczorek and Osek, 2013; Smith and Fratamico, 2010; Luangtongkum et al., 2009). It's proposed that mutation is achieved by a single point mutation in the *gyr*A gene switching from threonine to isoleucine in the Gyrase A subunit at position 86 (Wieczorek and Osek, 2013; Yang et al., 2017). Resistance to other antibiotics is often mediated by the *Campylobacter* multidrug efflux pump (CME). CME contains different proteins such as a periplasmic protein CmeA, an inner membrane efflux transporter CmeB and an outer membrane protein CmeC (Bolton, 2015). Three types of operon, cme A, B and C, control expression of the CME encoding proteins, which is modulated by CmeR, a transcriptional repressor, probably by inhibiting the cj0561c gene, which encodes a putative periplasmic protein (Lin et al., 2002). Moreover, mutation of the CmeR protein reduced the capacity of *C. jejuni* to colonise broilers (Guo et al., 2008).

Table 1.7 *Campylobacter jejuni* and *Campylobacter coli* resistance to Ciprofloxacin (CIP)

Sample type	Country	Year	Species	Resistance to CIP [% (n)]
Clinical/Human	Canada	2002-2013	*C. jejuni, C. coli*	41.6 (440), 50 (38)
	Canada	2011-2013	*C. jejuni, C. coli*	30.8 (180), 41 (39)
	USA	2011-2015	*C. jejuni, C. coli*	25.8 (5048), 36.3 (576)
	USA	2011-2012	*Campylobacter spp.*	25.3 (1962)
	UK	2008	*Campylobacter spp.*	37.5 (803)
	Europe	2014	*C. jejuni, C. coli*	60.2 (11,585), 68.9 (1,500)
	Europe	2015	*C. jejuni, C. coli*	60.8 (13,696), 70.6 (1,754)
Ruminants/Cattle Faeces	USA	2012	*C. jejuni*	16.3 (22)
	USA	2012-2013	*C. jejuni, C. coli*	35.4 (320), 74.4 (115)
Broilers/Chicken, Faeces, cloaca	Europe	2014	*C. jejuni, C. coli*	69.8 (3317), 74.3 (767)
	China	2012-2016	*C. jejuni, C. coli*	100 (166), 100 (40)
Swine/ Pig, Faeces, carcasses, Caeca	China	2008-2014	*C. coli*	97 (970)
	Ghana	2013-2014	*Campylobacter spp.*	30.3 (66)
	Europe	2015	*C. coli*	62.1 (704)

Adapted from Sproston et al. (2018)

1.7.2 *Arcobacter* antibiotic resistance

Only a few antibiotic susceptibility studies targeting *Arcobacter* species have been carried out. Antimicrobial resistance and potential pathogenicity of *Arcobacter* species are therefore still poorly understood (Levican et al., 2013a; Karadas et al., 2013). Usually, *Arcobacter* infections in humans are self-limiting, however when antibiotics are recommended, the fluoroquinolones and tetracycline are the most frequent treatments prescribed (Son et al., 2007). In a study by Houf et al. (2004) isolates of *A. butzleri* and *A. cryaerophilus* isolated from poultry were resistant to erythromycin and ciprofloxacin, which is of significance because these two

antimicrobials are the most prescribed as antibiotics used to treat human *Campylobacteraceae* infection. In another study, 17 strains of *A. butzleri* and 13 strains of *A. cryaerophilus*, were highly resistant to penicillins, macrolides, chloramphenicol, trimethoprim, and vancomycin (Fera et al., 2003). Other studies have demonstrated that isolates of *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* were resistant to various antibiotics such as nalidixic acid, metronidazole, cephalothin, cefoperazone, and carbenicillin (On et al., 1996, On et al., 1995).

1.8 Aim of study

One of the objectives of this study was to investigate the survival of campylobacters in milk. Another goal was to isolate lytic bacteriophages with biocontrol potential from farm environments that could target campylobacters and arcobacters and in order to achieve this *Campylobacter* and *Arcobacter* hosts had to be isolated from farm environments and characterised, which included genome sequencing. To achieve these objectives, a novel genus of predatory bacteria was isolated that could prey on the pathogenic bacteria isolated during this study.

Chapter TWO

General Materials and Methods

2.1 Growth, storage media and chemical solutions

All culture media for both growth and storage, were prepared using reverse osmosis (RO) water and then sterilised by autoclaving at 121°C and 15 psi for 15 minutes. The supplements or selective antibiotics were added to agar media when tempered to 50°C in a water bath. The molten media were then poured into sterile plastic Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet. After they were dry, the plates containing media were stored at 4°C for up to four weeks. Liquid media were stored at room temperature after autoclaving and cooling, unless otherwise stated.

2.1.1 Blood agar (BA)

Blood agar base No. 2 CM0271 (Oxoid, Basingstoke, UK) containing: proteose peptone 15.0 g/l, liver digest 2.5 g/l, yeast extract 5.0 g/l, sodium chloride 5.0 g/l, agar 12.0 g/l, was prepared according to manufacturer's instructions. After the sterilisation, the base medium was cooled down to 45-50°C, then 5 % v/v defibrinated horse blood from (HB035; TCS; Buckingham; UK) was added, and mixed gently prior to pouring into Petri dishes. To reduce colony swarming the percentage of the agar was increased to 2% w/v. Any bubbles were removed by briefly passing a Bunsen flame over the molten medium in the Petri dish, to ensure clear viewing of the plates and accurate colony counting.

2.1.2 Modified *Campylobacter* blood-free selective agar (mCCDA)

Campylobacter blood-free selective agar CM0739 (Oxoid) containing: nutrient broth No.2 25.0 g/l, bacteriological charcoal 4.0 g/l, casein hydrolysate 3.0 g/l, sodium desoxycholate 1.0 g/l, ferrous sulphate 0.25 g/l, sodium pyruvate 0.25

g/l, agar 12.0 g/l, was prepared according to manufacturer's instructions. The base medium was supplemented with an extra 4 g of bacteriological agar No. 1 LP0011 (Oxoid) to give a final percentage agar of 2% (w/v) when prepared for colony counting experiments to reduce swarming. For experiments aimed to isolate *Campylobacter* spp. from slurry samples *Campylobacter* selective supplement (SR0155; Oxoid) was added to the culture media. A vial of supplement was re-suspended in 2 ml of sterile RO water and aseptically dispensed into 500 ml of tempered mCCDA, mixed well, then the medium poured into Petri dishes. The final concentration of cefoperazone and amphoteracin were 32 mg/l and 10 mg/l respectively. For experiments that involved the isolation of *Arcobacter* spp. from slurry samples, cefoperazone, amphotericin B, teicoplanin selective supplement (CAT) SR0155 (Oxoid) was added to the culture medium. A vial of supplement was re-suspended in 4 ml of sterile RO water and aseptically dispensing into 500 ml of tempered mCCDA, mixed well, then poured into Petri dishes. The final concentration of cefoperazone, amphotericin B, teicoplanin were 0.8 mg/l, 0.4 mg/l and 10 mg/l respectively.

2.1.3 New Zealand Casamino Yeast Medium (NZCYM) basal agar

NZCYM Broth 240410 (Difco; Oxford, UK) containing: pancreatic digest of casein 10 g/l, casamino acids 1 g/l, yeast extract 5 g/l, sodium chloride 5 g/l, magnesium sulphate, anhydrous 0.98 g/l, was prepared according to manufacturer's instructions and bacteriological agar No. 1 (Oxoid) was added to a final concentration of 1.5% w/v, after autoclaving the medium was allowed to cool to 50°C and poured into Petri dishes.

2.1.4 NZCYM overlay agar

NZCYM overlay agar was prepared as above but to a final bacteriological agar No. 1 (Oxoid) to a final concentration of 0.6% w/v. The agar was melted in a microwave and tempered to 50°C and then aseptically dispensed into 5 ml aliquots in sterile glass universal tubes prior to use.

2.1.5 Nutrient broth No. 2

Nutrient Broth No.2 CM0067 (Oxoid) containing: 'Lab-Lemco' powder 10 g/l, peptone 10 g/l, sodium chloride 5 g/l, was prepared according to manufacturer's instructions by adding 25 g powder to 1 litre of RO water. After autoclaving, the medium was stored in at room temperature for a maximum of eight weeks.

2.1.6 Nutrient Agar (NA)

Nutrient Broth No.2 CM0067 (Oxoid) containing: 'Lab-Lemco' powder 1 g/l, yeast extract 2 g/l, peptone 5 g/l, sodium chloride 5 g/l and agar 15 g/l, was prepared according to manufacturer's instructions by adding 28g powder to 1 litre of RO water. After autoclaving this was cooled to 50°C and poured into Petri dishes.

2.1.7 Brain Heart Infusion agar (BHI)

Brain heart infusion agar CM1136 (Oxoid) containing brain infusion solids 12 g/l, beef heart infusion solids 5 g/l, proteose peptone 10 g/l, sodium chloride 5 g/l, glucose 2 g/l, disodium phosphate 2.5 g/l and agar 10 g/l was prepared according to manufacturer's instructions by adding 47 g powder to 1 litre of RO water. After autoclaving this was cooled to 50°C and poured into Petri dishes.

2.1.8 Mueller-Hinton (MH) agar

Mueller-Hinton (MH) agar CM0337 (Oxoid) containing: beef, dehydrated infusion from 300 g/l, casein hydrolysate 17.5 g/l, starch 1.5 g/l and agar 17 g/l, was prepared according to manufacturer's instructions by adding 38 g powder to 1 litre of RO water. After autoclaving, this was cooled to 50°C and poured into Petri dishes.

2.1.9 Maximum Recovery Diluent (MRD)

Maximum Recovery Diluent CM0733 (Oxoid) containing peptone 1.0 g/l, sodium chloride 8.5 g/l, was prepared according to manufacturer's instructions, by suspending 9.5 g powder in 1 litre RO water and the mixture autoclaved.

2.1.10 Phosphate Buffer Saline (PBS)

Phosphate buffered saline BR0014 (Oxoid) was prepared from Dulbecco `A' Tablets containing: NaCl 0.8% (w/v), KCl 0.02% (w/v), Na2HPO40.115% (w/v) and KH2PO40.02% (w/v). Solutions were prepared by dissolving one phosphate buffered saline tablet in 100 ml of RO water. PBS buffer was stored at room temperature for a maximum of 8 weeks.

2.1.11 Salt Magnesium (SM) buffer

Salt magnesium buffer was prepared by dissolving NaCl (Thermo Fisher) 5.8 g/l, $MgSO_4.7H_2O$ (Fisher Scientific) 2 g/l, tris (hydroxymethyl) amino methane (Sigma-Aldrich, Dorset, UK) 6 g/l and gelatine (Sigma-Aldrich, Dorset, UK) to a final concentration of 0.01% w/v in RO water. The pH was adjusted to 7.5 then

the buffer was autoclaved and stored at room temperature for a maximum of eight weeks.

2.1.12 Magnesium Sulphate Stock solution

A 1 M $MgSO_4.7H_2O$ (Fisher Scientific) stock solution was prepared in RO water and autoclaved. This was added to bacterial cultures at a working dilution of 10 mM, to isolate or propagate bacteriophage, in order to stabilise the phage.

2.1.13 Bacterial storage medium

Bacterial storage medium was prepared by mixed with nutrient broth No.2 (2.1.5) with glycerol to a final concentration of 20% v/v. this was followed by autoclaving, the bacterial storage medium was then aseptically aliquots in 1 ml in a 1.5 ml cryovial tube (Thermo Fisher Scientific) and stored at room temperature for a maximum of eight weeks.

2.2 Bacterial strains

Bacterial strains used in this thesis, listed in (**Table 2.1**) .

Table 2.1 Bacterial strains used in this thesis

Organism	Strain	Source
V. cucullus	5Lx	Cattle isolate / Laboratory stock
A. skirrowii	A2S6	Cattle isolate / Laboratory stock
C. jejuni	PT14	Human isolate / Laboratory stock
C. jejuni	NCTC 11168	Human isolate / NCTC
C. jejuni	81-176	Human isolate / Laboratory stock
C. coli	RM2228	Human isolate / Laboratory stock
C. coli	S9	Cattle isolate / Laboratory stock
C. ureolyticus	NCTC 10941	Human isolate / NCTC

2.3 Growth conditions

2.3.1 Microaerobic incubation

Different methods were used to generate microaerobic growth conditions according to the circumstances required. For most of the work described plates were incubated in 3.5 litre jars (Oxoid, Basingstoke, UK). A vacuum pump was used to reduce pressure was -22 psi and then the gas pressure was restored with a gas mix containing 5% v/v hydrogen, 85% v/v nitrogen and 10% v/v carbon dioxide (BOC limited, Surrey, UK). This procedure generated an atmosphere containing approximately 5.6% v/v oxygen, 3.6% v/v hydrogen, 7.3% v/v carbon dioxide and 83% v/v nitrogen. The jar was then placed in an incubator at the required temperature. For *Venatorbacter cucullus* and *C. ureolyticus* this was 37°C. For *A. skirrowii* the jars were incubated at 30°C. This method was also used to prepare jars for storage at 4°C. *Campylobacter jejuni* and *C. coli* isolates were 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) set at 42°C.

2.3.2 Aerobic incubation

Different temperatures were used to grow bacterial strains aerobically. For example, *A. skirrowii* was incubated at 30°C and *V. cucullus* at 37°C.

2.4 Storage

2.4.1 Long-term storage

For long-term storage at -80°C, bacterial strains were firstly grown under optimum conditions on BA (2.1.1). A sterile cotton swab was then used to collect the pure growth from the plates, which was dispersed into 1 ml aliquots of bacteriological storage medium (2.1.13) in 1.5 ml cryovial tubes. These were stored at -80°C until ready for use. The cryovial was thawed partially and approximately 10 µl of the frozen suspension removed to a blood agar plate using a sterile plastic loop. This was then incubated depending on the bacterial growth requirements. These stocks were revived regularly to check for viability and contamination.

2.4.2 Short-term storage

For short-term storage, a subculture was taken from the -80°C frozen stock to plate of BA (2.1.1). This was incubated at optimum conditions for each bacterium, which could then be stored at 4°C under ether microaerobic conditions for *Campylobacter* or aerobically for *A. skirrowii* for a maximum of four weeks.

2.5 Bacterial enumeration

All the bacteria described in this thesis were enumerated by a modification of the Miles Misra technique (Miles et al., 1938). Ten-fold serial dilutions from the bacterial suspension were prepared into PBS (2.1.10). Triplicate 10 µl aliquots of each dilution were dispensed on the surface of a mCCDA (2.1.2) or BA plate (2.1.1) containing 2 % agar. The plates were then allowed to dry in proximity of a Bunsen burner. Afterwards they were inverted and incubated under optimum conditions for each bacterial strain. The dilution that gave a number of colonies between 3

and 30, were counted and the average of the five spots was determined. The colony forming units per ml value (CFU/ml) was then calculated by multiplying the average number of colonies by the dilution factor and then by 100.

2.6 Confirmatory tests

Different primary tests were used to confirm the identity of the bacterial isolates described in this thesis.

2.6.1 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 10 µl of PBS (2.1.12) on a glass microscope slide (BDH Lab supplies, Leicestershire, UK) using a 10 µl inoculation loop. The bacterial suspension was air-dried followed by fixing with heat from 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. Then, the slide was immersed in 70% ethanol v/v (ThermoFisher) for one minute in order to decolourise the stained bacterial cells, followed by rinsing off excess ethanol with RO water. Finally, the 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 oil immersion using a light microscope at 100x magnification.

2.6.2 Oxidase test

Oxidase detection strips (MB02666; Oxoid) were used in this test. Each strip is impregnated with NNN'N' tetramethyl-p-phenylene-diamine dihydrochloride to detect the bacterial cytochrome oxidase enzyme activity. A single colony was removed by loop to oxidase detection strips, after 5 to 10 seconds the presence of the deep blue colour indicated a positive result and either light blue or no colour indicated a negative result.

2.6.3 Absence or present of aerobic growth

With all bacterial isolates, this test was carried out by taking a loopful of pure culture and using it to inoculate duplicate BA (2.1.1) plates. These were then incubated under aerobic and microaerobic conditions at the required temperature for example 30, 37, or 42°C. The growth was checked daily for up to 7 days.

2.7 Sample collection

Cattle slurry samples were collected from University of Nottingham - Dairy Centre located at Kingston on Soar, Nottingham LE12 5RY coordinates (52.839240, -1.249674). The slurry samples were collected in 50 ml sampling pots with aseptic technique and put into a sterile polythene bag then into a sampling box. The samples were then transferred immediately to the laboratory and kept at 4°C to be processed the next day.

2.8 Bacteria isolation from cattle slurry

A mix of 1 g cattle slurry sample and a 9 ml of maximum recovery diluent was prepared (2.1.9). This was considered as 10^{-1} dilution and serial ten-fold dilutions were made from this suspension. *Campylobacter* spp. were isolated by plating 100 µl from the 10^{-3} and 10^{-4} dilutions, in triplicate, on mCCDA supplemented with *Campylobacter* selective supplement (2.1.2). To isolate *Arcobacter* spp. 100 µl of the same dilutions were spread on mCCDA in triplicate but this time the media was supplemented with CAT (2.1.2). Plates were then incubated under microaerobic conditions (2.3.1) for 48 h at 42°C for *Campylobacter* spp. isolation work and at 30°C for *Arcobacter* spp. isolation. For both purposes, single colonies which had a typical *Campylobacter* or *Arcobacter* morphology on mCCDA media were sub-cultured on BA (2.1.1) and incubated under similar conditions before Gram stains of selected colonies were carried out to confirm typical *Campylobacter* and *Arcobacter* cell morphologies. Sub-culture was repeated for another four times to purify the single colony. Pure isolates were stored for the long term in bacterial storage media (2.1.13) at -80 °C in sterile 1.5 ml cryogenic tubes.

2.9 Confirming bacterial identity by molecular techniques

2.9.1 Genomic DNA extraction

All bacterial genomic DNA were isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions. In brief, cells from individual purified isolates were harvested from a quarter of an abundant lawn grown on BA plates (2.1.1) into 1.5 ml of PBS (2.1.10). The suspensions were then centrifuged at 12,000 x g for 2 minutes at room temperature using a Heraeus Pico 17 bench-top centrifuge. Next, the pellet was suspended in 180 µl of Lysis

Solution T (BB6678: composition confidential) and 20 µl of RNase A solution (R6148: composition confidential) were added followed by incubation at room temperature for 2 minutes. A 20 µl aliquot of 20 mg/ml Proteinase K (Sigma-Aldrich) stock solution was then added and the mixtures was incubated for 30 minutes at 55°C. Then, 200 µl of Lysis Solution C (B8803: composition confidential) was added to each sample and thoroughly vortexed for 15 seconds to achieve a homogenised mixture followed by a further incubation at 55°C for 10 minutes. Columns were prepared by adding 500 µl of Column Preparation Solution (C2112: composition confidential) then centrifuged in a Heraeus Pico 17 bench-top at 12,000 x g for 1 minute. The eluates were then discarded. An aliquot of 200 µl of absolute ethanol (Fisher Scientific) was added to each lysate samples and mixed well by vortexing for 5-10 seconds. This was then loaded onto the prepared column and centrifuged at 6,500 x g for 1 minute using a Heraeus Pico 17 bench-top centrifuge. The eluate and collection tube were both discarded, while all columns were placed into new 2 ml collection tubes. Next, 500µl of Wash Solution 1 (W0263: composition confidential) was added to the columns and all the tubes were again centrifuged for 1 minute at 6,500 x g. The collection tubes were discarded and replaced and 500 µl of Wash Solution 1 was added to the column for a second wash. The columns were then centrifuged at maximum speed of a Heraeus Pico 17 bench-top centrifuge for 3 minutes to dry the column. The collection tube was discarded and replaced. The DNA was eluted by carefully adding 200 µl of Elution Solution (B6803: 10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) directly to the centre of each column. Finally, tubes were then centrifuged at 6,500 x g for 1 minute in a Heraeus Pico 17 bench-top centrifuge in order to collect the eluted DNA. The genomic DNA samples were then stored at 4°C for short-term storage or -20°C for long-term storage.

2.9.2 Polymerase chain reaction (PCR)

2.9.2.1 Primers

The primers used to amplify the V3-V4 region of the bacterial 16S rRNA are listed in (**Table 2.2**).

Table 2.2 The primers used to amplify the V3-V4 region of bacterial 16S rRNA genes

Primer Name	Primer Sequence (5' to 3')	Tm (°C)	Amplicon size (bp)	References
314F	CCTACGGGNGGCWGCAG	61.2	~450-bp	(Hugerth et al., 2014)
805R	GGACTACHVGGGTWTCT	52.7	~450-bp	

2.9.2.2 PCR amplification of the 16S rRNA gene

The PCR reaction was conducted using DreamTaq Green 2x PCR Master Mix (applied Biosystems, 850 Lincoln Centre Drive, Foster City, California,USA) (ThermoFisher) in a BIOER XP thermal cycler machine. A Master Mix containing 2 U of DreamTaq DNA polymerase, 2x DreamTaq Green buffer, 0.4 mM of dNTP each and 4 mM of magnesium chloride along with two dyes (blue/yellow) to monitor electrophoresis progress was prepared. The reaction was prepared by adding 12.5 µl of PCR master mix with 30- 50 ng of template DNA, 10 pmol of forward and reverse primer in a final volume of 25 µl, RNAase free water was used during preparation of the PCR mix. The reaction was carried out using the following parameters: initial denaturation at 95°C for five minutes; the PCR cycle, starting from amplification including denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 2 minutes. This cycle was repeated 35 times. A final extension period at 72°C for ten minutes completed the reaction. Amplified PCR products were verified by agarose gel electrophoresis (2.9.3.1), and then stored at either 4°C for short term or -20°C for long term storage.

2.9.2.3 Cleaning PCR products

A Wizard SV Gel and PCR clean up system from Promega (2800 Woods Hollow Road · Madison, WI 53711-5399 USA) was used to remove debris, extcess nucleotides and primers. An equal volume of membrane binding solution was added to the PCR product. This mixture was transferred into the SV minicolumn. Prior to the centrifuge, 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 solvent was discarded and 700 µl of Membrane Wash Solution was added onto the SV minicolumn. Again, the column was centrifuged in a Biofuge Pico bench top centrifuge (Hettich Lab Technology, North America, USA) at 13,000 x g for one minute and the flow through was discarded. A second 500 µl 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 of Wash Solution was removed and the column was centrifuged in a Biofuge Pico bench top centrifuge at 13,000 x g, with open lids to evaporate ethanol residual which can interfere with the samples. The SV minicolumn was then transferred into a new 1.5 ml fresh Eppendorf tube and 50 µl of nuclease free water was applied to the column. The column was incubated at room temperature for one minute to elute the DNA sample. This was followed by centrifuging in a Biofuge Pico bench top centrifuge (Hettich Lab Technology, North America, USA) at 13,000 x g for one minute. The eluate contained the pure PCR product. The DNA concentration was measured using a Nano-drop spectrophotometer. Purified PCR products were stored at either 4°C for short term or -20°C for long term storage.

2.9.3 Gel electrophoresis

2.9.3.1 Agarose gel electrophoresis

DNAs extracted from bacterial isolates were routinely analysed by agarose gel electrophoresis (0.8% agarose) unless otherwise stated. Briefly, the agarose gel was prepared by adding 0.8% w/v agarose (Invitrogen) to 50 ml of 1x TAE buffer (40 mM tris-acetate adjusted with glacial acetic acid to pH 8, 1 mM disodium ethylene diamine tetra acetic acid) in a 250 ml sterile conical flask. The mixture was melted in a microwave and allowed to cool to approximately 50°C. Ethidium bromide (Fisher Scientific, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG) was added at a final concentration of 0.4 µg/ml and mixed by gently swirling the flask. The mixture was then poured into a gel casting tray with an appropriately sized comb. The gel was allowed to set at room temperature for approximately 35 minutes. Carefully, the end rubbers and comb were removed from the casting tray. The casting tray was then placed in a gel electrophoresis tank containing TAE buffer, and DNA samples mixed with 1 x blue/ orange loading dye (Promega) were loaded into the wells. A 100 bp DNA ladder (Promega,2800 Woods Hollow Rd., Madison, Wisconsin, USA) was also loaded to estimate the size of DNA samples. A negative control was also loaded in to one of the gel wells to check for contamination. The gel was run at 75 V for approximately 50 minutes until the bromophenol blue dye reached more than 1/2 length of the gel. Finally, gels were visualised under UV light using a Gel Doc XR+ System (Bio-Rad, Hertfordshire, UK).

2.9.3.2 Purification of amplified 16S rRNA gene sequences from agarose gels

PCR amplification products were separated by agarose gel electrophoresis and visualised using a long-wavelength UV transilluminator. The fragments were excised from the gel using a sterile scalpel and transferred into a pre-weighed Eppendorf tube. The weight of the excised gel slice was determined using a digital balance and the amplified DNA extracted from gel slice using wizard SV Gel and PCR clean up system (section 2.9.2.3). After estimating the gel weight, membrane binding solution was added at the ratio of 10 µl of solution per 10 mg of agarose gel. This was then incubated at 60°C until the gel was completely dissolved. Next, melted gel mixture was transferred SV minicolumn. The DNA samples were then purified as described in (2.9.2.3) and stored at 4°C for a short term or -20°C for long term storage.

2.10 DNA sequencing and analysis

2.10.1 Dye-terminator DNA sequencing

Purified PCR products were sequenced using dye-terminator chemistry with custom primers (Eurofins Value Read Service, Germany). PCR amplified 16s rRNA gene sequences were compared with other 16s rRNA gene sequences using the BLAST-N algorithm to search the 16 rRNA database (Camacho et al., 2009).

2.10.2 Genome sequencing of bacterial isolates

Genomic DNA from *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* S12 were isolated as described previously (2.9.1). DNA sequencing were performed using

the Illumina MiSeq platform (The University of Nottingham, Sutton Bonington Campus, UK). Sequence libraries were prepared following the Illumina Nextera™ tagmentation protocol (Illumina, Cambridge, UK). The libraries were sequenced using the v3 Illumina cassette. The data consisted of 3-4 million 100-250 bp paired-end sequence reads. Initial processing of the raw data and *de novo* assembly was performed using CLC Genomic Workbench v. 11.0.1 (Qiagen, Aahus, Denmark) by Prof. I. Connerton.

2.10.3 Annotation of *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* S12 isolates

The complete genome of *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* S12 were annotated automatically using the program Prokka version 1.07 (Seemann, 2014) running on a virtual unix machine (unbuntu). Frame shifts or any errors in the sequence assembly were confirmed using a combination of translated Basic Local Alignment Search Tool (BLAST; (Camacho et al., 2009), to observe protein translations for each nucleotide sequence and Artemis (Rutherford et al., 2000), to examine the six reading frames and determine which bases were responsible for the frame shifts. Once all potential frameshifts were confirmed the complete genome of *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* S12 were deposited in GenBank under the accession number CP034309.1, CP040239.1and CP040464.1 respectively. Genome comparisons were made between the genome sequences of *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* S12 and the database genomes of representative bacterial strains using mVISTA (Frazer et al., 2004).

2.11 Transmission electron microscopy

The transmission electron microscopy (TEM) was performed for some of the bacterial isolates discussed in this thesis. The TEM technique used included two staining steps. The first step was sample fixing, which aimed to render the bacteria non-viable and stabilise the bacterial cell wall. This involved adding a loopful of bacterial culture to 600 µl of fixative solution in 1.5 ml Eppendorf tube, which has been prepared in advance through mixing 2.5 ml 0.2 M cacodylate buffer (pH 7.4), 1.9 ml distilled water into 600 µl 25% v/v EM glutaraldehyde. The suspension was then incubated at room temperature for 1 hour. The tube was then centrifuged using a Biofuge Pico bench top centrifuge at room temperature at 10,000 x g for 1 minute. Using a sterile 1 ml tip, the supernatant was removed to a disposable tube under fume hood section. An aliquot of 1 ml of 0.1 M cacodylate buffer was added without disturbing the pellet to wash the pellet then incubated for 10 minutes at room temperature and then centrifuged at room temperature at 10,000 x g for one minute. The supernatant was discarded, and the pellet was then resuspended into 700 µl of 0.1 M cacodylate buffer twice. The sample was then stored at 4°C until required. The second step was staining. An aliquot of 13 µl of fixed bacterial suspension was transferred onto the formvar carbon film on copper 200 mesh grid (Emgrid Australia Pty Ltd PO Box 118, The Patch Vic 3792, Australia) and incubated at room temperature for 5 minutes. The suspension was removed by using filter paper and 13 µl of 2% w/v uranyl acetate was added onto the grids for 30 seconds. After staining, uranyl acetate was removed by using filter paper Whatman No.1 (Camlab Ltd, Unit 24, Norman Way Industrial Estate, Over Cambridge, CB24 5WE, United Kingdom). The sample was then washed twice to improve the image quality by adding 13 µl of distilled water to the grid, then removing using filter paper. The grid was then ready to be examined by TEM.

2.12 Statistical analysis

For statistical analysis, the one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of two or more independent (unrelated) groups. The (ANOVA) was performed using Microsoft Excel 2016.

Chapter Three

Survival of *Campylobacter* spp. and bacteriophage in milk

3.1 Introduction

Milk is a nutritious liquid secreted by female mammals for the sustenance of their young, it consists of an emulsion of fat in an aqueous solution of protein, lactose and inorganic salts (Bianchini et al., 2014). Milk from cows' forms a major part of the human diet in many countries, however, is an excellent medium for bacterial growth and can therefore be a source of pathogens (Robinson et al., 2014). Heat treatment known as pasteurisation has done much to make milk a safe product but occasional failure of the process (Fernandes et al., 2015), or the desire of people to consume raw milk can lead to large outbreaks of diarrhoeal and other illnesses for example those caused by *Campylobacter spp. Campylobacter* spp. detected in milk or in milk filters include: *C. jejuni*, *C. hyointestinalis* ssp. *hyointestinalis*, *C. concisus*, *C. fetus* ssp. *fetus*, *C. coli* and *C. lari* (Bianchini et al., 2014; Del Collo et al., 2017). Most cases of Campylobacterosis associated with milk consumption are caused by *C. jejuni* (Robinson et al., 2014). ISO 10272 provides general guidance for the preparation of test samples for milk and milk products (https://www.iso.org/obp/ui/#iso:std:iso:10272:-1:ed-2:v1:en), formulated in response to the recognition that milk is a source of *Campylobacter*. The advent of molecular techniques has meant that the more unusual species, that cannot be detected by routine culture, have been identified in cattle stool samples and in milk, one of these species is *C. ureolyticus* that is a known gastrointestinal pathogen, and for which a source had not previously been identified (Koziel et al., 2012). Little research has been carried out on the survival of *Campylobacter* in milk since the 1980s and none reported on the more recently described species such as *C. ureolyticus*. In addition to the role of milk in the diet, it can also be used as protective medium for preserving bacteria and bacteriophage when freeze drying. The ability of *Campylobacter* bacteriophage survival in milk still not fully understood, then the work in this chapter will clarify bacteriophage survival in milk. Milk is also a potential medium for administering bacteriophage in phage therapy

applications, ensuring stability and protecting phage particles from acid and digestive enzymes. It is unknown whether immunoglobulins, proteins lipids or other components of milk might inactivate the phage or inhibit their ability to bind their target bacteria. To answer these questions, basic survival tests on bacteriophage in milk before further research can be carried out.

3.2 Material and Methods

3.2.1 *Campylobacter* recovery experiments

3.2.1.1 Preparation of *Campylobacter jejuni* PT14 suspension

Campylobacter jejuni PT14 was grown (section 2.3.1) as confluent lawns on BA plates (section 2.1.1) and incubated at 42°C under microaerobic conditions. Three individual samples were prepared by suspending one half of the lawn into 10 mM $MgSO_4$ (w/v) from 1 M stock (section 2.1.12) to give a suspension of approximately 10^8 CFU/ml. The optical density at 600 nm was taken to standardise the suspensions and the actual viable count was determined by serial dilution in MRD (section 2.1.9) by the Miles and Misera method (section 2.5). The diluted samples were applied as triplicate 10 µl aliquots onto mCCDA agar (section 2.1.2), allowed to dry and incubated under microaerobic conditions.

3.2.1.2 Preparation and inoculation of UHT whole milk with *Campylobacter*

The pH of 3 individuals 500 ml UHT whole milk cartons with valid expiry dates, were adjusted to be between 6.53 ± 0.2 to 7.45 ± 0.04, by adding 500 µl of 10 M NaOH solution, to each. UHT milk was chosen for these experiments to avoid variability of indigenous flora. The *Campylobacter* suspension (section 3.1.1), was used to inoculate 25 ml volumes of the pH adjusted milk. Tenfold serial dilutions were performed in the pH adjusted milk, with the highest dilution containing approximately 1 cell/ml. A negative control with no *Campylobacter* added was also prepared. The dilutions containing approximately, 0, 10^3, 10^2, 10^1 and 1 CFU/ml were used for the experiment. One ml of milk from each of the samples was removed to marked sterile glass universal tube for initial viable count (section 3.2.1).

3.2.1.3 Enumeration of *Campylobacter* in different fractions of UHT milk

The remaining 24 ml of the milk samples from (section 3.2.2) were centrifuged at 20,000 x g for 40 min at 4°C in Oakridge tubes. The fat layer from each tube was removed and collected into marked sterile glass universal tubes (weighed) using a sterile spatula. This was resuspended in 5 ml PBS (section 2.1.10). One ml from the remaining supernatant was collected to marked sterile glass universal tubes. The rest of the supernatant was discarded. The pellets were re-suspended in 5 ml MRD (section 2.1.9) and transferred to marked sterile glass universal tubes with a further 5 ml of MRD being used to collect any remaining pellet. All layer partitions were stored microaerobically at 4°C, until used in the experiment. The viable count of *Campylobacter* from each of the milk partitions were determined using the Miles and Misra technique (section 2.5). For each dilution of *Campylobacter* there were three biological replicates and three technical replicates.

3.2.2 *Campylobacter* survival in UHT milk

Survival of *Campylobacter* inoculated in milk and MRD samples were studied under different parameters. During the experiments, samples were stored under different conditions. First, microaerobic conditions at 4°C. Second, aerobic conditions at 4°C. Finally, aerobic conditions at room temperature. All time point samples were collected and the viable counts tested at 8 hours, accept for the aerobic treatment condition at room temperature, where samples were collected and processed at 30 minute intervals for the first and second time point samples then samples were collected after 1 hour for the last three samples.

3.2.2.1 Preparation of *Campylobacter jejuni* PT14 suspension

Three different suspensions of *C. jejuni* PT 14 were prepared as biological replicates as described previously in 3.2.1.

3.2.2.2 UHT whole milk and MRD samples preparations:

Three 10 ml milk samples contained approximately 10^6 CFU/ml present three biological replicates were prepared from 500 ml Tesco UHT Whole Milk. The pH and the room temperature were recorded. Three parallel MRD replicates were prepared from the same suspension. The negative control treatment was also prepared for both milk and MRD (section 2.1.9). A 20 µl sample for each time point was removed (0, 8, 16, 24, and 48 hours) for micraerobic conditions. Samples stored aerobically were collected at 0, 30, 60, 120, 180 and 360 min, and the viable counts determined (section 2.2.2). The inoculated Petri dishes were incubated under microaerobic and aerobic conditions at 42°C, for 48 h. Three biological replicates and three technical replicates were obtained using 96-well microtitre plates with the Miles and Misa technique (section 2.5) used for enumeration.

3.2.3 The heat resistance of campylobacters during pasteurisation

3.2.3.1 Preparation the *Campylobacter* jejuni (PT14) suspension

Preparation the *C. jejuni* PT14 suspension was carried out as described in (section 2.2.1).

3.2.3.2 UHT whole milk sample preparation and pasteurisation

Three 10 ml milk samples contained approximately 10^8 CFU/ml to represent three biological replicates were prepared from 500 ml Tesco UHT Whole Milk. The pH and the room temperature were recorded. Three MRD replicates were prepared and the same suspension was used each time. The negative control treatment was also prepared for both milk and MRD (section 2.1.9). Then, 1.3 ml from the milk and MRD replicates were removed to 1.5 ml marked sterile glass vials. Before the heat treatment 200 µl of milk and MRD were moved from replicates as 0 time point samples and used to obtain the viable count as described above. The pasteurisation was complete when the temperature reached 72°C for 25 seconds and was stable. After that the samples were cooled down to the 4°C. The initial temperature (4°C), heating time to 72°C and cooling time from 72°C to 4°C were recorded. After pasteurisation, samples were taken from milk and MRD samples, to enumerate *Campylobacter*. The inoculated Petri dishes were incubated under microaerobic and aerobic conditions at 42°C, for 48 h. Three biological replicates and three technical replicates were obtained using 96 microplates with the Miles and Misa technique (section 2.5) used for enumeration.

3.2.4 *Campylobacter* bacteriophage CP30A recovery experiments

3.2.4.1 Bacteriophage propagation

Propagation of bacteriophage was carried out using the full plate lysis method. Prior to the propagation, an overnight growth of *C. jejuni* PT14 as a host was swabbed from a BA (section 2.1.1) plate into sterile 10 mM magnesium sulphate solution was prepared from 1 M stock solution (section 2.1.12) to contain approximately 8 $\log_{10}$ CFU/ml and subsequently divided into 500 µl aliquots. This

was mixed with 100 µl of CP30A bacteriophage at approximately 7 log_{10} PFU/ml and incubated at room temperature for 10 minutes for the phage to absorb to the bacteria. The top layer agar (section 2.1.4) was melted in a microwave, dispensed in 5 ml aliquots in sterile universal bottles and tempered to 50°C in a water bath. The *Campylobacter* and bacteriophage mixed suspension was then transferred into 5 ml of melted NZCYM overlay agar and thoroughly mixed. This was poured onto the surface of a NZCYM agar plate (section 2.1.3) and allowed to set. The plates were then incubated at 42°C under microaerobic conditions, overnight. The propagated bacteriophage was harvested with 5 ml of SM buffer (section 2.1.11), dispensed onto the surface of the plate, and incubated at 4°C on a gyratory shaker with 60 cycles per minute, overnight. After incubation, SM buffer containing bacteriophage was recovered, and bacterial cells and cell debris were removed by filtering the sample through a 0.2 µm filter. Finally, the filtered bacteriophage sample was stored at 4°C in sterile plastic universals.

3.2.4.2 Efficiency of CP30A plaquing (EOP)

Bacteriophages were enumerated by determining their efficiency of plaquing against their host strain. Lawns of host *Campylobacter* strains were prepared (section 3.2.4.1). but without addition of bacteriophage. Instead the bacteriophage suspension was ten-fold serially diluted with SM buffer down to a dilution factor of 10^{-8}, and 10 µl 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 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 following equation.

PFU/ml = number of plaques/ dilution factor/ volume of sample

3.2.5 UHT whole milk samples preparations

Suspensions of 10-fold diluted bacteriophage CP30A between 10^8, 10^7, 10^6 PFU/ml were used to inoculate 25 ml of UHT whole milk in 40 ml Oakridge tubes. To validate the results, three biological replicates for each bacteriophage concentration were prepared. A negative control was also prepared with 25 ml of UHT whole milk with no phage. The samples were then centrifuged at 10000 x g for 10 min and the supernatant used for bacteriophage enumeration. The efficiency of CP30A plaquing assay was used to enumerate (section 3.4.2.2).

3.2.6 *Campylobacter ureolyticus*

3.2.6.1 *C. ureolyticus* growth

Campylobacter ureolyticus NCTC 10941 was grown in the same way as *C. jejuni* PT14 (section 3.3.1.1) but incubated at 37°C instead of 42°C.

3.2.6.2 *C. ureolyticus* motility

Motility tests were performed on fresh *C. ureolyticus* and *C. jejuni* PT14 to confirm that *C. ureolyticus* was not motile (O'Donovan et al. 2014). Cultures of each were grown on BA at 37°C under microaerobic conditions. A semi-solid motility agar plate made from Mueller-Hinton broth (section 2.1.8) contained 0.4 % agar No. 1 (Oxoid) was inoculated with the culture using a sterilised pipette tip to stab the centre of the plate. Three replicates for *C. ureolyticus* NCTC 10941 and *C. jejuni* PT14 were prepared, and one plate with no cells as negative control. The plates were incubated at 37°C under microaerobic conditions for 72 hours and then the diameter of the motility halo was measured. This confirmed that *C. ureolyticus* NCTC 10941 (no halo) was indeed non motile whilst *C. jejuni* PT14 was fully motile (large halo).

3.3 Results

3.3.1 *Campylobacter* survival in UHT milk

3.3.1.1 *Campylobacter* recovery experiments

This experiment was performed to validate the methods that were used to isolate *Campylobacter* from milk samples. Statistically significant differences ($p < 0.05$) were assessed using a one-way single factor (ANOVA) (section 2.12). The average *Campylobacter* counts (log_{10} CFU/ml) recovered immediately after addition of *Campylobacter* cells (Table 3.1). *Campylobacter ureolyticus* NCTC 10941, *C. jejuni* NCTC 11168 and *C. coli* RM2228 showed no significant changes in viable counts (p=0.1, 0.3 and 0.3, respectively), between the number of viable *Campylobacter* cells added, compared to the number of the cells that were recovered. In contrast, *C. jejuni* PT14 and *C. jejuni* 81-176 showed significant differences (p=0.0004 and 0.003, respectively), between viable counts before and after addition to milk.

Table 3.1 Recovery of *Campylobacter* strains after addition to UHT milk

Campylobacter	**Average *log₁₀* CFU/ml number added to milk**	**Average log_{10} CFU/ml number recovered from milk**
C. jejuni PT 14	2.64(0.03)*	2.43(0.00)*
C. jejuni NCTC 81-176	2.97(0.05)*	2.67(0.06)*
C. jejuni NCTC 11168	2.66(0.12)*	2.56(0.10)*
C. coli RM2228	2.72(0.20)*	2.53(0.15)*
C. ureolyticus NCTC 10941	2.71(0.04)*	2.62(0.05)*

***The standard deviation**

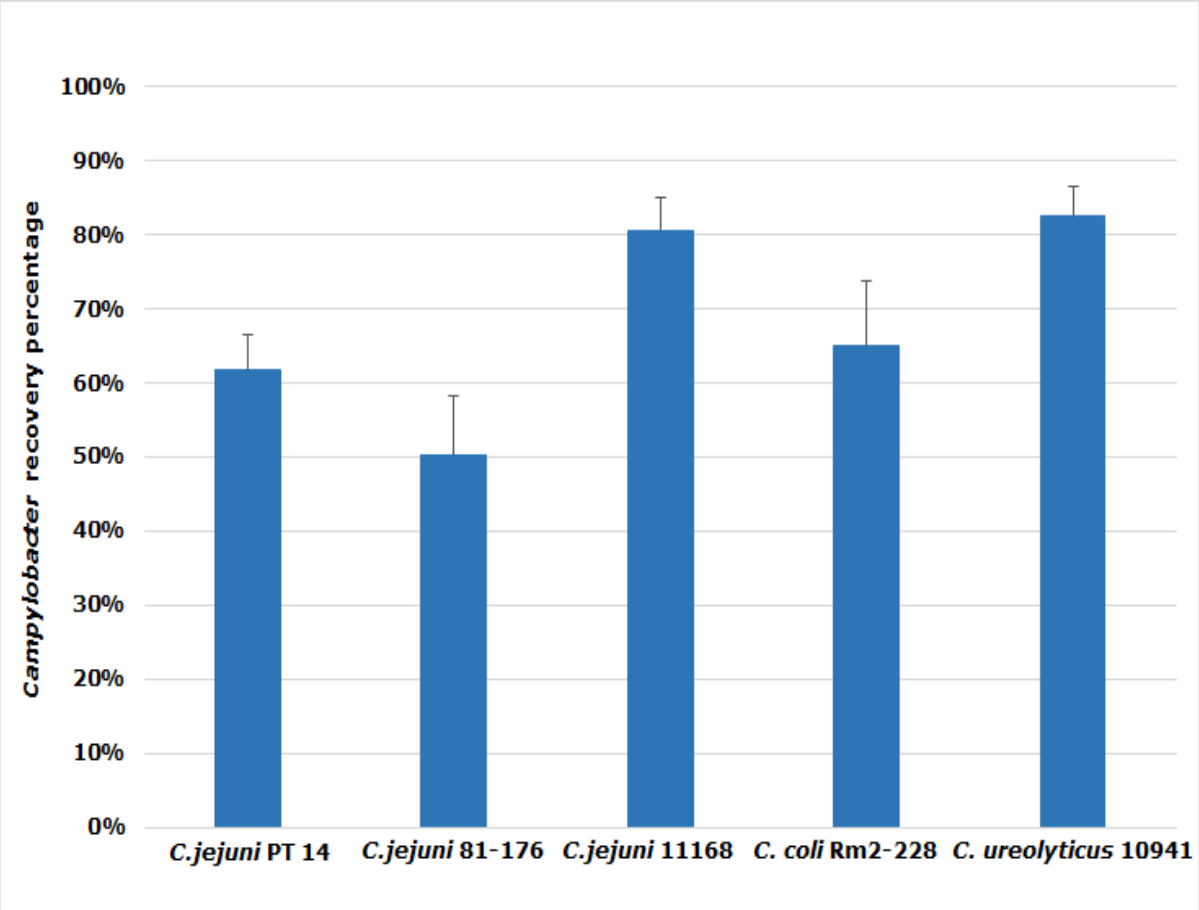


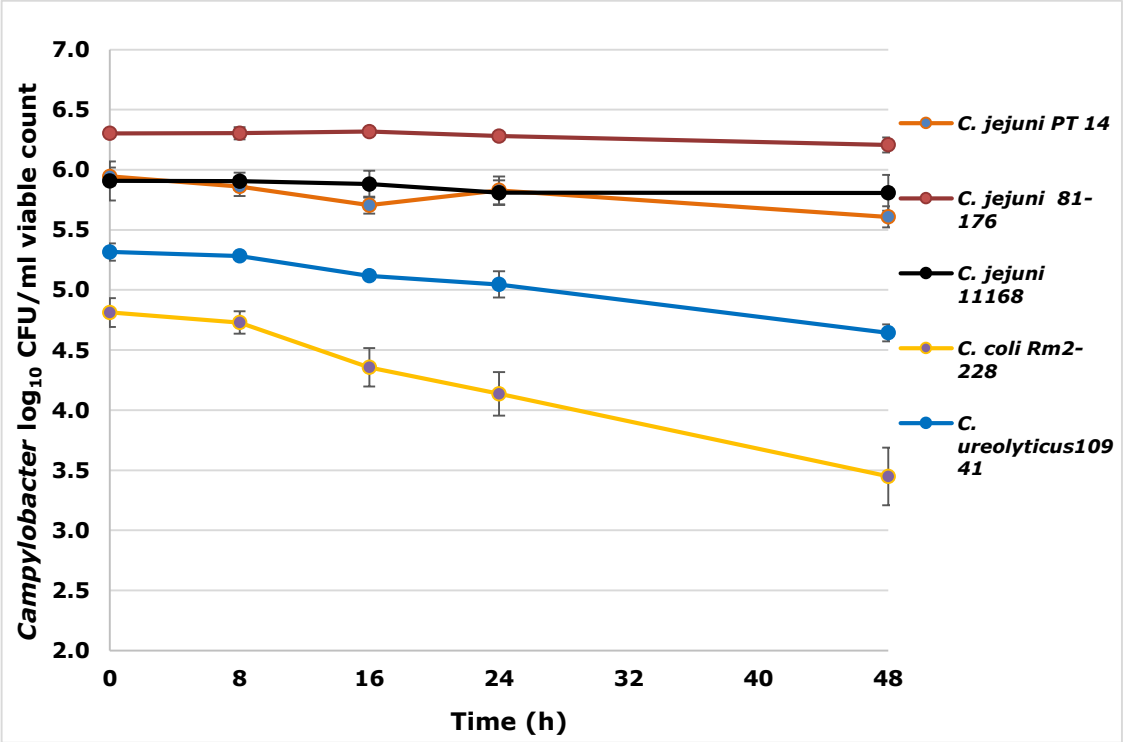
Figure 3.1 The percentage recovery of *Campylobacter* inoculated into milk

Despite the significant loss of viable *Campylobacter* cells following addition to milk and recovery for C. *jejuni* PT14 and *C. jejuni* 81-176, greater than 50% recovery was considered acceptable for the purpose of these experiments.

