Acanthamoeba as a model for the investigation of the molecular mechanisms of *Campylobacter jejuni* pathogenesis and survival in the environment

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Declaration

I declare that the work reported in this thesis is entirely my own and has been carried out at Kingston University, UK.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University.

Ana Vieira

Abstract

Campylobacter jejuni is a foodborne pathogen recognised as the leading cause of human bacterial gastroenteritis. Undercooked poultry products and contaminated water are considered as the most important sources of infection. Antimicrobial therapy is warranted only for immunocompromised patients and, although most people recover from this disease, others may develop rare neurodegenerative disorders such as Guillain-Barre Syndrome (GBS). The latter affects the nerves of the body leading to paralysis and requires extensive medical treatment. The wide use of antibiotics in medicine and in animal husbandry has led to an increased incidence of antibiotic resistance in *Campylobacter* over the last decade. Investigation of the molecular mechanisms of antibiotic resistance is considered important to control the spreading of resistant bacteria.

CmeABC RND-type multidrug efflux (MDR) pump and the *tetO* gene found on pTet plasmids mediate tetracycline resistance in *Campylobacter*. CmeABC MDR pump consists of three components: an outer membrane protein CmeA, an inner membrane drug transporter CmeB and a periplasmic protein CmeC.

Even though *C. jejuni* strains G1 and 11168H do not contain the pTet plasmid, the former was shown to be more resistant to tetracycline (Tet). Comparison of the genome of the G1 strain with that of the reference strain, 11168H, revealed a remarkable difference between the nucleotide sequences of their *cmeB* genes. In addition, it was observed that the transfer of the pTet plasmid from *C. jejuni* 81-176 to the G1 strain increased the level of Tet resistance above that of the former strain carrying this plasmid. This finding suggests that CmeB of strain G1 has a higher capacity to excrete this drug than its analogue in *C. jejuni* strains 81-176 and 11168H and thus, the former strain could be considered as an efflux pump variant with increased resistance to antibiotics. In this study we demonstrate that contribution of MDR pumps to antibiotic resistance might be dependent on the sequence variation of CmeB.

Although antibiotic resistance is the main function of MDR pumps, these pumps may have other physiological roles, such as in virulence. An important mechanism of bacterial pathogenesis is the survival of *Campylobacter* inside environmental hosts. As a host of pathogenic microorganisms, the protozoan *Acanthamoeba* is a good model for the investigation of bacterial survival in the environment and the molecular mechanisms of pathogenicity. The endosymbiotic relationship between this eukaryotic organism and microbial pathogens may contribute to persistence and spreading of the latter in the environment, which has significant implications for human health.

Although some studies suggest that *Acanthamoeba* supports *Campylobacter* survival in the environment, the type of interaction between these microorganisms needs to be elucidated. Also, the bacterial factors involved in this interaction remain unknown. Using a modified gentamicin protection assay it was found that *C. jejuni* 81-176 is able to survive and multiply inside this eukaryotic host. Thus, since these microorganisms can co-exist in the same environments (e.g. in poultry farms) the risk of infection with this foodborne pathogen is elevated. It is also reported that the CmeABC MDR pump is beneficial for the intracellular survival and multiplication of *C. jejuni* within *A. polyphaga.*

However, this MDR pump was found to be dispensable for *C. jejuni* biofilm formation, motility and oxidative stress. Moreover, it was observed that capsule production is also required for the interaction between *C. jejuni* and with amoebae. Due to their role in antibiotic resistance and virulence of *C. jejuni*, MDR pumps could be considered as good targets for the development of antibacterial drugs against this pathogen.

During the course of this study, a new chimeric *C. jejuni* strain was created due to horizontal gene transfer between two different strains, 81-176 and G1, which were growing together. This finding emphasises how easily *Campylobacter* can exchange its genetic material and thus adapt to the surrounding environment.

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List of Abbreviations

AGE	*Acanthamoeba* granulomatous encephalitis
AK	*Acanthamoeba* keratitis
AMR	Antimicrobial resistance
AR	Antibiotic resistance
BHI	Brain-heart infusion
BLAST	Basic local alignment search tool
bp	Base pairs
***cam*^{r}**	Chloramphenicol resistance gene
CBA	Columbia blood agar
CDC	Centres for Disease Control and Prevention
CDT	Cytholetal-distending toxin
c.f.u	Colony forming units
CL	Contact lenses
Cme	*Campylobacter* multidrug efflux
CNS	Central nervous system
CPS	Capsular polysaccharide structures
CV	Crystal violet
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleotide triphosphates
EC	Extracellular/ly
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
EPI	Efflux pump inhibitor
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and drug administration
FLA	Free-living amoebae
FQ	Fluoroquinolone
GBS	Guillan-Barré Syndrome
GFP	Green fluorescent protein
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
IC	Intracellular/ly
ICM	Intracellular multiplication
ICS	Intracellular survival
***kan*^{r}**	Kanamycin resistance gene
LB	Luria-Bertani
LOS	Lipooligosaccharide

MDR	Multidrug efflux pump
MH	Muller-Hinton
MIC	Minimal inhibitory concentration
ND	Not detected
NS	Not significant
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PYG	Peptone yeast glucose
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SOC	Super Optimal broth with Catabolite repression
t.p.	Time-point
TBE	Tris/Borate/EDTA buffer
Tet	Tetracycline
wt	Wild-type

CHAPTER 1: Introduction

1.1 The *Campylobacter* genus

Campylobacter was originally described as a member of the genus *Vibrio* as they were both curved cells with a microaerobic nature (Veron, 1973). In 1913, a microorganism responsible for abortion of pregnant ewes was discovered and designated as *Vibrio fetus*. However, the classification of this organism was deemed unsatisfactory due to the large difference in the G+C content of the DNA between this organism and that characteristic of *Vibrio* species (Veron, 1973). Therefore in 1973, Sebald and Veron proposed a new genus, *Campylobacter*, comprising Gram-negative and curved bacteria (0.2 to 0.8μm wide and 0.5 to 5μm long). These microorganisms are motile by means of a single polar flagellum, non-spore forming, microaerophillic (5-10% O_2 and 3-5% CO_2), with optimal growth between 30°C and 42°C. As opposed to the genus *Vibrio* that contains DNA with G+C content between 40 and 53%, *Campylobacter* G+C content varies between 26 and 36% (Wassenaar & Newell, 2006). These organisms are found ubiquitously in the environment and can colonise the intestine of wild birds (Dasti *et al*., 2010).

Campylobacter comprises 17 species with valid published names (Zhou *et al*., 2013) (Fig. 1.1). *Campylobacter* can colonise a diverse range of hosts, from farm livestock to humans and can adapt to various host environments (Zhou *et al*., 2013). Some members of this genus, such as *C. coli*, *C. upsaliensis* and *C. lari*, can cause human disease, but the most common is *C. jejuni* which is the leading cause of human gastroenteritis worldwide. Whilst some species, for instance *e.g. C. coli,* cause severe animal diseases, others are non-pathogenic (Zhou *et al*., 2013).