3.3.1.2 Survival of *Campylobacter* in UHT milk under microaerobic conditions at 4°C

For the first experiment, triplicate 10 ml of UHT milk samples, together with triplicate MRD controls, were inoculated with *Campylobacter* cells and incubated under microaerobic conditions, at 4°C. The survival of *Campylobacter* under these conditions (Figure 3.2) A for inoculated UHT milk and (Figure 3.2) B for inoculated MRD control.

A)



B)

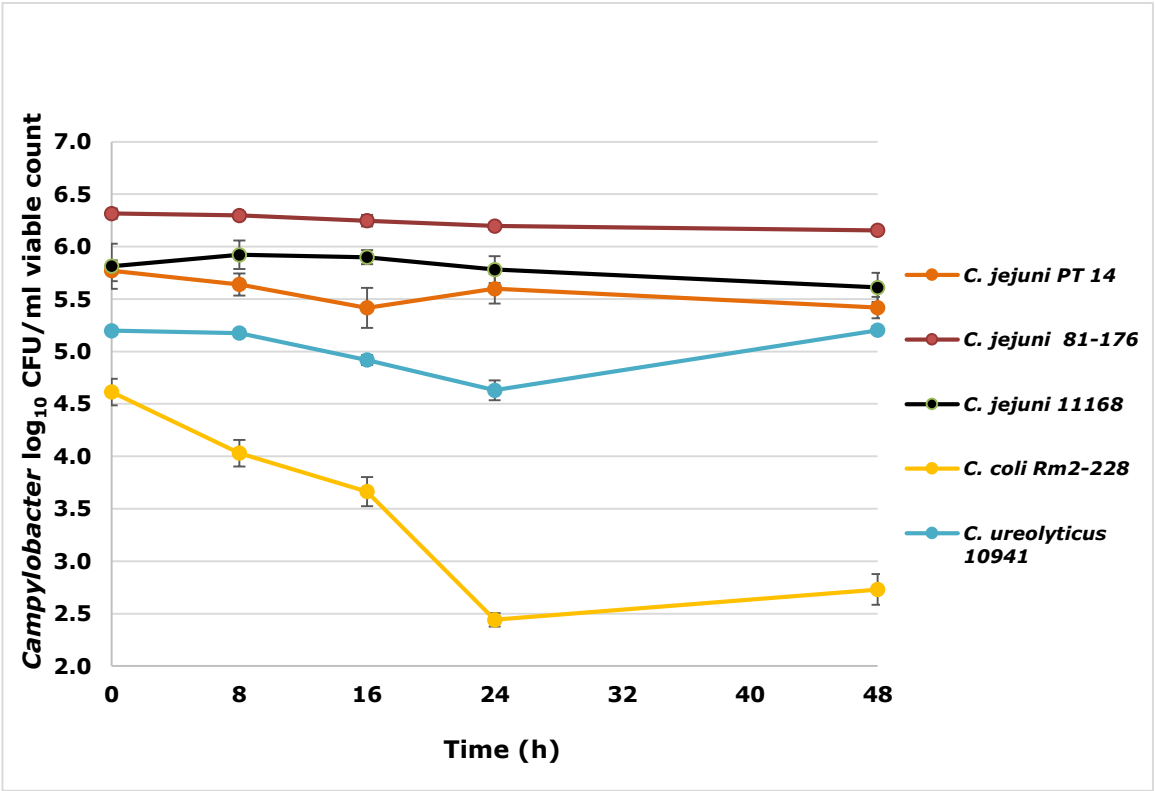


Figure 3.2 *Campylobacter* survival in milk under microaerobic conditions at 4°C (A Inoculated UHT Milk) (B Inoculated MRD control)

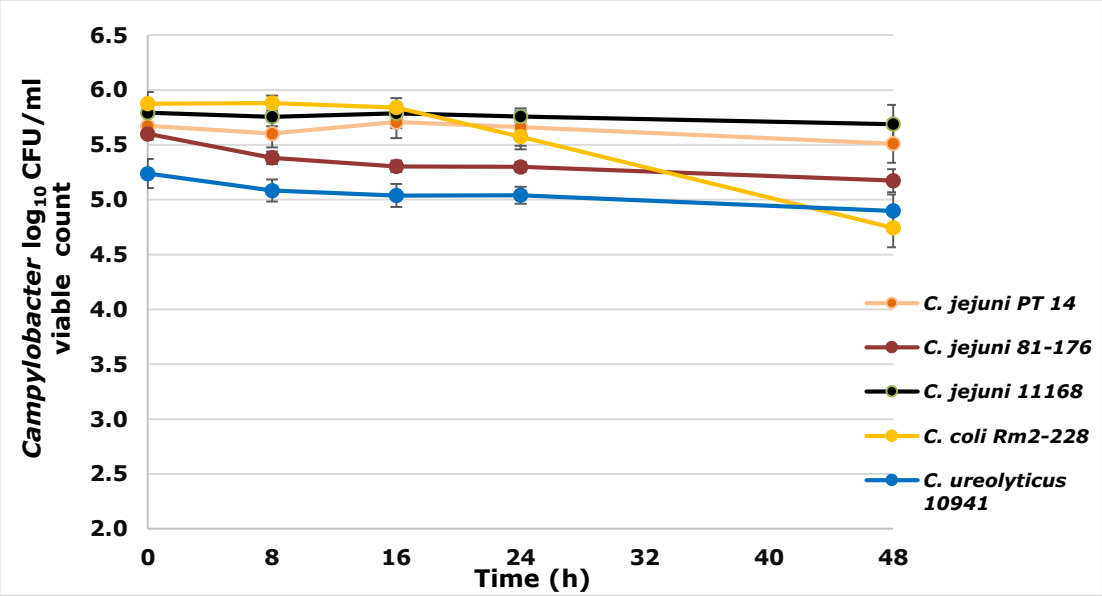
Experimental results (Figure 3.2) A Showed that the different *Campylobacter* strains and species varied in their ability to survive in milk under microaerophilic conditions at 4°C. Statistically significant differences ($P < 0.05$) were assessed using a one way single factor (ANOVA) (section 2.12). The *C. coli* RM2228 and *C. ureolyticus* 10941 strains, showed the greatest decline in average $\log_{10}$ viable counts from 4.81 and 5.32 at 0 h to 3.45 and 4.64 at 48h respectively following incubation in milk ($P = 0.0009$ and 0.0003 respectively). In contrast, two *C. jejuni* strains (81-176 and 11168) showed no significant difference in viability between 0 and 48 h ($P= 0.06$ and $P= 0.47$). The third *C. jejuni* strain (PT14), showed a statistically significant ($P=0.007$) reduction in viability, in UHT milk, over 48 h. Comparison of inoculated milk to MRD inoculated controls under the same conditions, showed a similar pattern of survival over the 48 h of incubation with the exception of *C. coli* RM2228, which declined by approximately 2 $\log_{10}$ CFU/ml over the first 24 h in MRD and viability was significantly different between 0 and 48 h ($P=0.0001$). For *C. ureolyticus* 10941, the viable count between 0 and 48 h did not show any significant difference ($P=0.82$). Two of the *C. jejuni* strains (PT14 and 81-176) showed a significant difference between 0 and 48 h in MRD ($P=0.01$ and 0.007 respectively) whilst the third *C. jejuni* (11168) showed no difference in viability ($P= 0.24$).

Statistical analysis of the UHT milk and MRD *Campylobacter* counts in the 48 h samples from each strain showed that there was significant difference for *C. jejuni* PT14, *C. jejuni* 81-176 and *C. jejuni* 11168 ($P=0.07$, 0.24 and 0.16). In contrast, *C. coli* RM2228 showed a significant reduction ($P=0.01$), in MRD, compared to milk after incubation for 48 h under microaerobic conditions at 4 °C. Conversely the *C. ureolyticus* 10941 strain exhibited a significant decrease ($P=0.0003$) in viable count in UHT milk compared to MRD under the same conditions.

3.3.1.3 Survival of *Campylobacter* in UHT milk under aerobic conditions at 4°C

The second experiment was similar to the first except that the samples were incubated under aerobic conditions and the results in (Figure 3.3) A and B.

A)



B)

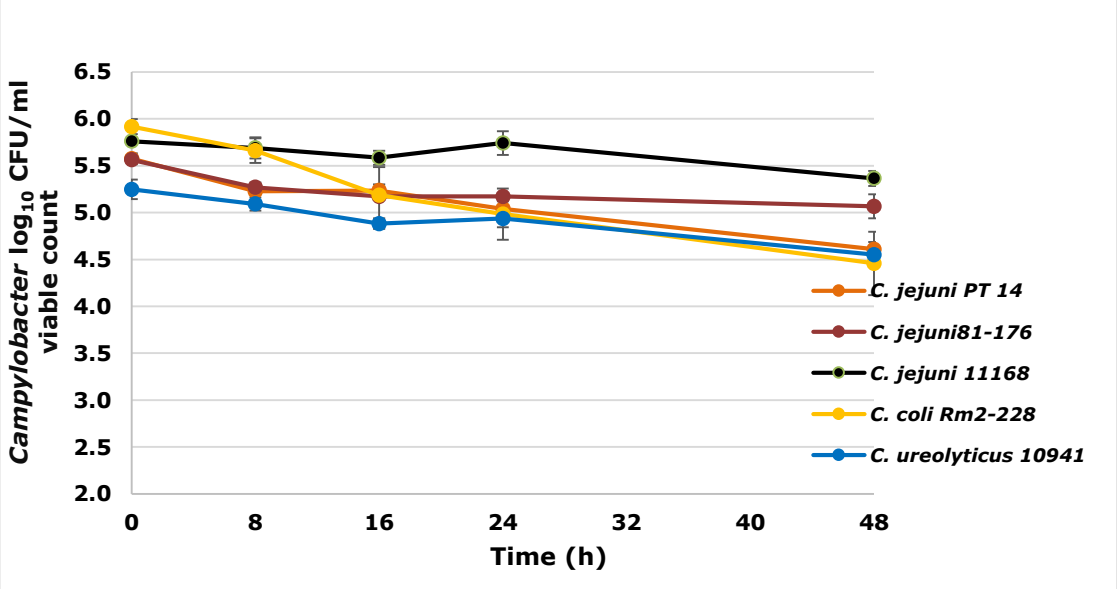


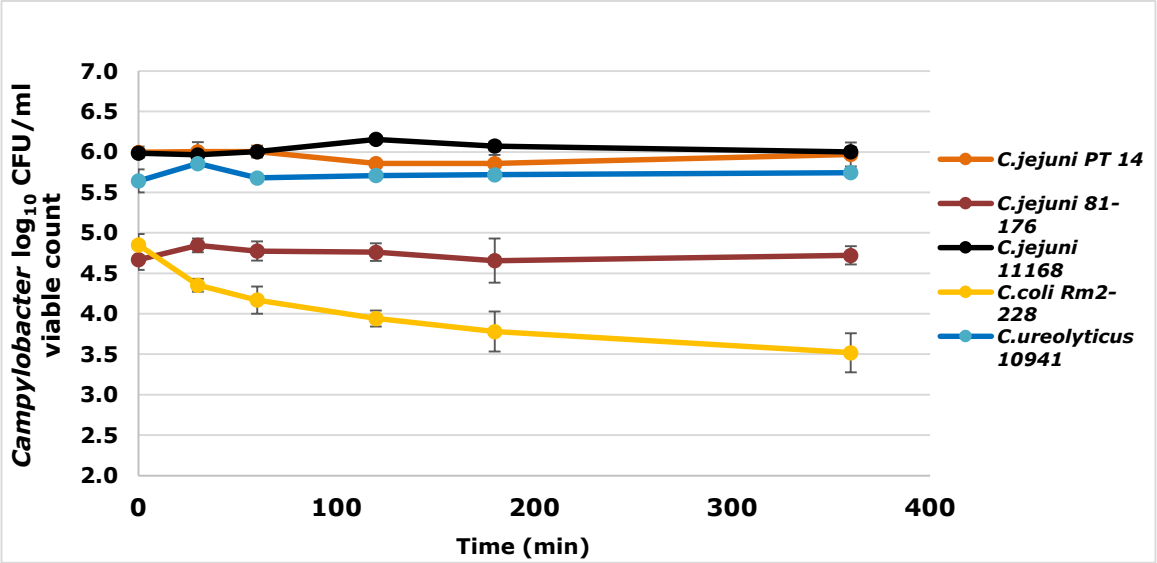
Figure 3.3 *Campylobacter* survival in milk and MRD under aerobic conditions at 4°C (A) Inoculated UHT milk (B) Inoculated MRD control

Two of the *C. jejuni* strains (PT14 and 11168) showed significant difference in viability between 0 and 48 h (*P*= 0.22 and 0.043 respectively). The third *C. jejuni* strain (81-176) and the *C. ureolyticus* strain showed only moderate differences following incubation in milk from an average $\log_{10}$ count of 5.6 and 5.24 at 0 h to 5.17 and 4.9 at 48 h respectively. However, a statistically significant reduction (*P*=0.003) was observed when *C. ureolyticus* was inoculated in UHT milk and incubated aerobically for 48 h at 4°C. In contrast, *C. coli* RM2228, exhibited a much greater reduction in viability of > 1 $\log_{10}$ CFU/ml, between 16 and 48 h (*P*= 0.001). Comparison of the UHT milk aerobic survival with MRD control aerobic survival, revealed that all the strains declined more in MRD than in UHT milk, with *C. jejuni* PT14 and C. *ureolyticus* showing the greatest difference. All were significantly different between UHT milk and MRD after 48 h incubation (*C. jejuni* PT14 *P*= 0.0001, *C. jejuni* 11168 *P*= 0.001, *C. coli* RM2228 *P*= 0.002, *C. jejuni* 81-176 *P*= 0.003 and C. *ureolyticus P*=0.0004).

3.3.1.4 Survival of *Campylobacter* in UHT milk under microaerobic conditions at 23 ± 0.5°C

The third experiment was similar to the second using aerobic conditions, but the incubation temperature was raised to 23 ± 0.5°C. The results are presented in (Figure 3.4) A and B.

A)



B)

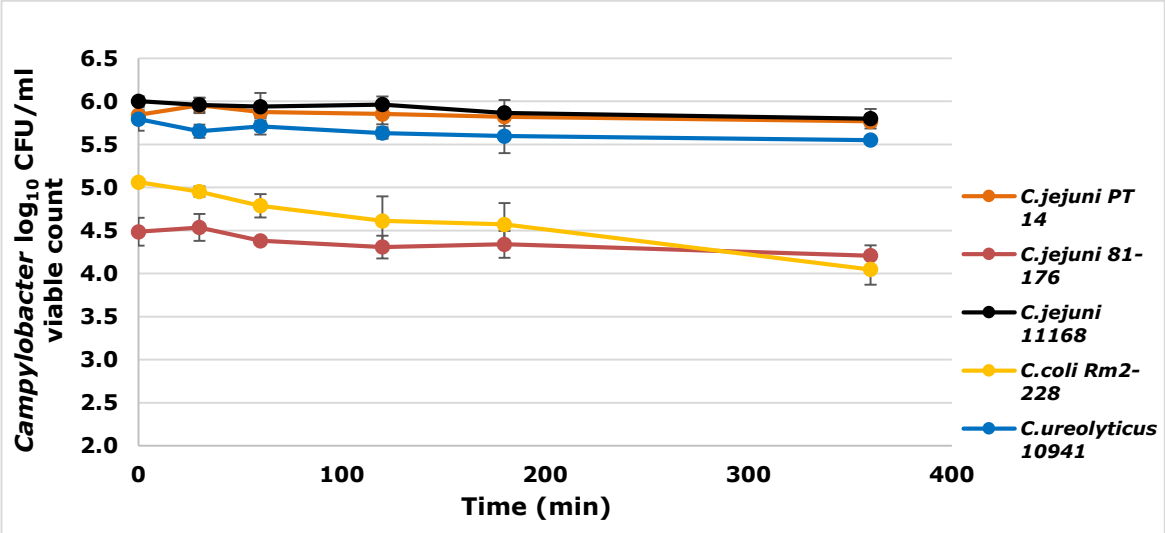


Figure 3.4 *Campylobacter* survival in milk and MRD under aerobic conditions at 23 ± 0.5°C (A Inoculated UHT milk) (B Inoculated MRD control)

In UHT milk, only *C. coli* RM2228 showed a rapid reduction in viable count of cells after 360 min of incubation, under aerobic conditions at 23 ± 0.5°C. A similar pattern was observed for the inoculated MRD samples with *C. coli* RM2228 declining the most and the other strains being relatively stable over the incubation period.

Finally, the comparison between incubation in UHT milk and incubation in MRD showed no significant difference (*P*=0.93 and 0.051 respectively) for both *C. jejuni* PT14 and 11168 from an of 6.0 and 5.64 at 0 minutes. There was also no significant difference in the average $\log_{10}$ count before and after the 360 minutes incubation period at 23°C (5.97 and 5.74 respectively). In contrast, *C. coli* RM2228 showed a more rapid decline in UHT milk than MRD (*P*=0.037). Conversely viable counts of *C. jejuni* 81-176, and *C. ureolyticus* 10941 were significantly higher (*P*=0.007 and 0.001 respectively) in UHT milk than MRD.

3.3.2 The heat resistance of campylobacters during pasteurisation

Heat treatment of 1 ml of *C. jejuni* PT14 suspensions in milk containing 10^6 CFU/ml at 72°C, in triplicate for 25 seconds followed by cooling to 4°C resulted in complete loss of viability.

3.3.3 Survival of *Campylobacter* CP30A bacteriophage in milk

Campylobacter CP30A bacteriophage recovery experiments were designed to assess the validity of experimental methods used to isolate *Campylobacter* bacteriophage from milk and test its suitability as a medium for administering bacteriophage in phage therapy. Table 3.2 shows the different CP30A titres used in the experiments and the average titre of the bacteriophage recovered after the experiments. *Campylobacter* CP30A bacteriophage was added to milk at different

dilutions containing approximately 10^6, 10^7 and 10^8 PFU/ml. The results clearly show that there was no significant difference between number of phage added and the number recovered (*P*= 0.65, 0.44 and 0.74 respectively).

Table 3.2 Recovery of bacteriophage after inoculation in milk at different titres

CP 30A bacteriophage concentration PFU/ml	Average $\log_{10}$ PFU/ml titre added to milk	Average $\log_{10}$ PFU/ml titre recovered from milk	Recovery percentage %	STDEV.S
10^6	5.97	5.89	81.11	0.24
10^7	7.02	6.86	68.27	0.05
10^8	7.95	7.88	83.15	0.16

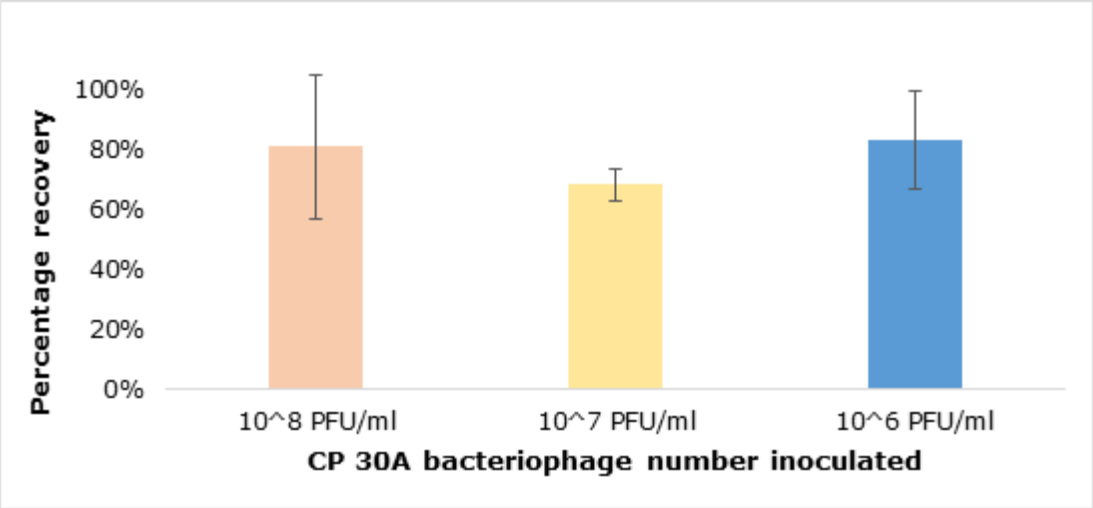


Figure 3.5 Recovery of bacteriophage CP 30A after inoculation in milk at different titres

These data indicate that *Campylobacter* bacteriophage can be efficiently recovered from milk and that milk has no inhibitory effect on *Campylobacter* bacteriophage.

3.4 Discussion

A review of the literature revealed that the majority of studies reporting the survival of *Campylobacter* in milk were published in 1980's (Doyle and Roman, 1982a). Since that time, there have been major developments in culture techniques and the discovery of many new species of *Campylobacter,* for example *C. ureolyticus*. As bovine milk has been suggested as a source of *C. ureolyticus* associated with human disease (O'donovan et al., 2014), it is clearly important to investigate the survival characteristics of this species and compare it to other *Campylobacter* species. Early studies of survival of *C. jejuni* strains in milk did not address different atmospheric conditions or temperatures, and used raw milk containing indigenous microflora. The experiments described in this study are the first to assess the survival of different *Campylobacter* strains and species including *C. ureolyticus,* in UHT milk, comparing aerobic and microaerobic conditions at refrigeration and room temperatures. The strains tested in this study all proved to be able to survive for considerable lengths of time, both aerobically and anaerobically, at refrigeration temperatures. Different study was proposed the sterilisation failure or contamination post-sterilisation, would prove a considerable reason for *Campylobacter* outbreak associated with consumption of milk. In this study, even at room temperature the *Campylobacter* strains were able to survive in considerable numbers, for more than 5 h, under aerobic conditions. It was interesting to note that in many cases, the strains tested survived better in UHT milk than in MRD, which contains peptone in an isotonic solution, and is considered an ideal medium for protecting microorganisms from stress (Straka and Stokes, 1957). It was also interesting to note that *C. coli* RM2228 declined in viability in UHT milk more quickly than the other strains. This strain was originally isolated from an avian host, whereas the other strains were isolated from human hosts. Further studies of the survival of many different strains would need to be conducted, to establish a link between host and survival in milk.

To sum up, in this study the survival of *Campylobacter* under different conditions in UHT milk, compared to MRD, as a standard buffer, had not previously been studied. These experiments can help in understanding the mechanisms of outbreaks of *Campylobacter* related to consumption of raw milk and in the food chain in general.

The results in this study have demonstrated the ability of *Campylobacters* to persist and survive under what are perceived to be unfavourable conditions for this bacterium. *Campylobacter ureolyticus* in milk could not only survive but showed evidence of increasing numbers of viable cells. Further studies to isolate campylobacters from milk or cattle carcases could allow further understanding of how these foodborne pathogens enter the human food chain and the possible development of rapid detection methods. More refined experiments, together with survival curve modelling, will be needed to better understand how the campylobacters differ when incubated in milk and experimental buffers, and how this is affected by oxygen tension and temperature.

These results show that bacteriophage survive can in milk under aerobic conditions. This basic knowledge is important in extending work on the isolation and preservation of bacteriophage and in determining the most appropriate media to be used for the delivery of bacteriophage products in animal phage therapy applications.

Chapter Four

Genomic characterisation of *Arcobacter spp.* and *Campylobacter spp*. cattle isolates

4.1 Introduction

Arcobacter and *Campylobacter* spp. are zoonotic pathogens present in cattle intestines, that may contaminate raw drinking milk (RDM) and dairy products (Hansson et al., 2019; Mungai et al., 2015). Since it was most likely that candidate bacteriophages would be isolated using *Arcobacter* and *Campylobacter* spp. hosts from the same environment, examples of these bacteria were isolated from bovine slurry (Giacometti et al., 2015). The genomic characterisation of these bacterial isolates is described in this chapter.

There are relatively few studies that report the prevalence and characterisation of isolates of *Arcobacter* and *Campylobacter* spp. from cattle compared to the number of studies reporting their prevalence in poultry. Despite this, a large number of outbreaks of human illnesses are caused by these zoonotic pathogens (Guévremont et al., 2014; Serraino et al., 2013; Yesilmen et al., 2014; Del Collo et al., 2017).

A description of the genus *Arcobacter* is given in Chapter 1 (section 1.1.4). *Arcobacter* spp. have been detected in faecal samples from healthy cattle and other ruminants with no clinical symptoms observed (Shah et al., 2011). *Arcobacter* varies in prevalence with one study in Belgium showing a range of 5.9 to 11% in dairy cattle, but it was more prevalent in calves and young animals (18.9 and 27.3% respectively). In a different study in Galicia (northwest of Spain) which included 89 dairy farms, *Arcobacter* spp. was isolated from 68.5% of the farms and 41.7% of faecal samples (Vilar et al., 2010a).

At least 27 different species have been described within the genus *Arcobacter*, Moreover, some species have been reassigned to different genera (Pérez-Cataluña et al 2018). Of the species that remain, *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been most associated with human disease. The same species have been isolated from bovine samples with *A. butzleri* being most frequently isolated, followed by *Arcobacter cryaerophilus* and *Arcobacter skirrowii* (Collado et al.,

2010; Fisher et al., 2014; Collado and Figueras, 2011; Collado et al., 2008). The absence of standard methods to isolate *Arcobacter* may have had a significant impact on the range of different species that have been detected (Van Driessche et al., 2005; Collado and Figueras, 2011). *Arcobacter* spp. was first isolated from bovine foetuses by Ellis et al. (1977), this was followed by isolation of what was named as "*Campylobacter cryaerophila"* by (Neill et al., 1985) which then renamed and reclassified as *A. cryaerophilus* after *Arcobacter* genus was proposed by (Vandamme et al., 1991). Not long after that *A. skirrowii* was isolated in 1992 from preputial fluids of bulls (Vandamme et al., 1992b).

Modern genome sequencing techniques have led to great improvements in our understanding of epidemiology and infection. Several sequences of *A. butzleri* and A. *cryaerophilus* are available but only one closed genome sequence of *Arcobacter skirrowii* (LMG 6621) is currently available. This isolate from lamb faeces isolated in the United Kingdom showed the bacterial chromosome length was 1,969,846 bp with a G+C content of 27.7% (Miller and Yee). Three genomic islands were identified encoding different functions and a type III Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Cas system. There were no plasmids identified in the LMG 6621T genome (Miller and Yee, 2018).

Most cases of campylobacteriosis are attributed to *C. jejuni* but there were 246,158 confirmed cases of human campylobacteriosis due to *C. coli* reported in the EU in 2017 equating to 24% of the total number reported cases (EFSA, 2018). Considering outbreaks in the same study, 66% of cases that were caused by contaminated milk and dairy products were attributed to *Campylobacter* spp. other than *C. jejuni* (EFSA, 2018). *Campylobacter coli* have been identified in 3 to 10 % of human *Campylobacter* cases in different various countries (Sheppard et al., 2009; Cody et al., 2012; Nohra et al., 2016).

A description of the genus *Campylobacter* and how species of the genus are involved human disease is given in Chapter 1 (section 1.1.4). Epidemiological

studies have shown that dairy cattle are a reservoir for *Campylobacter* so further research is required to reduce the impact of bovine *Campylobacter* spp.in human disease (Guévremont et al., 2014). Cattle can be colonised by *C. jejuni* and/or *C. coli* (Arsenault et al., 2012). Studies have also identified other species of *Campylobacter* such as *C. hyointestinalis* colonising cattle (Oporto and Hurtado, 2011; Guévremont et al., 2008). A study in United States of America showed that from a total of 181 *Campylobacter* isolates from cattle faeces, 71 were *C. jejuni,* 132 were *C. coli*, and 10 were other *Campylobacter* spp. (Sanad et al., 2011). In Japan, *C. coli* were detected in 3.6% (9/250) of beef cattle and from 16.0% (4/25) of beef farms (Haruna et al., 2013). In a French study, 12.8% of isolates were identified as *C. jejuni* and 3.7% identified as *C. coli* (Chatre et al., 2010). Most campylobacteriosis studies worldwide have focused on the role of *C. jejuni*. There is however still an important gap in understanding of the impact of *C. coli* and other campylobacters in the zoonoses cycle (Nohra et al., 2016; Elhadidy et al., 2019).

The prevalence of campylobacters other than *C. jejuni* and *C. coli* such as *C. hyointestinalis* may be underestimated for several different reasons including: the isolation technique, inappropriate antibiotics in selective media or unsuitable recovery conditions such as insufficient incubation period or unsuitable temperature and gas mixture (Lastovica and Allos, 2008). The *C. hyointestinalis* species is divided to two subspecies: *C. hyointestinalis* subsp*. hyointestinalis* and *C. hyointestinalis subsp. Lawsonii (BLOCH et al., 1995)*. *Campylobacter hyointestinalis* was first isolated from swine with proliferative ileitis and other enteric diseases (Gebhart et al., 1983).

As previously (section 1.2.2), the sequencing of whole genomes has significantly advanced our understanding of bacterial zoonosis particularly with regard to *C. jejuni* for which many genomes available. The first reported *C. coli* genome sequence (RM2228) was a chicken isolate that was demonstrated to be resistant

to a range of antibiotics (Fouts et al., 2005a). The chromosome length was 1,684,122 with a G+C content of 31.37 % and contained a single plasmid approximately 178 kb in size. The first fully assembled genome sequence of the gentamicin resistant *C. coli* CVM N29710 from chicken had a chromosome length was 1,673,221 bp and a G+C content of 31.4 % and a plasmid with multiple antibiotic resistance genes (Chen et al., 2013). The first complete genome assembled of a clinical isolate *C. coli* strain (15-537360) had a chromosomal length of 1,658,751 bp with a G+C content of 31.5 % and also included a conjugative plasmid with a type IV secretion system, but no antibiotic resistance genes were identified (Pearson et al., 2013).

The first complete genome sequence for both *C. hyointestinalis* subsp. *hyointestinalis* strain LMG 9260 and *C. hyointestinalis* subsp. *lawsonii* strain LMG 15993 were published in 2016 (Miller et al., 2016). The bacterial chromosome for *C. hyointestinalis* subsp*. hyointestinalis* LMG 9260, a human isolate, was 1,753,385 bp in length with a G+C content of 34%. The chromosome for *C. hyointestinalis* subsp. *lawsonii* strain LMG 15993 (from swine) was 1,753,277 bp in length and had a G+C content of 33.5%. In the *C. hyointestinalis* subsp. *lawsonii* genome, variable genes in integrated elements such as clustered regularly interspaced short palindromic repeats **(CRISPR)** arrays and Mu-like phage were identified. Insertion sequences **(IS)** were identified in both strains. (Miller et al., 2016).

The aim of this study then to characterise and analysing the genome sequence of selected isolates of *Arcobacter* and *Campylobacter* to use as candidate bacteriophage hosts. These were *A. skirrowii* A2S6, *C. coli* S9, and *C. hyointestinalis* S12. It was also to compare these isolates with known sequences from isolates from different sources to identify any source-specific genes associated with cattle.

4.2 Materials and Methods

4.2.1 Whole genome sequencing, assembly and annotation of cattle isolates

Whole genomic DNA for all strains in this study were isolated as described in 2.9.1. DNA sequencing were performed using the Illumina MiSeq platform at the University of Nottingham (Sutton Bonington Campus; UK) as described in 2.10.1. The data for *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* subsp. *hyointestinalis* S12 was processed and annotated as described in 2.10.2 and 2.10.3 The complete annotated sequence of *A. skirrowii* A2S6, *C. coli* S9 and *C. hyointestinalis* subsp. *hyointestinalis* S12 were deposited in GenBank under the accession NZ_CP034309.1, NZ_CP040239.1 and NZ_CP040464.1 respectively.

4.3 Results

4.3.1 *A. skirrowii* A2S6 chromosomal features

4.3.1.1 Structure and genes of *A. skirrowii* A2S6

The assembled circular chromosome of *A. skirrowii* A2S6 was determined to be 1,877,752 bp in length with 1,953 genes and an average G+C content of 27.8%. The sequence contained 4 copies of the ribosomal RNA operon and 44 tRNA genes. The chromosome of *A. skirrowii* A2S6 was found to have one duplicate region of 1,090 bp spanning from nucleotide positions 7, 463, 61 to 7, 474, 51. Figure 4.1 shows the genome map of the *A. skirrowii A2S6* chromosome and indicates the positions of the duplicated sequence. *Arcobacter skirrowii* A2S6 is currently the second *Arcobacter* from the species of *skirrowii* that has been completely assembled in NCBI database and has accession number CP034309.1.

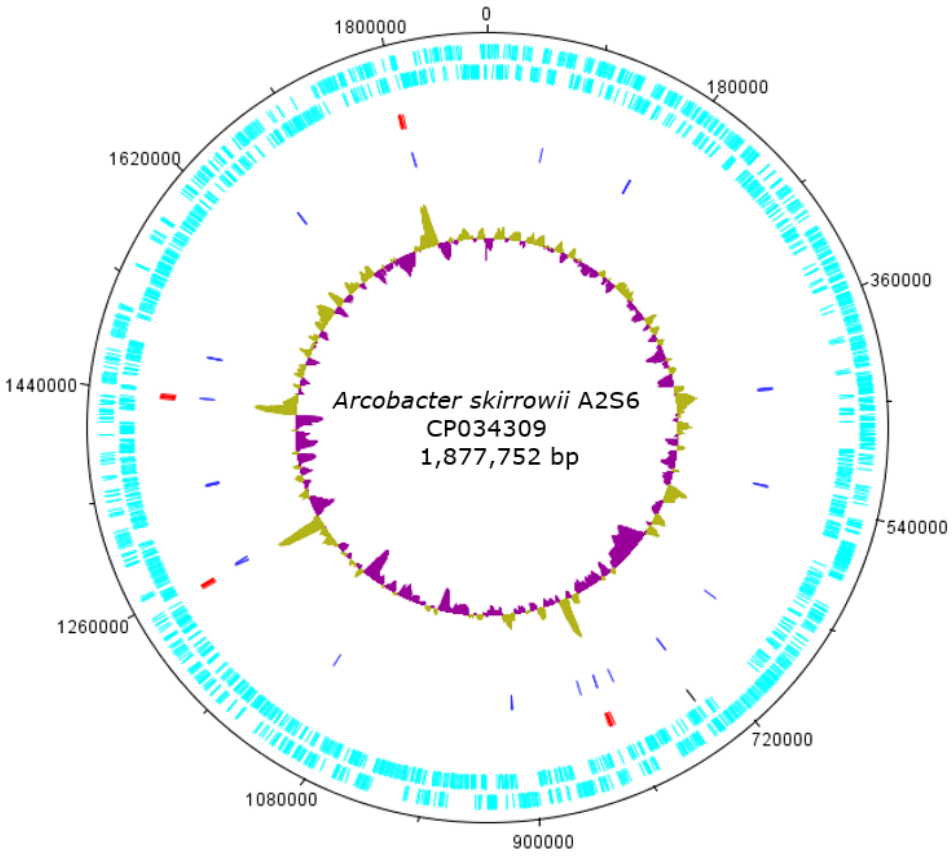


Figure 4.1 Circular representation of *A. skirrowii A2S6* chromosome
(Light blue represents coding DNA sequences) (Red shows rRNA and Dark blue represents tRNA sequences) (Black arcs show the regions of duplicate sequences) (The inner circle is a % G+C plot)

The *A. skirrowii* A2S6 genome contains 3 homopolymeric G:C tracts, which are defined as containing more than 7 consecutive G or C residues and are presented in **Table 4.1** . Hompolymeric tracts have been observed to vary in *Campylobacter spp*. to alter the reading frames of genes to effectively turn them on and off.

Table 4.1 Homopolymeric repeats present in *A.skirrowii A2S6* genome

Position	Homopolymeric G/C tract	Gene ID/Region affected	Putative function	Locus
393340-393347	G(8)	intergenic	N/A	
1133858-1133865	C(8)	intergenic	N/A	
1746691-1746697	C(7)		hypothetical protein	EI285_09095

4.3.1.2 Nucleotide sequence statistics of *A. skirrowii* A2S6

The distribution of nucleotides (Figure 4.2)is typical of an AT-rich *Arcobacte* genome

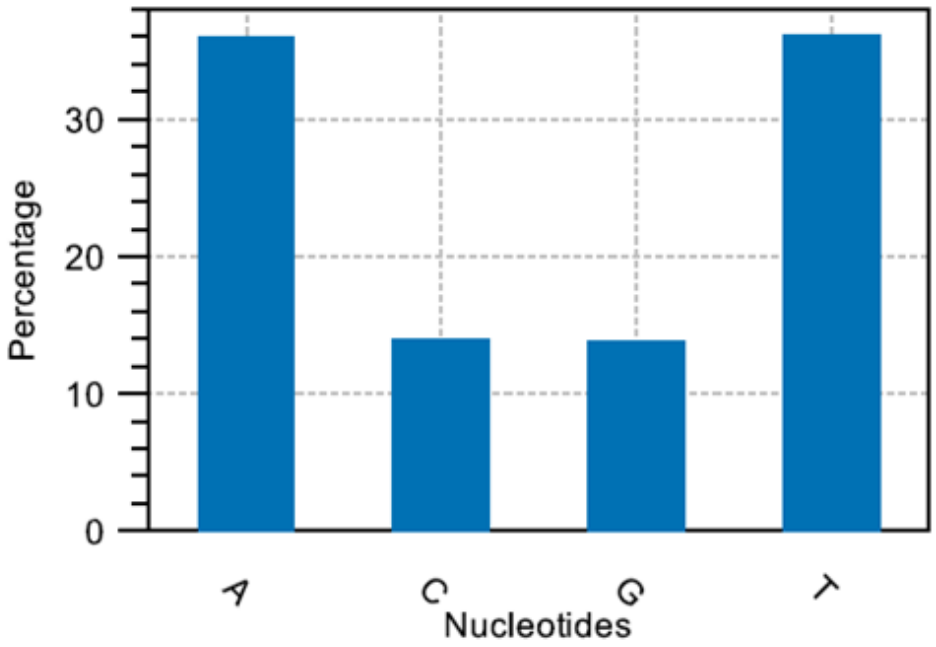


Figure 4.2 Nucleotide distribution histogram for the *A. skirrowii A2S6* chromosome

The frequency of codon usage reflects the tRNA pools available in the genome. The nucleotide frequency in codon positions (**Table 4.2**). Adenine residues show similar distribution at each position. Thymine residues are most frequent at the third position. Cytosine residues were the least commonly occurring nucleotide overall, and in the first and third positions. Guanosine residues were the least common in the third position. The codon AAA, which encodes the amino acid lysine was the most abundant codon occurring 50,456 times and the codon CGG, which encodes arginine, was the least abundant occurring 21 times.

Table 4.2 Frequency of nucleotide codon positioning for the *A. skirrowii A2S6* chromosome

Codon position	A	C	G	T
1.position	0.38	0.11	0.30	0.21
2.position	0.37	0.17	0.13	0.33
3.position	0.42	0.05	0.09	0.44

4.3.1.3 Comparison with the nearest *A. skirrowii* sequence neighbour

The program LAGAN shuffle available from mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml) was used to compare and identify the similarity between *A. skirrowii* A2S6 and the nearest neighbour from the same species available in the NCBI database. The complete genome sequence for *A. skirrowii* A2S6 cattle strain was *A. skirrowii* CCUG 10374, which was selected for comparison using mVISTA. (Figure 4.3)the alignment of the genomes where it is evident that large regions of low sequence identity with *A. skirrowii A2S6* are present. The mVISTA browser function allowed analysis of the genome data for genes present and absent that distinguish *A. skirrowii* A2S6 and *A. skirrowii* CCUG 10374. There were a total of 243 genes represented in *A. skirrowii* A2S6 but absent in *A. skirrowii* CCUG 10374 (Appendix 1). Of these 103 genes encoded hypothetical proteins.

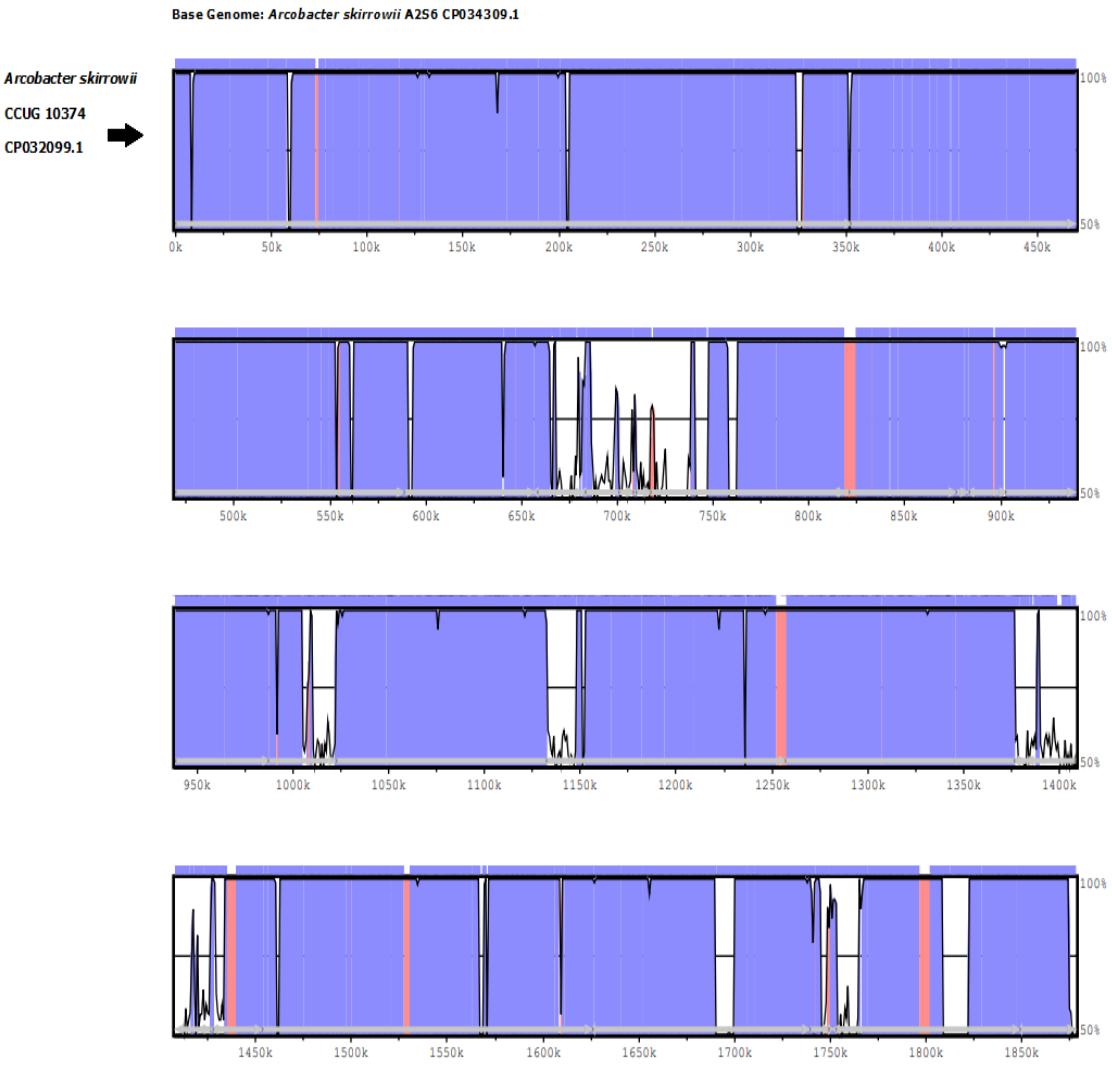


Figure 4.3 Chromosomal alignment of *A. skirrowii A2S6* and *A. skirrowii* CCUG 10374

Plot showing the percentage identity and locations of missing genes across the *A. skirrowii* A2S6 chromosome aligned with and *A. skirrowii* CCUG 10374. Purple represents conserved regions; pink represents conserved non-coding sequences (CNS) and white represents missing or relocated genes. Generated by mVISTA with the LAGAN function. There were 342 genes absent from the *A. skirrowii* A2S6 genome but present in *A. skirrowii* CCUG 10374. Of these 89 were hypothetical proteins (Appendix 2).

4.3.1.4 Plasmids

No plasmids were identified in the *A. skirrowii* A2S6 genome.

4.3.1.5 Taxonomic characterisation of *A. skirrowii* A2S6 by whole genome BLAST

The nearest neighbour to *A. skirrowii* A2S6 with whole genome sequenced, as determined by NCBI Genome neighbour report was found to be *A. skirrowii* CCUG 10374. The symmetric identity and gapped identity with *A. skirrowii* A2S6 were 83.36 % and 98.67% respectively which present the second member of *skirrowii* species have been uploaded to NCBI website under a session number NZ_CP034309. *Arcobacter skirrowii* A2S6 strains fall within one of four genomic groups, which is represented by *A. skirrowii* strain L403. (Figure 4.4) the dendrogram, based on genomic BLAST, of *A. skirrowii* A2S6.

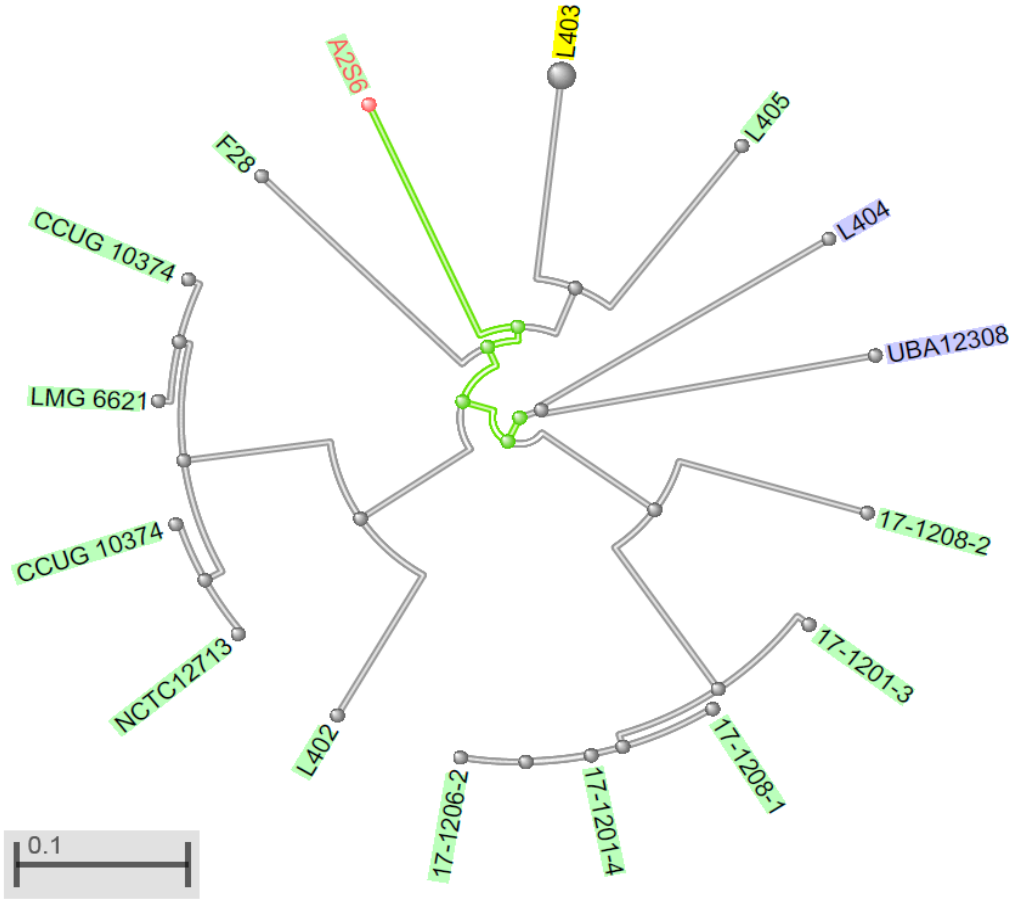


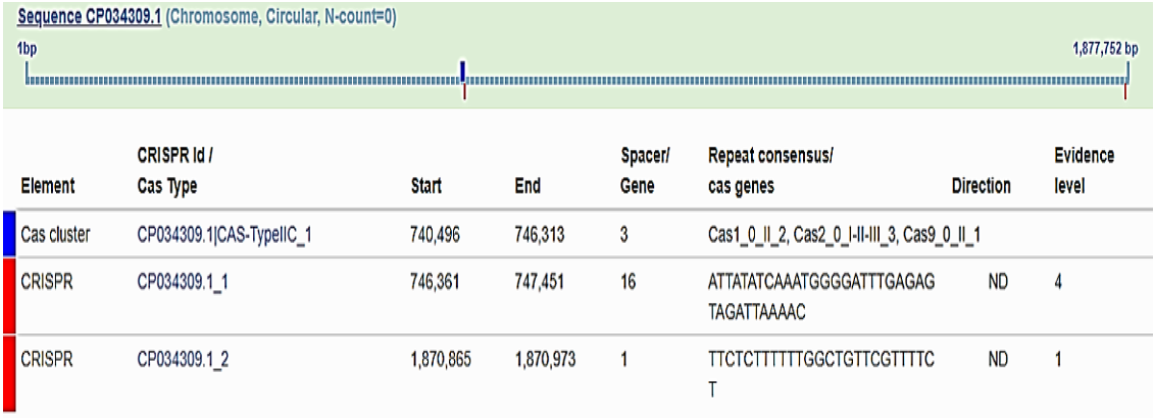
Figure 4.4 Phylogenetic comparison of *A. skirrowii A2S6* with other *A. skirrowii* strains

Arcobacter skirrowii A2S6 is in the red colour. Comparisons are based on genomic BLAST searches and the dendrogram was calculated using the Genome feature of the NCBI website.

4.3.1.6 CRISPR sequences of *A. skirrowii* A2S6

Clustered regularly interspaced short palindromic repeats (CRISPR) loci are arrays of short repeats named direct repeats (DR), followed by noticeable short sequences known called spacers. The CRISPR sequences, present in many bacteria and archaea, in parallel with *cas* genes, are proposed to provide host resistance against infection with lytic phage and foreign plasmids in (Marraffini, 2013).

Arcobacter skirrowii A2S6 has two pairs of CRISPR arrays in the chromosome. (Figure 4.5) the positions of the CRISPR arrays and *cas* genes in *A. skirrowii* A2S6. The first CRISPR array is from position 746361 to 747451. The second CRISPR array is truncated from position 1870865 to 1870973. This CRISPR array did not have any *cas* genes in the vicinity.



Element	CRISPR Id / Cas Type	Start	End	Spacer/ Gene	Repeat consensus/ cas genes	Direction	Evidence level
Cas cluster	CP034309.1|CAS-TypeIIC_1	740,496	746,313	3	Cas1_0_II_2, Cas2_0_I-II-III_3, Cas9_0_II_1		
CRISPR	CP034309.1_1	746,361	747,451	16	ATTATATCAAATGGGGATTTGAGAG TAGATTAAAAC	ND	4
CRISPR	CP034309.1_2	1,870,865	1,870,973	1	TTCTCTTTTTTGGCTGTTCGTTTTC T	ND	1

Figure 4.5 the positions of the CRISPR arrays and *cas* genes in *A. skirrowii A2S6* genome, red represents CRISPR arrays and blue the *cas* cluster

Table 4.3 shows the sequences of direct repeats and spacers as calculated by the CRISPRs web server: (https://crisprcas.i2bc.paris-saclay.fr/MainDb/StrainList/CP034309.1). The length of direct repeats and spacers appear to vary between the two pairs of arrays (Abby et al., 2014).

Table 4.3 Direct repeat and spacer sequences within the CRISPR 1 array of *A. skirrowii A2S6*

CRISPR 1		
Regions	**Direct Repeats**	**Spacer**
746361	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	ATAAAAACTGACTACCAACAAT TAAAAAAA
746427	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	ATATCTTTATATTCTATATTGTT TATAATA
746493	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	GCTTGCTTTATATATTAACATG TATATTAT
746559	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	TCGCAGAGCCTACAAATATCTT TAATAAT
746624	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	CCTTGAGTTTTTTTAACTTTATT ACCCTTAT
746691	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	ATTAAAGCTAATTGAATAGGTA AAATCAGGG
746758	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	TCATAGAGTGGGTAAGAAAAC ATAAGCAG
746823	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	TTAACAATTATGCAAACTTAAA TAACTTAA A
746890	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	TAGCAAAAATTTCAAACACAAA AATGTTT
746955	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	AAAGGCACAGTGGTACAAACT ATGAGAGA
747020	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	CTGCACTTTTTACTATTGGAAC TGCATTGC
747086	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	ATCCTATGAGAAGTGGAATAG CTGGAATGT
747152	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	ACTATATAAATAAAATTCTCTAT CTCCATA
747218	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	TCGTTTAATATTGTATTTTAAAT TTCTATC
747284	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	CACATAATCCTTTTATATATTCA ACATCTT
747350	ATTATATCAAATGGGGATTTG AGAGTAGATTAAAAC	CTACTTTTTTAAAACAATTAGA GAGTAGTT
747416	ATTATATCAAATGGGGATTTG AGAGTAGATTGTAAA	

Table 4.4 Direct repeat and spacer sequences within the CRISPR 2 array of *A. skirrowii A2S6*

CRISPR 2		
Regions	**Direct Repeats**	**Spacer**
1870865	TTCTCTTTTTTGGC TGTTCGTTTTCT	CGATATTGAAACTAAAGTTTCAAATCTCG TGCTCACAGATTAGCTTCTTATGAATTC
1870948	TTCTCTTTTTTTGC TGTTCGTTTTCT	

Nucleotide BLAST results from CRISPR direct repeats consensus sequence in *A. skirrowii* A2S6 in (Appendix 3) shows that the DR consensus sequence of the first CRISPR is present in two different *Arcobacter* spp. genomes: the *A. thereius* LMG 24486 chromosome and the *A. pacificus* LMG 26638 chromosome with 100% for both cover and identity and the same e- value of 8 X 10^{9}.

4.3.1.7 Prophage insertions of *Arcobacter skirrowii* A2S6

The *A. skirrowii* A2S6 chromosome contained three different prophage insertions. These prophage insertions were classified as incomplete (score < 70; by http://phaster.ca/). The first region position was between 676,005-683,290 bp and was 7.2 kb in length which orientated in both directions. The second region was between 721,479-730,691 bp and was 9.2 kb in length. It was located in the forward directions. The third prophage insertion region was 9.9 kb and extended from1,017,007 to 1,026,955 bp, orientated in the reverse strand, the three.

Figure 4.6 Prophage insertions of *Arcobacter skirrowi*i A2S6

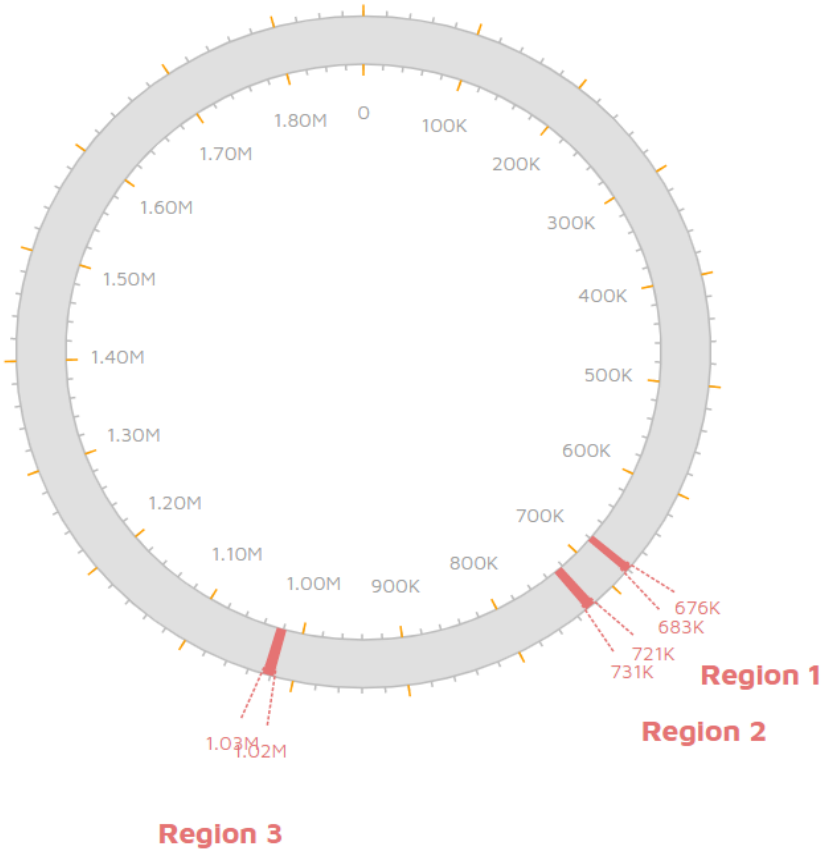


Figure 4.7 shows the three regions of prophage insertions in *A. skirrowii* A2S6 mapped on the chromosome

Nucleotide BLAST analysis of the three prophage regions showed that *A. skirrowii* CCUG 10374 was the only *Arcobacter* genome that had similar sequences to the first and third prophage sequences whilst the second sequence showed no similarity. Examining the Shuffle-LAGAN analysis using mVISTA shown in Figure 4.3 showed that the expected prophages were represented as regions of no sequence identity.

4.3.1.8 Virulence Factors of *A. skirrowii* A2S6

To date, the potential pathogenicity mechanisms of the genus *Arcobacter* are not fully understood with a limited number of studies available (section 1.4). Proposed pathogenicity gene markers from the *A. butzleri* RM4018 genome (Ferreira et al., 2016b, Miller et al., 2007) identified due to their role in pathogenicity in other microorganisms, which include: *cadF, cj1349, ciaB, mviN, pldA, tlyA ,hecA, hecB, irgA.* The locus tags of homologues of these proposed virulence markers were determined by BLAST search, in *A. skirrowii* A2S6 (**Table 4.5**).

Table 4.5 Homologues of proposed virulence gene markers found in *A. skirrowii* A2S6

Genes name	Function	*A. skirrowii* A2S6 locus tag	*% Identity to *A. butzleri* RM4018
cadF	Adherence/ internalisation to host cells	EI285_RS07655	28%
ciaB	Invasion protein	EI285_RS02790	83%
cjl349	Adherence to fibronectin	EI285_RS00810	69%
irgA	iron-regulated outer membrane protein	EI285_RS08660	36%
hecA	Encodes for a filamentous haemagglutinin	No match	
hecB	Encoding for a haemolysin activation protein	No match	
mviN	Peptidoglycan synthesis	EI285_RS02745	82%
pldA	phospholipase A	EI285_RS05575	58%
tlyA	Haemolysin/ adherence	EI285_RS04050	73%

*BLASTP comparison of protein sequences

4.3.1.9 Antibiotic resistance genes of *A. skirrowii* A2S6

The Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) was used to predict the resistome of *A. skirrowii* A2S6 from protein, genome, or nucleotide data based on homology and SNP models by CARD, with stingent criteria: perfect and strict hit only. The results identified the *adeF* gene in *A. skirrowii* A2S6 giving fluoroquinolone and tetracycline resistance by an efflux mechanism. The identity of matching region was 42% over the length of reference sequence (99.15%).

4.3.2 *C. coli* S9 chromosomal features

4.3.2.1 Structure and genes of *C. coli* S9

The assembled circular chromosome of *C. coli* S9 was 1,713,481 bp in length with 1,806 genes and an average G+C content of 31.37%. The sequence contained three copies of the ribosomal RNA operon and 44 tRNA genes. Figure 4.8 shows the genome map of the *C. coli* S9 chromosome. The genome contains 18 homopolymeric G:C tracts, which are defined as containing more than 7 consecutive G or C residues.

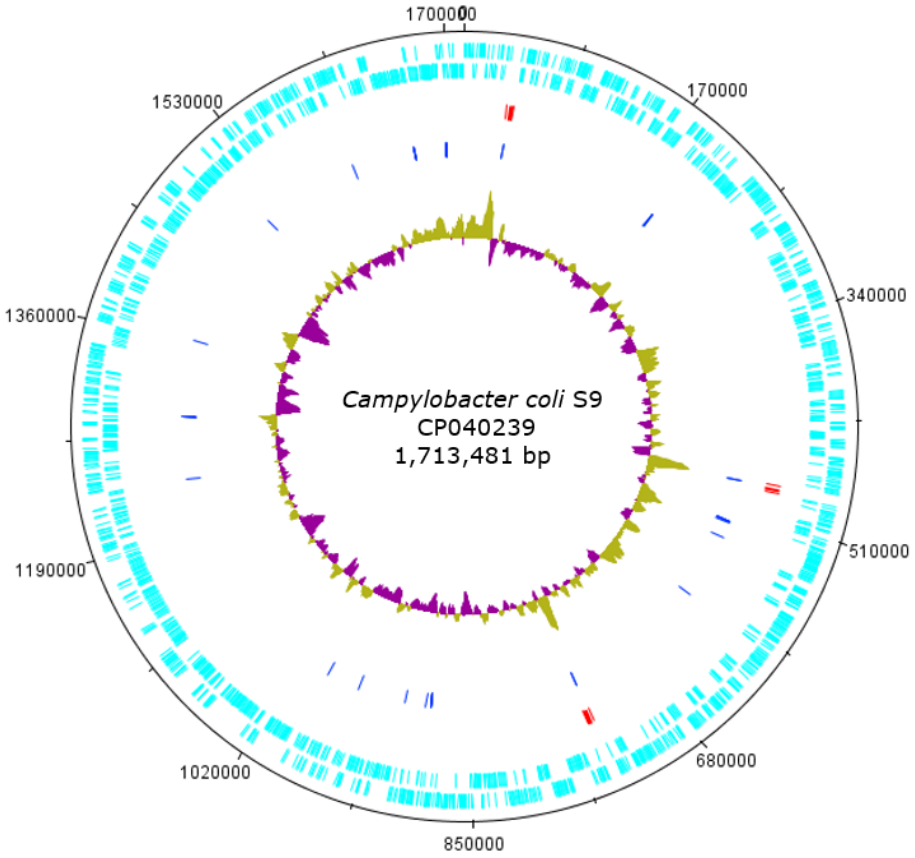


Figure 4.8 Circular representation of *C. coli* S9 chromosome (Light blue represents coding DNA sequences) (Red indicates rRNA and dark blue represents tRNA sequences) (The inner circle is a % GC plot)

Table 4.6 Homopolymeric repeats present in *C. coli* S9 genome

Position	Homopolymeric G/C tract (no.)	Locus_ tag	Putative function
65378-65388	C (11)	FD987_00240	Iron binding protein
486878-486886	G (9)	Intergenic	
648010-648020	G (10)	FD987_03345	DUF2920 family protein
893257-893265	C (9)	Intergenic	
1041400-1041408	C (9)	FD987_05345	Restriction endonuclease
1051701-1051710	C (10)	FD987_05380	Hypothetical protein
1232324-1232333	C (10)	Intergenic	
1306188-1306197	G (10)	FD987_06685	DUF4910 domain-containing protein
1314166-1314175	C (10)	FD987_06730	DUF2920 family protein
1315393-1315402	C (10)	FD987_06740	DUF2920 family protein
1318748-1318756	G (9)	FD987_06755	Formyl transferase
1321277-1321285	G (9)	FD987_06770	"3-oxoacyl-ACP synthase"
1328888-1328895	G (8)	FD987_06810	motility accessory factor
1333336-1333341	G (9)	FD987_06835	formyl transferase
1354740-1354748	C (9)	FD987_06910	DUF2920 family protein
1375610-1375619	C (10)	Intergenic	
1441623-1441632	G (10)	FD987_07320	hypothetical protein
1515825-1515833	G (9)	Intergenic	

4.3.2.2 Nucleotide sequence statistics *C. coli* S9

The distribution of nucleotides, shown in Figure 4.9 is typical of an AT-rich *Campylobacter* genome. The nucleotide frequency in codon positions (Table 4..

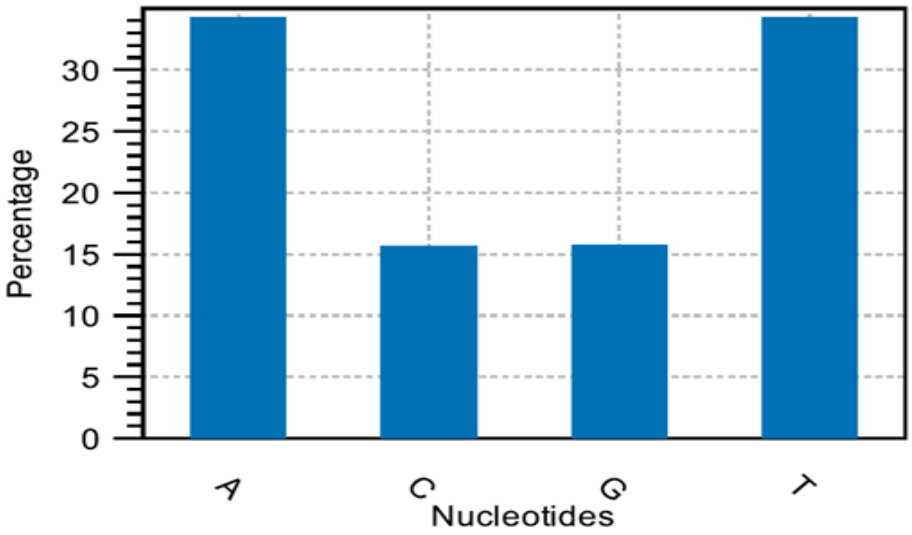


Figure 4.9 Nucleotide distribution histogram for the *C. coli* S9 chromosome

Codon usage indicates adenine residues have no significant difference in distribution at any position. Cytosine residues were the least frequent nucleotide, particularly in the third positions. Guanosine residues were the most frequent nucleotide in first position and least frequent in the third position. Thymine residues showed a different distribution from the other nucleotides and was most frequent at the third position.

The codon AAA, which encodes the amino acid lysine was the most frequent codon repeated 40,572 times and the codon CGG, which encodes arginine, had the lowest abundance with 57 instances.

Table 4.7 Frequency of nucleotide codon positioning for the *C. coli* S9 chromosome

Codon position	A	C	G	T
1. position	0.36	0.13	0.30	0.21
2. position	0.36	0.17	0.14	0.33
3. position	0.35	0.11	0.11	0.43

4.3.2.3 Comparison with nearest *C. coli* sequence neighbour

The LAGAN shuffle comparison program using mVISTA was used to identify the similarity between the *C. coli* S9 and its nearest neighbour *C. coli* BFR-CA-9557. Figure 4.10 shows the alignment of *Campylobacter coli* BFR-CA-9557 genome, where it is evident that large regions of low sequence identity with *C. coli* S9 are present. The mVISTA browser function allowed the analysis of the genome data to identify genes which present or absent in *C. coli* BFR-CA-9557compared to *C. coli* S9 (Appendix 1). This showed that a total of 69 genes were present in *C. coli* S9 and absent in *C. coli* BFR-CA-9557. This set included encoded 21 hypothetical proteins (Appendix 4).

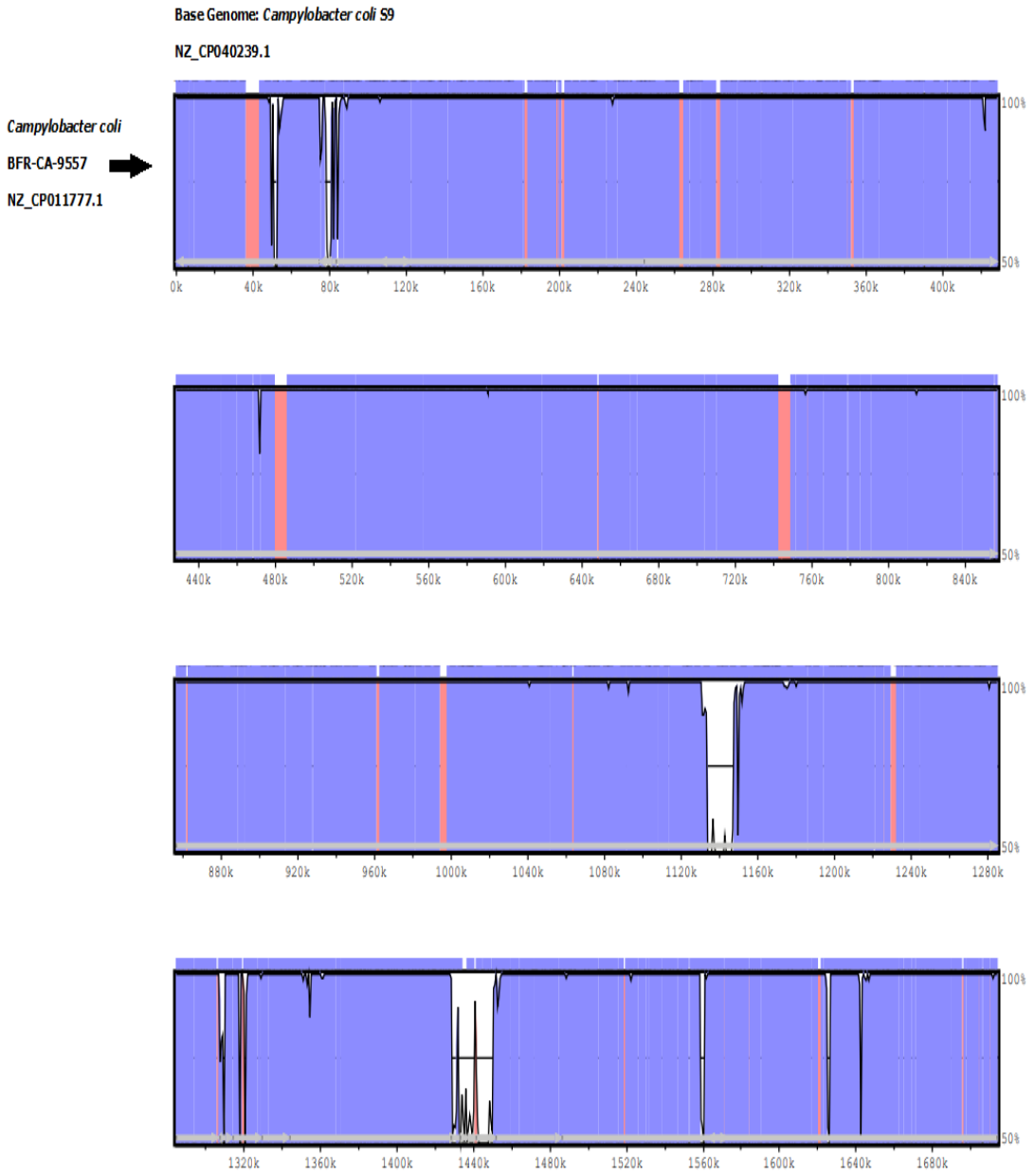


Figure 4.10 Chromosomal alignment of *C. coli* S9 and *C. coli* BFR-CA-9557. Plot showing the percentage identity and locations of missing genes across the *C. coli* S9 chromosome aligned with and *C. coli* BFR-CA-9557. Purple represents conserved regions; pink represents conserved non-coding sequences (CNS) and white represents missing or relocated genes. Generated by mVISTA with the LAGAN function. The genes absent from *C. coli* S9 but present in *C. coli* BFR-CA-9557 are shown in (Appendix 4).

4.3.2.4 Plasmids of *C. coli* S9

4.3.2.4.1 Identification of plasmids

Three plasmids were identified associated with the genomic DNA of *C. coli* S9. These were named pCcS9_1 pCcS9_2 and pCcS9_3.

4.3.2.4.2 Plasmid pCcS9_1, and Plasmid pCcS9_2

The sequence for plasmid pCcS9_1 has been uploaded to the NCBI website with the accession number CP040240.1. It has a G+C content of 31.83% and is 3321 bp in length. The pCcS9_1 plasmid contains 19 open reading frames. The plasmid pCcS9_2, NCBI accession no CP040241.1, is 3351 bp in length and has a G+C content of 31.24% with 17 open reading frames. Nucleotide BLAST, (with align two sequences option) showed a high degree of similarity between these two plasmids having approximately 95 % identity.

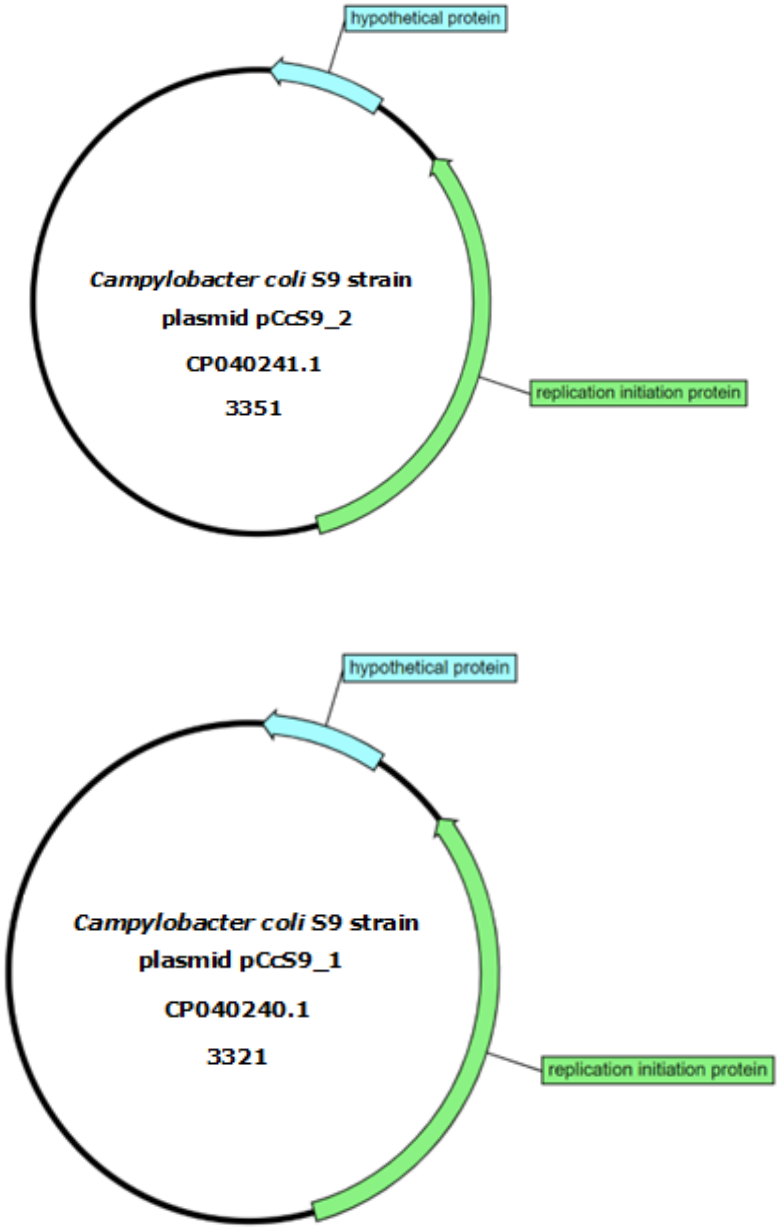


Figure 4.11 Structure of plasmid pCcS9_1 and plasmid pCcS9_2

4.3.2.4.3 Comparison of Plasmid pCcS9_1 with Plasmid pCcS9_2 and other nearest plasmid DNA sequences from *C. coli*

BLASTN analysis for both plasmids, identified 10 nearest neighbours with high DNA sequence identity. (Figure 4.12) distance tree analysis shows Plasmid pCcS9_1 Comparison and Plasmid pCcS9_2 and the relation to other similar plasmids. These plasmids are typical of the cryptic plasmids found in *C. coli* and *C. jejuni* and which have been used to construct shuttle vectors for gene manipulation such as expressing green fluorescent protein (Miller et al., 2000).

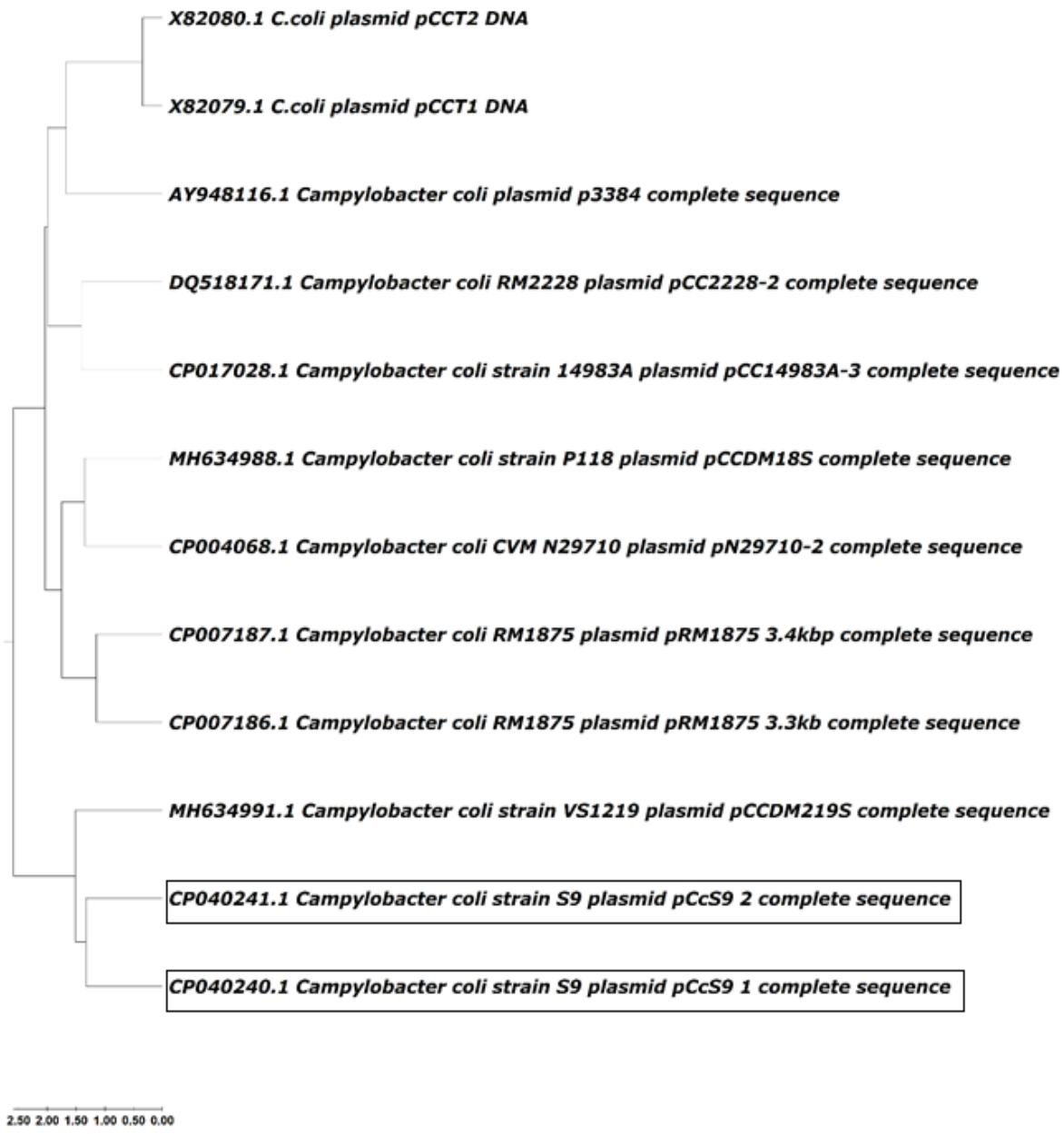


Figure 4.12 Evolutionary relationships taxa of Plasmid pCcS9_1 and Plasmid pCcS9_2 with nearest neighbour's plasmids belong to *C. coli*

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 19.71906880 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. Codon

positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3707 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018), this tree was generated using (Molecular Evolutionary Genetics Analysis) MEGA X software https://www.megasoftware.net/

4.3.2.4.6 Plasmid pCcS9_3 Structure and genes

Plasmid pCcS9_3, NCBI accession no. CP040242.1, has a G+C content of 29.09%, 103 open reading frames and is 25776 bp in length. Figure 4.13 shows the plasmid pCcS9_3, including 31genes.

4.3.2.4.7 Plasmid pCcS9_3 Comparison with nearest plasmid DNA sequence neighbours from *C. coli*

BLASTN analysis of Plasmid pCcS9_3, was carried out with 10 nearest neighbours with high DNA sequence identity from *C. coli*. The distance tree and the relation to other similar plasmids is shown in Figure 4.13

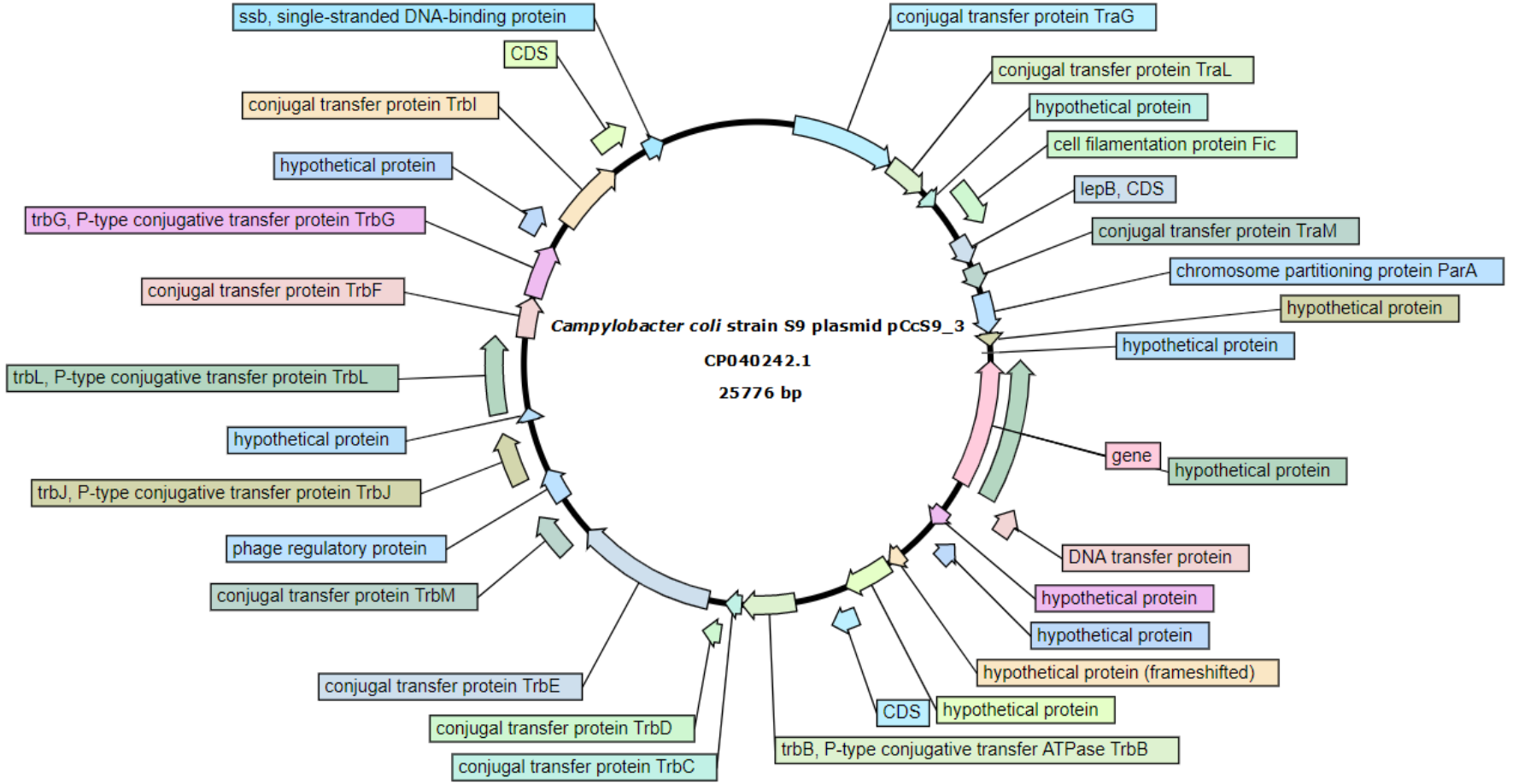


Figure 4.13 Plasmid pCcS9_3

Figure 4.13 Evolutionary relationships taxa of Plasmid pCcS9_3 with nearest 10 neighbour's plasmids belong to *C. coli*

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 50.43766930 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 30303 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018), https://www.megasoftware.net/.

4.3.2.5 CRISPR sequences of *C. coli* S9

No CRISPR arrays were detected in the *C. coli* S9 genome using the CRISPRs web server: (https://crisprcas.i2bc.paris-saclay.fr/MainDb/StrainList/CP040239).

4.3.2.6 Taxonomic characterisation of *C. coli* S9 by whole genome BLAST

The nearest neighbour to *C. coli* S9, as determined by NCBI Genome neighbour report was *C. coli* BFR-CA-9557. The symmetric identity and gapped identity with *C. coli* S9 were 94% and 98.97% respectively. The *C. coli* S9 strain fell within a genomic group representing non-poultry *C. coli* strains. (Figure 4.14) the dendrogram, based on genomic BLAST, of *C. coli* S9.

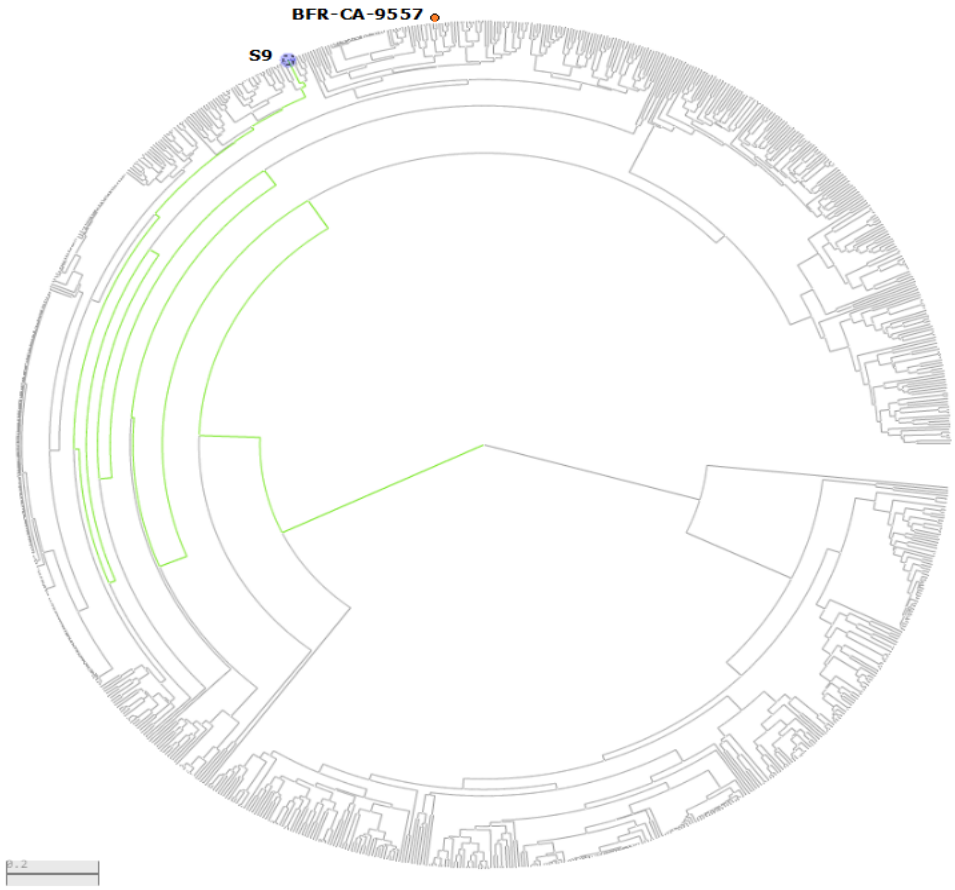
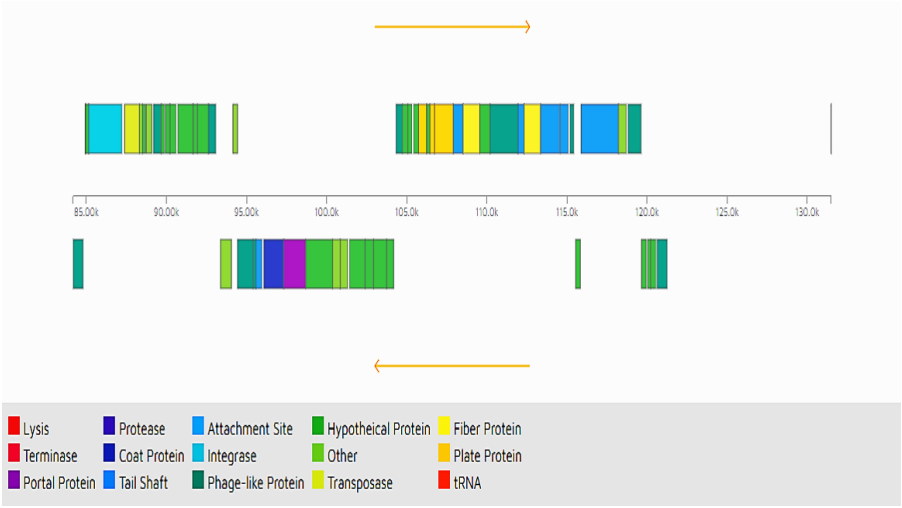


Figure 4.14 Phylogenetic comparison of *C. coli* S9 with other *C. coli* strains
Campylobacter coli S9 is in the blue circle. Comparisons are based on genomic BLAST searches and the dendrogram was calculated using the Genome feature of the NCBI website.