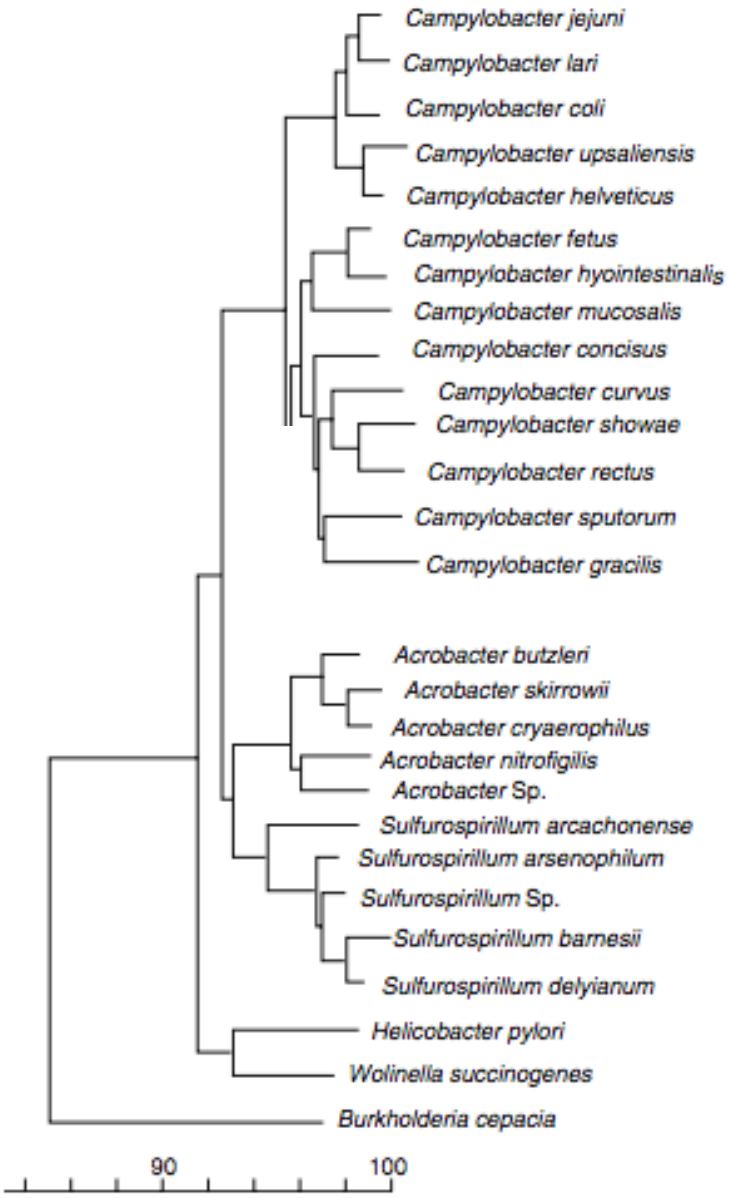


Figure 1.1. Phylogenetic tree of the family *Campylobacteraceae*. Fourteen distinct *Campylobacter* species and their closest neighbours, based on the 16S rRNA gene sequence similarity, are present in this tree. *Arcobacter* and *Sulfurospirillum* are Gram negative spiral-shaped bacteria and belonging to the Family *Campylobacteraceae*. *Burkholderia cepacia* was used as an outgroup organism (Wassenaar & Newell, 2006).

1.2. Overview of *Campylobacter jejuni*

C. jejuni is one of the most important food-borne pathogens in the world and, as mentioned above, is the leading bacterial cause of human gastroenteritis (Zhou *et al*., 2013; Havelaar *et al*., 2015). Most commonly, the disease is associated with consumption of undercooked poultry and contaminated water (Young *et al*., 2007). As a natural host and important food source of humans, the chicken is a good model for the

investigation of *Campylobacter* mechanisms of infection. *In vitro*, human intestinal epithelial cell lines are those most commonly used to examine pathogenic processes. *C. jejuni* is highly fastidious and sensitive to unfavourable growth conditions such as adverse pH or temperature (Altekruse *et al*., 1999). For its protection, when in stress conditions, *C. jejuni* is able to enter a dormant, but viable non-culturable state after transformation from a rod or spiral cell shape into a spherical or coccoid form (Fig. 1.2) (Ikeda & Karlyshev, 2012).

The first full genome sequence of *C. jejuni* (strain NCTC 11168) was published in 2000 (Parkhill *et al*., 2000). *C. jejuni* has a small genome (1.6 megabases) and displays extensive genetic variation. *Campylobacter* is naturally competent, meaning that it can take up DNA from the environment, leading to recombination between the genomes of the different species and the generation of even more genetic diversity among them (Young *et al*., 2007). Horizontal transfer may occur during host colonisation or *in vitro* growth, with both plasmids and chromosomal DNA, and may lead to the spread of new bacterial characteristics, such as antibiotic resistance, even in the absence of selective pressure (Young *et al*., 2007). To date, at least 117 *Campylobacter* complete genomes have been sequenced, enhancing the study of this pathogenic organism (NCBI, 2017). Availability of the whole genome sequences of several *Campylobacter* strains may allow great opportunities to develop improved preventive measures and novel treatment strategies.

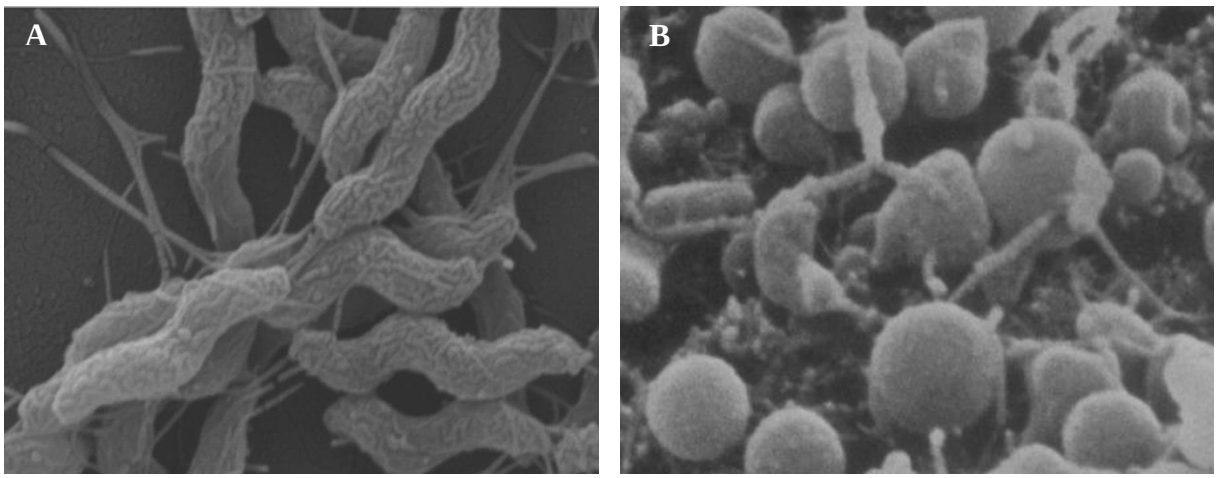


Figure 1.2. Scanning electron microscopy of *C. jejuni* cells (1 µm) (A) spiral shape (Xie *et al*. 2011) and (B) coccoid form (NG *et al*., 1985).

1.3. Clinical features of *Campylobacter jejuni* infection

Campylobacteriosis is a zoonotic disease caused mostly by *C. jejuni* and *C. coli* that can be transmitted directly or indirectly between animals and humans (CDC, 2014). Several environmental reservoirs can lead to human infection by *C. jejuni* (Fig. 1.3). In developed countries, contaminated animal products are the primary source of human infection, most commonly, after the consumption of undercooked chicken or unpasteurised milk (Young *et al*., 2007). *C. jejuni* can also infect humans directly through drinking water, where *Campylobacter* can associate with protozoans, such as freshwater amoebae, and form biofilms (Fig. 1.3) (Young *et al*., 2007).

Campylobacter infections are generally mild, but can be fatal among very young children, the elderly, or immunosuppressed individuals (EFSA, 2014). The most common clinical symptoms of *Campylobacter* infections include diarrhoea (frequently bloody), abdominal cramps, fever and vomiting with an infective dose being as low as 500-800 bacteria (Young *et al*., 2007). The symptoms typically last between three to six days, and non-immunocompromised individuals infected with *Campylobacter* generally recover without any specific treatment (WHO, 2016). For these individuals, antimicrobial therapy is not required, and all that is normally required is electrolyte

replacement and anti-dehydration therapy such as drinking extra fluids (CDC, 2014; WHO, 2016). Antimicrobial treatment (macrolides are the most effective antibiotics) is recommended for the individuals presenting invasive cases of this disease (when bacteria invade the intestinal mucosa and damage the tissues) and for those with immune systems severely weakened (WHO, 2016). Campylobacteriosis can be prevented by cooking all avian products thoroughly, avoiding cross-contamination in the kitchen and good hand hygiene control by washing hands with soap before preparation of food. Measures to reduce the prevalence of *Campylobacter* in poultry include enhanced biosecurity and reduction in antibiotic use in animals (CDC, 2014; WHO, 2016). However, in a minority of individuals infected with *Campylobacter*, serious illness may develop. This includes reactive arthritis and neurological disorders, such as Guillan-Barré Syndrome (GBS), a condition of the peripheral nervous system that can lead to paralysis and requires intensive medical care (Bolton, 2015). It is estimated that 1 in 5,000 campylobacterioisis individuals develop GBS (about 112 GBS cases annually in the United Kingdom are associated with *Campylobacter* infection) (Tam & O'Brien, 2016). In addition, complications such as bacteraemia, hepatitis, pancreatitis and miscarriage have been reported with various degrees of frequency (WHO, 2016).