4.3.2.7 Prophage insertions

One prophage insertion was detected in the *C. coli* S9 chromosome, orientated in both directions. This prophage insertion was classified as "Intact" (score > 90) using the web-based program http://phaster.ca/. The region is positioned between 84181 and 131496 bp and was 47.3 kb in length (Figure 4.15). The prophage sequence could be identified as a region of no sequence identity in Figure 4.10 chromosomal alignment of *C. coli* S9 and *C. coli* BFR-CA-9557.

A



B

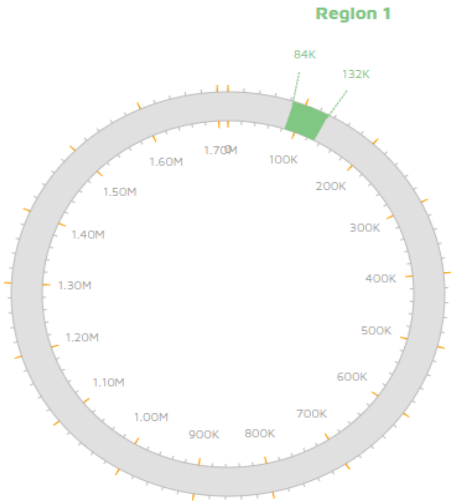


Figure 4.15 (A Prophage insertions in *C. coli* S9) (B Position in *C. coli* S9 chromosome)

4.3.2.8 Virulence factors of *Campylobacter coli* S9

The current knowledge regarding the pathogenicity of *Campylobacter* infection is described in Chapter 1.3. Genes thought to be involved are detailed in (Tables 1.1 to 1.3) The locus tags of each of these genes identified in *C. coli* S9 and a comparison of the protein sequence identity with nearest neighbour *C. coli* BFR-CA-9557 (Table 4.8)

Table 4.8 Virulence factors present in *Campylobacter coli* S9

Virulence factor	Gene	Locus Tag *C. coli* S9	Identity to *C. coli* BFR-CA-9557 %*
Adhesion/	*cadF*	FD987_07545	99.7
Invasion	*flaC*	FD987_03765	99.9
	flhB	FD987_04345	100
	fliR	FD987_05995	97
	fliQ	FD987_08565	100
	ciaB	FD987_04835	99.5
	ciaC	FD987_06370	100
	flhA	FD987_04650	99.9
	fliP	FD987_04205	99.6
Chemotaxis	*cheA*	FD987_01785	99
	cheV	FD987_01790	99.7
	cheW	FD987_01780	100
	cheY	FD987_05705	99.2
Motility	*flgE*	FD987_00230	99.8
	flaB	FD987_03765	99.6
	flaA	FD987_06890	94.4
	fliM	FD987_00640	100
	fliF	FD987_01960	100
	flgI	FD987_07470	100
	rpoN	FD987_03565	100
	fliK	FD987_04350	99.3
	fliA	FD987_00645	100
	fliY	FD987_00635	99.6
	flgH	FD987_03605	99.1
Other	*cdtB*	FD987_01820	99.63

*BLASTP comparison of protein sequences

4.3.2.9 Antibiotic Resistance Genes of *C. coli* S9

Antibiotic resistance in *Campylobacter* is discussed in (section 1.7.1). The Resistance Gene Identifier was used to predict the resistome of *C. coli* S9 from protein, genome, or nucleotide data based on homology and SNP models by CARD.

The results showed that *C. coli* S9 had the OXA-61 gene which is one of the AMR gene family encoding OXA beta-lactamase. This resistance gene functions by an antibiotic inactivation mechanism giving resistance to the antibiotic classes cephalosporins and penams. The identity of the matching region was 99.22% and the length of reference sequence was 100%

In addition, the genome of the *C. coli* S9 included multidrug and bile resistance response genes (Table 4.9) which are generally present in all *Campylobacter*.

Table 4.9 Multidrug and bile resistance genes in *C. coli* S9

Gene	Product	Function	locus_tag
cmeB	permease subunit CmeB	transporter	FD987_02205
cmeC	outer membrane subunit CmeC	efflux pump	FD987_02200
cmeR	transcriptional regulator CmeR	transcriptional repressor	FD987_02215

The gene *cmeB* encodes the multidrug efflux RND transporter permease subunit CmeB, which works as part of CME efflux pumps. This consists of the inner membrane efflux transporter *(CmeB)*, and *cmeC* encoding an outer membrane CME efflux pump. The *cmeR* gene encodes CmeR, the CME efflux pump transcriptional repressor (Lin et al., 2002). (Figure 4. AMR genes family for *C. coli* S9

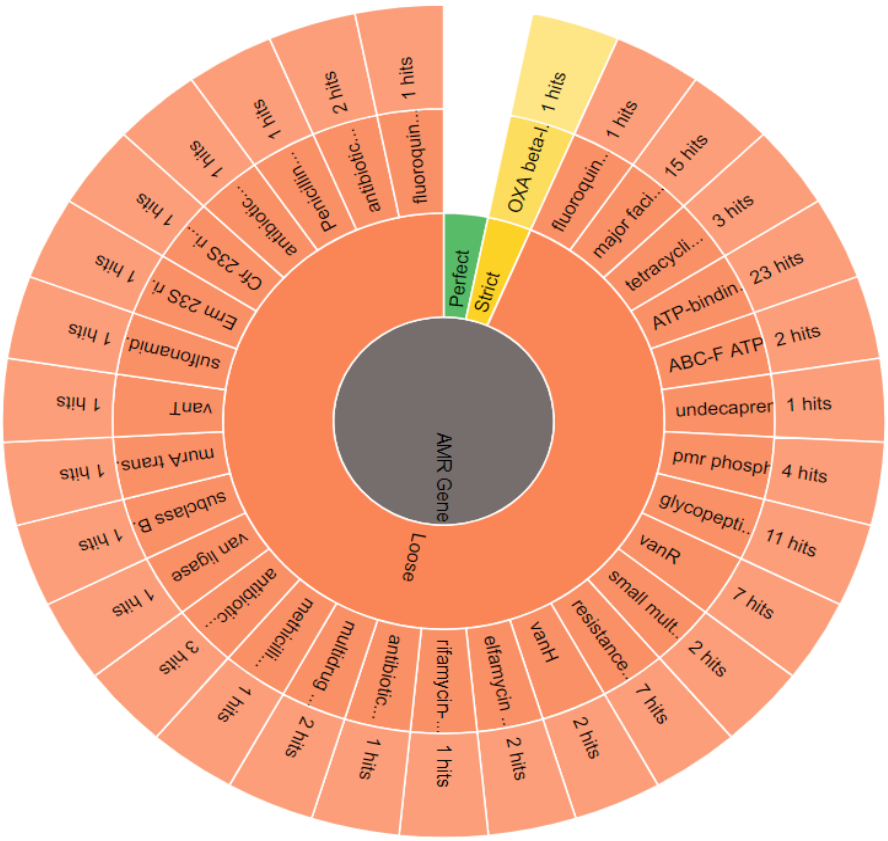


Figure 4.17 AMR gene family for *C. coli* S9

This figure was generated using the CARD: RGI online application and Perfect, Strict & Loose, complete genes only RGI Criteria.https://card.mcmaster.ca/rgi/results/Ju3pdQ5JlpyNHR21qIfhG5daIUHCX1jyqBYTDBm8

4.3.3 *C. hyointestinalis* subsp. *hyointestinalis* S12 chromosomal features

4.3.3.1 Structure and genes

The assembled circular chromosome of *C. hyointestinalis S12* is 1,752,184 bp in length with 1,811 genes and an average G+C content of 34.28. The sequence contains 3 copies of the ribosomal RNA operon and 43 tRNA genes. Two duplicated regions were identified, the first was 1,599 bp in length and the second was 2,927 bp. The first started from nucleotide position 1,171,550 to 1,173,149 and second started at nucleotide position 1, 90,767 to 1,193,694. (Figure 4.the genome map of the *C. hyointestinalis* S12 chromosome and indicates the positions of the duplicated sequences. The median chromosomal length of *C. hyointestinalis* strains listed in NCBI is 1.779 Mb, while the *C. hyointestinalis* S12 is the largest *C. hyointestinalis* chromosome complete sequences and the first with a single plasmid published in NCBI database.

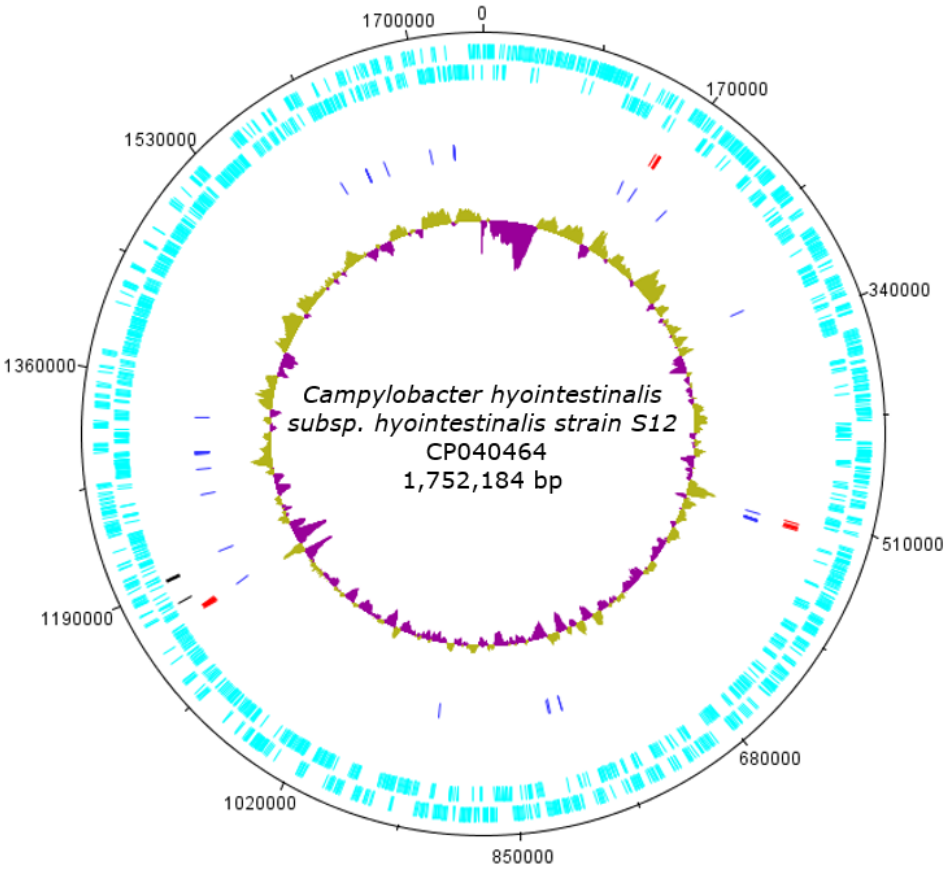


Figure 4.18 Circular representation of *C. hyointestinalis S12* chromosome

Light blue represents coding DNA sequences (Black arcs show the regions of duplicate sequences) (Red shows rRNA and Dark blue represents tRNA sequences) (The inner circle is a % G+C plot) The *C. hyointestinalis* S12 genome was found to contain 56 homopolymeric G:C tracts, defined as containing more than 7 consecutive G or C residues (Table 4.

Table 4.10 Homopolymeric repeats present in the *C. hyointestinalis* S12 genome

Position	G/C tract	Putative function	Locus
8720-8728	G(9)	intergenic	
19852-19861	G(8)	intergenic	
21920-21929	G(9)	glycosyltransferase famliy	FFA43-00095
27380-27388	G(8)	aceetyltransferase	FFA43-00120
34080-34088	G(9)	methionyl-tRNA formyltransferase	FFA43-00155
40544-40552	G(9)	PIG-L famliy deacetylase	FFA43-00195
41900-41908	C(8)	intergenic	
43048-43056	C(9)	N-acetyl sugar amidotransferase	FFA43-00210
45535-45543	G(9)	acylneuraminate	FFA43-00220
47227-47234	G(8)	hypothetical protein	FFA43-00225
49447-49454	G(8)	intergenic	
54036-54043	G(8)	hypothetical protein	FFA43-00260
55283-55292	G(10)	hypothetical protein	FFA43-00265
56510-56559	G(9)	hypothetical protein	FFA43-00275
65645-65653	G(9)	glycosyltransferase famliy 2	FFA43-00320
105816-105825	G(10)	formyl transferase	FFA43-00600
154265-154267	C(12)	intergenic N A	
161128-161136	G(9)	intergenic	
173050-173058	G(9)	intergenic	
250852-250861	G(10)	intergenic	
284391-284399	C(9)	TonB-dependent receptor	FFA43-01565
427898-427906	G(9)	intergenic	
474542-474550	G(9)	intergenic	
515758-515767	C(10)	intergenic	
522901-522909	G(9)	intergenic	
524705-524713	G(9)	intergenic	
631301-631308	C(9)	intergenic	
633286-633294	C(9)	intergenic	
647121-647131	G(10)	GGDEF domain-containing protein	FFA43-03470
722941-722949	C(9)	intergenic	
723138-723146	G(9)	intergenic	
775780-775789	C(9)	hypothetical protein	FFA43-04110
786380-786389	G(10)	intergenic	
830766-830773	C(8)	MBOAT family protein	FFA43-04370
853098-853106	G(9)	DUF2334 domain-containing	FFA43-04485
884539-884547	C(9)	intergenic	
1156144-1156151	C(8)	intergenic	
1163285-1163294	G(10)	intergenic	
1206299-1206306	C(8)	intergenic	
1208128-1208136	C(9)	intergenic	
1244496-1244504	G(9)	intergenic	
1318551-1318559	G(9)	AAC(3) famliy N-acetyltransferase	FFA43-06775
1349008-1349016	G(9)	intergenic	
1365757-1365764	C(8)	intergenic	
1480180-1480188	C(9)	3-oxoacyl-ACP synthase	FFA43-07555
1485507-1485515	C(9)	DUF4910 domain-containing	FFA43-07580
1560558-1560566	G(9)	hypothetical protein	FFA43-07975
1560747-1560756	G(10)	intergenic	
1562529-1562537	G(9)	class 1 SAM-dependent	FFA43-07990
1596066-1596074	C(9)	methytransferase domain-	FFA43-08165
1635490-1635498	G(9)	intergenic	
1660776-1660784	C(9)	intergenic	
1674792-1674800	C(9)	EAL domain-containing protein	FFA43-08570
1689550-1689558	C(9)	methyl-accepting chemotaxis	FFA43-08635
1722293-1722301	G(9)	intergenic	

4.3.3.2 Nucleotide sequence statistics

The distribution of nucleotides (Figure 4.19) was typical of an AT-rich *Campylobacter* genome. The median G+C content of *C. hyointestinalis* genomes listed on the NCBI website is 34% the nucleotide frequency in codon positions (Table 4.11).

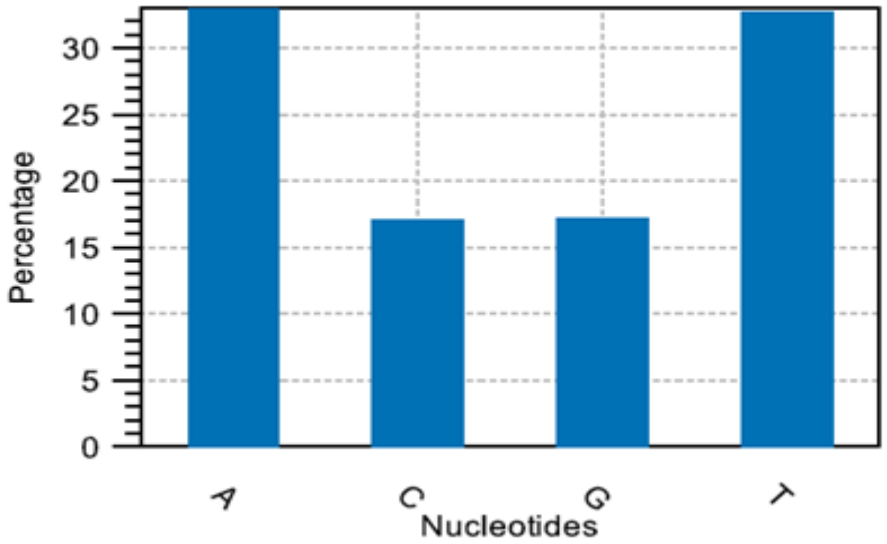


Figure 4.19 Nucleotide distribution histogram for the *C. hyointestinalis* S12 chromosome

Adenine residues were the most highly represented and distributed equally at each position whilst thymine residues were most frequent at the third than the second position. Guanosine residues were the least common in the third position and significantly more frequent in the first position. Cytosine residues were the least commonly occurring nucleotide overall, with no significate difference in frequency detected amongst each of the positions. The codon AAA, which encodes the amino acid lysine was the most abundant codon occurring 38,889 times and CGG, which encodes arginine, was the least abundant at 114.

Table 4.11 Frequency of nucleotide codon positioning for the *C. hyointestinalis* S12 chromosome

Codon position	A	C	G	T
1. position	0.37	0.13	0.31	0.18
2. position	0.35	0.17	0.15	0.33
3. position	0.36	0.15	0.13	0.37

4.3.3.3 Comparison with nearest *C. hyointestinalis* DNA sequence neighbours

The LAGAN shuffle comparison was used to identify the similarity between the *C. hyointestinalis* S12 and the nearest neighbour from the same species and subspecies. There was only one *C. hyointestinalis* subsp. *hyointestinalis* complete assembled genome sequences available in the NCBI database at the time of analysis. This was *C. hyointestinalis* subsp. *hyointestinalis* LMG 9260. Figure 4.16 shows the alignment of the two genomes, where it is evident that there were large regions of low sequence identity present. The mVISTA browser function identified of genes which were present and absent in C. *hyointestinalis* LMG 9260 compared to *C. hyointestinalis* S12.

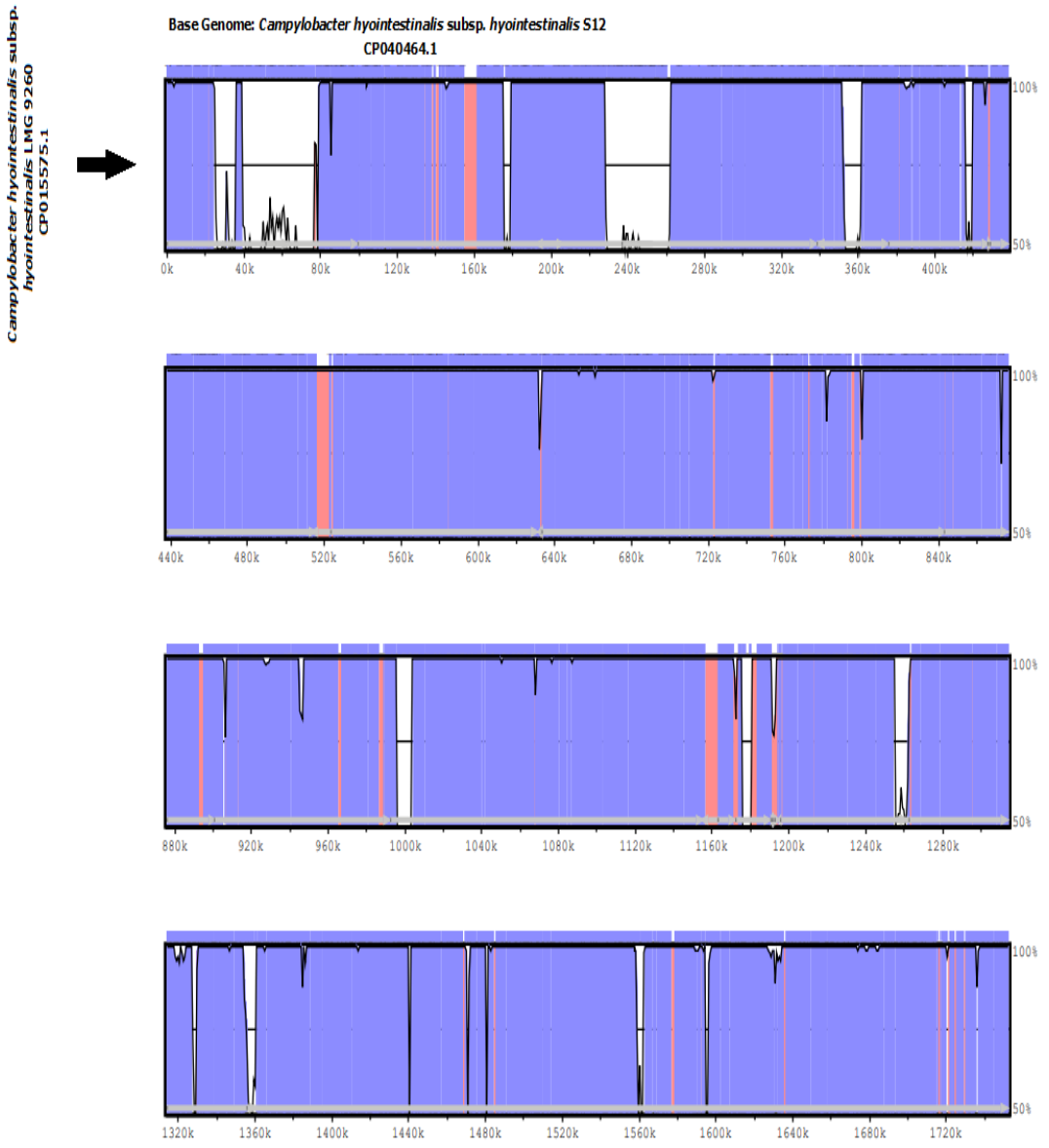


Figure 4.16 Chromosomal alignment of *C. hyointestinalis* S12 and *C. hyointestinalis* LMG 9260. Plot showing the percentage identity and locations of missing genes across the *C. hyointestinalis* S12 chromosome aligned with and *C. hyointestinalis* LMG 9260. Purple represents conserved regions; pink represents conserved non-coding sequences (CNS) and white represents missing or relocated genes. Generated by mVISTA with the LAGAN function

Table 4.12 Nucleotide sequence similarity of *C. hyointestinalis* S12 compared to other *C. hyointestinalis* strains

Strain	*Symmetric identity %	Gapped identity %
C. hyointestinalis subsp. *hyointestinalis* LMG 9260	89.13	98.39
C. hyointestinalis MGYG-HGUT-02307	89.11	98.38
C. hyointestinalis subsp. *lawsonii* CCUG 27631	76.36	94.73

*Identities were calculated by whole genomic BLAST

A total of 182 genes were identified to present in *C. hyointestinalis* S12 and absent in *C. hyointestinalis* LMG 9260 with 52 being hypothetical proteins (Appendix 6). The absent genes from *C. hyointestinalis* S12 but present in *C. hyointestinalis* LMG 9260 are shown in Appendix 7.

4.3.3.4 Plasmid pCh1 (S12)

4.3.3.4.1 Structure and genes

Analysis of the whole genome sequence of the *C. hyointestinalis* S12 cattle strain revealed the presence of one plasmid. This was the first recorded plasmid sequence belonging to *C. hyointestinalis* in the NCBI data base (accession number CP040465.1). The plasmid (Figure 4.21) was 6,796 bp in length and had a G+C content of 29.86 %.

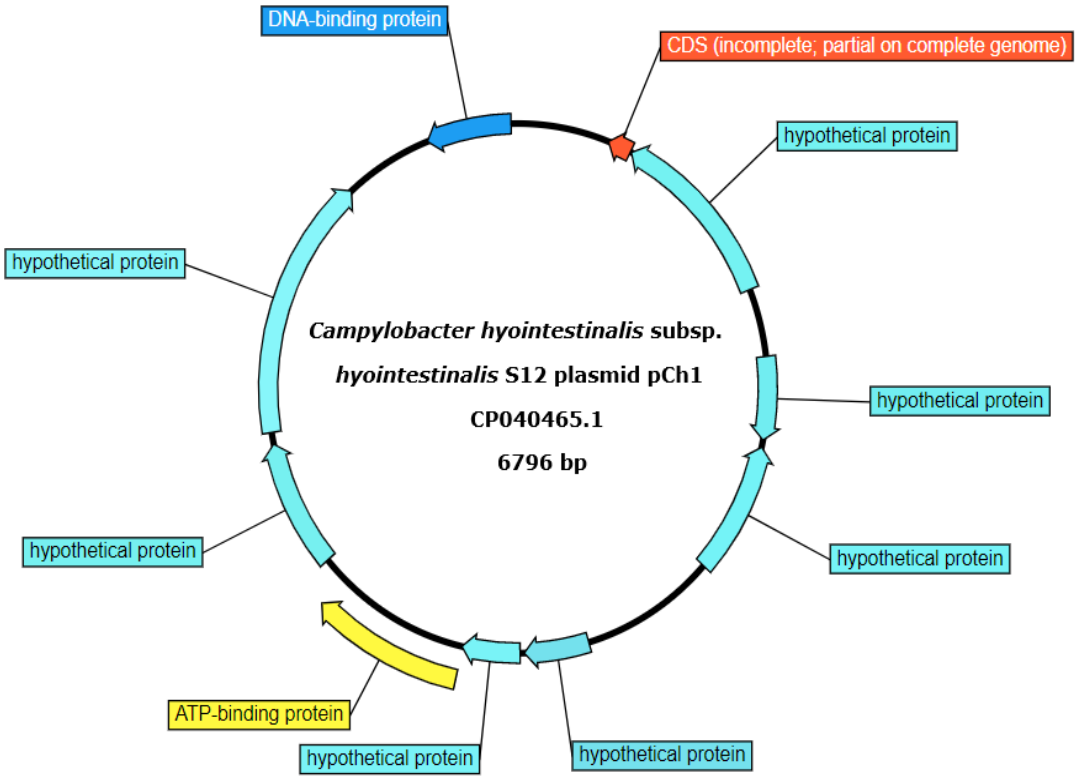
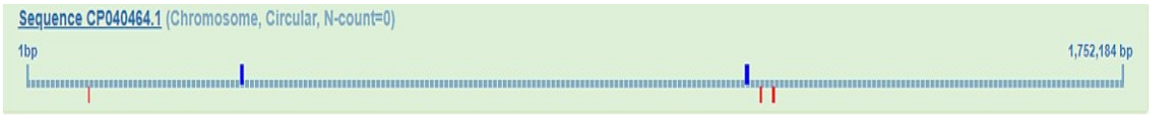


Figure 4.21 shows the structure of plasmid pCh1, which contained 37 open reading frames, including 11 genes encoding for 9 proteins

4.3.3.5 CRISPRs of *C. hyointestinalis* S12

Campylobacter hyointestinalis S12 also has three CRISPR arrays in the chromosome (Figure 4.22). Table 4. shows the sequences of direct repeats and spacers as calculated by the CRISPRs web server: (https://crisprcas.i2bc.paris-saclay.fr/MainDb/StrainList/CP034309.1). The length of direct repeats and spacers appear to vary between the two pairs of arrays (Abby et al., 2014) .



Element	CRISPR Id / Cas Type	Start	End	Spacer/ Gene	Repeat consensus/ cas genes	Direction	Evidence level
CRISPR	CP040464.1_1	99,674	99,793	1	TTTAGATTTAGTTCTTTAGTACGAATTTATTCGTA	ND	1
Cas cluster	CP040464.1|CAS-TypeIIC_1	341,841	346,085	3	Cas1_0_II_2, Cas2_0_I-II-III_3, Cas9_0_II_1		
Cas cluster	CP040464.1|CAS-TypeIB_2	1,147,311	1,152,909	6	Cas1_0_I-II-III-V_1, Cas2_0_I-II-III_2, Cas3_0_I_4, Cas4_0_I-II_3, Cas5_1_IB_5, Cas7_2_IB_6		
CRISPR	CP040464.1_2	1,171,550	1,173,149	24	GTTTCAAATCCTAAAAGGATAAATTTATAC	+	4
CRISPR	CP040464.1_3	1,190,837	1,193,694	43	GTTTCAAATCCTAAAAGGATAAATTTATAC	+	4

Figure 4.22 the positions of the CRISPR arrays and *cas* genes in details on *Campylobacter hyointestinalis* S12 genome. Red represents CRISPR arrays and blue cas clusters.

Table 4.13 Direct repeat and spacer sequences in the *C. hyointestinalis* S12 genome

Region	Direct Repeat	Spacer
	CRISPR 1	
99674	TTTAGATTTAGTTCTTTAGTACG AATTTATTCGTA	TAATTTAAAAGAATATAAATTTCTAAAGCGT GAGCAAAAGCGAACGGACT
99759	TTAGTATTTAGTTCTTTAGTACG AATTTATTCGTA	
1171550	GTTTAAAATCCTAAAAGGATAAA TTTATAC	AACATTACACCAGCGACATCAGTTGCAAGA TCAAATCT
1171618	GTTTCAAATCCTAAAAGGATAAA TTTATAC	GTTTATGATCTCTATCGTATCGTTCTCATCA CGGA
1171683	GTTTCAAATCCTAAAAGGATAAA TTTATAC	GCTTGGCTTGACGCTCAAGAGCAAAAAGC AAAAAA
1171748	GTTTCAAATCCTAAAAGGATAAA TTTATAC	AGCAACTTTTCAGATTAATACCATAGTGAA ATT
1171811	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TTCAAGCCAAAGTATATCAAGGGCGAACCT CTGCA
1171876	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CCCAAACCCAGTGACCATTTCAAATACACC TATAC
1171941	GTTTCAAATCCTAAAAGGATAAA TTTATAC	AAAAGCCTTGGAAAACACTACAGGCTCGA CGTGATTTTT
1172010	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TCAAGGCATCAAATGAACAATAGAGCAGCA AGAC
1172074	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TCTGTTTATTTATCGTCTGATGCTGATAGAT AAAA
1172139	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CCCTTTCTGTTTGCGATATCTGCGCTAAAT TCAGGTA
1172206	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TGAAATAGCTAAGTTCGACTAGATTATTATT TATTC
1172272	GTTTCAAATCCTAAAAGGATAAA TTTATAC	AAAACCAAACCACGTTTTTAAGGCTATTTA AACA
1172336	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TTCAAAGGGGAAGGTAAAATGGTTTTTTGA TTATC
1172401	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CATAATCACAAAAATTCGTAGAGAATTTGG CGATGAAA
1172469	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CCTAGGCACCCCTCAAACAGAGGAGAGTA TCTATAA
1172535	GTTTCAAATCCTAAAAGGATAAA TTTATAC	ATGCTCAAACTCAGCAACAAAGCAATTATA GCTA
1172599	GTTTCAAATCCTAAAAGGATAAA TTTATAC	GTTATCGGAGAGAATGAATTTGCAAAGGTA ATATA
1172664	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CGTAGTAGTCTGCTTTGTCGATGAAAAGGC TTAAAA
1172730	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CTATTATTTTTAGCCCTTTTGCTTTATTATC ATCA
1172795	GTTTCAAATCCTAAAAGGATAAA TTTATAC	ATTGCAAGATTTTTACCTATGCCTTTCTCAT TTGC
1172860	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TTGATTATAAACTTAGTTGCAATTAGTTCTT ATT
1172924	GTTTCAAATCCTAAAAGGATAAA TTTATAC	CGCCGCTTTTAAATCAGATTGATAATGTCT TTTA
1172988	GTTTCAAATCCTAAAAGGATAAA TTTATAC	TCTCATTATCTTTTTCAAGATTATTAATTGT ATTAT

1173054	GTTTCAAATCCTAAAAGGATAAATTTATAC	TAGAGTTTCAAGTTTAATACTACTATTTTTAATTTC
1173120	GTTTCAAATCCTAAAAGGATAAATTTATAC	

CRISPR 2

1171550	GTTTAAAATCCTAAAAGGATAAATTTATAC	AACATTACACCAGCGACATCAGTTGCAAGATCAAATCT
1171618	GTTTCAAATCCTAAAAGGATAAATTTATAC	GTTTATGATCTCTATCGTATCGTTCTCATCACGGA
1171683	GTTTCAAATCCTAAAAGGATAAATTTATAC	GCTTGGCTTGACGCTCAAGAGCAAAAAGCAAAAAA
1171748	GTTTCAAATCCTAAAAGGATAAATTTATAC	AGCAACTTTTCAGATTAATACCATAGTGAAATT
1171811	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTCAAGCCAAAGTATATCAAGGGCGAACCTCTGCA
1171876	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCCAAACCCAGTGACCATTTCAAATACACCTATAC
1171941	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAAAGCCTTGGAAAACACTACAGGCTCGACGTGATTTTT
1172010	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCAAGGCATCAAATGAACAATAGAGCAGCAAGAC
1172074	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCTGTTTATTTATCGTCTGATGCTGATAGATAAAA
1172139	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCCTTTCTGTTTGCGATATCTGCGCTAAATTCAGGTA
1172206	GTTTCAAATCCTAAAAGGATAAATTTATAC	TGAAATAGCTAAGTTCGACTAGATTATTATTTATTC
1172272	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAAACCAAACCACGTTTTTAAGGCTATTTAAACA
1172336	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTCAAAGGGGAAGGTAAAATGGTTTTTTGATTATC
1172401	GTTTCAAATCCTAAAAGGATAAATTTATAC	CATAATCACAAAAATTCGTAGAGAATTTGGCGATGAAA
1172469	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCTAGGCACCCCTCAAACAGAGGAGAGTATCTATAA
1172535	GTTTCAAATCCTAAAAGGATAAATTTATAC	ATGCTCAAACTCAGCAACAAAGCAATTATAGCTA
1172599	GTTTCAAATCCTAAAAGGATAAATTTATAC	GTTATCGGAGAGAATGAATTTGCAAAGGTAATATA
1172664	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGTAGTAGTCTGCTTTGTCGATGAAAAGGCTTAAAA
1172730	GTTTCAAATCCTAAAAGGATAAATTTATAC	CTATTATTTTTAGCCCTTTTGCTTTATTATCATCA
1172795	GTTTCAAATCCTAAAAGGATAAATTTATAC	ATTGCAAGATTTTTACCTATGCCTTTCTCATTTGC
1172860	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTGATTATAAACTTAGTTGCAATTAGTTCTTATT
1172924	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGCCGCTTTTAAATCAGATTGATAATGTCTTTTA
1172988	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCTCATTATCTTTTTCAAGATTATTAATTGTATTAT
1173054	GTTTCAAATCCTAAAAGGATAAATTTATAC	TAGAGTTTCAAGTTTAATACTACTATTTTTAATTTC
1173120	GTTTCAAATCCTAAAAGGATAAATTTATAC	

CRISPR 3		
1190837	GTTTCAAATCCTAAAAGGATAAATTTATAC	GTTTATGAAAATAAAATGTAAAGAAGTGTAAAGT
1190901	GTTTCAAATCCTAAAAGGATAAATTTATAC	CTGAAATTGCCCCTACTAAGAGTTGCTATAAGACTAA
1190968	GTTTCAAATCCTAAAAGGATAAATTTATAC	TACGGCGAGTTTGTAGATGGCTCGTCAAACCTACTGC
1191035	GTTTCAAATCCTAAAAGGATAAATTTATAC	CATATACAAATTTACCAAACAATGAAAAACATAAAA
1191101	GTTTCAAATCCTAAAAGGATAAATTTATAC	TGGAAACGCAATAGCAAGTAATTCACTAGCAATCAC
1191167	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAAATGATAGAGATAATAGATGAATATAACCTAT
1191231	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTAAATTTAATGATAAAGGAAATACCGATGAAAAAGA
1191298	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGAAGTTTTTAAAAATCTCAACCTAGAAGCTTTCAG
1191364	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTATTCTTTCAATCTCCGGCTTATTTTTTTTCATTTC
1191431	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCGTTGCCCCAAAACTAAAAGACAGTTTTTAAAAAAAC
1191499	GTTTCAAATCCTAAAAGGATAAATTTATAC	CATATTACCACTATTATCTACTATGTACCACATAT
1191564	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCAACAGTACCAAGTGATTTAGACATAGCGGTATTGAA
1191632	GTTTCAAATCCTAAAAGGATAAATTTATAC	ATACTAGTCATGATAAGAACTCCGAAGAAGAGTT
1191696	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTATGTCAGCAGTGCCGACGCTCTCATTGGCGCTG
1191761	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTTGGCAAATAGTTAAGTAAATAAAACCAAATCAAA
1191827	GTTTCAAATCCTAAAAGGATAAATTTATAC	GACAACTTGGAGCTTAGAAAACTTCATAAAAACCA

CRISPR 3		
Region	**Direct Repeat**	**Spacer**
1191892	GTTTCAAATCCTAAAAGGATAAATTTATAC	ATCACGCTTGGGCTAGTTATCGTAGTAGCCTCAGCTA
1191959	GTTTCAAATCCTAAAAGGATAAATTTATAC	AGAGTTCATAGATATGACTTTTCGAGAGTATGATAA
1192025	GTTTCAAATCCTAAAAGGATAAATTTATAC	AATGGCAAAGGCTATCTTGGAGTTACAAAAAAAC
1192089	GTTTCAAATCCTAAAAGGATAAATTTATAC	CAAGCTGGAAATTCGCCCTAATTTGTTTCTCCCTGAAA
1192157	GTTTCAAATCCTAAAAGGATAAATTTATAC	ATATATAGTTTGGGATAAAAAAGAAGATGGCAAACC
1192223	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCGTTGGCTTTCTGTTCATCTGATAAATCGTAAGTCCG
1192291	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCAATATCTTTTATCTTGATTTCAAGTGGTTTTA
1192355	GTTTCAAATCCTAAAAGGATAAATTTATAC	ACTATAGTTTTGCTCTGCAACACCTTTGGATACA
1192419	GTTTCAAATCCTAAAAGGATAAATTTATAC	CATTACAAAACTTACTCTAAAAGCAAAATATATGC
1192484	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAAACCAAACCACGTTTTTAAGGCTATTTAAACA

1192548	GTTTCAAATCCTAAAAGGATAAATTTATAC	TGGTCAGATATCGACTTTGCAAGGAACTTGATAAATA
1192615	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCTTTCTGTTTGCGATATCTGCGCTAAATTCAGGTA
1192681	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCCTGAGTATTGGCTAGTTTCAGGGAGAAAAATTCA
1192747	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGAGCGAAAAAGGGCGTATGTTATTGCCTACACCGA
1192813	GTTTCAAATCCTAAAAGGATAAATTTATAC	CTTTAAAGCCTTCATTTTCTTTTATGCTATCTAT
1192877	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGCTTCCTTATACGCAAGTTCAAACTCAAAACTTA
1192942	GTTTCAAATCCTAAAAGGATAAATTTATAC	CTACTCATCTCAAATGCAGTAGCACCAGTAGCAAG
1193007	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTAAAAAACAAGCTCCACAGCACTACAAAGAGAG
1193071	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAGGAGATGGAAAATGAACACGCAAACTATGCAAAGT
1193138	GTTTCAAATCCTAAAAGGATAAATTTATAC	GCGAACAGAGCGAAGAAAGTAGTGAAAGTGCAAATA
1193204	GTTTCAAATCCTAAAAGGATAAATTTATAC	AGCCTTGGAAAACACTACAGGCTAGACGTGACTTTC
1193270	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAATCTTCAGTCGGAAAAGCTTCTTTAAGCAACA
1193334	GTTTCAAATCCTAAAAGGATAAATTTATAC	AGTTTGGCAATGCAGGTAGAGTTTTAGCTATCTCTGA
1193401	GTTTCAAATCCTAAAAGGATAAATTTATAC	AATTTGATCCGTTGATTGTTGAAACTAAAATCGTAAC
1193468	GTTTCAAATCCTAAAAGGATAAATTTATAC	TTCTGCTTGAAAGTCAATTGTGGCAACATTTTGGCT
1193534	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCGACTTATAGCTATGGAATCCTTAGAAAGTCTTG
1193599	GTTTCAAATCCTAAAAGGATAAATTTATAC	TAGAGTTTCAAGTTTAATACTACTATTTTTAATTTC
1193665	GTTTCAAATCCTAAAAGGATAAATTTATAC	

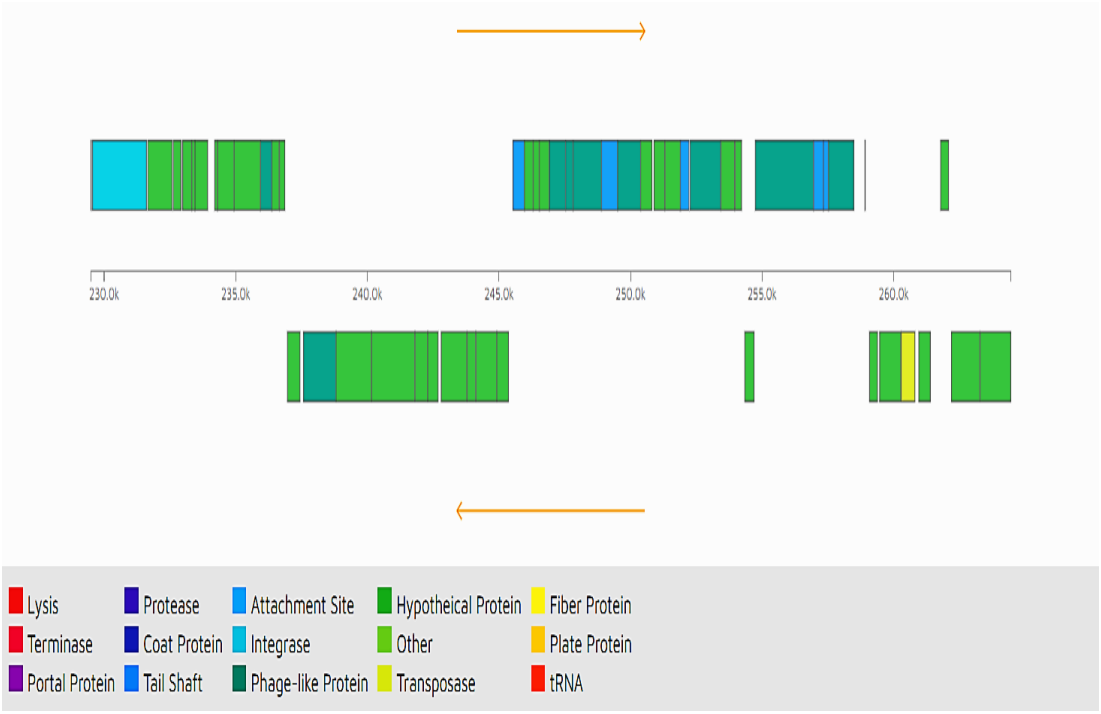
Noticeably, the results revealed that chromosome has two cas gene clusters, the first classed as a CAS-TypeIIC_1 which started from position 341,841 to 346,085. This includes the genes: Cas9_0_II_1, (341,841-344,318), Cas1_0_II_2 (344,872 - 345,765) and Cas2_0_I-II-III_3 (345,765 - 346,085), all of them in the forward orientation. The second cas gene cluster may be classed as a CAS-TypeIB_2, which starts from position 1,147,311 to 1,152,909. This includes 6 genes: Cas1_0_I-II-III-V_1 (1,147,311-1,148,303), Cas2_0_I-II-III_2 (1,148,313-1,148,591), Cas4_0_I-II_3 (1,148,626 1,149,123), Cas3_0_I_4 (1,149,154-1,151,334), Cas5_1_IB_5 (1,151,276-1,151,992) and Cas7_2_IB_6 (1,151,989-1,152,909),

all of them in reverse orientation. Appendix 8 details the nucleotide BLAST results from the CRISPR direct repeats consensus sequence of *C.hyointestinalis* S12*.* This shows that the DR consensus sequence of the CRISPRs arrays are present in the genomes two other *Campylobacter* species. The *C. fetus* subsp. *testudinum* 772, *C. fetus* subsp. *testudinum* Sp3 and *C. fetus* subsp. *testudinum* pet-3 chromosomes have 100% for both coverage and identity and same e-value with *C. hyointestinalis* S12 DR consensus sequence from the first CRISPR array. While, only *Campylobacter hyointestinalis* MGYG-HGUT-02307 have 100% coverage and identity with 2e-06 for the second and the third CRISPRs arrays DR consensus sequences. Nucleotide BLAST results from CRISPR Number 1 spacers of *C. hyointestinalis* S12 are listed in Appendix 9. Nucleotide BLAST results from CRISPR Number 2 spacers of *C. hyointestinalis* S12 are listed in Appendix 10. Nucleotide BLAST results from CRISPR Number 3 spacers of *C. hyointestinalis* S12 are listed in Appendix 11. In addition, Nucleotide BLAST X results from the CAS-Type IB genes sequences of *C. hyointestinalis* S12 are recorded in Appendix 12.