A vaccine to protect against *Campylobacter jejuni* was recently approved for human clinical trials by the U.S. Food and Drug Administration (FDA) (Zuraw, 2014). This vaccine is a conjugate containing polysaccharides from *C. jejuni* capsule joined to a carrier protein which will enhance immunogenicity, as carbohydrate antigens cannot directly activate naïve T cells. This is because most capsular polysaccharides are thymus-independent (TI) antigens meaning that helper T cells are needed to generate robust, long-lived antibody responses (Guerry *et al*., 2012). This vaccine strategy effectively converts a TI antigen into a thymus dependent (TD) antigen, allowing

boosting of the immune response, IgG antibody class switching, and the generation of memory cells possessing antibody with higher avidity for CPS (Guerry *et al*., 2012). In summary, by conjugating CPS to carrier proteins it is possible to induce a T-dependent immune response against these antigens. This vaccine is currently in Phase I trials where it is being tested for safety and immunogenicity (Riddle & Guerry, 2016).

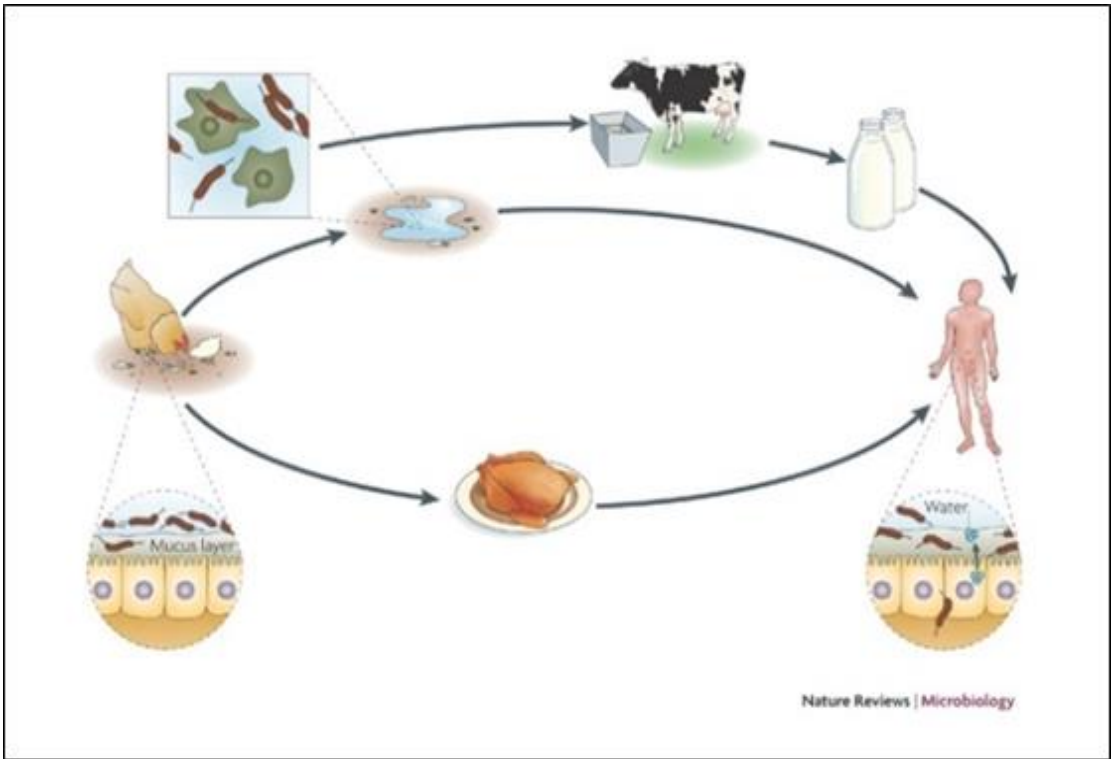


Figure 1.3. The sources and outcomes of *C. jejuni* infection. Several environmental reservoirs can lead to human infection by this bacterium, such as contaminated water and chicken products. *C. jejuni* can enter the water and associate with freshwater protozoa prolonging the risk of infection. *C. jejuni* can invade the gut epithelial layer resulting in inflammation and diarrhoea (Young *et al*., 2007).

1.3.1. Epidemiology

There is evidence to suggest an increase in the global incidence of campylobacteriosis in the past decade (Havelaar *et al*., 2015; Kaakoush *et al*., 2015). In 2015, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU) and has been so since 2005 (Fig. 1.4).

The number of reported confirmed cases of human campylobacteriosis in Europe in 2014 was 229,213 with a hospitalisation rate of 31.2% and a 0.03% case-fatality rate (59 reported deaths), costing approximately 2.4 billion euros a year to public health systems and through loss of productivity (EFSA, 2016a). In 2015 a total of 4,362 food-borne outbreaks were reported and *Campylobacter* caused 8.9% of the outbreaks (EFSA, 2016a). Broiler meat was the main food vehicle implicated in the reported *Campylobacter* outbreaks (EFSA, 2016a). In the United States (US) campylobacteriosis is estimated to affect 1.3 million persons every year, occurring especially during the summer with an average of 76 deaths a year (CDC, 2014). Overall, in the US it is estimated that costs to the health service and to patients from *Campylobacter* infections is $69.6 million annually (Havelaar *et al*., 2015).

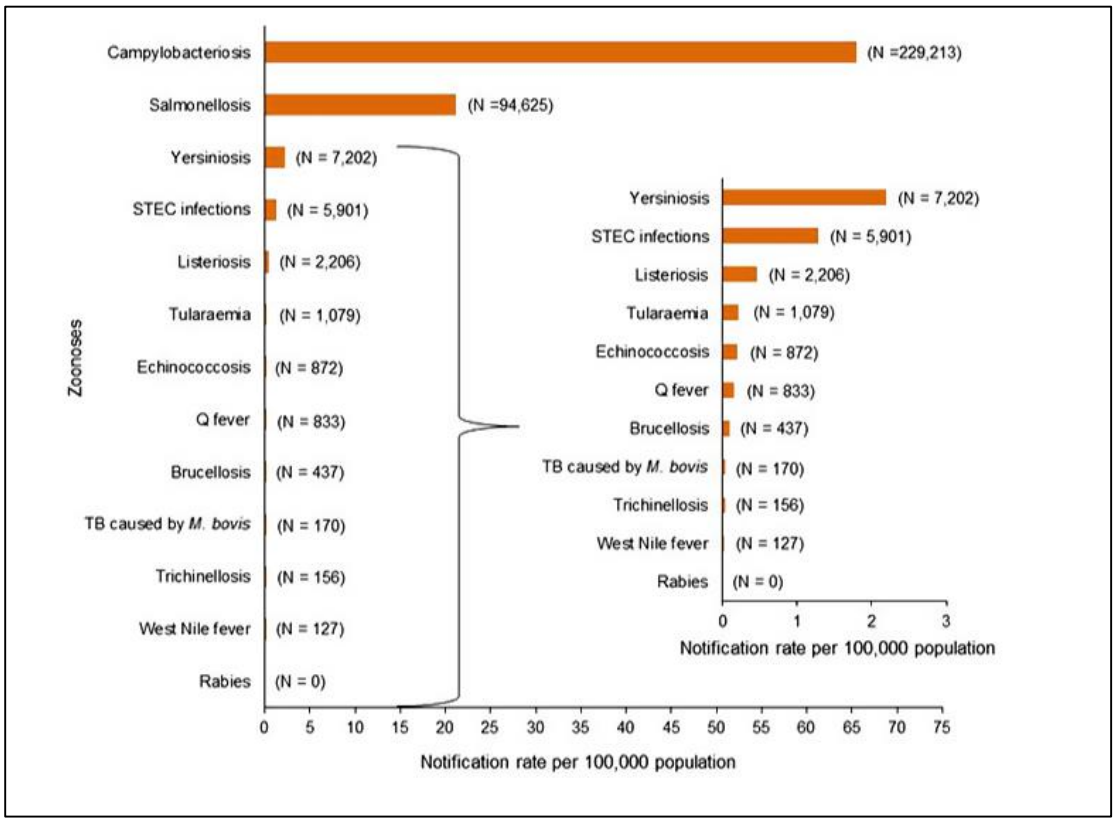


Figure 1.4. Reported numbers and notification rates of confirmed human zoonoses cases in the EU, 2015. In 2015, *Campylobacter* continued to be the most commonly reported gastrointestinal bacterial pathogen in humans in the European Union (EU). The number of reported confirmed cases of human campylobacteriosis was 229,213 with an EU notification rate of 65.5 per 100,000 population, a 5.8% decrease compared with the rate in 2014 (EFSA, 2016a).