4.3.3.6 Prophage insertions

A single prophage insertion was present in *C. hyointestinalis* S12 (Fig 4.23 A and B), orientated in both directions that correlated with a region of sequence non-identity with *C. hyointestinalis* LMG 9260 (Figure 4.16). The prophage had an approximate position of 229499-264445 kb and was 34.9 kb in length. This prophage insertion was classified as incomplete (score > 90) by the online program http://phaster.ca/.

A



B

Figure 4.23 A) Prophage insertion in *C. hyointestinalis* S12 in both strands of the DNA and B) position in chromosome

4.3.3.5 Taxonomic characterisation of *C. hyointestinalis* S12 by whole genome BLAST

The nearest neighbour to *C. hyointestinalis* S12, according to the NCBI Genome neighbour report was *C. hyointestinalis* LMG 9260, which exhibits a symmetric identity and gapped identity with *C. hyointestinalis S12* of 89.13 % and 98.39% respectively. The *C. hyointestinalis S12* genome represents one of four complete *C. hyointestinalis* genomes available at the NCBI database at the time of analysis. All four of these chromosomes were approximately 1,750,000 bp in size. (Figure 4.17) the dendrogram, based on genomic BLAST, of *C. hyointestinalis* S12 phylogeny.

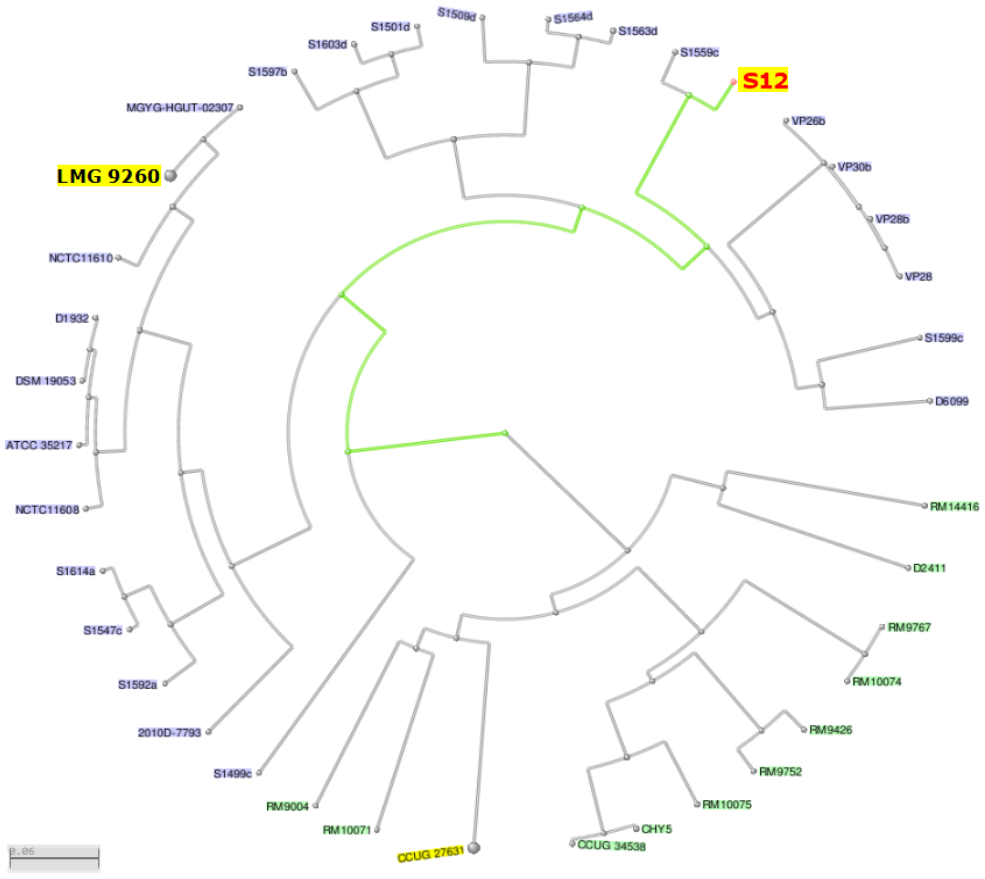


Figure 4.17 Phylogenetic comparison of *C. hyointestinalis* S12 with *C. hyointestinalis* LMG 9260 *Campylobacter hyointestinalis* S12 is red coloured comparisons are based on genomic BLAST searches and the dendrogram was calculated using the Genome feature of the NCBI website.

4.3.3.6 Virulence Factors

As a member of the genus *Campylobacter* most of the genes which potentially encode virulence factors are common to both *C. hyointestinalis* and *C. coli*. BLAST analysis of the genome sequence of *C. hyointestinalis* S12 using revealed similar results to those obtained for *C. coli* S9 (section 4.8).

Table 4.14 Virulence factors identified in *C. hyointestinalis* S12

Virulence factor	Gene	Locus Tag *C. hyointestinalis* S12	Identity to *C. hyointestinalis* LMG 9260 %*
Adhesion/	*cadF*	FFA43_07125	98.8
Invasion	*flaC*	Not found	
	flhB	FFA43_03045	98.8
	fliR	FFA43_01805	100
	fliQ	FFA43_00990	100
	ciaB	FFA43_06450	97.7
	ciaC	Not found	
	flhA	FFA43_01515	98.5
	fliP	FFA43_05180	99.6
Chemotaxis	*cheA*	FFA43_07410	98.45
	cheV	FFA43_07415	98.8
	cheW	FFA43_07405	99.4
	cheY	FFA43_06745	99.2
Motility	*flgE*	FFA43_08695	99.4
	flaB	FFA43_08150	87.3
	flaA	FFA43_05910	98
	fliM	FFA43_07775	100
	fliF	FFA43_01525	100
	flgI	FFA43_02165	100
	rpoN	FFA43_05905	99
	fliK	FFA43_00055	100
	fliA	FFA43_07770	100
	fliY	FFA43_07780	99.6
	flgH	FFA43_03855	98.7
Other	*cdtB*	FFA43_00370	99.6

4.3.3.7 Antibiotic Resistance Genes

The Resistance Gene Identifier was used to predict the resistome of *C. hyointestinalis* S12 from protein, genome, or nucleotide data based on homology and SNP models by CARD, using the stingent criteria: perfect and strict hit only option used to detect the antibiotic resistance genes. The results showed that *C. hyointestinalis S12* has the *gyrA* allele, which is one of the AMR gene family encoding for fluoroquinolone resistance. This resistance gene functions by an antibiotic target alteration mechanism. The identity of the matching region was 77.78% and the length of reference sequence was 100%.

4.4 Discussion

This work provides new genomic information on *Campylobacter* and species from cattle, which have been somewhat neglected. This was essential for their intended use as hosts for phage isolation. All the isolates (*A. skirrowii A2S6, C. coli S9 and C. hyointestinalis S12)* results demonstrate that, all three strains had a genomic structure typical of their relatives with sequenced genomes. The presence of homopolymeric repeats is common in *Campylobacter* (Clark et al., 2016, Miller et al., 2010), but the number identified in *C. hyointestinalis* appears particularly high. There is little information published related to *Arcobacter* species. Interestingly, the taxonomic analysis revealed that each of the three strains grouped with isolates from a similar source. The two *Campylobacter* strains had plasmids which has been highlighted in many other campylobacters (Boukerb et al., 2020, Chen et al., 2013, Marasini et al., 2018). The *A. skirrowii* A2S6 genome did not include any plasmids, however plasmids have been reported in different species of *Arcobacter* (On et al., 2019). This study identified a completely new plasmid in *C. hyointestinalis* named pCh1, which had not been reported before in any *C. hyointestinalis* that have been sequenced (Miller et al., 2016). All three sequenced strains had prophage sequences. Prophages in *Campylobacter* are common (Liu et al., 2016, Marasini and Fakhr, 2016), and also have been reported in many *Arcobacter* species (Miller et al., 2007, Millar and Raghavan, 2017, Rovetto et al., 2017). The campylobacters had the expected virulence factors (Bolton, 2015, Mm Hassan, 2019). The virulence of *Arcobacter* is completely unknown and there is little evidence of virulence factors corresponding to those of campylobacters in their genomic sequence. In humans, *Arcobacter* have been associated with gastrointestinal disease. Arcobacters are also thought to contribute to abortion in cattle as they can be isolated from aborted cattle foetuses. They can be isolated from pets with no disease symptoms, but they are considered as opportunistic agents (Ferreira et al., 2016b, Di Blasio et al., 2019, Pejchalová et al., 2016). They

seem to be generally harmless organisms living in cattle and chickens which occasionally cause disease in humans that is largely self-limiting. Whilst the *A. skirrowii* A2S6 strain had only one AMR associated gene it was important because fluroquinalones are used to treat human clinical cases (see 1.7.2). Further work should undertake to further explore the role of arcobacters in the increase in antibiotic resistance.

From the total results in this chapter (Chapter 4) which provides a strong base for the bacteriophage isolation experiments (Chapter 5) and considerably adds to our knowledge of the genomic characteristics of *Arcobacter* and *Campylobacter* from cattle sources.

Chapter 5

Isolation and characterisation of *Campylobacter* and *Arcobacter* specific bacteriophages from cattle slurry samples

5.1 Introduction

Bacteriophage, or phage, are viruses that target bacteria. Phages can be present in any environmental system containing their bacterial hosts having an important role in biological processes related to their environments (Kakasis and Panitsa, 2019). Phage are known to be highly host specific, which can be to a species or even one strain. Bacteriophage are believed to be the most abundant and diverse organisms in nature (Keen, 2015).

The majority of phages (more than 95%) that are isolated and classified belong to the order Caudovirales. These include the families Siphoviridae, Myoviridae and Podoviridae, which all have double stranded DNA, isometric heads and tails (Kabwe et al., 2020, Al-Zubidi et al., 2019, Sharp, 2001).

The bacteriophage life cycle can divided into virulent or temperate types. Virulent or lytic phages, are phage that replicate through the lytic cycle. The phages attach to the bacterial host and then inject their genomic material which redirects the host's molecular machinery to synthesize virus particles before the final destruction of the host cell to release new virions (Sausset et al., 2020). Lysis of the cell wall by virulent phages is controlled by two types of protein, the holins and the lysins, both of which play pivotal roles in the final stages of the lytic cycle (Cisek et al., 2017). Temperate or lysogenic phages, have the capability to integrate their genome into the host DNA and stay dormant as prophage. Here they are replicated with the host genome but under specific circumstances, such as host stress, can shift into the lytic cycle to replicate new virions and exit the cell (Sausset et al., 2020). The importance of the lysogenic cycle on the host cells is that the lysogen can encode genes that confer phenotypic advantages to the host bacteria, including antibiotic resistance, virulence factors, and resistance to superinfection (Sausset et al., 2020).

Bacteriophages that attack *Campylobacter* have been reported over the last sixty years, although changes in *Campylobacter* taxonomy have sometimes made the host phage relationships unclear (Connerton et al., 2011). However, the first lytic phages that can infect what are reclassified as *C. coli* and *C. fetus* were isolated from cattle and pigs between 1964 -1968 (Fletcher and Bertschinger, 1964, Firehammer and Border, 1968, Fletcher, 1965). The first temperate bacteriophages and their host were isolated from aborted sheep foetuses by Bryner et al. (1982). The majority of *Campylobacter* bacteriophage isolated possess double-stranded DNA, with icosahedral heads and tails, belonging to the *Myoviridae* family (Connerton et al., 2008). Lysogenic or temperate bacteriophage of *Campylobacter* are variably observed as prophages in genome sequence data. For *C. jejuni* these include a Mu-like bacteriophage (Fouts et al., 2005b), which was notably absent in the first genomic sequence of the type strain *C. jejuni* NCTC 11168 (Parkhill et al., 2000). Moreover, Mu-like prophage have been demonstrated to mediate genomic rearrangements in *C. jejuni* DNA that can lead to phage resistance and the generation of infectious bacteriophage CampMu (Scott et al., 2007).

Virulent *Campylobacter* bacteriophages can reduce the intestinal carriage of *Campylobacter* by broiler chickens (Atterbury et al., 2005). Using phage as an intervention in controlled trials (Loc Carrillo et al., 2005; Wagenaar et al., 2005; El-Shibiny et al., 2009) and in broiler house applications (Kittler et al., 2013) has demonstrated reductions in the intestinal load of *Campylobacter* by approximately 2 $\log_{10}$ CFU/g (Carvalho et al., 2010; Connerton et al., 2011; Hammerl et al., 2014; Richards et al., 2019). This represents a potential reduction in the risk of infection from contaminated poultry meat (Crotta et al., 2017). No *Arcobacter* phage have not been described to date. The aim of study was to extend the availability of phages for

the control *Campylobacter* in dairy production and to attempt to isolate *Arcobacter* phages which would be expected to be present alongside their hosts in cattle.

5.2 Material and Methods

5.2.1 Collection of cattle slurry samples

Cattle slurry samples were collected from Centre of Dairy Research and Innovation located Sutton Bonington, Nottingham, LE12 5RY, coordinates (52.839240, -1.249674) on two occasions (9/9/2018 and 12/04/2019). Slurry samples were collected from different farm locations including: Site 1 "Silage pit"; Site 2 "Crud Stackable deep litter bedding mature"; Site 3 "Dairy cow and shed effluent"; site 4 "Heifers shed effluent" and Site 5 "Growing heifer (11 to 15 meters) and cow effluent". The sampling locations are shown in Figure 5.1. The sample from Site 5 was collected by means of a 100 ml plastic pot attached to 20 m plastic rope, which allowed collection from a 5-6 m depth. These silage, effluent and other slurry samples were collected in 50 ml sampling pots, placed in a sterile polythene bag, and then placed in a cooled sample box. The samples were then transferred immediately to the laboratory and kept at 4^{o}C until the next day for isolation.

Figure 5.1 Birds Eye view of University of Nottingham - Centre of Dairy Research and Innovation A) Site locations 1-5, B) Maximised view of sample 5 location

5.2.2 Host lawn preparation

Fresh subcultures of *C. jejuni* PT14, *C. hyointestinalis* S12 and *A. skirrowii* A2S6 described and characterised in Chapter 4, were made on BA (2.1.1) plates and incubated under microaerobic conditions at 42°C for 24 h (*C. jejuni* PT14), microaerobic conditions at 37°C for 48 h (*C. hyointestinalis* S12) or microaerobic conditions at 30°C for 48 h (*A. skirrowii* A2S6). Bacterial growth from each strain was harvested in a 10 ml solution of 10 mM MgSO4 and 1 mM $CaCl_2$ using sterile swab. The cell density was then adjusted to approximately 10^8 CFU /ml, which approximated to growth from half of the plate having an OD_{600} of approximately 0.350 for *C. jejuni* PT14. For *C. hyointestinalis* S12 and *A. skirrowii* A2S6 three whole plates were

harvested in the same amount of solution. An aliquot of 500 µl of each suspension was added to 5 ml of molten NZCYM overlay agar (section 2.1.4) at 50°C mixed and quickly added to the surface of pre-warmed NZCYM basal agar (section 2.1.3). Plates were then left to set.

5.2.3 Detection and isolation of bacteriophages, non-enrichment method

To each slurry sample, 1 g was mixed with 9 ml of SM buffer (section 2.1.11). The mixtures were then incubated at 4°C for 24 h with slow rotation to elute the phage into the buffer. A 1 ml aliquot of the first dilution was removed and centrifuged at 3,000 x *g* for 3 minutes, the supernatant from which was then subjected to second centrifugation step for 5 minutes at 13,000 x *g*. The second supernatant was then passed through a 0.45 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) and a 0.22 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) to remove any remaining bacterial cells. After that, 10 µl aliquots of 10 fold serial dilutions were dispensed onto a lawns of the host strain lawns (section 5.2.2) in triplicate with positive control (lawn only) and negative control (media only). All the plates were then incubated under microaerobic conditions for 48 h, *C. jejuni* PT14 plates incubated at 42°C, while the *C. hyointestinalis* S12 were incubated at 37°C and *A. skirrowii* A2S6 plates were incubated at 30°C. Any visible plaques of lysis were monitored after the incubation period. Any spots that showed lytic reactions were harvested with a pipette tip then suspended in SM buffer (section 2.1.11) for plaque purification.

5.2.4 Plaque purification

Primary plaques were sequentially propagated to ensure single clones were obtained. Single, well isolated plaques were picked with a pipette tip and suspended in 500 µl SM buffer (section 2.1.11). The suspensions were then passed through a 0.45 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) and a 0.22 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany). After which serial dilutions of the supernatant were performed, and 10 µl of each dilution in triplicates were dispensed on NZCYM overlay plates (section 2.1.4) containing the bacterial host isolates in the top layer. Plates were then incubated according to the host requirements as mentioned above. The process was repeated selecting well isolated plaques from the dilution series. Finally, after five rounds of purification, a single purified plaque of suspected bacteriophages was suspended in 100 µl SM buffer (section 2.1.11) and stored at 4°C.

5.2.5 Bacteriophage propagation and storage

For the propagation of bacteriophages host cells were prepared as described in (section 5.2.2) To 400 µl of the cell suspension, a 100 µl aliquot of a bacteriophage stock containing 8 $\log_{10}$ PFU/ml was then added and mixed. This mixture was incubated for 20 - 30 minutes under microaerobic conditions at the appropriate temperature for the host. Next, 5 ml NZCYM overlay agar aliquots (section 2.1.4) in sterile universal bottles were melted in the microwave, and kept in a water bath at 55°C. The incubated host cell and bacteriophage suspensions were then transferred to the overlay agar tube and mixed prior to pouring onto plates of NZCYM basal agar (section 2.1.3) and allowed to set. The plates were then incubated under microaerobic conditions for 48 hours at the appropriate temperature for the host. Successful lysis of host cells from the bacteriophage infection was observed by comparison to non-

infected host control plates by a reduction in turbidity. The propagated bacteriophages were then harvested, by addition of 5 ml of SM buffer (section 2.1.11) onto the surface of the plates. The plates were then incubated overnight at 4°C with shaking on a shaker platform with 60 cycles/minute. Following incubation, SM buffer containing bacteriophage was collected and filtered by passing through a 0.22 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany) to remove cell debris and residual agar. The filtered bacteriophage stocks were stored at 4°C in sterile plastic universals or Eppendorf tubes. Enumeration of bacteriophage obtained was determined by serial dilution and titration on host cell lawns prepared as described in (section 5.2.2).

5.2.6 Bacteriophage transmission electron microscopy

Transmission electron microscopy (TEM) was performed to examine bacteriophage morphologies. High titre bacteriophage suspensions (section 5.2.5) free of host material, increasing the chance to produce high quality images were prepared. The bacteriophages were fixed using a similar method to that used for bacterial transmission electron microscopy section (section 2.11). An aliquot of 13 µl of fixed phage suspension was transferred onto the formvar carbon film on copper 200 mesh grid and incubated at room temperature for five minutes. The suspension was removed by using filter paper and 13 µl of 2% w/v uranyl acetate was added onto the grids for 30 seconds. After staining, uranyl acetate was removed by using filter paper. The sample was then washed twice to improve the image quality by adding 13 µl of distilled water to the grid, then removing using filter paper. The grid was then ready to be examined by TEM.

5.2.6 Bacteriophages genomic DNA extraction

Different two methods have been applied to extract the phage genomic DNA in this study.

5.2.6.1 Phage DNA preparation using Wizard® DNA Clean-Up System

The isolation of phage DNA suitable for sequencing was achieved using the Promega Wizard® DNA Clean-Up System A7280. High titre bacteriophage suspension was prepared using 20 full plate lysates (section 5.2.5) to produce approximately 100 ml of 9 $\log_{10}$ PFU/ml. The phage suspension was filtered through a 0.22 µm-pore-size membrane filter (Minisart; Sartorius, Gottingen, Germany), and the filtered phage suspension centrifuged at 37,500 x *g* for 2 h at 4°C in 35 ml Oakridge tubes. The pellets were re-suspended in 0.5 ml of 5 mM $MgSO_4$ per tube (prepared from 1 M stock section 2.1.12). Aliquots of 1 µl 10 mg/ml DNase and 1 µl 10 mg/ml RNase were added and the suspension incubated at 37°C for 30 minutes. Following the incubation, 10 µl of 0.5 M EDTA and 5 µl of 10 mg/ml Proteinase K were added. Wizard® DNA Clean-Up Resin was dissolved by warming to 37°C for 10 minutes and then cooling to 25–30°C. One Wizard® Minicolumn was prepared for each sample by attaching the barrel of a 5 ml syringe to the Luer-Lok® extension of each Minicolumn. One ml of Wizard® DNA Clean-Up Resin was added to each sample and mixed by inverting several times and transferred into the syringe barrel. The sample forced through the column using the plunger. The column then was washed by pipetting 2 ml of 80% isopropanol into the syringe and gently pushed through the Minicolumn. The Minicolumn was placed into to a 1.5 ml Eppendorf tube then centrifuged for 2 minutes at 10,000 x *g* then transferred to a fresh Eppendorf tube. An aliquot of 50 µl of sterile RO water prewarmed to 65–70°C was added to the Minicolumn and

incubated for 1 minute at room temperature. To elute the bound DNA fragments, the Minicolumn was then centrifuged for 20 seconds at maximum speed and stored for at 4°C.

5.2.6.2 Phenol extraction for purification phage genomic DNA

High titre bacteriophage suspension was prepared as described in (section 5.2.6.1). Following centrifugation at 37,500 x *g* and resuspension of pellets, an aliquot of 400 µl phage suspension was transferred to a 1.5 ml Eppendorf tube. Lysis solution containing 25 µl 10% SDS, 50 µl 1 M Tris-HCl pH 8.0, 25 µl 0.5 M EDTA, pH 8.0 and 200 µg Proteinase K was added to the tube. The tube was then gently mixed by inverting the tube back and forth. The suspension was incubated for 20 minutes at 56-65°C. Next, organic extraction of the protein from the aqueous phase was achieved by adding a mix of a 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) to the tube. Without using the vortex, the tube was then mixed well for 2 minutes. The tube was centrifuged in a microfuge at maximum speed for 2 minutes. The top aqueous layer was transferred to a clean 1.5 ml Eppendorf tube. Residual phenol was removed by extraction two times with 100% chloroform. After removing the phenol, 50 µl of 3M NaOAc (pH 5.2) was added and mixed. Directly, a 1 ml volume of ice-cold 100% EtOH was added to the tube and mixed by repeatedly inverting the tube. The DNA was then moved to a fresh a 1.5 ml Eppendorf tube containing 70% EtOH at room temperature using a plastic loop, then centrifuged and the pellet dried by incubating at 55°C with lid open the dissolved in 200 µl TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The DNA was stored at 4°C until required.

5.2.7 Bacteriophage DNA sequencing

The DNA sequence of phage genomes were determined using the Illumina MiSeq next generation sequencing technology from libraries prepared using the Nextera™ tagmentation protocol and run on an Illumina v3 cassette to produce between 0.7 and 0.9 million sequence reads of 80 to 250 bps per genomic DNA preparation. These reads were used for *de novo* assembly of the phage genomes using the CLC Genomic Workbench Software package version 9.1 (Qiagen). The phage DNA sequence was annotated using Phaster (http://phaster.ca/) and HHpred (https://toolkit.tuebingen.mpg.de/tools/hhpred).

5.3 Results

5.3.1 Plaque formation and recognition

Visual examination of phage isolation plates following sample application produced some unexpected results. Samples applied to *C. jejuni* PT14 host lawns produced no plaques. However, severe damage to the lawns was observed on the surfaces of all the treatment plates incubated at 42°C compared to control plates of *C. jejuni* PT14.

No plaques were detected on *Arcobacter* plates for any samples incubated under microaerobic conditions at 30°C for 48 h, with monitoring every 12h. However, unusual circular zones were observed inside the 10 µl spot areas in all treatment plates compared to the positive control. These observations were hampered because *A. skirrowii* A2S6 did not form a uniform lawn on NZCYM. (Figure 5.2) The primary unusual circular pitting in *A. skirrowii* A2S6 lawn when inoculated with 10 µl of cattle slurry supernatant sample. The areas of pitting were reduced upon serial dilution implying the activity was particulate and could be diluted.

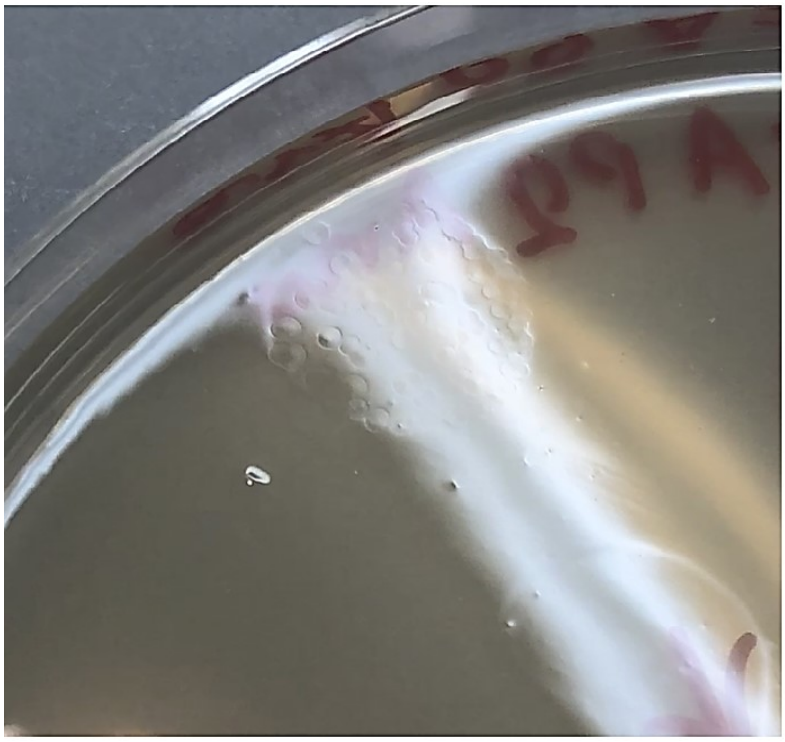


Figure 5.2 Circular zones resulted from slurry supernatant in *A. skirrowii* A2S6 lawn grown on NZCYM

The application of slurry filtrate samples to *C. hyointestinalis* S12 lawns also exhibited unusual plaque-like features when incubated under microaerobic conditions at 37°C for 48 h, with monitoring every 12 h. These unusual plaques could only be seen if the light source was tilted at an acute angle of approximately 45° when the plates were examined. These plaques on the *C. hyointestinalis* S12 lawn only appeared inside the sample application area and declined decimally through the dilution series implying they were particulate. (Figure 5.3) A and B shows these primary plaques on lawns of *C. hyointestinalis* S12. The sample from which these plaques were obtained was taken from Site 5 (section 5.2.1). The effect was reproduced among all replicates from this site giving a total of 15 replicates from three separate experiments. Plaque purification was carried out as described in (section 5.2.4) and propagation as described in (section 5.2.5).

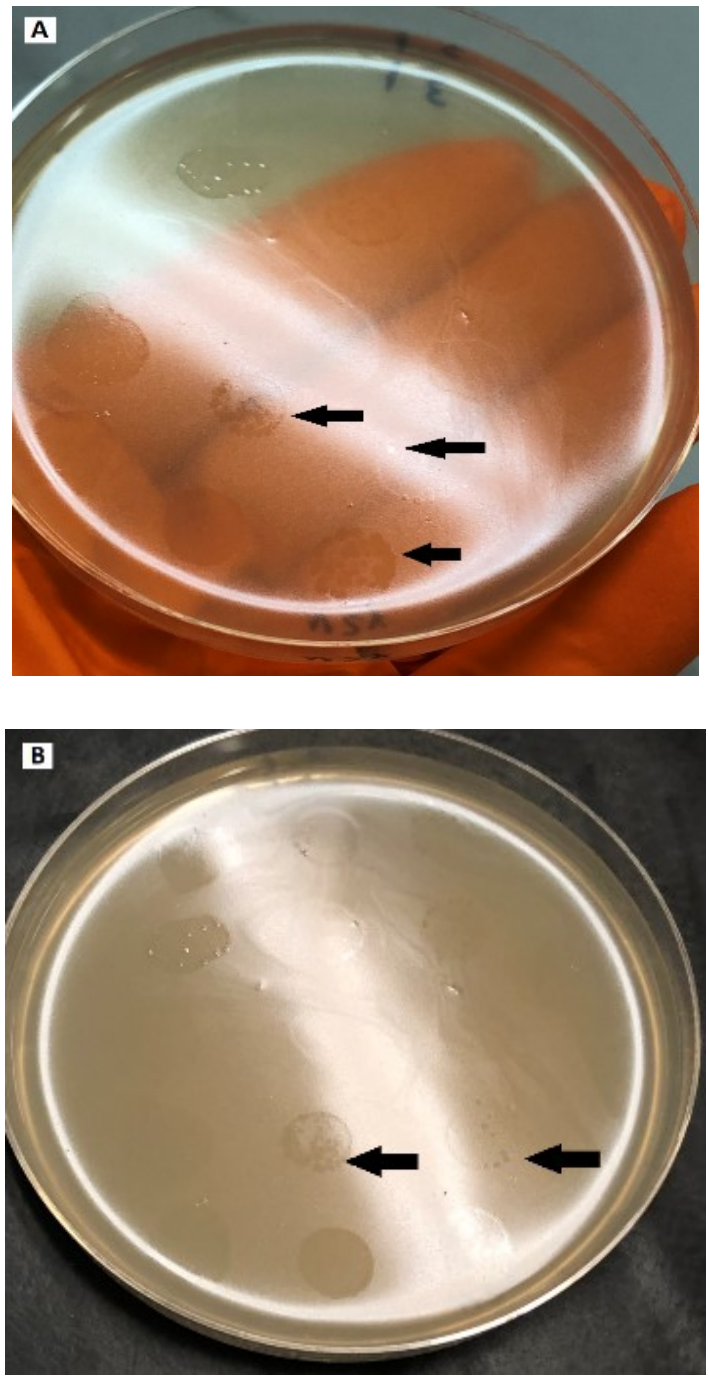


Figure 5.3 Serial dilutions of the filtered slurry supernatant applied as 10 μl droplets to *C. hyointestinalis* S12 lawn, resulted in an unusual, difficult to visualise plaques following incubation for 48h at 37°C (A lawn with coloured background) (B lawn with dark background)

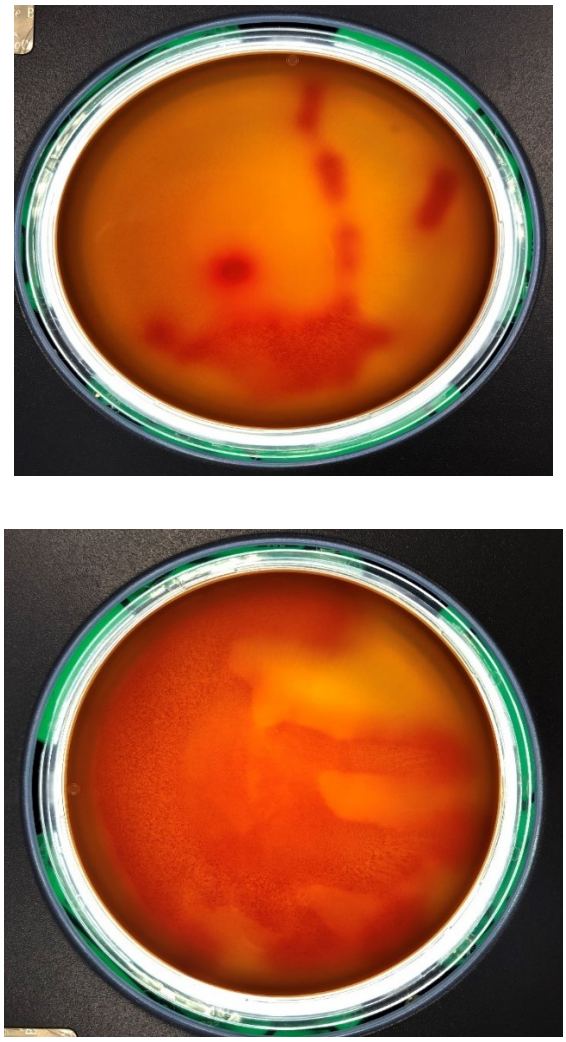


Figure 5.4 Two examples of haemolysis-like activity of the bacterial isolate when grown on BA independently of its host

5.3.2 Evidence that a bacterium was responsible for plaque formation

The propagated lytic material from the plaques in (section 5.3.1) was examined by TEM (section 5.2.6). The images shown in (Figure 5.5) A and B confirmed that the source of the lytic activity was bacterial rather than due to phage lysis. This was surprising as initial samples for TEM were prepared with the expectation of discovering bacteriophage and had been filtered two times through a 0.22 µm-pore-size membrane filter to remove all potentially pathogenic bacteria from the preparation but had not been formalin fixed to inactivate living bacteria. However, during the TEM session the clear observation of bacterial cells (Figure 5.5 A) resulted in ending the TEM session for safety reasons. Further TEM sessions with formalin fixed samples revealed the bacterial cell morphology (Figure 5.5 B).

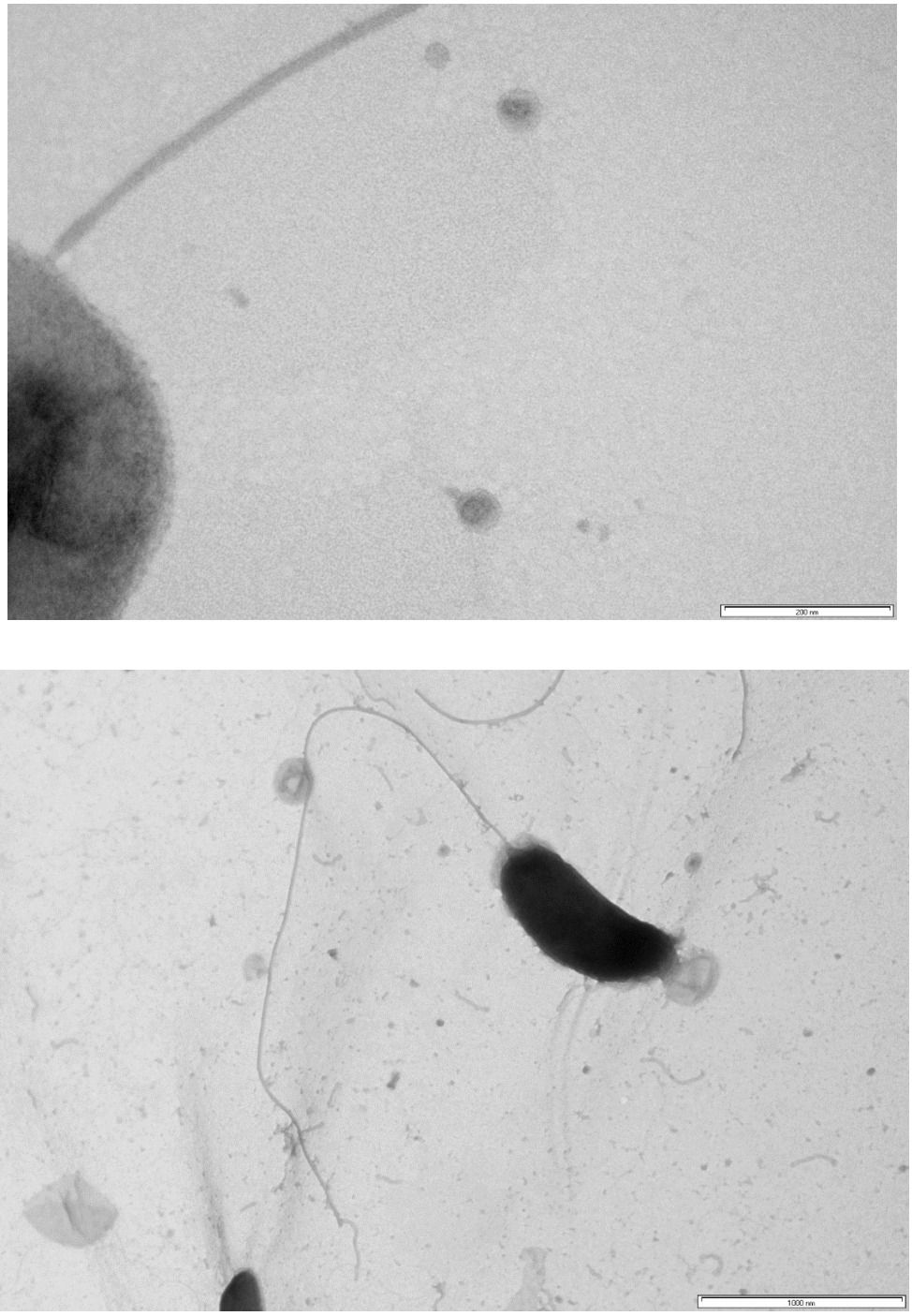


Figure 5.5 (A shows a part of a bacterial cell wall and flagella that were un-stained and un-fixed, as the sample had been treated as phage TEM sample) Bar represents 200 nm and (B TEM image of novel bacteria ASxL5, after stained with 2% w/v uranyl acetate for 30 seconds, bar represents 1000 nm)

Following this observation the purified, propagated stock from the plaques was stained using Gram's method which revealed the presence of a very small slender poorly staining, Gram-negative, curved rod-shaped bacteria (Figure 5.6). Subsequent it was discovered that the ASxL5 bacterium could be cultured on rich media such as BA or BHI (section 2.1.1 and 2.17) independent of it's host, although growth was poor. Colonies produced were very small, round, and transparent. Following storage of inoculated BA for seven days at 4°C an unusual haemolysis-like activity was observed (Figure 5.4). Further experiments were carried out on this novel bacterium and are described in Chapter 6.

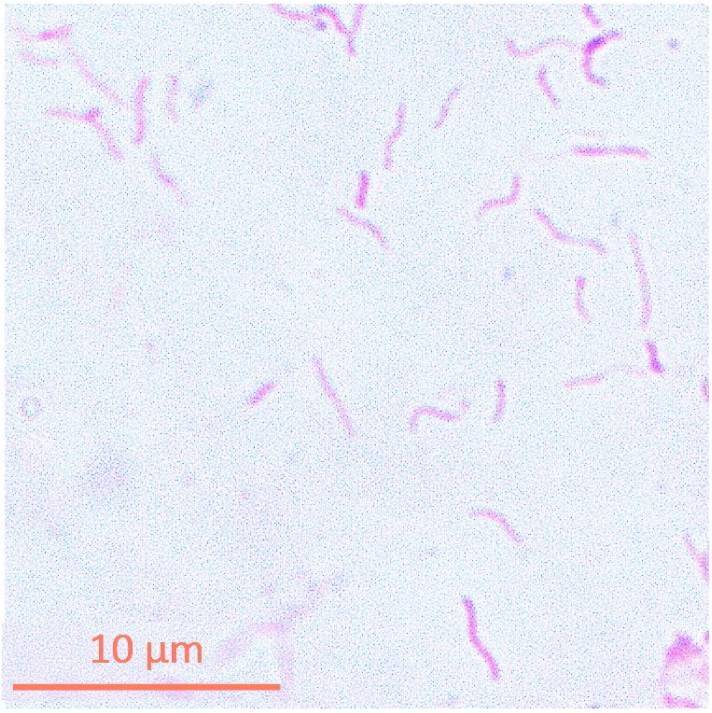


Figure 5.6 Light microscopy picture of Gram-stain of plaque forming bacteria ASxL5

5.3.3 Bacteriophage associated with predation by novel bacterium

Experiments were carried out using TEM (section 5.2.6) to attempt to understand the predation process by the novel bacterium ASxL5. This resulted in a further interesting observation shown in (Figure 5.7) which revealed phage particles associated with *C. hyointestinalis* S12 post-attack by the predatory bacteria. Images of *C. hyointestinalis* S12 alone did not show the phage particles. The appearance of the phage particles seemed to be associated with the stress of predation suggesting the particles represent excised lysogenic bacteriophages. (Figure 5.7) A and B show purified particles harvested post-attack of *C. hyointestinalis* S12, which have a long, contractile tail of 134 nm and icosahedral heads with diameters of 71 nm. This morphology indicates that these phages belong to the *Myoviridae* family of tailed phages. (Figure 5.7) B and C show phage particles that are broken or misassembled post-predatory attack of the host *C. hyointestinalis*, which may be a consequence of incomplete assembly before the host was consumed.

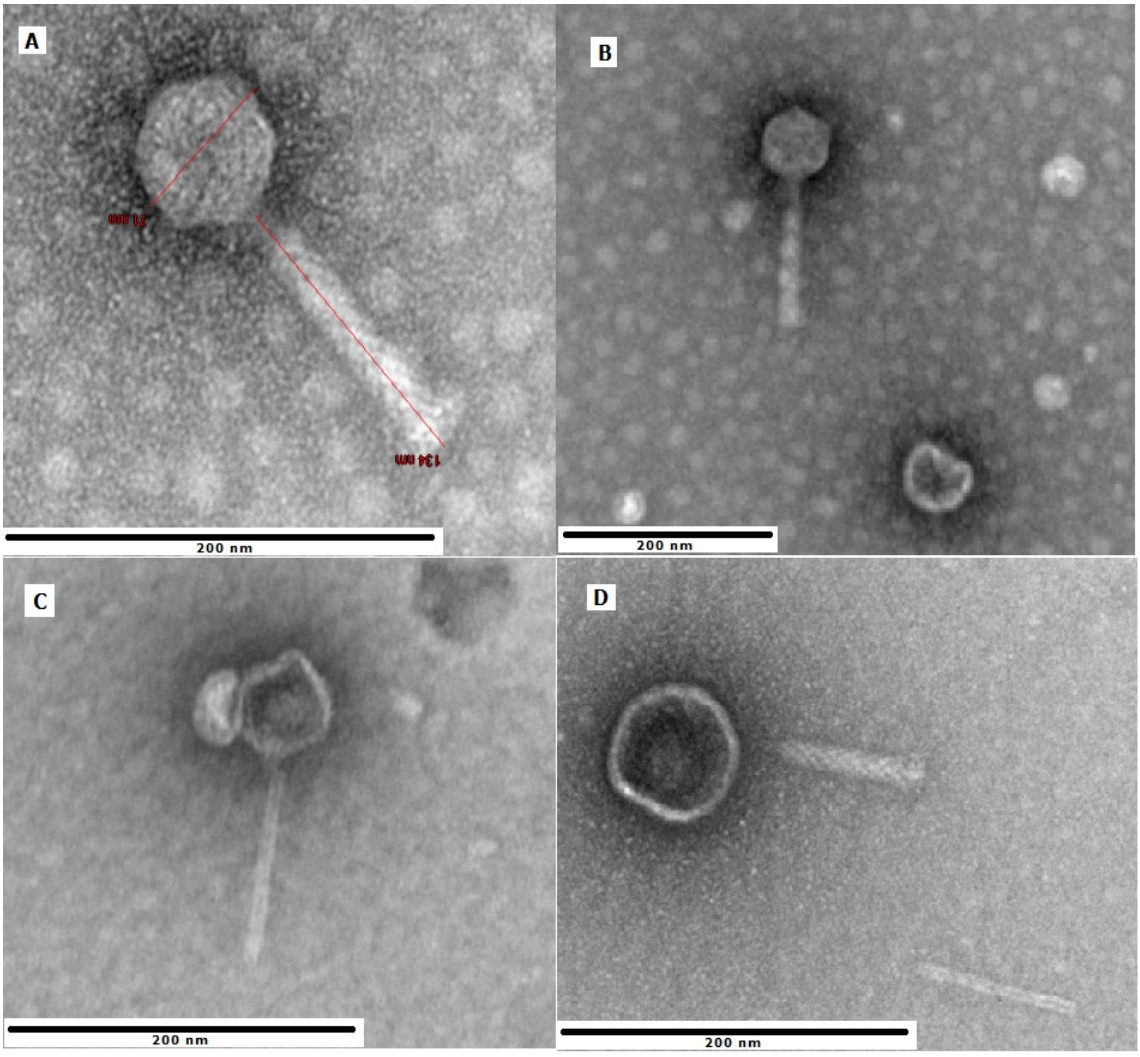


Figure 5.7 Transmission electron micrographs of lysogenic bacteriophages isolated from *C. hyointestinalis* S12 after attack by ASxL5 predatory bacterium (A and B show intact phage particles) (C and D show typical broken or misassembled phage particles)

5.3.4 Identification of an excised prophage

The whole genome sequencing of the DNA isolated from phage preparations of post-predatory attack of *C. hyointestinalis* S12 was undertaken using Illumina MiSeq next generation sequencing technology. The resulting reads were then assembled using the CLC Genomic Workbench Software package to produce a major linear contig of

33,817 bp with sequence heterogeneity at each end. The consensus sequence had nucleotide identity with a putative prophage sequence in the genome of *C. hyointestinalis* S12, which unequivocally defines the source of the phage as an excised prophage. The phage sequence was annotated using Phaster and HHpred, and the corresponding map (**Error! Reference source not found.**).