1.4. *Campylobacter jejuni* pathogenesis

Despite the significant health burden caused by *C. jejuni*, our current understanding of disease pathogenesis is still developing. Sequencing of the genome of many different *C. jejuni* strains in the recent years has started to accelerate research in *Campylobacter* genetics, pathogenesis and host immunity in response to infection (Backert & Hofreuter, 2013).

To establish infection in humans and animal hosts, *C. jejuni* must bypass the mechanical and immunological barriers of the gastrointestinal (GI) tract (Young *et al*., 2007). *C. jejuni* is able to penetrate the mucus layer of the GI epithelium to further interact with the underlying epithelial cells (Young *et al*., 2007). High motility of this pathogen was shown to be crucial for the infection process (Morooka *et al*., 1985). The establishment of infection of epithelial cells by *C. jejuni* can be divided into three distinct processes: (i) adherence to the enterocytes, (ii) invasion into the intestinal epithelium cells and (iii) survival inside a defined intracellular compartment (Fig. 1.5) (Backert & Hofreuter, 2013). There is considerable evidence that *C. jejuni* can disrupt the tight junctions of epithelial cells which thus allows the bacterium to move to the basolateral surface and either re-invade these cells or be taken up by macrophages (Poly & Guerry, 2008). The main focus in the field of *C. jejuni* research has been to identify the bacterial factors mediating the efficient interaction with cultured epithelial cells, but these studies depend on the bacterial strain, cell line choices, and variation in the experimental procedures (Backert & Hofreuter, 2013).

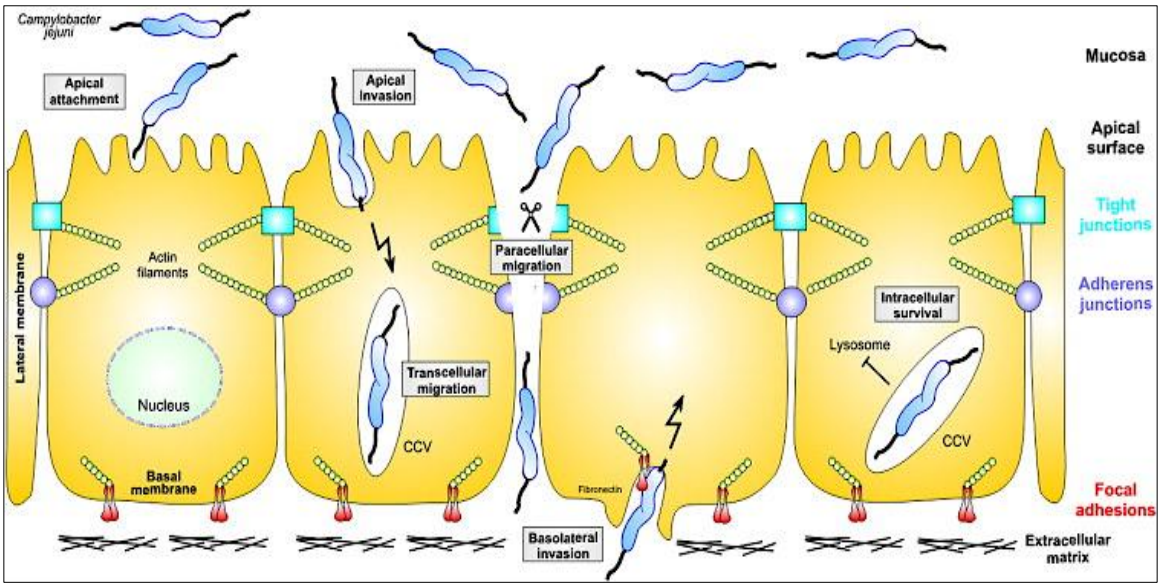


Figure 1.5. Hypothetical model of *C. jejuni* mechanism of infection. This pathogen can interact with, invade into, transmigrate across and survive within polarised intestinal epithelial cells (Backert & Hofreuter, 2013).

1.4.1. Virulence factors

The pathogenicity of *C. jejuni* depends on its capability to interact with and, subsequently, invade host cells (Lugert *et al*., 2015). Each of these steps in the pathogenesis of *C. jejuni* infection depends upon the expression of a combination of several virulence factors such as capsular polysaccharide, flagellar apparatus, cytolethal distending toxin and post-translational glycosylation (Zilbauer *et al*., 2008; Bolton, 2015).

Motility of *C. jejuni* is known to be a crucial factor for invasion and intestinal colonisation by this pathogen (Yao *et al*., 1994). *C. jejuni* motility is mediated by a powerful flagellar apparatus, which enables this bacterium to move with high velocity inside a particular microenvironment and to be able to reach potential target cells (Lugert *et al*., 2015). The flagellum comprises a hook-basal body (composed by several proteins such as FlhA, FliR, FliY, MotA/B) and an extracellular filament structure (composed by the proteins FlaA and FlaB) (Bolton, 2015). Some studies suggest that this apparatus also functions as a type III secretion system, transporting virulence

factors into the host cell (Konkel *et al*., 2004). *Campylobacter* uses chemotaxis, a mechanism by which motile bacteria sense and move towards more favourable conditions, to locate colonisation sites in the avian gut (Chang & Miller, 2006). Proteins associated with *Campylobacter* chemotaxis include CheA/B/R, transducer-like proteins, the response regulator controlling flagellar rotation CheY and the AL-2 biosynthesis enzyme LuxS (Hamer *et al*., 2010; Hermans *et al*., 2011).

The adherence of *C. jejuni* to the intestinal epithelial cells is essential for host colonisation and is mediated by several components on the bacterial surface named adhesins (Lugert *et al*., 2015). An important adhesin that mediates *C. jejuni* adherence to fibronectin is the outer membrane protein CadF (Konkel *et al*., 1997). Other important adhesins include the *Campylobacter* adhesion protein CapA, the N-linked glycosylated lipoprotein JlpA, the fibronectin-like protein FlpA, the periplasmic protein with chaperone and protease activity HtrA, the periplasmic binding protein PEB1, the N-oligosaccharyltransferase PglB and the putative global posttranscriptional regulator CsrA (Pei & Blaser, 1993; Jin *et al.*, 2001; Szymanski *et al*., 2002; Ashgar *et al*., 2007; Fields & Thompson, 2008; Flanagan *et al*., 2009; Baek *et al*., 2011). Besides these, several other adhesins have also been reported (Rubinchik *et al*., 2014). A virulence plasmid termed pVir encodes genes that are involved in DNA uptake and transport of proteins by a putative type VI secretion system. Two of those genes, *comB3* and v*irB11*, were reported to also be required for *C. jejuni* adherence to intestinal cells (Bacon *et al*., 2000).

Further to adhesion, the invasion process of *C. jejuni* is considered to be the most important stage that causes damage to the host cells (Lugert *et al*., 2015). Many proteins have been associated with the process of invasion. These include the flagella secreted *Campylobacter* invasion antigens CiaB, CiaC and CiaI; the invasion associated protein IamA; a lipoprotein involved in iron acquisition CeuE, a small acidic protein FspA and

the outer membrane CadF (Park & Richardson, 1995; Carvalho *et al*., 2001; Monteville *et al*., 2003; Konkel *et al*., 2004; Poly *et al*., 2007; Christensen *et al*., 2009; Buelow *et al*., 2011). The chaperone HtrA and the antimicrobial peptide resistance protein VirK were also shown to be required for *C. jejuni* invasion (Novik *et al*., 2009; Baek *et al*., 2011). Secretion of the cytolethal-distending toxin (CDT) by *C. jejuni* is highly important for its invasion to host cells (Asakura *et al*., 2008). CDT is a tripartite toxin composed of three subunits encoded by the *ctdA, cdtB* and *cdtC* genes that are all required for the toxin to be functionally active (Asakura *et al*., 2008). CDT causes eukaryotic cells to arrest in the G2/M phase of their cell cycle, preventing them from entering into mitosis and, therefore, leading to cell death (Asakura *et al*., 2008). The *Campylobacter* glycome composed of carbohydrate structures, such as lipooligosaccharides (LOS) and polyssacharide capsule (CPS) and by N-linked protein glycosylation systems was shown to be required for adhesion and invasion of this pathogen (Karlyshev *et al*., 2005a; Louwen *et al*., 2008). More specifically, the *galE* gene, which encodes a UDP-glucose 4-epimerase and is involved in LOS synthesis, was shown to be required for adherence and invasion of epithelial cells (Fry *et al*., 2000). A capsular polysaccharide transport protein KpsM, a capsule biosynthesis protein KpsE and an N-linked general protein glycosylation pathway were also shown to reduce not only host cell invasion, but also chicken colonisation (Bacon *et al*., 2000; Karlysehv *et al*., 2004; Bachtiar *et al*., 2007). The CPS varies in sugar composition and linkage, leading to capsular structure diversity among different *C. jejuni* strains (Bacon *et al*., 2001). In addition due to variation in sugar composition, the CPS can be modified with ethanolamine, glycerol, and O-methyl phosphoramidate (MeOPN), and this modification may be advantageous at some points during the *C. jejuni* lifestyle and disadvantageous at others, proving to be a key determinant in virulence (Guerry *et al*., 2012; Maue *et al*., 2013).

Among the factors known to be required for intracellular survival of *Campylobacter* within host cells, are superoxide dismutase SodB; aspartate ammonia lyase AspA; fumarate reductase flavoprotein FrdA; iron transporter FeoB; *Campylobacter* invasion antigen CiaI; guanosine-3-pyrophosphohydrolase SpoT; polyphosphate kinase Ppk1; heptosyltransferase WaaF (required for LOS formation); sensor kinase CprS; and a virulence protein VirK (Gaynor *et al*., 2005; Naikare *et al*., 2006; Candon *et al*., 2007; Novik *et al*., 2009; Svensson *et al*. 2009; Naito *et al*., 2010; Novik *et al*., 2010; Buelow *et al*., 2011; Liu *et al*., 2012). Intracellular survival of *C. jejuni* within macrophages has been confirmed in different studies (Kiehlbauch *et al*., 1985; Day *et al*., 2000; Hickey *et al*., 2005).

C. jejuni possesses a rigorous stress control response which enables it to adapt to different oxygen conditions, heat shock, reduced pH and nutrient starvation (Bolton, 2015). *Campylobacter* may encounter some of these conditions during food processing or storage, and resistance to these is important for its survival, transmission and infection in humans. SpoT is responsible for the stringent control of the *C. jejuni* stress survival response (Gaynor *et al*., 2005). *Campylobacter* oxidative stress regulator CosR and the metalloregulatory proteins Fur and PerR are also essential to control the oxidative stress response (Belzer *et al*., 2011; Hwang *et al.*, 2011). Proteins that are required for oxygen stress include the catalase KatA (that converts hydrogen peroxide to water and oxygen when the former is too high in the cell cytoplasm); the alkyl hydroperoxide reductase AhpC; the thiol peroxidase Tpx; cytochrome c peroxidases; the iron-binding protein Dps; the antioxidant superoxide dismutase SodB that confers protection against the superoxide anion; the iron-induced ferredoxin FdxA and DnaJ which is involved in the heat shock response (Konkel *et al*., 1998; Baillon *et al*., 1999; van Vliet *et al*., 2001; Ishikawa *et al*., 2003; Atack *et al*., 2008; Bingham-Ramos & Hendrixson, 2008; Atack & Kelly, 2009; Palyada *et al*., 2009).

1.5. Antibiotic resistance in *Campylobacter jejuni*

Antimicrobial resistance (AMR) has increasingly become a problem in recent years since not only has the discovery of novel antibiotics slowed drastically, but, at the same time, antibiotic use is rising, causing microbes to evolve and resist these drugs (O´Neill, 2014). Antimicrobial resistance currently claim at least 50,000 lives each year across Europe and the US alone, where in other areas of the world a similar scenario was observed (O´Neill, 2014). The major cause for this increasing AMR is the heavy use of antimicrobial drugs in humans and animals when most of the time they are unnecessary, or not properly used (O´Neill, 2014).

When clinical therapy is warranted for *Campylobacter* infections, macrolides, (erythromycin) and fluoroquinolones (FQ) (ciprofloxacin) are the most frequently used antimicrobials and, although in practice not often used, tetracyclines have been suggested as an alternative choice of treatment (Wieczorek & Osek, 2013). However, *Campylobacter* is becoming increasingly resistant to these antibiotics, especially to FQ and tetracycline (Tet). *Campylobacter* isolates very commonly show resistance to these antibiotics in many countries (Luangtongkum *et al*. 2009; Bolinger & Katheriou, 2017). For this reason, macrolides are still the most effective antibiotics against *Campylobacter* infections whilst FQ are losing effectiveness against this pathogen (Bolinger & Katheriou, 2017).

Antibiotics have been widely used in food-producing animals, contributing to the emergence of antibiotic-resistant bacteria in many poultry products that are further consumed by humans. The latter can develop resistance to antibiotics with consequences to human health. Because antibiotics enable animals to grow faster and to gain weight more efficiently, their use in growth promotion became a common practice in animal rearing (Markus & van Lankveld, 2014). Concerns about increasing antibiotic resistance and transfer of antibiotic resistance genes from animal pathogens to human

pathogens, led to the withdrawal of antibiotics as growth promoters in the EU since January 2006 (Castanon, 2007). The major goal of the European ban on antibiotic growth promoters is to reduce antibiotic resistance traits in the microbial flora of farm animals (Castanon, 2007). Although European countries banned the use of tetracycline in animals in 1972, this antibiotic is still used in the USA and Canada as a growth promoter in animal feeds and tetracyclines are still licensed for therapeutic use in poultry in the UK (Wieczorek & Osek, 2013; Markus & van Lankveld, 2014). In fact, according to Piddock *et al*. (2008) chlortetracycline was the most commonly used therapeutic antibiotic in poultry production).

Monitoring AMR to ciprofloxacin, erythromycin and tetracycline in *Campylobacter* became mandatory in the major food-producing animals. There is evidence that antimicrobial resistance in *Campylobacter* isolated from chicken meat is widespread and increasing and that *Campylobacter* isolates resistant to tetracycline are highly prevalent in many countries (Piddock *et al*., 2008; EFSA, 2016b), including the UK (Wimalarathna *et al*., 2013). The widespread antimicrobial resistance in the *Campylobacter* population probably results from horizontal gene transfer, where bacteria can acquire genetic material, including antimicrobial resistance genes, from relatively distant lineages (Wimalarathna *et al*., 2013). The frequently high level of Tet resistance in *Campylobacter* is most likely due to the presence of the tetracycline resistance gene *tetO*, carried by a transferable plasmid pTet, which facilitates tetracycline resistance transmission (Wieczorek & Osek, 2013).

The *Campylobacter* multidrug resistance virulence factors will be discussed in detail in Section 1.6.2 of the introduction.

1.6. Antibiotic resistance mechanisms

There are several mechanisms of antibiotic resistance in Gram-negative bacteria, where synergy between antibiotic efflux and a second mechanism (e.g. antibiotic resistance genes) is well established (Iovine, 2013). In general, antibiotic resistance mechanisms include modification of the antibiotic target or its expression; antibiotic failure in reaching its target; antibiotic efflux out of the cell and modification or inactivation of the antibiotic (Iovine, 2013). These distinct mechanisms provide resistance against different classes of antibiotics, such as aminoglycosides, β-lactams, fluoroquinolones, macrolides and tetracyclines.