The sequence heterogeneity at the left and right ends of the phage sequence is similar to that observed for transposable phage Mu from *E. coli*, whereby non-Mu host DNAs adjacent to the site of integration are packaged with the Mu genome on the basis of the head-full mechanism during the lytic phase. The number of bases flanking the phage genome is between 60 to 150 kb for the left-hand end and 0.5 to 3 kb for the right-hand end (Symonds et al., 1987). Due to the sequence heterogeneity the assembly program could not extend the contig but upon examining individual sequence reads extending from the left and right-hand ends of the phage genome enabled the positions in the *C. hyointestinalis* S12 genome from where they were excised to be determined, (**Table 5.1 The locations of flanking sequences from the excised Mu-like phages from *C. hyointestinalis* S12**. The locations of flanking sequences outside the prophage region implies that the sequences have been transposed, and that the element is a functional transposable phage that responds to stress.

Annotation of the phage sequence revealed predicted functions consistent with a functional phage, (**Error! Reference source not found.**). Most notable were r ecognisable functions associated with the ability of phage Mu to transpose, a DDE-type integrase and the DNA transposition protein B. Structural similarities predicted by HHpred also highlighted specific orthologues of phage Mu: the Mu middle operon regulator (*mor*), Mu assembly protein and the Mu baseplate protein.

Table 5.1 The locations of flanking sequences from the excised Mu-like phages from *C. hyointestinalis* S12

Sequences at breakpoint	Chromosome Locus (nt)	Putative Function (implied mechanism)
CGTAAAAC/TGTGA	64,022	Glycosyltransferase (transposition)
AATAAAAC/TTAAT	227,963	Intergenic (native position)
TACGATTT/TGTGA	227,610	Hypothetical (transposition)
GGGTATTT/TGTGA	258,741	Intergenic (transposition)
TTCACTTC/TGTGA	828,021	Hydroxymethylbilane (transposition)
GTAGCTTC/TGTGA	1,082,730	Ankyrin repeat (transposition)
GTAGCTTC/TGTGA	1,323,730	Diaminopimelate decarboxylase (transposition)
CTGAGACT/TGTGA	1,391,337	Flavodoxin (transposition)
TTGAGTAT/TGTGA	1,727,388	Hypothetical (transposition)
CTATATTT/TGTGA	227,897	Intergenic (internal inversion)
ACCTTTCT/TGTGA	231,320	Integrase (internal inversion)

The DNA sequences in bold are host flanking sequences. Nucleotide locations relate to the genome sequence of *C. hyointestinalis* S12 (CP040464)

The direction of translation is indicated by the arrows and the location within the nucleotide sequence indicated by the numbers below. Unidentified open reading frames are marked as URFs.

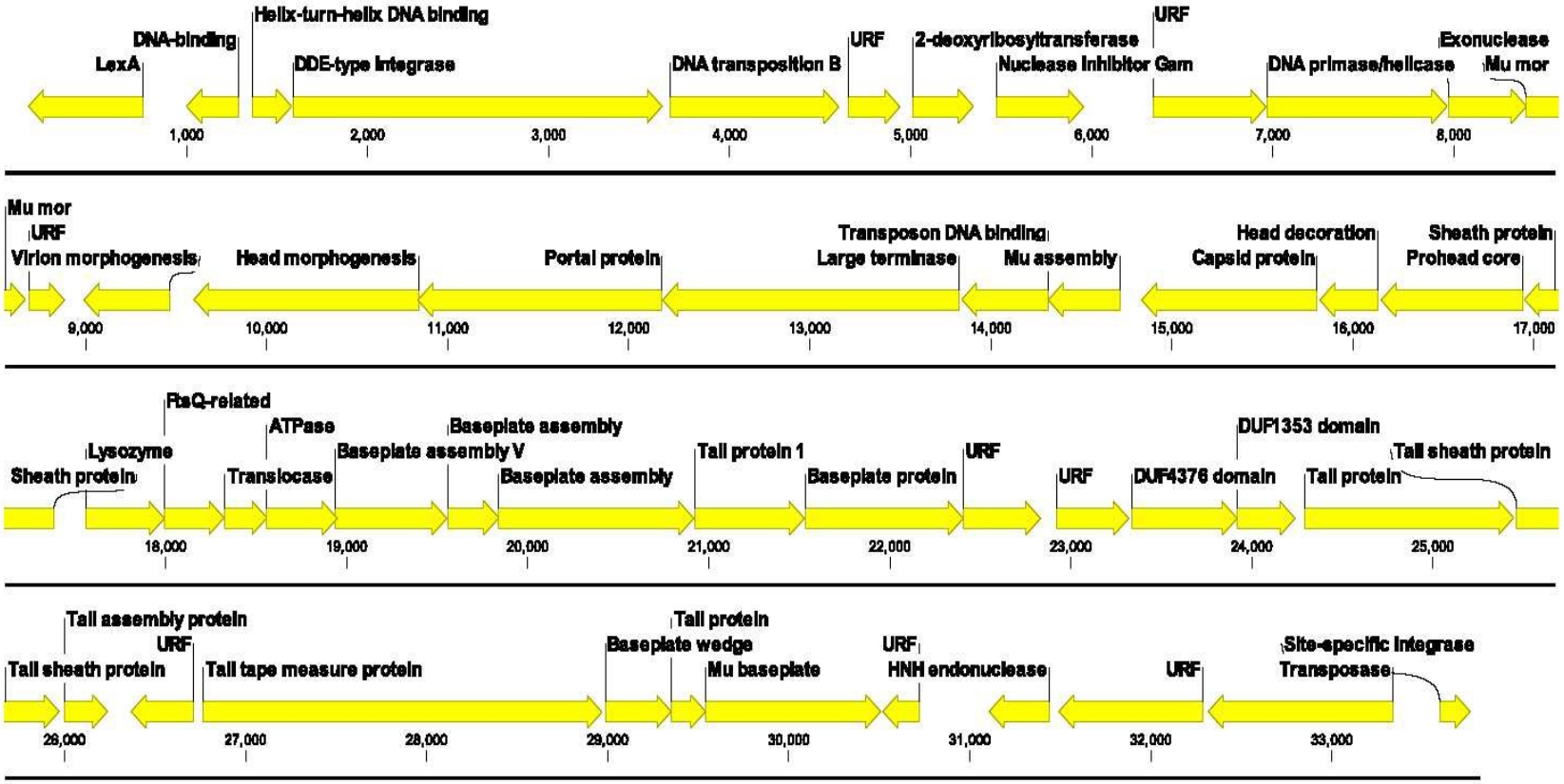


Figure 5.8 Map of the open reading frames present in the sequence of Mu-like phage from *C. hyointestinalis*

5.4 Discussion

Plaques observed on bacterial lawns often represent lytic activity which is frequently caused by lytic bacteriophage and occasionally can linked to predatory bacteria. In this study, it was observed that supernatants from cattle slurry samples produced cloudy plaques on lawns of *C. hyointestinalis* S12 isolated from the same environment. Faecal matter and manure samples of some farm animals like chicken and pigs were good sources of *Campylobacter* bacteriophages (Brathwaite et al., 2013; El-Shibiny et al., 2005) so the expectation of this study was that cattle slurry would likewise be a source of useful bacteriophage from various *Campylobacter* species likely to be present. The isolation methods were successfully tested on faecal material from pigs but the same methods as applied to cattle slurry, did not produce the abundant phage populations anticipated.

There are no previous studies, that use cattle faecal or slurry samples as sources of *Campylobacter* specific phages. The unusual lytic activity that prevented adequate lawn formation could be one reason behind this. The unusual plaques generated by the predatory bacteria may play a role as the predator may play to trigger the excision of lysogenic phage. All these results represent a unique phenomenon, which has only been observed during the work in this study. It is assumed that microbiological diversity and environmental conditions can represent potent effectors of changes in bacterial populations in closed ecological systems such as farm slurry tank.

5.5 Conclusion

Unusual lytic activity was found to be caused by a predatory bacterium ASxL5. This were confirmed by Gram stain and by TEM imaging. Bacteriophages observed following predation by ASxL5 were lysogenic phage released from the *C. hyointestinalis* S12. The *C. hyointestinalis* S12 genome includes a prophage insertion (section 4.3.3.6) was integrated in genome. However, the sequence data from released phage DNA exhibited a larger size of genomic than what would be expected.

Chapter Six

Characterisation of novel predatory bacteria isolated from bovine slurry

6.1 Introduction

A predatory bacterium is one that demonstrates the ability to pursue and kill other living bacteria to obtain biosynthetic materials and energy (Pérez et al., 2016). This is distinct from the universal recycling of the nutrients from dead microorganisms and from parasitic interactions where bacteria form close associations with their hosts without killing them. Predatory bacteria have evolved diverse life cycles to exploit abundant food sources in the niches where they are found, for example in marine habitats (Linares-Otoya et al., 2017). They are a taxonomically diverse group connected only by their unique bactericidal life cycle (Pérez et al., 2016). Examples of predatory bacteria are found in several different phyla including: *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and *Firmicutes* (Pasternak et al., 2013). However, the most well-studied predatory bacteria are, *Bdellovibrio* and *Bdellovibrio*-and-like organisms (BALOs) Sockett (2009). Predatory bacteria are promising sources of new bioactive compounds and antibiotics (Korp et al., 2016).

Predatory bacteria are suggested to enhance microbial diversity, have the potential to prevent dysbiosis and have a positive effect on ecosystem health, productivity and stability (Johnke et al., 2020). Despite these positive attributes, there are few studies of new predatory bacteria because of difficulties in culturing the bacteria, and the need for careful observation of cellular interactions in order to understand their complex lifecycles. This information is not readily available from *in silico* analysis.

In an era of increased antimicrobial resistance novel strategies such as the use of bacteriophage, that target bacterial pathogens, are being investigated (Vila et al., 2019). The ASxL5 bacterium was isolated from cattle slurry collected from the University of Nottingham Dairy Centre, Nottinghamshire, in 2019 using techniques for phage isolation (El-Shibiny et al., 2005). The aim of the investigation was to

isolate organisms that had potential as biocontrol agents. *Campylobacter hyointestinalis*, a zoonotic pathogen that is increasingly associated with enteric disease in humans (Wilkinson et al., 2018), was prevalent in all cattle slurry samples and was used as a target host.

6.2 Materials and Methods

6.2.1 Isolation of host bacteria

Bacterial isolates to use as potential hosts were isolated and identified as described in (section 2.7, 2.8) from all cattle slurry samples.

6.2.1 Isolation of ASx5L

The ASxL5 bacterium was isolated from cattle slurry using *C. hyointestinalis* S12 as host using techniques described in Chapter 5.2.

6.2.3 Phenotypic characterisation of ASx5L

Once it was established that a bacterium was responsible for the lytic plaques rather than a bacteriophage, attempts were made to cultivate the organism independently from the host and characterize it further. Weak growth that improved on subculture was obtained BHI (section 2.1.7) and BA (section 2.2.1) with aerobic incubation at 37°C. Antibiotic sensitivity was tested was carried out on BHI agar incubated aerobically at 37°C using discs with the following antibiotics (Oxoid): amoxycillin and clavulanic acid 30 µg; cefotaxime 30 µg; streptomycin 10 µg; ciprofloxacin 5µg; ceftazidime 30 µg nalidixic acid 30 µg; imipenem 10 µg; azithromycin 15 µg; chloramphenicol 30 µg; cefoxitin 30 µg; tetracycline 30 µg; nitrofurantoin 300 µg; aztreonam 30 µg; ampicillin 10 µg; cefpodoxime 10 µg; trimethoprim-sulfamethoxazole 25 µg. Salt tolerance was established by cultivation aerobically at 37°C on BHI agar plates to which additional NaCl was added to give a range of concentrations up to 10.

6.2.4 Microscopy

ASxL5 was cultured aerobically by spreading uniformly on BA for 24 h at 37°C and harvested into 1 ml of 3 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer, fixed for 1 h at room temperature then centrifuged at 10,000 *g* for 3 min. The pellet was then re-suspended gently into 600 µl of 0.1 M cacodylate buffer. The fixed ASxL5 suspension was transferred onto Formvar/ carbon film on copper 200 mesh grids. The bacteria stained with 0.5% (w/v) uranyl acetate for 1 min and examined by TEM using a TEI Tecnai G2 12 Biotwin microscope. The predator prey interaction was also examined by TEM as described above combining equal numbers of prey and predator in NZCYM broth (BD Difco™, Fisher Scientific UK Ltd, Loughborough) and incubating for 48 h at 37°C, under microaerobic conditions for *Campylobacter* or aerobic conditions for *E. coli.* Prey and predatory bacteria were examined independently to establish any changes in cell morphology arising as a consequence of predation.

6.2.5 Host range determination

Overnight cultures of ASxL5 were grown by spreading growth on BHI or BA plates using a sterile swab. The ASxL5 cells were collected and suspended in MRD (CM0733, Oxoid) and then placed at 4°C for 7 d, to starve the cells. NCTC reference or laboratory stock bacteria cultures were inoculated into BHI broth or NBroth No 2 (section 2.5.1), incubated overnight, centrifuged at 13,000 *g* and re-suspended in MRD to an OD_{600} of 0.4. The cultures were: *Bacillus subtilis* NCTC 3610, *Citrobacter freundii* NCTC 9750, *Enterobacter aerogenes* NCTC 10006, *Enterococcus faecalis* NCTC 775, *Escherichia coli* NCTC 86, *Klebsiella oxytoca* 11466, *Leuconostoc mesenteroides* NCTC 10817, *Listeria monocytogenes* NCTC 4885, *Paenibacillus macerans* NCTC 6355, *Providencia stuartsii* NCTC 10318, *Pseudomonas fluorescens* SMDL, *Rhodococcus hoagie* NCTC 1621, *Salmonella*

enterica Montevideo NCTC 5747, *Serratia liquefaciens* NCTC 10861, *Staphylococcus aureus* NCTC 8532, *Streptococcus pneumoniae* NCTC 7465, *Yersinia enterocolitica* NCTC 10460. *Campylobacter* hosts were incubated microaerobically at 37°C on BA plates and then suspended in NZCYM broth. *Campylobacter* hosts tested were: *C. coli* 12667 NCTC, *C. jejuni* 12662, *C. jejuni* PT14, *C. jejuni* NCTC 11168, *C. helveticus* NCTC 12472, *C. lari* NCTC 11458, *C. upsaliensis* NCTC 11541, *C. hyointestinalis* NCTC 11608. Cells were collected in MRD, centrifuged at 13,000 *g* and re-suspended in MRD to an OD_{600} of 0.4. An aliquot of 0.5 ml of the suspensions was added to 5 ml aliquots of molten NZCYM top agar (section 2.1.4) and poured on to 1.2 % NZCYM baseplates (section 2.1.3). Once set and dried, serial dilutions of ASxL5 were dispensed as 20 µl droplets in triplicate onto each lawn plate. The incubation temperature and atmosphere were dependent on the test bacteria's requirements.

6.2.6 16s rRNA and Whole Genome Sequence Determination

The DNA was prepared from bacterial isolates using GenElute™ Bacterial Genomic DNA Kit (section 2.9.1). The PCR amplification of 16S rRNA gene and sequence determination of the product was carried out using standard methods (section 2.9.2). The DNA for whole genome sequencing was extracted using the PureLink™ Genomic DNA Kit (Fisher Scientific, Loughborough, UK). The genome sequence of ASxL5 was determined using a combination of Illumina MiSeq consisting of 250 bp paired-end reads using libraries prepared from the Nextera tagmentation kit, and long reads of 2 to 20 kb from the PacBio (Pacific Biosciences) platform performed at the Nu-Omics DNA Sequencing Research Facility, Northumbria University. The genome was assembled using CLC Genomics Workbench 12.0.3 (Qiagen, Aarhus, Denmark). ASxL5 cultures were deposited at National Collection of Type cultures (UK) and the Netherlands Culture Collection of Bacteria (NCCB). Genomes of

related organisms used for comparisons were: *Thalassolituus oleivorans* MIL-1 (Golyshin et al. 2013; accession HF680312, complete); *Bacterioplanes sanyensis* NV9 (accession CP022530, complete); *Oceanobacter kriegii* DSM 6294 (accession NZ_AUGV00000000, incomplete); *Marinomonas communis* DSM 5604 (accession ASM436330v1, incomplete) and *Thalassolituus* sp. C2-1 (accession NZ_VNIL01000001, incomplete). The Ortho average nucleotide identity (OrthoANI) and amino acid identity (AAI) were determined using software available online (Lee et al., 2016, Rodriguez-R and Konstantinidis, 2014). The digital estimates for DNA–DNA hybridization values using formula 2 from these five genomes from related organisms were computed by using the online software tool gbdp2_blastplus available at http://ggdc.dsmz.de/ (Meier-Kolthoff et al., 2013). Functional annotation of the ASxL5 genome using orthology assignment was carried out by using the BlastKOALA KEGG online tool for functional characterisation of genome sequences (Kanehisa and Goto, 2000) and COGs were determined by the eggNOG-mapper online tool (Huerta-Cepas et al., 2017).

6.3 Results

6.3.1 ASxL5 is a predatory Bacterium with Unusual Cell Morphology

The ASxL5 bacterium was isolated from bovine slurry because it formed plaques on *C. hyointestinalis* host similar to those produced by bacteriophage. It was an unexpected finding because part of the phage isolation procedure involved filtration through a 0.2 µm filter designed to remove bacterial cells. Microscopic examination of the material extracted from the plaques revealed small Gram-negative curved rod-shaped bacteria. Axenic culture was achieved independent of prey cells on rich solid media such as BHI and BA, with weak growth that improved on subculture using heavy inocula. Colonies were small reaching 2 mm in diameter after 72 h and were beige, translucent, circular, convex and shiny. It was not possible to carry out most standard biochemical tests as ASxL5 could not be reliably cultured in liquid medium suggesting a complex life cycle with possible dependence on biofilm formation. ASxL5 was aerobic, oxidase and catalase positive and able to tolerate 5% NaCl. It was resistant to streptomycin 10 µg, but sensitive to all other antibiotics tested. The characteristics of ASxL5 are summarized in **Table 6.1**. The ASxL5 bacterial cells were examined by (TEM Figure 6.1) .

Table 6.1 Phenotypic characteristics of ASx5L

Characteristic		ASxL5
Growth temperature:	42°C	++
	37°C	+++
	25°C	++
	4°C	+
Catalase		+
Oxidase		+
pH range:	3-9 (tested)	4-9
Salt tolerance:	0.5-10 % (tested)	0.5 to 5%
Cell dimensions:	Grown on BA 48 h	
	length	1.63 µm (±0.42)
	width	0.37 µm (±0.08)
	Incubated with *C. jejuni* 48 h	
	length	2.09 µm (±0.69)
	width	0.30 µm (±0.06)
	Incubated with *E. coli* 48 h	
	length	4.99 µm (±2.45)
	width	0.63 µm (±0.11)

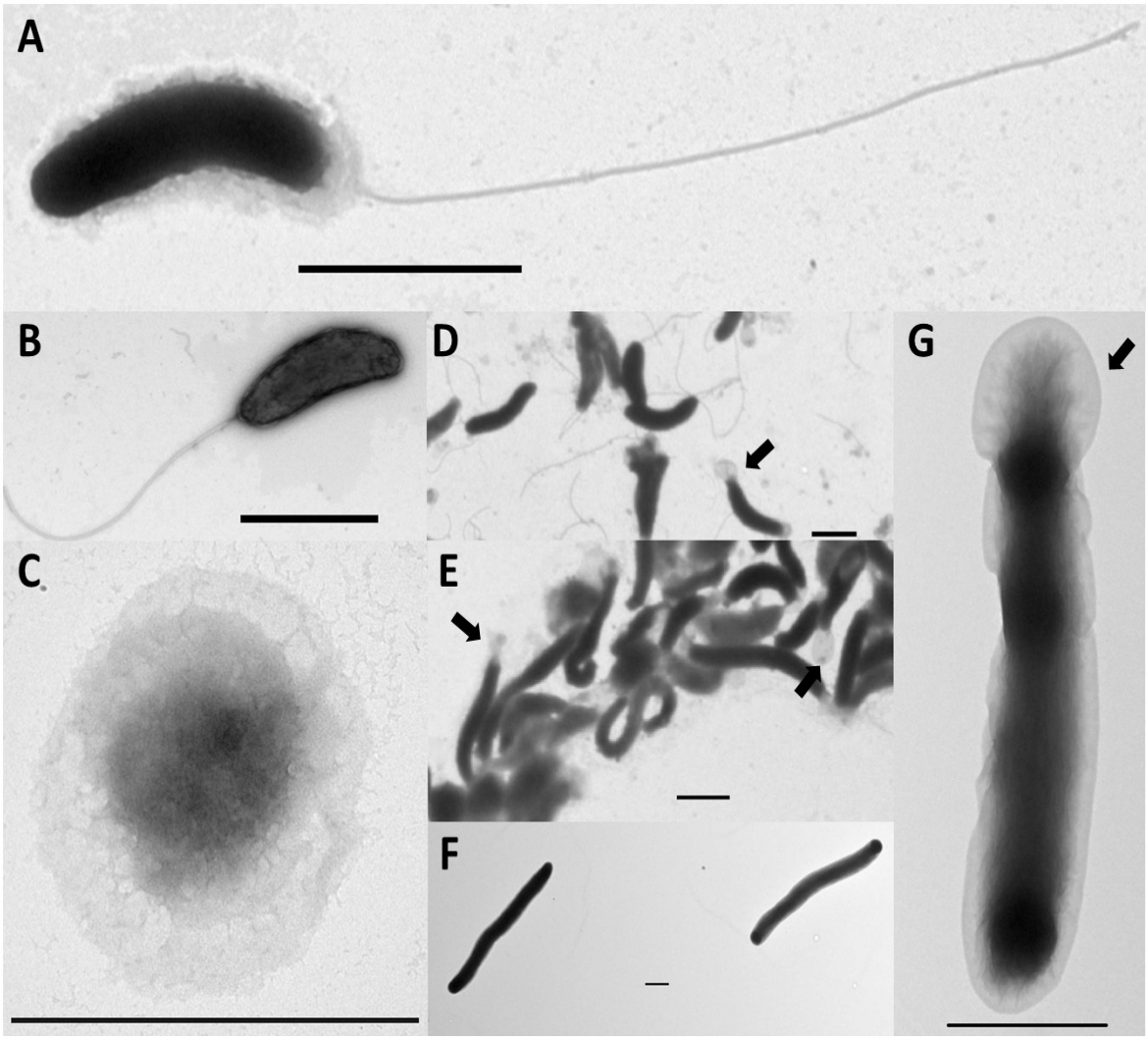


Figure 6.1 TEM of ASx5L showing: (A ASx5L showing long polar flagellum) (B typical ASx5L cell) (C coccal ASx5L cell following prolonged incubation without nutrients) (D group of ASx5L cells showing unusual apical structure indicated with arrow)(E group of ASx5L cells incubated with *Campylobacter* prey showing increased cell length compared with those grown without prey (panel D) also showing apical structures) (F large filamentous aflagellate, ASx5L cells, following incubation with *E. coli* prey)(G single ASx5L cell following incubation with *E. coli* showing unusual apical structure. Bar represents 1 μm)

Host independent grown on BA, the ASxL5 cells were small curved bacteria with an average length of 1.63 μm (±0.4) and width of 0.37 μm (±0.08), with a single long (up to 5 μm) polar flagellum. Approximately 1.6 % of cells appeared to have a width of less than 0.2 μm which would allow passage through a filtration device. An unusual structural extension resembling a cowl (latin *cucullus*), was observed at the apex of some cells (arrow Figure 6.1 D, E and G). This appeared to be composed of excess outer membrane, possibly due to a rapid reduction in size of the periplasmic envelope, with the outer membrane remaining intact, giving a "baggy" appearance. Prolonged incubation of ASxL5 without nutrients (in PBS), at 4 °C, resulted in most, but not all, of the cells exhibiting coccal morphology (Figure 6.1 C). When ASxL5 was grown for 48 h with *C. jejuni* as prey, the mean cell sizes were significantly longer and narrower, than cells grown without host, (2.1 μm by 0.3 μm; p = 0.0003 and p = 4 x 10^{-15}, by ANOVA respectively; **Table 6.1** and Figure 6.1E). In contrast when ASxL5 was grown for 48 h with *E. coli* as prey, the mean cell sizes were longer and wider than when grown without prey (5.0 μm by 0.63 μm; *p* = 0.00016 and *p* = 2 x 10^{-15}, by ANOVA respectively, **Table 6.1**). Cell length was variable, often showing filamentation (Figure 6.1F). ASxL5 cells showed a complete absence of flagella when incubated for 48 h with either *C. jejuni* or *E. coli* as prey. The name *Venatorbacter cucullus* gen. nov. sp. Nov was proposed for the ASxL5 bacterium (full description following section 6.4).

6.3.2 The 16s rRNA and Genome Sequence of ASxL5 Reveal a Relationship with Marine Bacteria

Determination of the 16S rRNA gene sequences enabled database searches to establish the sequence resembled those in the class *Gammaproteobacteria* and were most closely aligned with marine bacteria in the family *Oceanospirillaceae* (Figure 6.2).

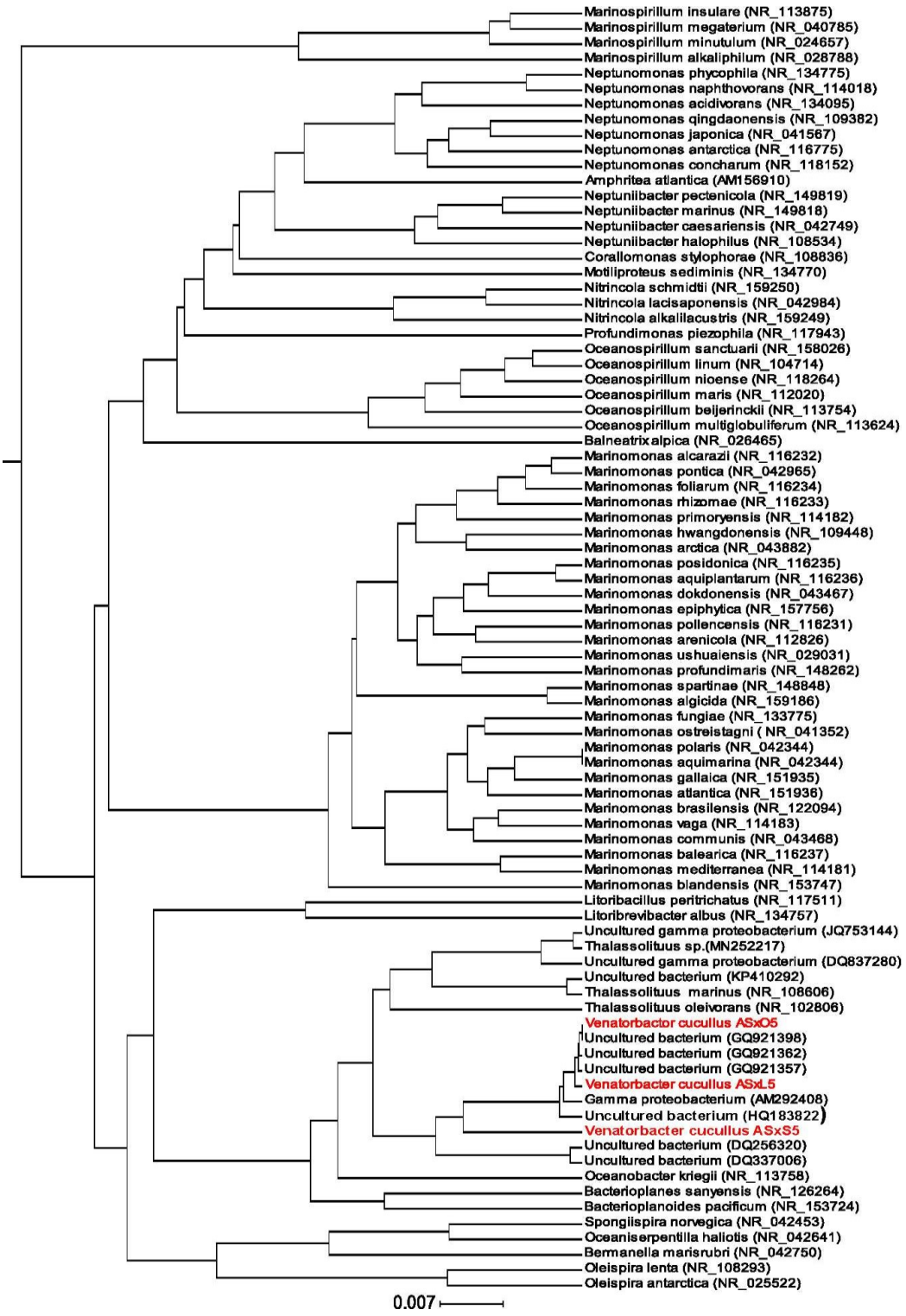


Figure 6.2 Phylogenetic tree using 16S rRNA sequences highlighting the position of *Venatorbacter cucullus* gen. nov. sp. nov. strain A (red) relative to relative to uncultured and marine bacteria genera within the family *Oceanospirillaceae*. Genbank accession numbers are presented in parentheses. Sequences were aligned using CLC Genomics Workbench with default parameters and phylogenetic relationship inferred using the Unweighted Pair Group Method using Arithmetic averages (UPGMA), with 100 bootstrap replicates.

The 16S rRNA sequences were notably diverged from predatory bacteria belonging to the family *Bdellovibrionaceae* (Deltaproteobacteria). The *Venatorbacter cucullus* ASxL5 bacterium had 3 copies of the 16S rRNA genes with two being identical to each other and the third differing by 3 bases. Two further predatory bacterial isolates from the same location with similar morphology and phenotypic characteristics (*V. cucullus* ASx5S and *V. cucullus* ASx5O) were not identical, but clustered with *V. cucullus* ASxL5 and uncultured bacterial database sequences, separate from other genera in the *Oceanospirillaceae* (Figure 6.2). The whole genome sequence of *V. cucullus* ASxL5 was determined and appears in the NCBI database under the accession number CP046056. The genome size of *V. cucullus* ASxL5 was 2 831 152 bp with a G+C ratio of 56.1%. The genome sequence contained 2 653 CDSs (total), of which 2 567 were predicted to encode proteins, and of these 1596 could be assigned a putative function (60.2%). The genome contained 67 RNA genes comprising of 9 rRNAs (3 each 5S, 16S and 23S) together with 57 tRNAs. The genetic characteristics of ASxL5 were compared to the available genomes of the closest relatives identified from the 16s rRNA gene sequences (Table 6.2). Ortho Average nucleotide identity (OrthoANI) Lee et al. (2016) comparing ASxL5 with related taxa gave values ranged between 64.48 and 79.39. This analysis is better suited to more closely related isolates from the same genus (Qin et al., 2014)but is included for completeness. Instead AAI is frequently recommended for comparing different genera because resolution is progressively lost at the nucleotide level for more distantly related populations (Rodriguez-R and Konstantinidis, 2014). Comparative AAI values ranged between 45.67 and 74.84. The closest related available genome sequence (incomplete), determined by AAI was that of *Thalassolituus* sp. C2-1 (accession NZ_VNIL01000001). This strain was isolated from a deep-sea sediment of the Mariana Trench, but no phenotypic information regarding this strain is available for comparison at present. This organism has a much larger genome at 4.36 Mb compared to 2.82 Mb for ASxL5. The estimates for digital DNA–DNA hybridization comparing ASxL5 with the 4

available genomes from related genera are also presented in (Table 6.2). Estimated digital DNA–DNA hybridization values between 19.8 and 28.9%, and differences in G+C contents of between 2.7 and 11.2%, respectively. These data together with the other genomic characterizations indicate that ASxL5 is clearly distinct from its relatives in the Oceanospirillaceae for which genomic data are available.

The genome sequences of ASx5S and ASx5O were determined and respectively appear in the NCBI database under the Genbank accession numbers CP045550 and CP046055. Although the sequences exhibited marked similarity as may be expected from bacteria of the same putative species, the genome sequences revealed a notable difference in that ASx5S contained an inversion of approximately 0.9 Mb compared to the other predatory bacterial isolates. (Figure 6.3) a graphical representation of the whole genome alignments of the isolates.

Table 6.2 Genomic comparison of ASx5L with closely related genera for which genomic sequence is available

Genome	**Ortho ANI**	**AAI**	**G+C ratio**	**16S RNA identity**	**Genome size (Mb)**	**DDH[3]**
ASxL5	100	100	56.1	100	2.82	-
Thalassolituus sp. C2-1	79.39	74.84	53.1	95.93	4.36	20.7
Thalassolituus oleivorans MIL-1	70.75	67.61	53.2	95.03	3.9	20.7
Bacterioplanes sanyensis NV9	70.47	66.11	53.4	94.64	4.29	19.9
Oceanobacter kriegii DSM 6294	72.08	53.78	55.3	94.14	4.5	19.8
Marinomonas communis DSM 5604	64.48	45.67	44.9	90.56	3.85	28.9

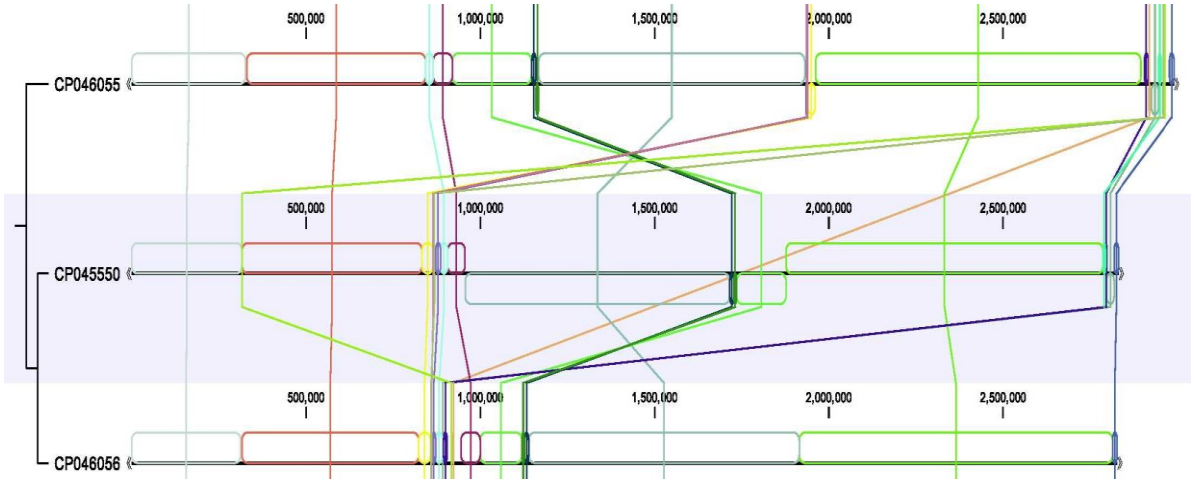


Figure 6.3 Whole genome sequence alignments of the predatory bacteria of the Oceanospirillaceae, the genome sequences are indicated by their Genbank accession numbers and were aligned using CLC Genomics Workbench 20.0.3

Examination of the component genes present in ASxL5 genome using the KEGG database (Kanehisa and Goto, 2000) reveals metabolic pathways typical of an aerobic *Gammaproteobacterium*. ASxL5 contains a total of 75 genes assigned to bacterial motility proteins including those involved in chemotaxis, flagella assembly and type IV pilus systems. Within the last category are 9 out 10 genes are responsible for twitching motility in a range of other organisms. The genome of ASxL5 contained the complete ectoine biosynthesis pathway involved in the protective response to osmotic stress (Czech et al., 2018), as might be expected for a halophilic organism. The genome also contains the complete pathways for many cofactors and vitamins including the riboflavin synthesis pathway. Hydrocarbon utilization pathways were incomplete. A comparison of the distribution of genes in COG categories for ASxL5 with the two most related genomes available, *T. olerverans* and *T. sp.* is presented in (Figure 6.4). The most notable difference between ASxL5 and the other two genomes is the greater number of genes involved in amino acid transport and metabolism in ASxL5. On

the whole the smaller genome of ASxL5 contained proportionally less genes from each COG category compared to the larger related genomes.

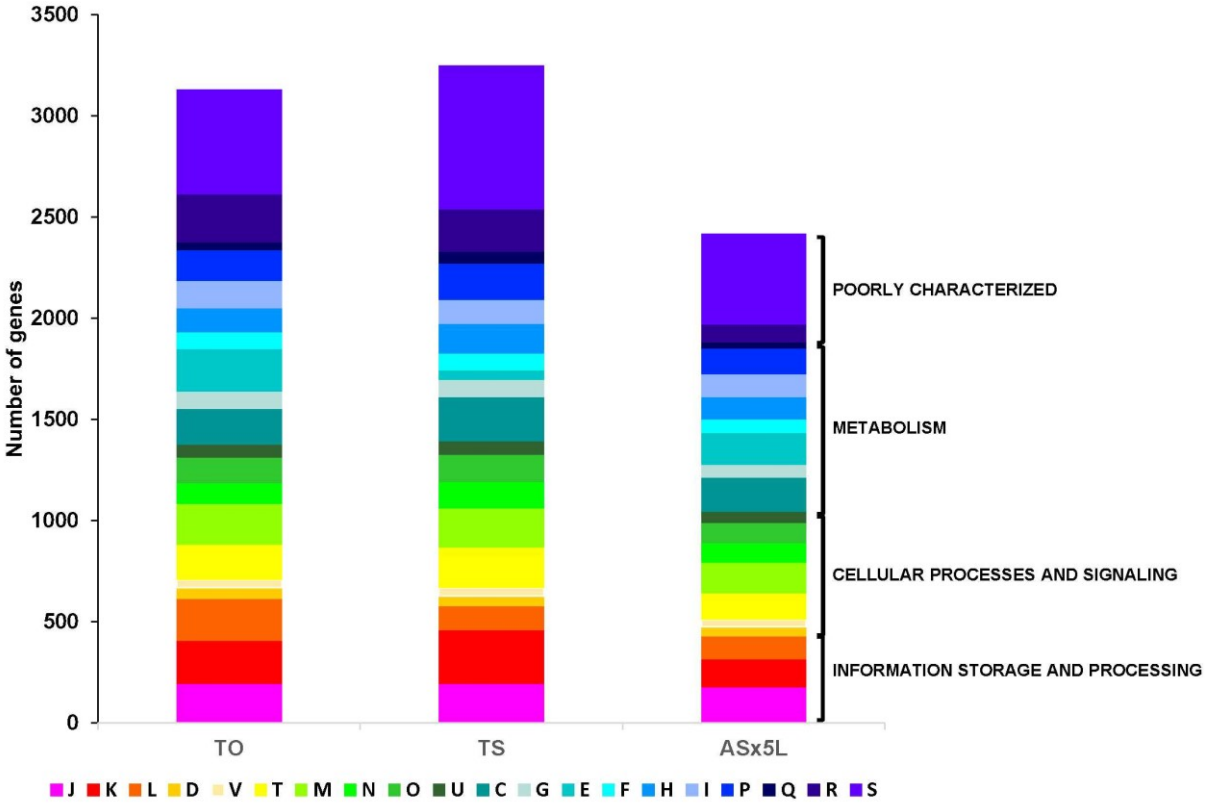


Figure 6.4 Functional class distribution of predicted genes according to the clusters of orthologous groups of proteins of ASx5L compared to *T. oleivorans* MIL-1 (TO) and *Thalassolituus* sp. C2-1 (TS) J, translation, ribosomal structure and biogenesis; K, transcription; L, replication, recombination and repair; D, cell cycle control, cell division, chromosome partitioning; V, defence mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, posttranslational modification, protein turnover, chaperones; U, Intracellular trafficking, secretion, and vesicular transport; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, Function unknown

6.3.3 ASxL5 Preys on *Campylobacter* species and Other Gram-Negative Hosts

The predatory activity of the ASxL5 bacterium was investigated to determine host range. The bacterium was able to form plaques on *Campylobacter* species including: *C. hyointesinalis* 11608, *C. jejuni* PT14, *C. jejuni* 12662, *C. jejuni* NCTC 11168; *C. coli* NCTC 12667; *C. helveticus* NCTC 12472; *C. lari* NCTC 11458 and *C. upsaliensis* NCTC 11541. Testing of a wider selection of Gram-negative and Gram-positive bacteria revealed that ASxL5 could also form plaques on *Escherichia coli* NCTC 86, *Citrobacter freundii* NCTC 9750 and *Klebsiella oxytoca* 11466. The microscopic interaction with *E. coli* NCTC 86 is shown in (Fig 6.5 A-D) whilst the interaction with *C. jejuni* PT14 and *C. hyointestinalis* S12 are shown in (Fig 6.5 E-H). The attack mechanism appeared to be different between the preys types tested, with one or more *E. coli* cells becoming attached to each ASxL5 cell, positioned laterally along the extended cell before adsorption. In contrast ASxL5 appeared to attach to campylobacters via a single contact point, often with the predator cell apex, making contact near the *Campylobacter* cell apex (Fig 6.5 H).

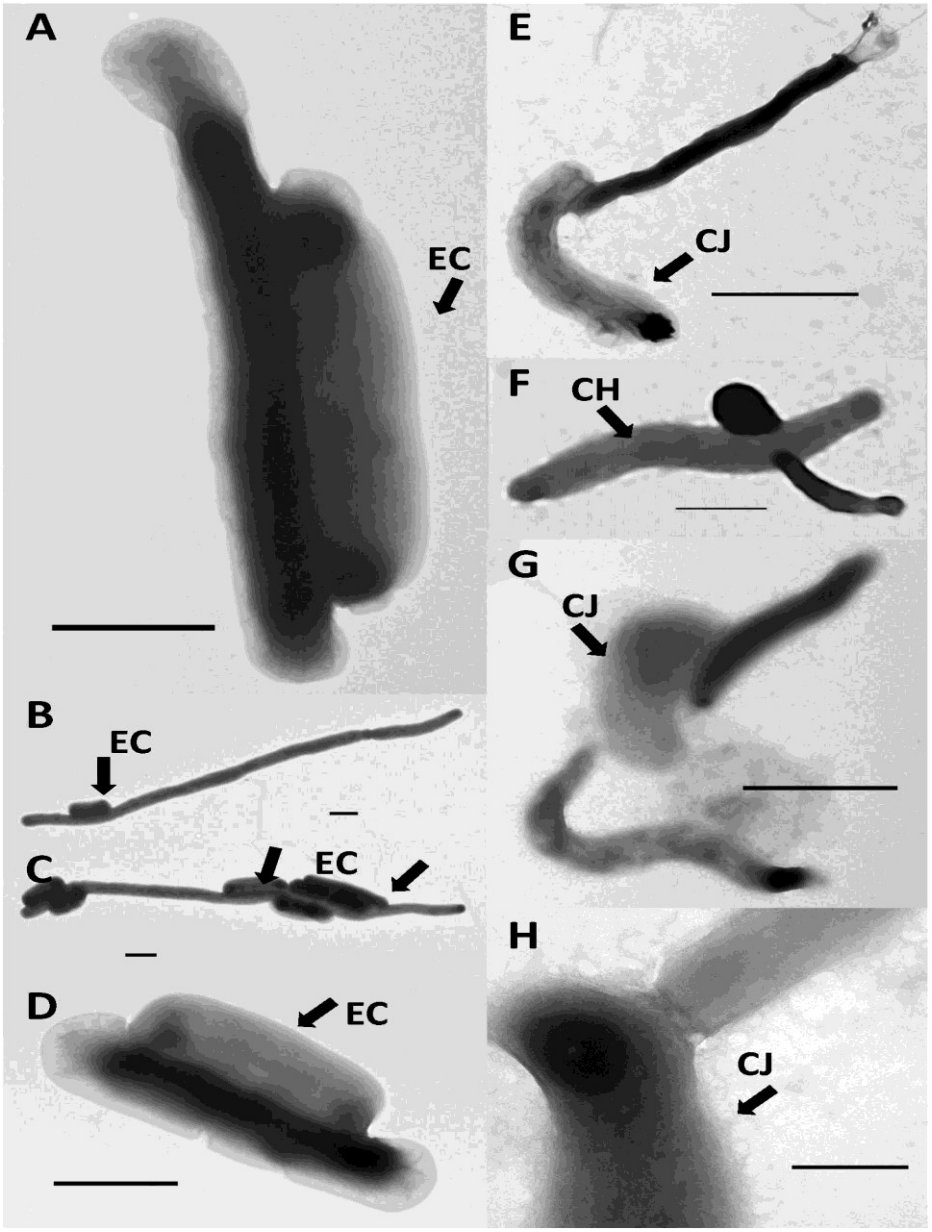


Figure 6.5 TEM of ASx5L interacting with prey showing (A-D with *E. coli* prey; E-H with *C. jejuni* prey) (A typical cell ASx5L attached to single *E. coli* (EC) cell) (B filamentous ASx5L attached to single EC cell) (C filamentous ASx5L cells attached to multiple EC cells) (D smaller ASx5L cell attached to single *E. coli* (EC) cell) (E single ASx5L cell attached to *C. jejuni* (CJ) cell) (F ASx5L attacking a *C. hyointestinalis* (CH) cell) (G two ASx5L cells attacking a CJ cell) (H close view of attachment point of ASx5L, close to apex of CJ cell (bar 0.2 μm)) Bar represents 1 μm in A-G

6.4 Discussion

Predatory bacteria have evolved to exploit abundant prey sources; it is becoming apparent that they are widespread in many different environments (Pérez et al., 2016) . In this study the ASxL5 bacteria was isolated from slurry using phage isolation methods because of the organism's small cell size and the genomic relatedness of ASxL5 to members of the marine bacterial family *Oceanospirillaceae* was surprising, even though the organism was halotolerant being able to grow on 5% salt containing medium. Water quality analysis of the slurry revealed the sodium chloride level to be less than 0.1%. The slurry is therefore far from a marine environment - geographically and chemically. The presence of at least two related, but non-identical isolates from the same source, provided evidence that these predators were thriving in this non-marine environment. Moreover, microbiome analysis revealed identical 16S rRNA sequences to be in the top 50 most abundant operational taxonomy units (OTUs) in the slurry collected in this study and several uncultured bacteria were identified in the Genbank database that had similar 16s rRNA sequences to the ASxL5 bacterium. Three of these (GQ921362, GQ921357 and GQ921396; Figure 6.6) were all isolated from fracture water, from a depth of 1.3 km depth in a South African gold mine in 2009, while a further two (DQ256320 and DQ337006) were obtained from subsurface water (in South Africa) in 2005 (16S rRNA sequences Blasted into Gene bank data base; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The most closely related 16S rRNA sequence relative to ASxL5 is a partial 16S rRNA sequence that was obtained from enrichment culture of sandy sediment, obtained from a beach in Northern France in 2006 (accession number AM292408) Alain et al. (2012). A further closely related 16s rRNA sequence from an uncultured bacterium, HQ183822.1, was obtained from a collection pool leached from a municipal landfill site in China (Liu et al., 2011).

Clearly, the ASxL5 bacteria is not highly represented in taxonomic databases but it is likely that these sequences from uncultured bacteria represent similar organisms to ASxL5, which are distributed worldwide, often in challenging environments. The closest relatives to ASxL5 from whole genome phylogenetic analysis were: *Thalassolituus* sp. C2-1, *Thalassolituus marinus*, *T. oleivorans*. and *Oceanobacter kriegii*. All these cultures were isolated from marine environments and utilise hydrocarbons (Bowditch et al., 1984, Yakimov et al., 2004, Choi and Cho, 2013) and from the literature, *T. marinus*, *T. olevorans* and *O. krieggi* are motile, halotolerant, oxidase positive curved rods but have few other phenotypic characteristics were common with ASxL5. However, the phenotypic characteristics of *Thalassolituus* sp. C2-1.are unknown.

In this study examination of the coding content of the ASxL5 genome provided functional insights into the phenotypic characteristics. The presence of genes that encode type IV pili (Tfp) are of particular interest as these facilitate cell movement referred to as social gliding or twitching without flagella over surfaces. The Tfp have other functions including predation, pathogenesis, biofilm formation, natural DNA uptake, auto-aggregation of cells and development (Wall and Kaiser, 1999). The presence in the ASxL5 genome of numerous copies (18 in total) of genes encoding diguanylate cyclase (enzyme that catalyses the conversion of 2 guanosine triphosphate to 2 diphosphate and cyclic di-GMP) and the presence of the corresponding diguanylate cyclase phosphodiesterase (catalyses the degradation of cyclic di-GMP to guanosine monophosphate; 6 copies present) was of interest because cyclic-di-GMP is an important second messenger involved in many processes including: biofilm development and detachment, motility, attachment and virulence (Wall and Kaiser, 1999, Dow et al., 2006). In *Bdellovibrio bacteriovorus* it has been shown to control the switch between free-living and predatory lifestyles (Hobley et al., 2012).

Most research into predatory bacteria has centred on *Bdellovibrio*, *Bdellovibrio*-like organisms and *Myxococococcus* species. These and other known examples of predatory bacteria form a taxonomically diverse group. Despite this diversity, a group of signature protein families that reflect the phenotype of 11 known predatory bacteria has been identified (Pasternak et al., 2013, Pasternak et al., 2015). The two genes in this study that are frequently associated with predatory bacterial genomes were those encoding O-antigen ligase (*waa*L) and tryptophan 2,3-dioxygenase (*kyn*A). The former was present in the ASxL5 genome sequence, but the latter was not. The transcriptional regulator gene *gnt*R was absent in the predator group examined, but three *gnt*R genes could be identified in ASxL5. The availability of more diverse predatory bacterial genomes will enable the development of finer resolution analyses in the future that can take into account evidence of functional and environmental differences between group members. The most remarkable features of *Venatorbacter cucullus* gen. nov. sp. nov. as captured by TEM images, are its unique flexible morphologies that facilitate interactions with prey bacteria. The type of interaction observed is different from other predatory bacteria and has not been identified or reported previously (Fig

6.7) A proposed predatory life cycle of ASxL5 (Fig 6.6).

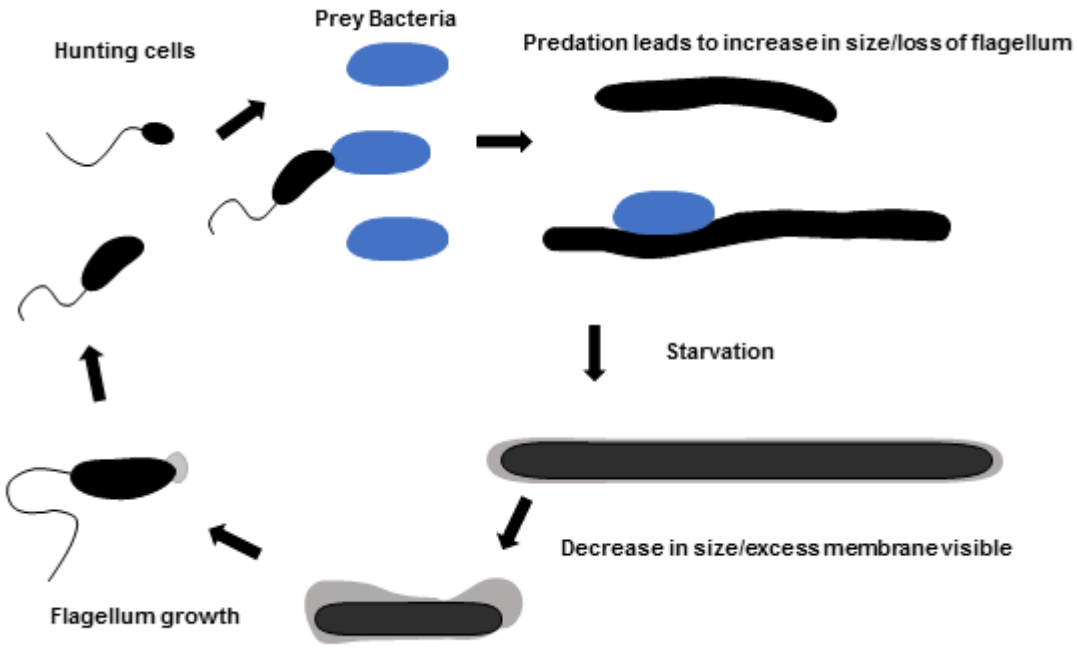


Figure 6.6 Proposed life cycle of *Venatorbacter cucullus* gen. nov. sp. nov.

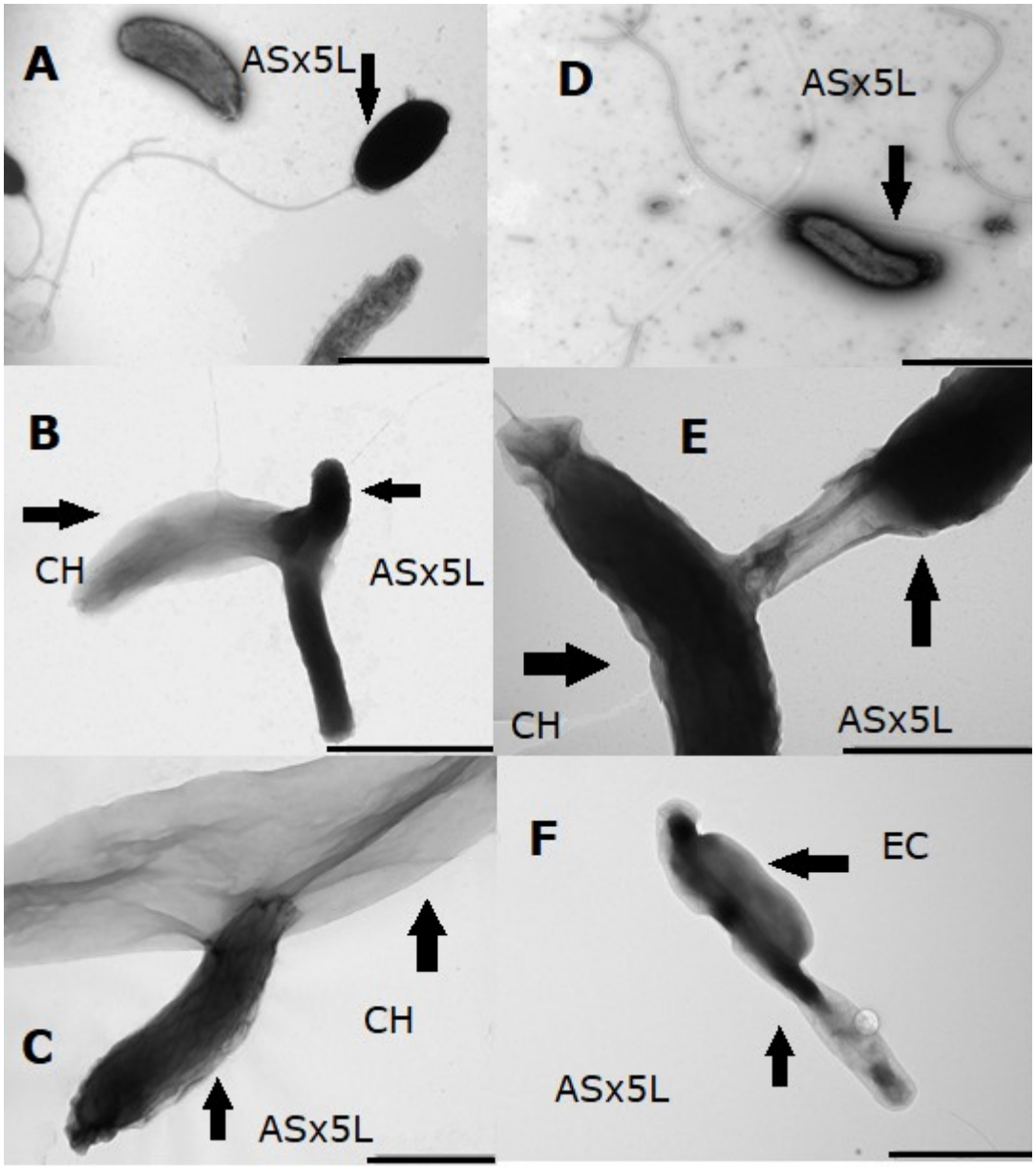


Figure 6.7 TEM of ASx5L and different type interaction between predatory and prey bacterium has not been identified or reported previously (A, D ASx5L predator) (B,C and E ASx5L attacking a *C.hyointestinalis* (CH) cell) (F ASx5L cell attached to single *E. coli* (EC) cell)

There are few examples in the literature of similar apical structures to those we report here, but these include those of *Terasakiispira papahanaumokuakeensis* an Oceanospirillaceae bacterium, that shows occasional apical enlargement(Zepeda et al., 2015) and the Alphaproteobacteria, *Terasakiella pusilla* previously in the genus *Oceanospirillum*, that exhibits what are described as "polar membranes" (Terasaki, 1979). The presence of coccal forms in older cultures is a frequent observation particularly for bacteria with curved morphology, such as *Vibrio*, *Campylobacter* and Helicobacter (Baker and Park, 1975, Ng et al., 1985, Reshetnyak and Reshetnyak, 2017) and probably represents a degenerative state. Further work is required to elucidate the precise life cycle of *Venatorbacter cucullus* gen. nov. sp. nov. to determine how it traps and feeds on its prey, and whether its genome encodes bioactive compounds that can be exploited for medicinal or biotechnological purposes.

Description of *Venatorbacter* gen. nov. *Venatorbacter* (Ven.a.tor, ba'c.ter, L. composed of venator from L. n. *venator*, 'hunter' and Gr. n. *bacter*, 'a rod'. *Venatorbacter*, 'a hunting rod'. Cells are aerobic, halotolerent, Gram-negative, motile rods. Catalase and oxidase activities are positive.

Description of *Venatorbacter cucullus* gen. nov. sp. nov. *Venatorbacter cucullus* (cu'cull.us.; L. n. *cucullus* meaning cowl).

In addition, the description features of the genus, cells are of 1.63 μm in length by 0.37 μm wide when grown on BA or BHI. Colonies on BHI agar are small reaching 2 mm in diameter after 72 h. They are beige, translucent, circular, convex and shiny. The type strain ASxL5 can use *E. coli*, *Klebsiella* spp. *Campylobacter* spp. and several other Gram-negative bacteria as prey. It was isolated in Nottinghamshire UK from bovine slurry and is deposited at National Collection of Type Cultures (UK): accession number NCTC 14397 and the Netherlands Culture Collection of Bacteria (NCCB) accession number NCCB 100775.

Chapter Seven

Discussion

7.1 General discussion and Conclusion

Campylobacter jejuni is the major cause of human bacterial gastroenteritis and is the most commonly reported foodborne bacterial disease in the EU and worldwide. Consumption of poultry products is thought to be the main source of infection but consumption of contaminated raw milk also plays a significant role in campylobacteriosis.

Chapter 3 of this thesis updated research undertaken in 1980s into the survival of *Campylobacter* spp*.* in milk but here using UHT milk without microflora. However, the consumption of raw milk (Davys et al., 2020) and outbreaks associated with milk (Kenyon et al., 2020) have prompted international interest in campylobacters from milk and dairy sources (Hansson et al., 2019, Jaakkonen et al., 2020). This work also included a study of the survival of C. *ureolyticus* thought to be associated with milk-borne outbreaks. Investigation into phage survival in milk was also carried out as this was completely unknown. Evidence from this work showed that campylobacters survive very well in milk, particularly C. *ureolyticus* which showed evidence of increasing numbers. The phage study provided evidence that milk would potentially be an ideal medium for administering bacteriophage therapy to bovine subjects being both cheap and safe.

Chapter 4 described the genomic characterisation of selected isolates of *Arcobacter* and *Campylobacter* from bovine slurry to use as candidate hosts in order to isolate bacteriophage from bovine samples. The DNA sequences were analysed and compared to isolates with known sequences in the NCBI database from isolates from various different sources. Genomic characterisation provides a strong base for the bacteriophage isolation experiments and considerably adds to our knowledge of the genomic characteristics of *Arcobacter* and *Campylobacter* from cattle sources. The *C. hyointestinalis* isolate was particularly interesting as it

seems to be distinctively cattle adapted being dissimilar to human and swine isolates in the database. The *C. coli* S9 strain fell within a genomic group representing non-poultry *C. coli* strains as expected. The *A. skirrowii* isolated was most related to a strain in the database isolated from water and in contrast to the *Campylobacter* isolates showed little evidence of pathogenic traits. Prophage were particularly prevalent in this strain potentially making it potentially resistant to some bacteriophage.

In Chapter 5 attempts were made to use the isolates described in Chapter 4 as hosts to isolate bacteriophage from bovine slurry. Despite rigorous attempts bacteriophage could not be directly isolated from the slurry. This was surprising due to the comparative ease in which bacteriophage can be isolated from pig or chicken sources. Small plaques thought to be phages were instead predatory bacteria described in chapter 6. A bacteriophage sequence was obtained during the sequencing of this bacteria which was probably a prophage released from the host due to the stress of predation. Clearly slurry is not an ideal environment for isolation of *Campylobacter* and *Arcobacter* bacteriophage. Whether they are present but in low and undetectable numbers or they are inactivated by inorganic or organic components of slurry or predated upon by the eukaryotic microflora (Pinheiro et al., 2007) is unknown.

Chapter 6 describes the taxonomic and microscopic characterisation of the predatory bacterium isolated during attempts to isolate bacteriophage (Chapter 5). The isolation of a completely new type of predatory bacteria was an important discovery. The proposed name for this predatory bacterium is *Venatorbacter cucullus.* The process of getting this new genus and species recognised has been initiated. This has been problematic as standard biochemical tests cannot be carried out on an organism that does not grow in broth medium. The way in which new bacteria are described is not well suited to predatory bacteria as there are

few examples. Why this morphologically unusual relative of marine bacteria is thriving in a slurry tank in central England is an intriguing mystery. Much further work needs to be done to understand this new bacterium which may have useful bioactive compounds that can be exploited for medicinal or biotechnological purposes.

In conclusion although most human disease associated with *Campylobacter* is thought to originate from poultry, the study of bovine campylobacters and arcobacters have been somewhat neglected. It is clear from this work that we do not have the full picture regarding their taxonomy, pathogenicity, survival and genetic characteristics. It is also clear that the microflora of slurry is complex and contains species and genera that have not previously been isolated.

The future work, will include plane to study this novel predatory bacteria in two line, the first is to complete the phenotypical studies that I established to find out more about properties of this properties bacteria including the prey rang, predating capacity, comparing with other predatory bacteria, etc.

The second line is the genotypical studies to have more understanding about the genomic structure of this novel predatory bacterium, which may demonstrates the role of this bacteria in the environment or If there any possible biomedical applications of their enzymes.

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Appendix

Appendix 1:

Genes present in *A. skirrowii A2S6* but absent in *A. skirrowii* CCUG 10374 studied

Gene	Product
EI285_00040	DMT famliy transporter
EI285_00045	Crp/Fnr famliy transcriptional regulator
EI285_00305	hyothetical protein
EI285_00310	nuclease
EI285_01150	DUF4885 domain-containing protein
EI285_01155	hyothetical protein
EI285_01765	mechanosensitive ion channel
EI285_01770	hyothetical protein
EI285_01885	diguanylate cyclase
EI285_02930	hyothetical protein
EI285_02935	hyothetical protein
EI285_02940	hyothetical protein
EI285_02945	lipolytic protein
tmK	dTMP kinase
EI285_02975	phosphoribosyltransferase
EI285_03210	DNA methyltransferase
EI285_03435	aldose 1-epimerase family protein
EI285_03490	methylated-DNA--[protein]-cysteine S-methyltransferase
EI285_03500	hyothetical protein
EI285_03540	hyothetical protein
EI285_03545	metal-dependent hydrolase
EI285_03550	hyothetical protein
EI285_03555	hyothetical protein
EI285_03565	tetratricopeptide repeat protein
EI285_03570	hyothetical protein
EI285_03575	hyothetical protein
EI285_03580	WYL domain-containing protein
EI285_03585	hyothetical protein
EI285_03590	DUF3482 domain-containing protein
EI285_03595	hyothetical protein
EI285_03600	hyothetical protein
EI285_03605	IS3 family transposase
EI285_03610	transposase
EI285_03615	DNA polymerase IV
EI285_03620	phage repressor protein
EI285_03625	gamma-glutamylcyclotransferase
EI285_03630	HU family DNA-binding protein
EI285_03635	hyothetical protein
EI285_03640	deoxyribonuclease

Appendix 1 continued

Gene	Product
EI285_03645	hyothetical protein
EI285_03650	IS256 family transposase
EI285_03690	hyothetical protein
EI285_03695	ATP-binding protein
EI285_03700	hyothetical protein
EI285_03705	hyothetical protein
EI285_03710	hyothetical protein
EI285_03715	hyothetical protein
EI285_03720	hyothetical protein
EI285_03725	DUF2493 domain-containing protein
EI285_03730	hyothetical protein
EI285_03735	hyothetical protein
EI285_03740	transcriptional regulator
EI285_03745	hyothetical protein
EI285_03750	hyothetical protein
EI285_03755	hyothetical protein
EI285_03760	Fic family protein
EI285_03765	hyothetical protein
EI285_03770	hyothetical protein
EI285_03775	NAD-dependent deacetylase
EI285_03780	DUF2779 domain-containing protein
EI285_03785	hyothetical protein
EI285_03790	hyothetical protein
EI285_03795	ATP-binding protein
EI285_03800	5'-nucleotidase
EI285_03805	M28 family peptidase
EI285_03810	hyothetical protein
EI285_03815	hyothetical protein
EI285_03820	DUF2779 domain-containing protein
EI285_03825	hyothetical protein
EI285_03830	hyothetical protein
EI285_03835	ATP-binding protein
EI285_03840	5'-nucleotidase
EI285_03845	M28 family peptidase
EI285_03850	hyothetical protein
EI285_03855	hyothetical protein
EI285_03865	hyothetical protein
EI285_03870	restriction endonuclease subunit S
EI285_03875	type I restriction - modification system subunit M
EI285_03880	hyothetical protein

Appendix 1 continued

Gene	Product
EI285_03885	restriction endonuclease subunit S
EI285_03890	hyothetical protein
EI285_03895	hyothetical protein
EI285_03900	hyothetical protein
EI285_03905	hyothetical protein
EI285_03910	winged helix-turn-helix transcriptional regulator
EI285_03915	type I restriction - modification system subunit R
EI285_03920	M48 family peptidase
EI285_03925	IS630 family transposase
EI285_03930	hyothetical protein
EI285_03935	hyothetical protein
EI285_03940	hyothetical protein
EI285_03945	hyothetical protein
cas9	type II CRISPR RNA-guided endonuclease Cas9
cas1	type II CRISPR-associated endonuclease Cas1
EI285_03985	transposase
EI285_03990	IS3 family transposase
cas2	type II CRISPR-associated endonuclease Cas2
EI285_04060	DUF4885 domain-containing protein
EI285_04065	DUF4885 domain-containing protein
EI285_04070	hyothetical protein
EI285_04075	hyothetical protein
EI285_04080	hyothetical protein
EI285_04200	hyothetical protein
EI285_04775	hyothetical protein
EI285_04780	DNA-dinding protein
EI285_05230	hyothetical protein
EI285_05255	GGDEF domain-containing protein
pglF	UDP-N-acetylglucosamine 4,6-dehydratase(configuration-retaining)
rfbC	dTDP-4-dehydrorhamnose 3,5-epimerase
EI285_05335	glycosyltransferase family 4 protein
EI285_05340	type II toxin-antitoxin system prevent-host-death family antitoxin
EI285_05345	NAD-dependent epimerase/dehydratase family protein
EI285_05355	glycosyltransferase
EI285_05360	oligosaccharide repeat unit polymerase
EI285_05365	glycosyltransferase family 1 protein
EI285_05370	glycosyltransferase
EI285_05375	polysaccharide biosynthesis protein
EI285_05380	glycosyltransferase
EI285_05385	nucleotidyl-sugar pyranose mutase

Appendix 1 continued

Gene	Product
EI285_05390	GDP-L-fucose synthase
gmd	GDP-mannose 4,6-dehydratase
EI285_05400	GDP-mannose mannosyl hydrolase
EI285_05405	mannose-1-phosphateguanylyltransferase/mannose-6-phosphate isomerase
EI285_05410	MarR family EPS-associated transcriptional regulator
EI285_06010	hyothetical protein
EI285_06015	hyothetical protein
EI285_06020	hyothetical protein
EI285_06025	hyothetical protein
EI285_06030	hyothetical protein
EI285_06035	ATP-dependent Clp protease proteolytic subunit
EI285_06040	hypothetical protein
EI285_06045	WYL domain-containing protein
EI285_06050	hyothetical protein
EI285_06055	DUF4062 domain-containing protein
EI285_06060	hyothetical protein
EI285_06065	hyothetical protein
EI285_06070	hyothetical protein
EI285_06095	XRE family transcriptional regulator
EI285_06100	type II toxin-antitoxin system HipA family toxin
EI285_06530	sel1 repeat family protein
EI285_07040	flavodoxin family protein
EI285_07275	hypothetical protein
EI285_07280	hypothetical protein
EI285_07285	hypothetical protein
EI285_07290	hypothetical protein
EI285_07295	hypothetical protein
EI285_07300	hypothetical protein
EI285_07305	hypothetical protein
EI285_07310	XRE family transcriptional regulator
EI285_07315	hypothetical protein
EI285_07320	site-specific integrase
EI285_07325	hypothetical protein
EI285_07330	hypothetical protein
EI285_07335	hypothetical protein
EI285_07350	type III restriction endonuclease subunit R
EI285_07355	Abi family protein
EI285_07360	DUF2130 domain-containing protein
EI285_07365	site-specific DNA-methyltransferase
EI285_07370	hypothetical protein

Appendix 1 continued

Gene	Product
EI285_07385	transposase
EI285_07390	IS3 family transposase
EI285_07395	DUF1016 domain-containing protein
EI285_07400	hypothetical protein
EI285_07405	site-specific integrase
EI285_07410	hypothetical protein
EI285_07415	linear amide C-N hydrolase
EI285_07420	ShlB/FhaC/HecB family hemolysin secretion/activation protein
EI285_07425	filamentous hemagglutinin N-terminal domain-containing protein
EI285_07430	hypothetical protein
EI285_07435	DNA-binding response regulator
EI285_07440	methyl-accepting chemotaxis protein
EI285_07450	response regulator
EI285_07455	response regulator
EI285_07465	YgiW/YdeI family stress tolerance OB fold protein
EI285_07470	hypothetical protein
EI285_07475	response regulator
EI285_07480	hypothetical protein
EI285_07510	hypothetical protein
EI285_07515	hypothetical protein
EI285_07520	hypothetical protein
EI285_07525	hypothetical protein
EI285_07530	hypothetical protein
EI285_07535	hypothetical protein
EI285_07540	hypothetical protein
EI285_07545	hypothetical protein
EI285_07550	hypothetical protein
EI285_07555	hypothetical protein
EI285_07560	hypothetical protein
EI285_07675	MBL fold metallo-hydrolase
EI285_07680	AraC family transcriptional regulator
EI285_08005	Flagellin
EI285_08220	hypothetical protein
EI285_08230	hypothetical protein
EI285_08420	transposase
EI285_08425	IS3 family transposase
EI285_08840	linear amide C-N hydrolase
EI285_08845	ShlB/FhaC/HecB family hemolysin secretion/activation protein
EI285_08850	filamentous hemagglutinin N-terminal domain-containing protein
EI285_08855	diguanylate cyclase
EI285_08860	hypothetical protein

Appendix 1 continued

Gene	Product
EI285_08865	DNA-binding response regulator
EI285_09065	Flagellin
EI285_09070	Flagellin
EI285_09075	DUF115 domain-containing protein
EI285_09090	poly(glycerol-phosphate) alpha-glucosyltransferase
EI285_09095	hypothetical protein
EI285_09100	class I SAM-dependent methyltransferase
EI285_09110	type II toxin-antitoxin system prevent-host-death family antitoxin
EI285_09115	type II toxin-antitoxin system VapC family toxin
EI285_09120	glycosyltransferase
EI285_09125	WxcM-like domain-containing protein
EI285_09130	N-acetyltransferase
EI285_09135	DegT/DnrJ/EryC1/StrS family aminotransferase
EI285_09140	glycosyltransferase family 61 protein
EI285_09145	hypothetical protein
EI285_09150	hypothetical protein
EI285_09155	MBL fold metallo-hydrolase
EI285_09160	hypothetical protein
EI285_09165	amino acid adenylation domain-containing protein
EI285_09170	acyl carrier protein
EI285_09175	ketoacyl-ACP synthase III
EI285_09180	SDR family oxidoreductase
EI285_09185	SDR family oxidoreductase
EI285_09190	acyl carrier protein
EI285_09195	hypothetical protein
EI285_09200	ketoacyl-ACP synthase III
EI285_09205	hypothetical protein
EI285_09215	winged helix-turn-helix transcriptional regulator
EI285_09240	sodium-dependent transporter
EI285_09245	deoxyribonuclease IV
EI285_09250	C4-dicarboxylic acid transporter DauA
EI285_09260	ATP-binding protein
EI285_09265	hypothetical protein
EI285_09270	succinate--CoA ligase
EI285_09275	UDP-N-acetylmuramate dehydrogenase
EI285_09280	glycerol kinase
EI285_09285	type I DNA topoisomerase
EI285_09290	YfcE family phosphodiesterase
Gene	
EI285_09295	biotin synthase
EI285_09745	FAD-dependent thymidylate synthase
EI285_09750	trimeric intracellular cation channel family protein
EI285_09755	hypothetical protein
purN	phosphoribosylglycinamide formyltransferase
ruvC	crossover junction endodeoxyribonuclease RuvC

Appendix 2:

Genes absent in *A. skirrowii A2S6* but present in *A. skirrowii* CCUG 10374examined

Gene	Product
ASKIR_0049	two-component system response regulator,putative CusR
ASKIR_0050	two-component system sensor histidine kinase,putative CusS
ASKIR_0051	putative copper resistance protein
ASKIR_0052	outer membrane efflux protein, TolC family,putative CusC
ASKIR_0053	putative copper/silver efflux system, membrane fusion protein CusB
ASKIR_0054	CusA family copper/silver efflux pump
ASKIR_0077	Fic family protein (DUF4172 domain)
ASKIR_0078	DUF262 domain-containing protein
ASKIR_0092	putative lipid asymmetry ABC transporter MlaABCDEF component MlaB
mlaD	lipid asymmetry ABC transporter MlaABCDEF, periplasmic component MlaD
mlaF	lipid asymmetry ABC transporter MlaABCDEF, ATPase component MlaF
mlaE	lipid asymmetry ABC transporter MlaABCDEF, permease component MlaE
ASKIR_0327	major facilitator superfamily transporter
ASKIR_0328	SAM-dependent methyltransferase
ASKIR_0329	TonB-dependent receptor
ASKIR_0330	transcriptional regulator, AraC family
ASKIR_0331	manganese efflux pump MntP
ASKIR_0332	major facilitator superfamily transporter
ASKIR_0333	major facilitator superfamily transporter
ASKIR_0334	ABC transporter, ATP-binding/permease components
ASKIR_0335	ABC transporter, ATP-binding/permease components
ASKIR_0336	TonB-dependent siderophore receptor
ASKIR_0337	transcriptional regulator, AraC family
ASKIR_0338	transcriptional regulator, RcnR/FrmR family
ASKIR_0339	divalent metal cation transporter
ASKIR_0361	Sel1 domain-containing protein
ASKIR_0393	SRPBCC domain-containing protein
ASKIR_0394	transcriptional regulator, AraC family
ASKIR_0395	acetyltransferase
ASKIR_0396	SRPBCC domain-containing protein
ASKIR_0397	chorismate mutase, type II
ASKIR_0398	transglutaminase family protein
ASKIR_0399	histidine phosphatase family protein
ASKIR_0400	helix-hairpin-helix domain-containing protein
ASKIR_0401	acetyltransferase
ASKIR_0402	RhuM family protein
ASKIR_0403	hypothetical protein
ASKIR_0404	type IIG restriction/modification system
ASKIR_0543	transcriptional regulator, XRE family

Appendix 2 continued

Gene	Product
ASKIR_0544	hypothetical protein
ASKIR_0545	YchJ family protein (SEC-C domain)
ASKIR_0555	phosphate ABC transporter, ATP-binding protein
ASKIR_0556	phosphate ABC transporter, permease protein
ASKIR_0557	phosphate ABC transporter, permease protein
ASKIR_0558	phosphate ABC transporter, periplasmic phosphate-binding protein
ybaK	cysteinyl-tRNA(Pro) deacylase
ASKIR_0594	putative membrane protein
ASKIR_0602	putative ribonuclease, YlaK/PhoH family
ASKIR_0625	acyltransferase
tmk	dTMP kinase
ASKIR_0677	virulence protein RhuM family protein
ASKIR_0678	toxin-antitoxin system, antitoxin component, HicB family
ASKIR_0679	toxin-antitoxin system, toxin component, HicA family
ASKIR_0704	hypothetical protein
ASKIR_0705	cold shock domain-containing protein
ASKIR_0706	hypothetical protein
ASKIR_0727	putative class D beta-lactamase
argDII	N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase
ASKIR_0739	nitroimidazol reductase (NimA) family protein
ASKIR_0740	benzoate:proton symporter BenE
ada	O6-methylguanine-DNA methyltransferase
glyA2	serine hydroxymethyltransferase
ASKIR_0743	hypothetical protein
ASKIR_0744	hypothetical protein
ASKIR_0745	hypothetical protein
ASKIR_0754	hypothetical protein
ASKIR_0755	hypothetical protein
ASKIR_0757	hypothetical protein
ASKIR_0758	hypothetical protein
ASKIR_0759	hypothetical protein
ASKIR_0760	hypothetical protein
ASKIR_0761	hypothetical protein
ASKIR_0762	hypothetical protein
ASKIR_0763	hypothetical protein
ASKIR_0764	hypothetical protein
ASKIR_0765	type IIP restriction/modification system, restriction endonuclease, PstI family
ASKIR_0766	type IIP restriction/modification system, DNA methyltransferase
ASKIR_0767	resolvase
ASKIR_0776	hypothetical protein

Appendix 2 continued

Gene	Product
ASKIR_0777	zeta toxin domain-containing protein
ASKIR_0778	hypothetical protein
ASKIR_0779	group II intron reverse transcriptase/maturase
ASKIR_0780	transcriptional regulator, XRE family
ASKIR_0781	hypothetical protein
ASKIR_0782	putative membrane protein
ASKIR_0784	putative membrane protein
ASKIR_0785	hypothetical protein
ASKIR_0786	hypothetical protein
ASKIR_0787	putative membrane protein
ASKIR_0788	transposase, IS256 family
ASKIR_0789	putative membrane protein
ASKIR_0790	hypothetical protein
ASKIR_0792	putative membrane protein
ASKIR_0793	putative membrane protein
ASKIR_0794	hypothetical protein
tssI1	type VI secretion system, syringe needle protein
tssD	type VI secretion system, inner tube protein
ASKIR_0797	hypothetical protein
ASKIR_0798	tetratricopeptide repeat protein
ASKIR_0799	putative membrane protein
tssA	type VI secretion system, baseplate protein
tssB	type VI secretion system, tubular sheath protein
tssC	type VI secretion system, tubular sheath protein
tssE	type VI secretion system, baseplate protein
tssF	type VI secretion system, baseplate protein
tssG	type VI secretion system, baseplate protein
tssH	type VI secretion system, ClpV1 family ATPase TssH
ASKIR_0807	type VI secretion system-associated FHA domain-containing protein TagH
tssJ	type VI secretion system, membrane platform protein
tssK	type VI secretion system, baseplate protein
tssL	type VI secretion system, membrane platform protein
tssM	type VI secretion system, membrane platform protein
ASKIR_0812	serine/threonine-protein kinase
tssI2	type VI secretion system, syringe needle protein
ASKIR_0814	putative chitinase
ASKIR_0815	hypothetical protein
ASKIR_0816	hypothetical protein
ASKIR_0818	putative chitinase
ASKIR_0819	tetratricopeptide repeat protein

Appendix 2 continued

Gene	Product
ASKIR_0820	putative membrane protein
ASKIR_0821	methyltransferase
ASKIR_0822	site-specific tyrosine recombinase, phage integrase family
sbcD	DNA repair exonuclease SbcCD, nuclease subunit
sbcC	DNA repair exonuclease SbcCD, ATPase subunit
ASKIR_0825	hypothetical protein
ASKIR_0826	ATP-binding protein (AAA domain)
ASKIR_0827	hypothetical protein
ASKIR_0828	DUF2779 domain-containing protein
ASKIR_0829	helicase
ASKIR_0830	putative membrane protein
ASKIR_0831	hypothetical protein
ASKIR_0832	hypothetical protein
ASKIR_0835	hypothetical protein
ASKIR_0836	putative membrane protein
ASKIR_0837	putative membrane protein
ASKIR_0838	hypothetical protein
ASKIR_0839	putative DNA-binding protein
ASKIR_0840	replication initiation protein
ASKIR_0846	hypothetical protein
ASKIR_0866	hypothetical protein
ASKIR_0870	hypothetical protein
ASKIR_0871	putative membrane protein
ASKIR_0872	hypothetical protein
cas6	CRISPR/Cas system-associated RAMP protein Cas6
ASKIR_0874	hypothetical protein
ASKIR_0875	CRISPR/Cas system-associated RAMP protein
ASKIR_0876	hypothetical protein
ASKIR_0877	CRISPR/Cas system-associated RAMP protein Cas7/Csm3, type III
ASKIR_0878	CRISPR/Cas system-associated RAMP protein, type III
ASKIR_0879	CRISPR/Cas system-associated RAMP protein
ASKIR_0880	CRISPR/Cas system-associated protein Csx1, type III
ASKIR_0881	Cas2 family CRISPR/Cas system-associated protein
cas1	CRISPR/Cas system-associated endonuclease Cas1
cas2	CRISPR/Cas system-associated endoribonuclease Cas2
ASKIR_0884	hypothetical protein
ASKIR_0885	porin family protein
ASKIR_0886	hypothetical protein
ASKIR_0887	putative membrane protein
ASKIR_0888	transcriptional regulator, YafY family

Appendix 2 continued

Gene	Product
ASKIR_0889	paraslipin family protein, SPFH superfamily
ASKIR_0890	putative membrane protein
ASKIR_0952	hypothetical protein
ASKIR_1111	putative membrane protein
ASKIR_1112	hypothetical protein
ASKIR_1127	UDP-glucose 4-epimerase
ASKIR_1128	toxin-antitoxin system, toxin component, MazF/PemK family
ASKIR_1129	hypothetical protein
ASKIR_1130	sugar transferase
ASKIR_1131	toxin-antitoxin system, transcriptional regulator HipB
ASKIR_1132	toxin-antitoxin system, toxin component, HipA family
ASKIR_1134	glycosyltransferase, family 2
ASKIR_1135	WbsX-like glycosyltransferase
ASKIR_1136	polysaccharide biosynthesis acetyltransferase
ASKIR_1137	hypothetical protein
ASKIR_1138	polysaccharide biosynthesis protein
ASKIR_1139	putative membrane protein
ASKIR_1140	HAD superfamily hydrolase, probable phosphatase
ASKIR_1141	hypothetical protein
ASKIR_1142	ATP-binding protein (AAA domain)
ASKIR_1143	acylneuraminate cytidylyltransferase family protein
ASKIR_1144	UDP-N-acetylglucosamine 4-epimerase
ASKIR_1145	acylneuraminate cytidylyltransferase family protein
ASKIR_1146	hypothetical protein
ASKIR_1147	putative KAP family NTPase
ASKIR_1148	hypothetical protein
ASKIR_1149	dTDP-4-amino-4,6-dideoxygalactose transaminase
ASKIR_1150	WxcM-like sugar acyltransferase
ASKIR_1151	WxcM-like domain-containing protein
ASKIR_1215	polyferredoxin-like protein
ASKIR_1216	TonB-dependent receptor
ASKIR_1250	toxin-antitoxin system, toxin component, HicA family
ASKIR_1251	hypothetical protein
ASKIR_1252	hypothetical protein
ASKIR_1280	RecQ family ATP-dependent DNA helicase
ASKIR_1281	site-specific tyrosine recombinase, phage integrase family (INT_Rci_Hp1_C domain)
ASKIR_1282	hypothetical protein
ASKIR_1283	HNH endonuclease
ASKIR_1284	hypothetical protein
ASKIR_1285	SprT-like domain-containing protein

Appendix 2 continued

Gene	Product
ASKIR_1285	SprT-like domain-containing protein
ASKIR_1286	hypothetical protein
ASKIR_1287	hypothetical protein
ASKIR_1288	hypothetical protein
ASKIR_1289	hypothetical protein
ASKIR_1290	hypothetical protein
ASKIR_1291	hypothetical protein
ASKIR_1292	type IIP restriction/modification system, cytosine-specific DNA methyltransferase
ASKIR_1293	hypothetical protein
ASKIR_1294	type IIP restriction/modification system, restriction endonuclease, Fnu4HI family
ASKIR_1295	KAP family NTPase
ASKIR_1296	hypothetical protein
ASKIR_1297	putative membrane protein
ASKIR_1298	hypothetical protein
ASKIR_1299	ATP-binding protein (AAA domain)
ASKIR_1300	hypothetical protein
ASKIR_1301	DUF4297 domain-containing protein
ASKIR_1302	transcriptional regulator, XRE family
ASKIR_1303	hypothetical protein
ASKIR_1304	hypothetical protein
ASKIR_1305	putative DNA-binding protein
ASKIR_1306	hypothetical protein
ASKIR_1307	hypothetical protein
ASKIR_1308	putative DNA-binding protein
ASKIR_1309	single-stranded DNA-binding protein
ASKIR_1310	putative membrane protein
ASKIR_1311	putative membrane protein
ASKIR_1312	P-type type IV conjugative transfer system ATPase TrbE/VirB4
ASKIR_1313	hypothetical protein
ASKIR_1314	P-type type IV conjugative transfer system protein TrbL/VirB6
ASKIR_1315	hypothetical protein
ASKIR_1316	hypothetical protein
ASKIR_1317	P-type type IV conjugative transfer system protein TrbF/VirB8
ASKIR_1318	P-type type IV conjugative transfer system translocation pore protein TrbG/VirB9
ASKIR_1319	P-type type IV conjugative transfer system translocation pore protein TrbI/VirB10
ASKIR_1320	hypothetical protein
ASKIR_1321	P-type type IV conjugative transfer system ATPase TrbB/VirB11
ASKIR_1322	P-type type IV conjugative transfer system coupling protein TraG/VirD4
ASKIR_1323	hypothetical protein
ASKIR_1356	putative membrane protein
ASKIR_1357	putative membrane protein

Appendix 2 continued

Gene	Product
ASKIR_1397	toxin-antitoxin system, toxin component, MazF/PemK family
ASKIR_1401	toxin-antitoxin system, toxin component, YafQ family
ASKIR_1402	toxin-antitoxin system, antitoxin component, RelB family
ASKIR_1404	hypothetical protein
ASKIR_1405	toxin-antitoxin system, toxin component, RelE/ParE family
ASKIR_1429	ATP-binding protein (AAA, DUF4143 domains)
ASKIR_1517	hypothetical protein
ASKIR_1518	putative membrane protein
ASKIR_1519	putative membrane protein
ASKIR_1520	hypothetical protein
ASKIR_1521	putative membrane protein
ASKIR_1522	hypothetical protein
ASKIR_1523	putative membrane protein
ASKIR_1524	hypothetical protein
ASKIR_1525	putative membrane protein
ASKIR_1526	hypothetical protein
ASKIR_1527	hypothetical protein
ASKIR_1528	putative membrane protein
ASKIR_1529	hypothetical protein
ASKIR_1530	hypothetical protein
ASKIR_1531	putative membrane protein
ASKIR_1532	hemagglutinin domain-containing protein
ASKIR_1533	hemolysin secretion/activation protein, ShlB/FhaC/HecB family
ASKIR_1540	ferritin-like protein
ASKIR_1541	acyl-CoA dehydrogenase
ASKIR_1542	phosphonate ABC transporter, periplasmic substrate-binding protein
ASKIR_1604	hypothetical protein
ASKIR_1757	MCP-domain signal transduction protein
ASKIR_1758	PAS sensor-containing signal transduction protein
ASKIR_1759	MCP-domain signal transduction protein
ASKIR_1760	PAS sensor-containing diguanylate cyclase/phosphodiesterase
ASKIR_1761	putative periplasmic substrate-binding protein
ASKIR_1808	TonB-dependent receptor
ASKIR_1844	sulfite exporter TauE/SafE family protein
maf1	motility accessory factor
maf2	motility accessory factor
ASKIR_1888	hypothetical protein
ASKIR_1889	4-alpha-L-fucosyltransferase