1.6.1. RND multidrug efflux pumps

Multidrug resistance efflux (MDR) pumps are widely distributed in bacterial species and constitute an important class of resistance determinants. These pumps allow for drug extrusion out of the cells and have been increasingly associated with clinically relevant AMR and are consequently a major threat to the public health (Sun *et al*., 2014). Although there are several types of multidrug efflux pumps, members of the resistance-nodulation-division (RND) multidrug efflux pump superfamily are the most clinically relevant (Blair *et al*., 2014).

The RND systems are highly conserved and are found in different bacterial species. These systems are found as a tripartite system always comprising an inner membrane transporter, an outer membrane protein channel and a periplasmic adaptor protein (Blair *et al*., 2014). Importantly, each of these three component proteins is essential for drug efflux, and the absence of even one component makes the entire complex totally non-functional (Nikaido & Takatsuka, 2009). RND proteins form a continuous channel across the Gram-negative cell envelope ensuring that the drug target is effluxed across

the periplasm and the outer membrane directly into the external medium using a proton-gradient as an energy source (Dinesh & Kumar, 2013). Efflux systems lower the intracellular antibiotic concentration, allowing bacteria to survive at higher antibiotic concentrations. In addition, overexpression of these systems may cause clinically relevant levels of AMR in Gram-negative pathogens (Blair *et al*., 2014). RND pumps are also known to interact synergistically with other resistance mechanisms. For instance, they are able to increase the outer membrane permeability barrier and thus, increase the levels of antibiotic resistance (Li *et al*., 2015).

Examples of RND efflux pumps that are able to confer resistance to a broad range of antimicrobial compounds which are found in Gram-negative pathogens are: AcrAB-TolC in *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica*, MexAB-OprM in *Pseudomonas aeruginosa*, AdeABC in *Acinetobacter baumannii*, MtrCDE in *Neisseria gonorrhoeae*, BpeAB-OprB in *Burkholderia pseudomallei*, VexAB-TolC in *Vibrio cholerae* and CmeABC in *Campylobacter jejuni* (Blair *et al*., 2014).

Resistance to several types of antimicrobial compounds by these efflux pumps clearly indicate great challenges in antibiotic development against Gram-negative bacteria. Nonetheless, if these systems can be antagonised by an efflux pump inhibitor (EPI) this could offer a good opportunity for adjuvant therapy to combat infections by these pathogens (Li *et al*., 2015). An ideal EPI would enhance the activity of multiple antibiotics and be relatively stable and non-toxic to the eukaryotic cells (Blair *et al*., 2014). Phenylarginine β-naphthylamide (PaβN) was the first identified as an EPI since it inhibits the conformational change required for the inner membrane transport to function. However, this compound is not clinically useful as it is toxic to eukaryotic cells (Blair *et al*., 2014). Another range of compounds shown to increase the intracellular accumulation of several antibiotics are the naphthylpiperazines (NMP), but, unfortunately, these compounds are also too toxic for clinical use as they posess

serotonin agonist properties (Sun *et al*., 2014). Although several types of compounds, such as quinolone derivatives, have been investigated as potential EPIs, none have been used in clinics (Sun *et al*., 2014).

1.6.2. The *Campylobacter* CmeABC multidrug efflux pump

Although genome sequencing predicts 14 efflux pumps in *Campylobacter* (e.g. CmeDEF, CmeG, Acr3), the best studied is the CmeABC (*Campylobacter* multidrug efflux) and this is described as the major efflux pump causing AMR (Iovine, 2013). CmeABC belongs to the family of RND pumps and includes an outer membrane protein CmeC, an inner membrane drug transporter CmeB and a periplasmic protein CmeA that bridges CmeB and CmeC (Iovine, 2013). It has been widely reported that CmeABC works synergistically with other resistance mechanisms to confer high-level antibiotic resistance (see Section 1.7). This MDR pump has been reported to be required for resistance in different *C. jejuni* strains to a broad range of structurally unrelated antimicrobial agents. Examples are the different categories of antibiotics (tetracycline, ampicillin, nalidixic acid, chloramphenicol, gentamicin, erythromycin, etc.); bile salts (cholic acid, taurocholic acid, etc.); surfactants (sodium dodecyl sulphate, benzalkonium chloride); disinfectants and antiseptics (chlorhexidine, cetylpyridinium chloride); metals (cobalt, zinc, copper) and other compounds such as triclosan, protamine and ethidium bromide (Lin *et al*., 2002; Pumbwe & Piddock, 2002; Mavri & Mozina, 2012). Bile salts are present in the small intestine for digestion of fats and are able to kill bacteria by destroying the lipid bilayer of their cell membrane (Gunn, 2000). The CmeABC MDR pump was shown to be essential for *Campylobacter* growth in bile-containing media, and consequently for colonisation of the chickens' intestinal tract by mediating resistance to bile salts (Lin *et al.*, 2003).

CmeR (encoded by *cmeR*) is the transcriptional repressor of the three genes *cmeA, cmeB* and *cmeC* (Lin *et al*., 2005). The *cmeR* gene is located upstream of *cmeA* and encodes a protein with a sequence and structure similar to TetR (Gu *et al*., 2007), a known transcriptional repressor that regulates the expression of the tetracycline resistance determinant encoded by the *tetA* gene (Cuthbertson & Nodwell, 2013). Additionally, it was shown that CmeR directly regulates the c*meABC* promoter region via specific binding to this promoter region (Lin *et al*., 2005). As inactivation of *cmeR* leads to overexpression of the *cmeABC* pump, it was observed that mutation in this regulator results in higher resistance to different antibiotics (Lin *et al*., 2005). Importantly, in addition to controlling the expression of CmeABC MDR pump, CmeR also modulates expression of different genes with diverse physiological functions and is required for optimal colonisation of chickens (Guo *et al*., 2008). The *Campylobacter* CosR response regulator that modulates oxidative stress response was also shown to repress *cmeABC* expression (Grinnage-Pulley *et al*., 2016). In *Campylobacter*, CmeR and CosR act as moderators to maintain balanced production of *cmeABC* and facilitate its adaptation to environmental changes (Lin *et al*., 2005; Grinnage-Pulley *et al*., 2016). Different point mutations in the *cmeR* resulted in an altered expression of the *cmeABC* MDR pump (Cagliero *et al*., 2007; Perez-Boto *et al*., 2015).

A second efflux system, CmeDEF, which has different substrate-binding properties, and, thus, is functionally distinct from CmeABC, has been identified in *Campylobacter* (Pumbwe *et al*., 2005). In this system, the inner membrane transporter CmeF that has homology with CmeB is linked to an outer membrane CmeD by a putative membrane protein CmeE (Pumbwe *et al*., 2005). Although little is known about this secondary efflux system, it is described as being expressed at low level and acts interactively with CmeABC in conferring resistance to antimicrobials and toxic compounds (Akiba *et al*., 2006). Inactivation of CmeF leads to increased susceptibility to several antibiotics and

CmeABC and CmeDEF are involved in maintaining cell viability since at least one of these efflux pumps is required for optimal growth of *Campylobacter*, showing a physiological role for these MDR pumps (Akiba *et al*., 2006). A new putative efflux transporter, CmeG, has recently been reported (Jeon *et al*., 2011). CmeG was shown to be required for optimal growth *in vitro*, antibiotic resistance, and also for oxidative stress resistance by mediating resistance to hydrogen peroxide (Jeon *et al*., 2011).

Because CmeABC is a good target for the development of intervention strategies to combat antibiotic resistance in *Campylobacter*, inhibition of this MDR pump by an EPI has been considered a novel approach to enhance drug accumulation inside the bacterial cell and thus, increase bacterial susceptibility to antibiotics (Kaatz, 2002). The EPI, Phe-Arg β-naphthyl-amide dihydrochloride, was shown to be effective at inhibiting the function of CmeABC pump (Martinez & Lin, 2006). However, several key aspects (e.g. toxicity, *in vivo* stability, production costs) should be addressed before EPIs can be used clinically and accepted in the medical community (Zeng *et al.*, 2010). A more realistic and promising approach currently being investigated to potentiate the activity of clinical antibiotics against *C. jejuni* infection is a CmeC vaccine as this was shown to be important for colonisation and is dramatically induced and immunogenic *in vivo* (Zeng *et al*., 2010). However, the CmeC vaccination regimen should be optimised to enhance CmeC-specific mucosal immune response for protection against *C. jejuni* infection (Zeng *et al*., 2010).

It became clear that the CmeABC MDR pump plays an important role in mediating AMR in *Campylobacter*, but the contribution of other efflux transporters remains to be elucidated. In addition, other natural functions of these efflux transporters in *Campylobacter* physiology await further investigation.

1.7. Other antibiotic resistance mechanisms in *Campylobacter*

Besides the CmeABC efflux pump, there are other major resistance mechanisms in *Campylobacter* (Fig. 1.6). Quinolones exert their antibacterial effect by preventing synthesis of the bacterial DNA causing cell death (Wieczorek & Osek, 2013). Fluoroquinolone resistance in *Campylobacter* is mainly mediated by point mutations in the quinolone resistance-determining region of the DNA Gyrase (*gyrA*), with the mutation They-86-Ile being the most common and the one responsible for ciprofloxacin resistance (Alfredson & Korolik, 2007; Iovine, 2013). Briefly, FQ form a stable complex with the intracellular enzymatic target DNA gyrase (encoded by *gyrA*), trapping it onto DNA and thus, leading to a decrease in DNA replication and transcription, and ultimately cell death (Iovine, 2013). The *C. jejuni mfd* (mutation frequency decline) gene encodes a transcription-repair coupling factor involved in DNA-repair and it has been reported that mutations in this gene also contribute to the development of FQ resistance in this pathogen (Han *et al*., 2008). Another mechanism of FQ resistance is efflux, reducing the intracellular concentration of FQ. Additionally, the CmeABC pump works synergistically with GyrA mutations in causing high-level FQ resistance (Luo *et al*., 2003; Ge *et al*., 2005; Yan *et al*., 2006).

Macrolides are antibiotics, mostly produced by *Streptomyces*, which interrupt protein synthesis in the bacterial ribosome by targeting the 50S subunit and inhibiting RNA-dependent protein synthesis (Wieczorek & Osek, 2013). Macrolide resistance in *Campylobacter* is the result of modification of the ribosome target-binding site by mutation of the 23S rRNA gene (Wieczorek & Osek, 2013). Efflux through CmeABC is also described as a macrolide resistance mechanism and so is its synergy with 23S rRNA mutations, the latter conferring high-level of resistance (Lin *et al.*, 2007; Gibreel *et al*., 2007). Additionally, a third mechanism of macrolide resistance involves altered membrane permeability mediated by expression of the major outer membrane porin

(MOMP). This porin creates transmembrane pores and allows diffusion of hydrophilic molecules, limiting the entry of most antibiotics with a high molecular weight (Iovine, 2013).

Aminoglycosides, such as gentamicin and kanamycin, are a class of antibiotics that inhibits bacterial protein synthesis (Iovine, 2013). Aminoglycoside resistance in *Campylobacter* is mediated by the aminoglycoside resistance gene *aphA-3*, a gene that encodes for a phosphotransferase, the latter modifying aminoglycoside antibiotics (Iovine, 2013). These enzymes bind to the decoding region in the A-site of the bacterial ribosomal 30S subunit, resulting in aberrant proteins by interfering with accurate proofreading (Wieczorek & Osek, 2013). Additionally, kanamycin resistance has been also linked to a *Campylobacter* plasmid that encodes tetracycline resistance, as it carries a kanamycin-phosphotransferase gene, *aph-7* (Taylor & Courvalin, 1988).

The β-lactams, such as penicillins, are a category of antibiotics that contain a β-lactam ring in their structure (Wieczorek & Osek, 2013). In *Campylobacter*, three mechanisms mediate resistance to these antibiotics: inactivation by the enzymes β-lactamases, such as the OXA-61, efflux out of the cell and decrease of the membrane permeability of most anionic antibiotics (Alfredson & Korolik, 2005; Iovine, 2013).

Tetracycline works by inhibiting bacterial protein synthesis. In *Campylobacter*, *tetO* encodes for TetO, a protein that mediates tetracycline resistance (Wieczorek & Osek, 2013). Once inside the cell, tetracycline reversibly binds to the 30S subunit of ribosomes and prevents the attachment of the charged aminoacyl-tRNA to the ribosomal site A thus, inhibiting protein synthesis (Dasti *et al*., 2007). The *tetO* gene is more frequently carried on transmissible plasmids; however, it can also be chromosome-encoded (Dasti *et al*., 2007). Besides efflux through CmeABC, this was also reported to work synergistically with TetO, conferring a high-level of Tet resistance (Gibreel *et al*., 2007).

Although mutations play a major role in the development of AR in *Campylobacter*, this pathogen can also acquire antibiotic-resistance determinants via horizontal gene transfer (HGT) (Luangtongkum *et al*., 2009). In *Campylobacter,* HGT is mainly mediated by natural transformation or conjugation, where the former is a major mechanism for the transfer of chromosomally encoded resistance and the latter plays a role in the transfer of plasmid-mediated resistance (Luangtongkum *et al.,* 2009). Transfer of DNA, especially antibiotic-resistance determinants, has been widely reported in *Campylobacter* and has been shown both *in vitro* in bacterial cultures (Wilson *et al.*, 2003; Jeon *et al*., 2008) and *in vivo* in the chicken intestine (de Boer *et al*., 2002; Avrain *et al*., 2004). It is highly likely that the transfer of the pTet plasmid in the intestinal tract of chickens is possible because it has been reported that this contributes to the spread of Tet resistance in this pathogen (Avrain *et al*., 2004).

As *Campylobacter* is naturally competent, there is considerable ground for the emergence of resistant mutants (Parkhill *et al*., 2000). Additionally, as this pathogen is a commensal of animal species, antimicrobial treatment in poultry farms may create an ideal opportunity for *Campylobacter* to evolve additional resistance mechanisms (Iovine, 2013). As macrolides are the drug of choice in the treatment of campylobacteriosis, further understanding of macrolide resistance mechanisms in *Campylobacter* is required to avoid the spread of this antibiotic resistance category, which is of great clinical concern in public health. Modern approaches such as genomics and proteomics are expected to provide new insights into the molecular mechanisms and help in our understanding of the development of AMR in *Campylobacter* (Wieczorek & Osek, 2013).

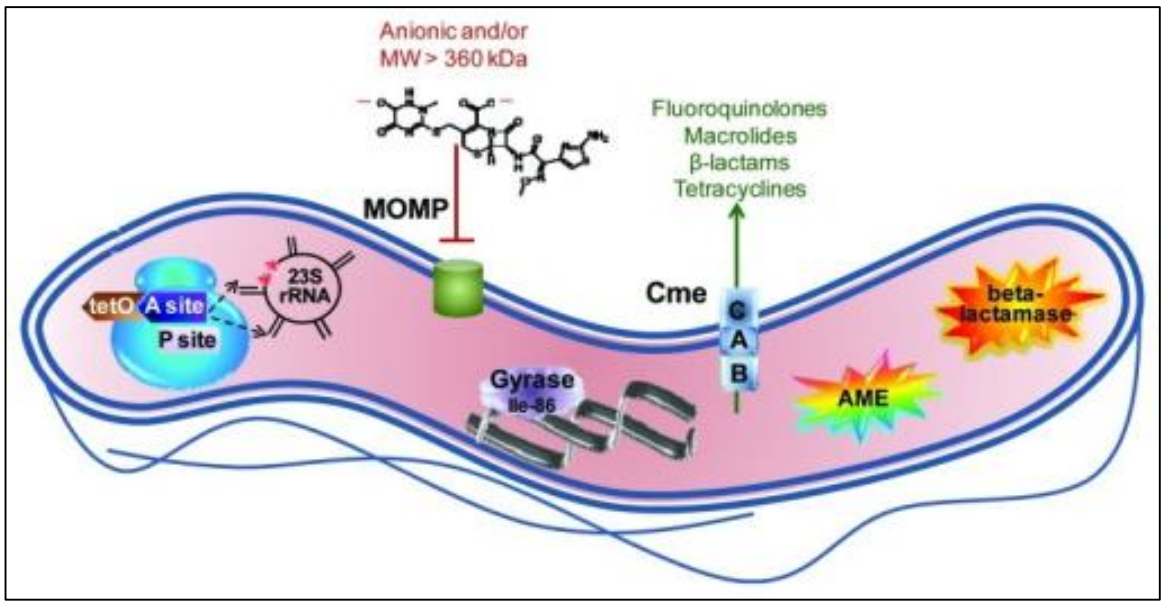


Figure 1.6. Major antibiotic resistance mechanisms in *Campylobacter*. The ribosome, shown in blue at the left, is the site of binding of the TetO protein (shown in brown) to the A site (shown in dark purple) confering tetracycline resistance. Point mutations in 23S rRNA (shown in black and indicated by red stars) confers resistance to macrolides. MOMP (shown in green) limits the entry of most antibiotics that are negatively charged or with a molecular weight larger than 360 kDa; The Thr-86-Ile substitution in DNA gyrase (shown in light purple) confers resistance to this antibiotic class. The MDR CmeABC (shown as stacked blue squares) contributes to resistance to several antibiotics and works synergistically with other resistance mechanisms, often leading to high-level resistance. Aminoglycoside-modifying enzymes (AME; shown as the multi-colored star burst) are the main means of aminoglycoside resistance**.** Finally, β-lactamases (shown as the orange star burst) contribute to β-lactam resistance (Iovine, 2013).