Appendix 2 continued

Gene	Product
ASKIR_1890	aminotransferase, DegT/DnrJ/EryC1/StrS family
ASKIR_1891	WbqC family protein
ASKIR_1892	methyltransferase
ASKIR_1893	SAM-dependent methyltransferase
fldA	flavodoxin
ASKIR_1895	leucine-rich repeat domain-containing protein
ASKIR_1896	SAM-dependent methyltransferase
ASKIR_1897	methyltransferase
ASKIR_1898	UDP-glucose 6-dehydrogenase
ASKIR_1899	glycosyltransferase, family 2
ASKIR_1900	glycosyltransferase, family 2
ASKIR_1901	dTDP-4-amino-4,6-dideoxygalactose transaminase
ASKIR_1902	WxcM-like sugar acyltransferase
ASKIR_1903	WxcM-like domain-containing protein
ASKIR_1904	Phosphonate metabolism-associated iron-containing alcohol dehydrogenase
ASKIR_1905	phosphonopyruvate decarboxylase
ASKIR_1906	phosphoenolpyruvate mutase /cytidylyltransferase
ASKIR_1907	putative polysaccharide biosynthesis protein
ASKIR_1908	acyl-CoA synthetase (AMP-forming) / AMP-acid ligase II
ASKIR_1909	short-chain dehydrogenase/reductase
ASKIR_1910	hypothetical protein
pseH	UDP-4-amino-4,6-dideoxy-beta-L-AltNAc o-acetyltransferase
ASKIR_1912	deacetylase, PIG-L family
ASKIR_1913	hypothetical protein
pseI	pseudaminic acid synthase
pseG	UDP-2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranosyl transferase
pesF	CMP-pseudaminic acid synthetase
pesC	UDP-2-acetamido-2, 6-dideoxy-beta-L-arabino-hex-4-ulose aminotransferase
ASKIR_1936	type II cytosine-specific DNA methyltransferase
ASKIR_1937	DUF1863 domain-containing protein
ASKIR_1938	putative membrane protein
ASKIR_1939	DUF1863 domain-containing protein
ASKIR_1940	type IIP restriction/modification system,restriction endonuclease, HaeIII family
ASKIR_1941	hypothetical protein
ASKIR_1942	type IIP restriction/modification system,cytosine-specific DNA methyltransferase
ASKIR_1943	putative histidine kinase
ASKIR_1944	transposase endonuclease subunit TnsA
ASKIR_1945	integrase (rve domain)
ASKIR_1946	transposition-related ATP-binding protein TniB
ASKIR_1947	transposition-related protein TniQ

Appendix 2 continued

Gene	Product
ASKIR_1948	ATP-binding protein (AAA domain)
ASKIR_1949	peptidase, S8 family
ASKIR_1950	type IIG restriction/modification system
ASKIR_1951	site-specific tyrosine recombinase, phage integrase family
ASKIR_1952	DUF4145 domain-containing protein
ASKIR_1953	hypothetical protein
ASKIR_1981	membrane-anchored protein, YitT family (DUF161,DUF2179 domains)
ASKIR_1997	Cupin domain-containing protein
ASKIR_2003	hypothetical protein
ASKIR_2028	phosphoethanolamine transferase
ASKIR_2029	DUF1924 domain-containing protein
ASKIR_2030	diheme cytochrome c
ASKIR_2031	diheme cytochrome c (N-terminal cytochrome bdomain)
ASKIR_2032	hypothetical protein
ASKIR_2033	two-component system response regulator
ASKIR_2034	two-component system sensor histidine kinase
ASKIR_2035	sulfatase
thy	thymidylate synthase complementing protein
ASKIR_2037	UPF0126 domain-containing membrane protein
ASKIR_2038	hypothetical protein
purN	phosphoribosylglycinamide formyltransferase 1
ruvC	RuvABC resolvasome, subunit RuvC

Appendix 3:

Nucleotide BLAST results from CRISPR spacers in *Arcobacter skirrowii A2S6*.CRISPAR 1 and 2

CRISPR 1

Regions	Direct Repeats	Spacer	Best Match Description	Cover %	Identity %	e-value
746361	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	ATAAAAACTGACTACCAA CAATTAAAAAAA	*Ruminococcus torques* ATCC 27756 Scfld0215	66%	100%	0.21
746427	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	ATATCTTTATATTCTATAT TGTTTATAATA	*Clostridium oryzae* strain DSM 28571 CLORY_contig000030	76%	100%	0.003
746493	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	GCTTGCTTTATATATTAAC ATGTATATTAT	*Lachnobacterium bovis* DSM 14045	70%	100%	0.83
746559	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	TCGCAGAGCCTACAAATA TCTTTAATAAT	*Aliiarcobacter faecis* strain AF1078 scaffold7.1	86%	100%	0.75
746624	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	CCTTGAGTTTTTTTAACTT TATTACCCTTAT	NA			
746691	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	ATTAAAGCTAATTGAATA GGTAAAATCAGGG	NA			
746758	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	TCATAGAGTGGGTAAGAA AACATAAGCAG	*Vibrio alginolyticus* NBRC 15630 = ATCC 17749 chromosome 1	68%	100%	0.19
746823	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	TTAACAATTATGCAAACTT AAATAACTTAAA	NA			
746890	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	TAGCAAAAATTTCAAACA CAAAAATGTTT	*Halobacillus massiliensis* strain Marseille-P3554	72%	100%	0.048
746955	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	AAAGGCACAGTGGTACAA ACTATGAGAGA	*Flavobacterium ummariense* strain DS-12	62%	100%	3.0
747020	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	CTGCACTTTTTACTATTGG AACTGCATTGC	*Paraliobacillus ryukyuensis* strain Marseille-P3391	70%	100%	0.053
747086	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	ATCCTATGAGAAGTGGAA TAGCTGGAATGT	*Ileibacterium valens* strain NYU-BL-A3 NODE_4_length_46638_cov_242.499_ID_7	63%	100%	0.83
747152	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	ACTATATAAATAAAATTCT CTATCTCCATA	*Halobacillus karajensis* strain DSM 14948	70%	100%	0.053
747218	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	TCGTTTAATATTGTATTTT AAATTTCTATC	*Cetobacterium ceti* strain ATCC 700028	90%	100%	0.013
747284	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	CACATAATCCTTTTATATA TTCAACATCTT	*Clostridium botulinum* B str. Eklund 17B (NRP)	100%	96.15%	0.013
747350	ATTATATCAAATGGGGAT TTGAGAGTAGATTAAAAC	CTACTTTTTTAAAACAATT AGAGAGTAGTT	*Carnobacterium jeotgali* MS3 BP18DRAFT_scf7180000000062_quiver.2_C	73%	100%	0.053
747416	ATTATATCAAATGGGGAT TTGAGAGTAGATTGTAAA					

CRISPR 2

Regions	Direct Repeats	Spacer	Cover %	Identity %	e-value
1870865	TTCTCTTTTTTGGCTGTTCGTTTTCT	CGATATTGAAACTAAAGTTTCAAATCTCG TGCTCACAGATTAGCTTCTTATGAATTC	NA		
1870948	TTCTCTTTTTTTGCTGTTCGTTTTCT				

NA: Not applicable

Nucleotide BLAST results from CRISPR direct repeats

CRISPR	DR Consensus sequence	*Arcobacter* spices	Cover %	Identity %	e-value
1	ATTATATCAAATGGG GATTTGAGAGTAGAT TAAAAC	*A. thereius* LMG 24486	100	100	8 X 10^9
		A. pacificus LMG 26638	100	100	8 X 10^9
2	TTCTCTTTTTTGGCTG TTCGTTTTCT	*A. porcinus* CCUG 56899	100	96.15	0.067
		A. cryaerophilus D2610	100	96.15	0.067
		A. skirrowii CCUG 10374 c	88	95.65	4.1

Appendix: 4

Genes present in *C. coli* S9 but absent in *C. coli* BFR-CA-9557 studied

Gene	Product
FD987_00185	hypothetical protein
FD987_00300	hypothetical protein
FD987_00305	hypothetical protein
FD987_00315	peptidase C39
FD987_00320	MobC family plasmid mobilization relaxosome protein
FD987_00325	mobilization protein
FD987_00330	replication initiation protein
FD987_00335	hypothetical protein
FD987_00340	bacteriocin
FD987_00350	hypothetical protein
FD987_00355	S24 family peptidase
FD987_00405	hypothetical protein
FD987_00430	deoxyribonuclease
FD987_00520	phage baseplate assembly protein V
FD987_00525	hypothetical protein
FD987_00625	hypothetical protein
FD987_01865	OXA-61 family class D beta-lactamase OXA-489
FD987_02470	hypothetical protein
FD987_02475	hypothetical protein
FD987_03865	hypothetical protein
FD987_03870	hypothetical protein
FD987_04185	hypothetical protein
FD987_05345	restriction endonuclease
FD987_05595	hypothetical protein
FD987_05795	glycosyltransferase family 2 protein
rfbH	lipopolysaccharide biosynthesis protein RfbH
FD987_05805	NAD-dependent epimerase/dehydratase family protein
FD987_05810	thiamine pyrophosphate-binding protein
rfbG	CDP-glucose 4,6-dehydratase
rfbF	glucose-1-phosphate cytidylyltransferase
FD987_05825	alpha-1,2-fucosyltransferase
FD987_05830	hypothetical protein
FD987_05835	DegT/DnrJ/EryC1/StrS family aminotransferase

Appendix 4 continued

Gene	Product
FD987_05840	NAD-dependent epimerase/dehydratase family protein
gmd	GDP-mannose 4,6-dehydratase
FD987_05850	mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase
FD987_05855	glycosyltransferase family 2 protein
waaF	lipopolysaccharide heptosyltransferase II
FD987_05865	glycosyltransferase family 25 protein
FD987_06690	class I SAM-dependent methyltransferase
FD987_06695	aminoglycoside N(3)-acetyltransferase
FD987_06700	acyl carrier protein
FD987_06705	hypothetical protein
FD987_06750	GNAT family N-acetyltransferase
FD987_06755	formyl transferase
FD987_06770	3-oxoacyl-ACP synthase
FD987_06910	DUF2920 family protein
FD987_07265	haloacid dehalogenase
FD987_07270	3-hydroxy-3-methylglutaryl-CoA lyase
FD987_07275	3-deoxy-manno-octulosonate cytidylyltransferase
FD987_07280	alpha-2,3-sialyltransferase
FD987_07285	hypothetical protein
FD987_07305	hypothetical protein
FD987_07310	glycosyltransferase
FD987_07325	polyhydroxyalkanoate biosynthesis repressor PhaR
neuC	UDP-N-acetylglucosamine 2-epimerase (hydrolyzing)
FD987_07335	acylneuraminate cytidylyltransferase family protein
FD987_07340	hypothetical protein
glf	UDP-galactopyranose mutase
galE	UDP-glucose 4-epimerase GalE
FD987_07355	UDP-glucose/GDP-mannose dehydrogenase family protein
FD987_07360	SDR family oxidoreductase
FD987_07365	glycosyltransferase family 2 protein
FD987_07760	GyrI-like domain-containing protein
FD987_07915	RloC protein
FD987_07920	restriction endonuclease subunit S
FD987_08270	DUF262 domain-containing protein
FD987_08275	hypothetical protein
FD987_08365	hypothetical protein

Appendix 5:

Genes absent in *C. coli S9* but present in *C. coli BFR-CA-9557*

Gene	Product
AB430_00025	hypothetical protein
AB430_00055	general secretion pathway protein GspF
AB430_00060	general secretion pathway protein GspE
AB430_00065	transformation system protein
AB430_00070	transformation system protein
AB430_00075	general secretion pathway protein GspD
AB430_00080	transformation system protein
AB430_00085	pyruvate-flavodoxin oxidoreductase
AB430_00090	hydrolase
AB430_00095	fibronectin-binding protein
AB430_00470	hypothetical protein
AB430_00475	hypothetical protein
AB430_00480	hypothetical protein
AB430_00545	chemotaxis protein
AB430_00710	transposase
AB430_00715	hypothetical protein
AB430_00720	adenine specific DNA methyltransferase
AB430_01465	hypothetical protein
AB430_01470	restriction endonuclease
AB430_02135	hypothetical protein
AB430_02140	hypothetical protein
AB430_02235	mcrBC 5-methylcytosine restriction system component
AB430_02895	DNA methylase N-4
AB430_02900	hypothetical protein
AB430_02970	hypothetical protein
AB430_03015	hypothetical protein
AB430_03020	hypothetical protein
AB430_03025	XRE family transcriptional regulator
AB430_03630	membrane protein
AB430_03635	hypothetical protein
AB430_03640	membrane protein
AB430_03645	ABC transporter ATP-binding protein
AB430_05350	hypothetical protein
AB430_05355	hypothetical protein

Appendix 5 continued

Gene	Product
AB430_05605	hypothetical protein
AB430_06540	restriction endonuclease
AB430_06790	hypothetical protein
AB430_06990	hypothetical protein
AB430_06995	asparagine synthase
AB430_07000	glycerol-3-phosphate cytidylyltransferase
AB430_07015	hypothetical protein
AB430_07455	hypothetical protein
AB430_07460	transcriptional regulator
AB430_07465	hypothetical protein
AB430_07515	hypothetical protein
AB430_07520	sugar-phosphate nucleotidyltransferase
AB430_07425	cytochrome C553
AB430_07650	baseplate assembly protein
AB430_07655	membrane protein
AB430_07755	hypothetical protein
AB430_08165	acetyltransferase
AB430_08170	acyl carrier protein
AB430_08175	SAM-dependent methyltransferase
AB430_08255	PseD protein
AB430_08270	transferase
AB430_08275	3-hydroxyacyl-CoA dehydrogenase
AB430_08280	hypothetical protein
AB430_08285	N-acetyl sugar amidotransferase
AB430_08290	methyltransferase
AB430_08735	adenylylsulfate kinase
AB430_08740	transporter
AB430_08745	sulfate adenylyltransferase
AB430_08750	sulfate adenylyltransferase subunit 2
AB430_08755	3'-5'-bisphosphate nucleotidase
AB430_08765	D-glycero-D-manno-heptose1-phosphate guanosyltransferase
AB430_08770	phosphoheptose isomerase
AB430_08775	dehydrogenase
AB430_08780	GDP-mannose 4,6-dehydratase
AB430_08785	pyridoxamine 5-phosphate oxidase
AB430_08790	GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase
AB430_08795	dTDP-4-dehydrorhamnose 3,5-epimerase
AB430_08805	hypothetical protein
AB430_08810	capsular biosynthesis protein
AB430_08995	30S ribosomal protein S9

Appendix 6:

A total of 182 genes were identified to present in *C. hyointestinalis* S12 and absent in *C. hyointestinalis* LMG 9260. Which encode 52 hypothetical proteins

Gene	Product
FFA43_00110	UDP-N-acetylglucosamine 4,6-dehydratase
FFA43_00115	LegC family aminotransferase
FFA43_00120	acetyltransferase
FFA43_00125	methionyl-tRNA formyltransferase
FFA43_00130	acetyltransferase
asnB	asparagine synthase (glutamine-hydrolyzing)
neuB	N-acetylneuraminate synthase
FFA43_00145	ORF6N domain-containing protein
neuC	UDP-N-acetylglucosamine 2-epimerase (hydrolyzing)
FFA43_00155	methionyl-tRNA formyltransferase
FFA43_00160	MaoC family dehydratase
FFA43_00165	GNAT family N-acetyltransferase
neuC	UDP-N-acetylglucosamine 2-epimerase (hydrolyzing)
FFA43_00195	PIG-L family deacetylase
neuC	UDP-N-acetylglucosamine 2-epimerase (hydrolyzing)
FFA43_00205	class I SAM-dependent methyltransferase
FFA43_00210	N-acetyl sugar amidotransferase
FFA43_00215	CBS domain-containing protein
FFA43_00220	acylneuraminate cytidylyltransferase family protein
FFA43_00225	hypothetical protein
FFA43_00230	acylneuraminate cytidylyltransferase family protein
FFA43_00235	glutamate-1-semialdehyde 2,1-aminomutase
FFA43_00240	serine acetyltransferase
FFA43_00245	flagellin modification protein PseA
FFA43_00250	SDR family oxidoreductase
FFA43_00255	ABC transporter ATP-binding protein
FFA43_00260	hypothetical protein
FFA43_00265	hypothetical protein
FFA43_00270	hypothetical protein
FFA43_00275	hypothetical protein
FFA43_00280	class I SAM-dependent methyltransferase
asnB	asparagine synthase (glutamine-hydrolyzing)
FFA43_00290	glycosyltransferase family 4 protein
FFA43_00295	hypothetical protein
FFA43_00300	peptidoglycan bridge formation glycyltransferase FemA/FemB family protein
FFA43_00305	hypothetical protein
FFA43_00310	glycosyltransferase family 4 protein
FFA43_00315	hypothetical protein
FFA43_00320	glycosyltransferase family 2 protein
FFA43_00325	class I SAM-dependent methyltransferase

Appendix 6 continued

Gene	Product
FFA43_00330	GtrA family protein
asnB	asparagine synthase (glutamine-hydrolyzing)
FFA43_00340	zinc-binding dehydrogenase
FFA43_00345	hypothetical protein
FFA43_00350	glycosyltransferase family 4 protein
FFA43_00355	UDP-N-acetylglucosamine 2-epimerase (non-hydrolyzing)
wecC	UDP-N-acetyl-D-mannosamine dehydrogenase
cdtB	cytolethal distending toxin nuclease subunit Ch-CdtB
cdtC	cytolethal distending toxin subunit Ch-CdtC
FFA43_00410	prepilin-type N-terminal cleavage/methylation domain-containing protein
FFA43_00600	formyl transferase
FFA43_00910	molybdopterin molybdotransferase MoeA
FFA43_00915	DUF2156 domain-containing protein
FFA43_00920	GNAT family N-acetyltransferase
FFA43_01215	hypothetical protein
FFA43_01220	hypothetical protein
FFA43_01225	hypothetical protein
FFA43_01230	DDE-type integrase/transposase/recombinase
FFA43_01235	bacteriocin
FFA43_01240	hypothetical protein
FFA43_01245	hypothetical protein
FFA43_01250	host-nuclease inhibitor protein Gam
FFA43_01255	hypothetical protein
FFA43_01260	hypothetical protein
FFA43_01265	DUF1018 domain-containing protein
FFA43_01270	hypothetical protein
FFA43_01275	hypothetical protein
FFA43_01280	phage virion morphogenesis protein
FFA43_01285	phage head morphogenesis protein
FFA43_01290	DUF935 family protein
FFA43_01295	hypothetical protein
FFA43_01300	DUF1804 family protein
FFA43_01305	DUF1320 domain-containing protein
FFA43_01310	hypothetical protein
FFA43_01315	hypothetical protein
FFA43_01320	hypothetical protein
FFA43_01325	hypothetical protein
FFA43_01330	lysozyme
FFA43_01335	hypothetical protein
FFA43_01340	hypothetical protein

Appendix 6 continued

Gene	Product
FFA43_01345	hypothetical protein
FFA43_01350	phage baseplate assembly protein V
FFA43_01355	baseplate assembly protein
FFA43_01360	baseplate assembly protein
FFA43_01365	phage tail protein I
FFA43_01370	hypothetical protein
FFA43_01375	hypothetical protein
FFA43_01380	hypothetical protein
FFA43_01385	DUF4376 domain-containing protein
FFA43_01390	DUF1353 domain-containing protein
FFA43_01395	phage tail protein
FFA43_01400	phage tail protein
FFA43_01405	phage tail assembly protein
FFA43_01410	hypothetical protein
FFA43_01415	phage tail tape measure protein
FFA43_01420	phage tail protein
FFA43_01425	phage tail protein
FFA43_01430	phage tail protein
FFA43_01435	hypothetical protein
FFA43_01440	HNH endonuclease
FFA43_01445	hypothetical protein
FFA43_01925	c-type cytochrome
FFA43_01940	glycosyltransferase family 4 protein
FFA43_01945	glycosyltransferase family 2 protein
FFA43_01950	polysaccharide deacetylase family protein
FFA43_01955	glycosyltransferase
FFA43_01960	glycosyltransferase family 1 protein
FFA43_01965	glycosyltransferase
FFA43_01970	polysaccharide deacetylase family protein
FFA43_01975	O-antigen ligase family protein
FFA43_01980	glycosyltransferase family 4 protein
FFA43_01990	lysophospholipid acyltransferase family protein
FFA43_02275	HAMP domain-containing histidine kinase
FFA43_02280	response regulator transcription factor
FFA43_02300	aryl-sulfate sulfotransferase
FFA43_02660	prepilin-type N-terminal cleavage/methylation domain-containing protein
FFA43_03390	autotransporter outer membrane beta-barrel domain-containing protein
FFA43_04145	hypothetical protein
FFA43_04420	AEC family transporter
FFA43_04570	hypothetical protein

Appendix 6 continued

Gene	Product
FFA43_04575	GGDEF domain-containing protein
FFA43_04740	hypothetical protein
FFA43_04745	hypothetical protein
hypA	hydrogenase maturation nickel metallochaperone HypA
hypE	hydrogenase expression/formation protein HypE
hypD	hydrogenase formation protein HypD
hypB	hydrogenase nickel incorporation protein HypB
FFA43_05185	beta-ketoacyl-ACP synthase
fabG	3-oxoacyl-ACP reductase FabG
FFA43_05195	thioester dehydrase
FFA43_05200	beta-ACP synthase
FFA43_05205	hypothetical protein
FFA43_05210	NAD(P)/FAD-dependent oxidoreductase
FFA43_05215	4-phosphopantetheinyl transferase family protein
FFA43_05220	hypothetical protein
FFA43_05225	outer membrane lipoprotein carrier protein LolA
FFA43_05230	acyl-CoA thioesterase
FFA43_05575	Crp/Fnr family transcriptional regulator
FFA43_05580	DUF2892 domain-containing protein
FFA43_06080	helix-turn-helix domain-containing protein
FFA43_06085	ATP-binding protein
FFA43_06095	hypothetical protein
FFA43_06170	hypothetical protein
FFA43_06270	hypothetical protein
FFA43_06425	hypothetical protein
FFA43_06465	hypothetical protein
FFA43_06470	hypothetical protein
FFA43_06475	hypothetical protein
FFA43_06480	hypothetical protein
FFA43_06485	hypothetical protein
FFA43_06490	conjugal transfer protein TraG
FFA43_06495	transposase
FFA43_06810	site-specific DNA-methyltransferase
FFA43_06955	DASS family sodium-coupled anion symporter
tcuB	tricarballylate utilization 4Fe-4S protein TcuB
tcuA	FAD-dependent tricarballylate dehydrogenase TcuA
FFA43_06970	hypothetical protein
FFA43_06975	GntR family transcriptional regulator
FFA43_06980	cupin domain-containing protein
FFA43_06985	winged helix-turn-helix transcriptional regulator

Appendix 6 continued

Gene	Product
FFA43_07090	major outer membrane protein
FFA43_07095	major outer membrane protein
FFA43_07335	DUF302 domain-containing protein
nhaA	Na+/H+ antiporter NhaA
FFA43_07505	MFS transporter
FFA43_07510	sel1 repeat family protein
FFA43_07555	3-oxoacyl-ACP synthase
pseF	pseudaminic acid cytidylyltransferase
FFA43_07970	TIM barrel protein
FFA43_07975	hypothetical protein
FFA43_07980	ATP-grasp domain-containing protein
FFA43_07985	class I SAM-dependent methyltransferase
FFA43_07990	class I SAM-dependent methyltransferase
FFA43_08145	GNAT family N-acetyltransferase
FFA43_08155	hypothetical protein
FFA43_08165	methyltransferase domain-containing protein
FFA43_08235	sel1 repeat family protein
FFA43_08315	type II toxin-antitoxin system PemK/MazF family toxin
FFA43_08320	hypothetical protein
FFA43_08325	restriction endonuclease subunit S
FFA43_08805	hypothetical protein
FFA43_08930	HrgA protein

Appendix 7:

Genes absent in *C. hyointestinalis* S12 but present in *C. hyointestinalis* LMG 9260 examined

Gene	Product
CHH_0008	IS605/IS607 family integrase/resolvase
CHH_0009	IS605/IS607 family transposase
CHH_0022	iron-containing alcohol dehydrogenase
CHH_0023	putative serine acetyltransferase
CHH_0024	methyltransferase
CHH_0025	aminotransferase, DegT/DnrJ/EryC1/StrS family
CHH_0026	PseG family hydrolase
CHH_0027	cytidylyltransferase, putative
CHH_0028	N-acetylneuraminate synthase
CHH_0029	putative Zn-peptidase, M28 family (DUF2172 domain)
CHH_0030	hypothetical protein
CHH_0031	putative tungsten cofactor oxidoreducase radical SAM maturase
CHH_0032	hypothetical protein
CHH_0033	methyltransferase
CHH_0034	hypothetical protein
CHH_0035	polysaccharide biosynthesis protein, putative
CHH_0036	aldolase/citrate lyase family protein
CHH_0037	3-deoxy-D-manno-octulosonate cytidylyltransferase
CHH_0038	methyltransferase FkbM family protein, putative
CHH_0039	maltose O-acyltransferase (MAT)-like acetyltransferase
CHH_0040	nucleoside-diphosphate-sugar epimerase
CHH_0041	maltose O-acyltransferase (MAT)-like acetyltransferase
CHH_0042	xenobiotic acyltransferase (XAT) family acetyltransferase
CHH_0043	SAM-dependent methyltransferase
CHH_0044	dehydrogenase, putative
CHH_0045	nucleoside-diphosphate-sugar epimerase
CHH_0046	cyclase family protein
CHH_0047	putative phosphatase, HAD family protein
CHH_0048	putative membrane protein
CHH_0049	glucose-1-phosphate cytidylyltransferase, putative
CHH_0050	CDP-glucose 4,6-dehydratase, putative
CHH_0051	dTDP-4-dehydrorhamnose 3,5-epimerase
CHH_0052	nucleoside-diphosphate-sugar epimerase
CHH_0053	glycosyltransferase, family 2
CHH_0054	SAM-dependent methyltransferase
CHH_0055	aminotransferase, DegT/DnrJ/EryC1/StrS family
CHH_0056	FemAB family protein
CHH_0057	polysaccharide deacetylase
CHH_0058	glycosyltransferase, family 2

Appendix 7 continued

Gene	Product
CHH_0059	glycosyltransferase, family 1
CHH_0060	hypothetical protein
CHH_0061	hypothetical protein
CHH_0062	hypothetical protein
CHH_0063	sugar transferase
CHH_0069	putative type II secretion system protein
CHH_0072	[FeFe] hydrogenase H-cluster maturation GTPase HydF
CHH_0073	[FeFe] hydrogenase H-cluster radical SAM maturase HydE
CHH_0074	[FeFe] hydrogenase H-cluster radical SAM maturase HydE
CHH_0075	hydrogenase, cytochrome b subunit
CHH_0076	[FeFe] hydrogenase, small subunit
CHH_0077	[FeFe] hydrogenase, large subunit
CHH_0174	acetyltransferase
CHH_0175	putative DUF2156 domain protein
moeA2	molybdopterin molybdenumtransferase
CHH_0177	putative selenium metabolism protein, YedE family
CHH_0211	hypothetical protein
CHH_0212	putative transcriptional regulator, XRE family (peptidase S24 LexA-like domain)
CHH_0214	hypothetical protein
CHH_0216	hypothetical protein
CHH_0217	hypothetical protein
CHH_0218	putative replication protein
CHH_0219	hypothetical protein
gmhB	D,D-heptose 1,7-bisphosphate phosphatase
waaD	ADP-L-glycero-D-mannoheptose-6-epimerase
waaE	D,D-heptose 1-phosphate adenosyltransferase / 7-phosphate kinase
gmhA	sedoheptulose 7-phosphate isomerase
waaF	heptosyltransferase II
CHH_0352	glycosyltransferase, family 1
CHH_0353	putative O-antigen ligase
CHH_0354	glycosyltransferase, family 9
CHH_0355	glycosyltransferase, family 1
CHH_0356	glycosyltransferase, family 9
CHH_0357	glycosyltransferase, family 1
CHH_0358	glycosyltransferase, family 9
CHH_0359	putative polysaccharide deacetylase
waaQ	heptosyltransferase III
CHH_0361	glycosyltransferase, family 2
waaM	lipid A biosynthesis lauroyl acyltransferase
waaC	heptosyltransferase I
ugd	UDP-glucose 6-dehydrogenase

Appendix 7 continued

Gene	Product
CHH_0422	IS605/IS607 family integrase/resolvase
CHH_0423	IS605/IS607 family transposase
CHH_0506	putative type II secretion system protein
CHH_0592	membrane-associated zinc metalloprotease, S2P/M50 family
CHH_0736	putative copper homeostasis protein, NlpE family
CHH_0810	hypothetical protein
CHH_0811	putative AAA domain protein
CHH_0823	toxin-antitoxin system, antitoxin component
CHH_0824	toxin-antitoxin system, toxin component, YafQ family
CHH_0825	putative restriction endonuclease
CHH_0826	hypothetical protein
CHH_0828	putative membrane protein
CHH_0829	hypothetical protein
CHH_0830	putative membrane protein
CHH_0831	zonula occludens toxin (Zot) family protein
CHH_0832	type II and III secretion system protein
CHH_0833	putative phage replication initiation protein
CHH_0833	putative phage replication initiation protein
CHH_0834	site-specific recombinase, phage integrase family
CHH_0837	outer membrane beta-barrel domain protein
CHH_0941	DASS family sodium/dicarboxylate symporter
CHH_0942	MatE efflux family protein
hypA	hydrogenase nickel insertion protein HypA
hypE	hydrogenase expression/formation protein HypE
hypD	hydrogenase expression/formation protein HypD
hypC	hydrogenase assembly chaperone HypC
CHH_0982	membrane protein, TerC family
CHH_0983	TerZ/TerD family protein
CHH_0984	TerZ/TerD family protein
CHH_0985	transcriptional regulator, XRE family
CHH_1055	type III restriction/modification system, mod subunit
CHH_1056	type III restriction/modification system, res subunit
CHH_1107	tellurite-resistance/dicarboxylate transporter (TDT) family protein
CHH_1108	cytochrome b
CHH_1109	dehydrogenase/reductase, iron-sulfur cluster subunit
CHH_1110	transcriptional regulator, Crp family
CHH_1111	putative oxidoreductase/sulfur reductase
CHH_1112	putative DUF2892 domain protein
CHH_1113	putative DUF2892 domain protein
CHH_1226	hypothetical protein

Appendix 7 continued

Gene	Product
CHH_1228	hypothetical protein
CHH_1231	putative membrane protein
CHH_1232	hypothetical protein
CHH_1233	hypothetical protein
CHH_1234	hypothetical protein
CHH_1274	putative membrane protein
CHH_1275	hypothetical protein
CHH_1276	hypothetical protein
CHH_1296	YopX family protein
CHH_1297	putative membrane-bound metal-dependent hydrolase (DUF457 domain)
CHH_1298	hypothetical protein
CHH_1299	putative membrane protein
CHH_1303	transcriptional regulator, AraC family
CHH_1307	IS605/IS607 family transposase
CHH_1308	IS605/IS607 family integrase/resolvase
fla1	flagellin
CHH_1466	autotransporter domain protein
CHH_1518	putative type II secretion system protein
CHH_1539	IS605/IS607 family transposase
CHH_1540	IS605/IS607 family integrase/resolvase
pseF	CMP-pseudaminic acid synthetase
CHH_1589	ATP-grasp domain-containing protein
CHH_1590	ATP-grasp domain-containing protein
CHH_1591	adenylylsulfate kinase
CHH_1592	phosphonopyruvate decarboxylase, putative
CHH_1593	phosphoenolpyruvate phosphomutase, putative
CHH_1594	radical SAM superfamily enzyme,MoaA/NifB/PqqE/SkfB family (SPASM domain)
CHH_1595	putative CDP-alcohol phosphatidyltransferase
pseG	UDP-2,4-diacetamido-2,4,6-trideoxy-beta-L-altropyranosyl transferase
CHH_1597	WbqC family protein
CHH_1598	SAM-dependent methyltransferase
CHH_1599	aminotransferase, DegT/DnrJ/EryC1/StrS family
CHH_1646	hypothetical protein
hsdS	type I restriction/modification system, S subunit
CHH_1681	hypothetical protein
CHH_1682	putative OmpA/MotB domain protein
CHH_1683	putative membrane protein
CHH_1764	hypothetical protein
CHH_1768	hypothetical protein
CHH_1769	ATPase, AAA family

Appendix 7 continued

Gene	Product
CHH_1770	relaxase
CHH_1771	putative toxin-antitoxin system, toxin component, RelE/ParE family
CHH_1772	putative toxin-antitoxin system, toxin component, RelE/ParE family
CHH_1773	hypothetical protein
CHH_1774	hypothetical protein
CHH_1775	site-specific recombinase, phage integrase family
CHH_1797	Mrr restriction system protein

Appendix 8:

Nucleotide BLAST results from CRISPR direct repeats consensus sequence in *Campylobacter hyointestinalis* S12

CRISPR	DR Consensus sequence	*Campylobacter* spices	Cover %	Identity %	e-value
1	TTTAGATTTAGTTCTTTAGTACGAATTTATTCGTA	*Campylobacter fetus* subsp. *testudinum* 772	100	100	2e-08
		Campylobacter fetus subsp. *testudinum* Sp3	100	100	2e-08
		Campylobacter fetus subsp. *testudinum* pet-3	100	100	2e-08
		Campylobacter hyointestinalis MGYG-HGUT-02307	100	100	2e-06
2 and 3	GTTTCAAATCCTAAAAGGATAAATTTATAC	*Campylobacter iguaniorum* 2463D	100	96.67	5e-04
		Campylobacter iguaniorum 1485E	88	96.67	5e-04

Appendix 9:

Nucleotide BLAST results from CRISPR Number 1 spacers in *C. hyointestinalis* S12

CRISPAR 1						
Regions	**Direct Repeats**	**Spacer**	**Best Match Description**	**Cover %**	**Identity %**	**Identity %**
99674	TTTAGATTTAGTTCTTTAGTACGAATTTATTCGTA	TAATTTAAAAGAATATAAATTTCTAAAGCGTGAGCAAA	*Campylobacter fetus* subsp. *testudinum* 772	100	100	4e-16
99759	TTAGTATTTAGTTCTTTAGTACGAATTTATTCGTA	AGCGAACGGACT				

Appendix 10:

Nucleotide BLAST results from CRISPR Number 2 spacers in *C. hyointestinalis* S12

CRISPAR 2						
Regions	**Direct Repeats**	**Spacer**	**Best Match Description**	**Cove r %**	**Identity %**	**Identity %**
1171550	GTTTAAAATCCT AAAAGGATAAA TTTATAC	AACATTACACCAGCG ACATCAGTTGCAAGA TCAAATCT	NA			
1171618	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GTTTATGATCTCTAT CGTATCGTTCTCATC ACGGA	NA			
1171683	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GCTTGGCTTGACGCT CAAGAGCAAAAAGCA AAAAA	NA			
1171748	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AGCAACTTTTCAGAT TAATACCATAGTGAA ATT	NA			
1171811	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTCAAGCCAAAGTAT ATCAAGGGCGAACCT CTGCA	*Campylobacter hyointestinalis* subsp. *hyointestinalis* LMG 9260	88	100	5e-07
1171876	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCCAAACCCAGTGAC CATTTCAAATACACC TATAC	NA			
1171941	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AAAAGCCTTGGAAAA CACTACAGGCTCGAC GTGATTTTT	NA			
1172010	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TCAAGGCATCAAATG AACAATAGAGCAGCA AGAC	NA			
1172074	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TCTGTTTATTTATCGT CTGATGCTGATAGAT AAAA	NA			
1172139	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCCTTTCTGTTTGCG ATATCTGCGCTAAAT TCAGGTA	NA			
1172206	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TGAAATAGCTAAGTT CGACTAGATTATTAT TTATTC	NA			
1172272	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AAAACCAAACCACGT TTTTAAGGCTATTTAA ACA	*Campylobacter hyointestinalis* subsp. *hyointestinalis* LMG 9260	100	97.06	4e-07

Appendix 10 continued

1172336	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTCAAAGGGGAAGG TAAAATGGTTTTTTG ATTATC	NA			
1172401	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CATAATCACAAAAAT TCGTAGAGAATTTGG CGATGAAA	NA			
1172469	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCTAGGCACCCCTCA AACAGAGGAGAGTAT CTATAA	NA			
1172535	GTTTCAAATCCT AAAAGGATAAA TTTATAC	ATGCTCAAACTCAGC AACAAAGCAATTATA GCTA	*Campylobacter hyointestinalis* subsp. *hyointestinalis* LMG 9260	100	100	9e-09
1172599	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GTTATCGGAGAGAAT GAATTTGCAAAGGTA ATATA	NA			
1172664	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CGTAGTAGTCTGCTT TGTCGATGAAAAGGC TTAAAA	NA			
1172730	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CTATTATTTTTAGCCC TTTTGCTTTATTATCA TCA	NA			
1172795	GTTTCAAATCCT AAAAGGATAAA TTTATAC	ATTGCAAGATTTTTA CCTATGCCTTTCTCA TTTGC	NA			
1172860	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTGATTATAAACTTA GTTGCAATTAGTTCT TATT	NA			
1172924	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CGCCGCTTTTAAATC AGATTGATAATGTCT TTTA	NA			
1172988	GTTTCAAATCCT AAAAGGATAAA TTTATAC					
1173054	GTTTCAAATCCT AAAAGGATAAA TTTATAC					
1173120	GTTTCAAATCCT AAAAGGATAAA TTTATAC					

Appendix 11:

Nucleotide BLAST results from CRISPR Number 3 spacers in *C. hyointestinalis* S12

CRISPAR 3						
Regions	**Direct Repeats**	**Spacer**	**Best Match Description**	**Cover %**	**Identity %**	**e-value**
1190837	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GTTTATGAAAATA AAATGTAAAGAAG TGTAAAGT	NA			
1190901	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CTGAAATTGCCCC TACTAAGAGTTGC TATAAGACTAA	NA			
1190968	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TACGGCGAGTTT GTAGATGGCTCG TCAAACCTACTGC	NA			
1191035	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CATATACAAATTT ACCAAACAATGAA AAACATAAAA	NA			
1191101	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TGGAAACGCAAT AGCAAGTAATTCA CTAGCAATCAC	NA			
1191167	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AAAATGATAGAGA TAATAGATGAATA TAACCTAT	NA			
1191231	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTAAATTTAATGA TAAAGGAAATACC GATGAAAAAGA	*Campylobacter hyointestinalis* subsp. *lawsonii* CCUG 27631	94	100	4e-09
1191298	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CGAAGTTTTTAAA AATCTCAACCTAG AAGCTTTCAG	NA			
1191364	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTATTCTTTCAAT CTCCGGCTTATTT TTTTTCATTTC	NA			
1191431	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCGTTGCCCCAAA ACTAAAAGACAGT TTTTAAAAAAAC	NA			

Appendix 11 continued

1191499	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CATATTACCACTA TTATCTACTATGT ACCACATAT	NA			
1191564	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCAACAGTACCAA GTGATTTAGACAT AGCGGTATTGAA	NA			
1191632	GTTTCAAATCCT AAAAGGATAAA TTTATAC	ATACTAGTCATGA TAAGAACTCCGAA GAAGAGTT	NA			
1191696	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTATGTCAGCAGT GCCGACGCTCTC ATTGGCGCTG	NA			
1191761	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTTGGCAAATAGT TAAGTAAATAAAA CCAAATCAAA	NA			
1191827	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GACAACTTGGAG CTTAGAAAACTTC ATAAAAACCA	NA			
1191892	GTTTCAAATCCT AAAAGGATAAA TTTATAC	ATCACGCTTGGG CTAGTTATCGTAG TAGCCTCAGCTA	NA			
1191959	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AGAGTTCATAGAT ATGACTTTTCGAG AGTATGATAA	NA			
1192025	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AATGGCAAAGGC TATCTTGGAGTTA CAAAAAAAC	NA			
1192089	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CAAGCTGGAAATT CGCCCTAATTTGT TTCTCCCTGAAA	*Campylobacter hyointestinalis* subsp. *hyointestinalis* LMG 9260	100	100	9e-11
1192157	GTTTCAAATCCT AAAAGGATAAA TTTATAC	ATATATAGTTTGG GATAAAAAAGAA GATGGCAAACC	NA			
1192223	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TCGTTGGCTTTCT GTTCATCTGATAA ATCGTAAGTCCG	NA			

Appendix 11 continued

1192291	GTTTCAAATCCTAAAAGGATAAATTTATAC	TCAATATCTTTTATCTTGATTTCAAGTGGTTTTA	NA			
1192355	GTTTCAAATCCTAAAAGGATAAATTTATAC	ACTATAGTTTTGCTCTGCAACACCTTTGGATACA	NA			
1192419	GTTTCAAATCCTAAAAGGATAAATTTATAC	CATTACAAAACTTACTCTAAAAGCAAAATATATGC	NA			
1192484	GTTTCAAATCCTAAAAGGATAAATTTATAC	AAAACCAAACCACGTTTTTAAGGCTATTTAAACA	*Campylobacter hyointestinalis* subsp. *hyointestinalis* LMG 9260	100	97.06	4e-07
1192548	GTTTCAAATCCTAAAAGGATAAATTTATAC	TGGTCAGATATCGACTTTGCAAGGAACTTGATAAATA	NA			
1192615	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCTTTCTGTTTGCGATATCTGCGCTAAATTCAGGTA	NA			
1192681	GTTTCAAATCCTAAAAGGATAAATTTATAC	CCCTGAGTATTGGCTAGTTTCAGGGAGAAAAATTCA	NA			
1192747	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGAGCGAAAAAGGGCGTATGTTATTGCCTACACCGA	NA			
1192813	GTTTCAAATCCTAAAAGGATAAATTTATAC	CTTTAAAGCCTTCATTTTCTTTTATGCTATCTAT	NA			
1192877	GTTTCAAATCCTAAAAGGATAAATTTATAC	CGCTTCCTTATACGCAAGTTCAAACTCAAAACTTA	*Campylobacter hyointestinalis* subsp. *lawsonii* CCUG 27631	100	100	3e-09

Appendix 11 continued

1192942	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CTACTCATCTCAAA TGCAGTAGCACCA GTAGCAAG	NA
1193007	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTAAAAAACAAGCT CCACAGCACTACAA AGAGAG	
1193071	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AAGGAGATGGAAA ATGAACACGCAAAC TATGCAAAGT	
1193138	GTTTCAAATCCT AAAAGGATAAA TTTATAC	GCGAACAGAGCGA AGAAAGTAGTGAAA GTGCAAATA	
1193204	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AGCCTTGGAAAACA CTACAGGCTAGAC GTGACTTTC	
1193270	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AAATCTTCAGTCGG AAAAGCTTCTTTAA GCAACA	
1193334	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AGTTTGGCAATGCA GGTAGAGTTTTAGC TATCTCTGA	
1193401	GTTTCAAATCCT AAAAGGATAAA TTTATAC	AATTTGATCCGTTG ATTGTTGAAACTAA AATCGTAAC	
1193468	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TTCTGCTTGAAAGT CAATTGTGGCAACA TTTTGGCT	
1193534	GTTTCAAATCCT AAAAGGATAAA TTTATAC	CCGACTTATAGCTA TGGAATCCTTAGAA AGTCTTG	
1193599	GTTTCAAATCCT AAAAGGATAAA TTTATAC	TAGAGTTTCAAGTT TAATACTACTATTTT TAATTTC	
1193665	GTTTCAAATCCT AAAAGGATAAA TTTATAC		

Appendix 12:

Nucleotide BLAST X results from CAS-Type IB genes sequences in *C. hyointestinalis S12*

CAS-Type IB genes	Best Match Description	Cover %	Identity %	e-value
Cas1_0_I-II-III-V_1	CRISPR-associated endonuclease Cas1 [Campylobacter hyointestinalis]	99	100	0.0
	CRISPR-associated endonuclease Cas1 [Campylobacter hyointestinalis]	99	99.09	0.0
	CRISPR-associated endonuclease Cas1 [Campylobacter fetus]	99	98.48	0.0
Cas2_0_I-II-III_2	CRISPR-associated endonuclease Cas2 [Campylobacter fetus]	99	98.91	2e-51
	MULTISPECIES: CRISPR-associated endonuclease Cas2 [Campylobacter]	99	100	2e-51
	CRISPR-associated endonuclease Cas2 [Campylobacter fetus]	99	98.91	8e-51
Cas4_0_I-II_3	Dna2/Cas4 domain-containing protein [Campylobacter hyointestinalis]	99	100	7e-119
	Dna2/Cas4 domain-containing protein [Campylobacter hyointestinalis]	99	99.39	2e-118
	MULTISPECIES: Dna2/Cas4 domain-containing protein [Campylobacter]	99	98.79	3e-118
Cas3_0_I_4	CRISPR-associated helicase Cas3 domain-containing protein [Campylobacter fetus subsp. fetus]	99	99.31	0.0
	CRISPR-associated helicase Cas3 domain-containing protein [Campylobacter fetus subsp. fetus]	99	99.03	0.0
	CRISPR-associated helicase Cas3 domain-containing protein [Campylobacter fetus subsp. fetus]	99	99.03	0.0
Cas5_1_IB_5	CRISPR-associated protein Cas5 [Campylobacter hyointestinalis]	99	100	3e-174
	CRISPR-associated protein Cas5 [Campylobacter hyointestinalis]	99	99.58	8e-174
	CRISPR-associated protein Cas5 [Campylobacter fetus]	99	99.58	1e-173

Appendix 12 continued

Cas7_2_IB_6	type I-B CRISPR-associated protein Cas7/Csh2 [Campylobacter hyointestinalis]	99	100	0.0
	type I-B CRISPR-associated protein Cas7/Csh2 [Campylobacter hyointestinalis]	99	99.67	0.0
	type I-B CRISPR-associated protein Cas7/Csh2 [Campylobacter hyointestinalis]	99	99.02	0.0