1.8. Other physiological functions of multidrug efflux pumps

Recently, several studies have identified different physiological roles for MDRs, indicating that they are not only involved in antibiotic resistance. This is because efflux pumps not only export antibiotics, but also host-derived antimicrobial agents, allowing the bacteria to adapt and survive in their ecological niches. Other functions of MDR efflux pumps include cell communication, stress response, fitness, biofilm formation, detoxification of intracellular metabolites and colonisation of both animal and plant hosts (Piddock, 2006; Poole, 2008; Martinez *et al*., 2009; Alvarez-Ortega *et al*., 2013; Dinesh & Kumar, 2013; Sun *et al*., 2014; Li *et al*., 2015; Blanco *et al*., 2016).

As previously mentioned, AcrAB-TolC and MexAB-OprM are homologs of *Campylobacter* CmeABC. Since MDR pumps are able to pump toxic bile salts out of the cells, they are able to promote the bacterial adaptation to the animal's intestinal tract (Sun *et al*., 2014). Although CmeABC was reported to be required for chicken colonisation by mediating resistance to the bile salts (Lin *et al*., 2003), several homologs of this efflux pump have been reported to be crucial for other pathogens to colonise and invade other host cells. For instance, the *Pseudomonas aeruginosa* MexAB-OprM, the *Klebsiella pneumoniae* AcrAB pump, the *Salmonella* AcrAB-TolC, the *Vibrio cholerae* efflux pump VexB, the *Burkholderia pseudomallei* BpeAB-OprB efflux pump, the *Neisseria gonorrhoeae* MtrCDE efflux system and the *Francisella tularensis* AcrAB were shown to be required for mice colonisation and in some cases for epithelial cell invasion and survival in macrophages (Hirakata *et al*., 2002; Jerse *et al*., 2003; Chan & Chua, 2005; Buckley *et al*., 2006; Nishino *et al*., 2006; Bina *et al*., 2008; Webber *et al*., 2009; Padilla *et al*., 2010). It was also reported that the *Pseudomonas* MexAB-OprM system contributes to the colonisation of tomato plants (Vargas *et al*., 2011).

In *Salmonella enterica* and *Burkholderia cenocepacia*, these efflux systems were demonstrated to be required for bacterial motility (Webber *et al*., 2009; Bazzini *et al*., 2011). Bacterial quorum sensing through efflux of auto inducers by this MDR was shown for *Burkholderia paseudomallei* and *cenocepacia* and for *Pseudomonas aeruginosa* (Evans *et al*., 1998; Chan & Chua, 2005; Bazzini *et al*., 2011). In the literature, there are some studies reporting on whether RND efflux pumps are required for biofilm formation, although MDR pumps have been reported to be required for biofilm formation (Kvist *et al*., 2008; Matsumura *et al*., 2011; Baugh *et al*., 2012). However, a recent publication reported to the contrary (Schlisselberg *et al*., 2015), thus suggesting further investigation regarding this topic is required.

In summary, it is clear that MDR pumps influence bacterial pathogenesis and may act as virulence determinants.

1.9. The *Acanthamoebae* genus

Protozoa are defined as a group of unicellular eukaryotic organisms that lack cell walls. A large group within the protozoa are the amoebae, which are ubiquitously distributed in the environment (Khan, 2006). *Acanthamoeba* is a genus of amoebae that was first described by Volkonsky in 1930 (Volkonsky, 1931). *Acanthamoebae* are opportunistic protists that are characterised by the presence of protoplasmic spine-like structures on its surface (known as acanthopodia), double wall cysts and an irregular outer layer (Khan 2006; Siddiqui & Khan, 2012a). They contain one or more contractile vacuoles (required for osmotic regulation), digestive vacuoles, lysosomes and several glycogen-containing vacuoles (Siddiqui & Khan, 2012a). *Acanthamoebae* move through the actin cytoskeleton that forms the cytoplasmic protrusions and it is relatively fast, with a motility rate of approximately 0.8μm/second (Siddiqui & Khan, 2012a). Food uptake in these organisms occurs by pinocytosis (engulfing of liquids/small particles by invagination of the plasma membrane) and/or by phagocytosis (engulfing large particles forming an internal compartment) (Khan, 2006).

Acanthamoebae are free-living amoebae (FLA) that are distributed worldwide and are the most prevalent protozoa found in the environment (Sandstrom *et al*., 2011). They have the ability to survive in diverse environments and have been isolated from public water supplies (swimming-pools, lakes, seawater, rivers), bottled water, ventilation ducts, air-conditioning units, sewage, soil, vegetables, surgical instruments, hospital units, mammalian cell culture and contact lenses (Sandstrom *et al*., 2011). *Acanthamoebae* are highly resistant organisms as they have the capacity to survive

under adverse conditions, such as extreme pH, high osmotic pressure, high temperature and food deprivation (Tosetti *et al*., 2014).

These eukaryotic organisms undergo two stages during their life cycle: an active trophozoite phase and a resistant cyst stage (Fig. 1.7). Throphozoites are normally 25-40μm in diameter and possess a large number of mitochondria (that generate the energy required for the metabolic activities. They exhibit acanthopodia on their surface that allow movement, feed on organic particles, or microbes, and are able to divide asexually via binary fission (parent cell mitotically divides into two daughter cells). When exposed to harsh conditions, *Acanthamoebae* adopt a reversible and dormant double-walled cyst stage with minimal metabolic activity and that are normally 13-20μm in diameter (Marciano-Cabral & Cabral, 2003). This process where the throphozoite encloses itself within a resistant shell is known as encystment (Khan, 2006; Siddiqui & Khan, 2012a). The outer walls of the cyst consist of proteins and polysaccharides, while the inner wall possesses cellulose (Khan, 2006). Cysts may be airborne, helping in the spread of *Acanthamoebae* in the environment and they remain viable for several years protecting, transmitting and maintaining the pathogenicity of this organism (Siddiqui & Khan, 2012a). When under favourable conditions, the excystment process is induced (throphozoites emerge from the cysts leaving behind the outer shell) and they are able to reproduce and complete the cycle (Siddiqui & Khan, 2012a).

Figure 1.7. The life cycle of *Acanthamoebae* spp. Under optimal environmental conditions, *Acanthamoeba* remains in the active throphozoite form (A), while, under harsh conditions, it changes to a resistant double-walled cyst form (B).

There are over than 20 unique species of *Acanthamoebae*, the most common being *A. polyphaga* and *A. castellanni* (Maycock & Jayaswal, 2016). Initially, they were classified based on the cyst size and shape; however, this classification has proved unreliable because cyst morphology may change depending on culture conditions (Marciano-Cabral & Cabral, 2003). Classification of *Acanthamoebae* species is now based on the sequence analysis of 18S ribosomal RNA coding DNA (18S rDNA) (Marciano-Cabral & Cabral, 2003; Kong, 2009). Comparison of 18S rDNA sequences allowed distinction of three morphological groups of *Acanthamoebae* and divided them into 15 unique sequence types (T1-T15 genotypes) (Kong, 2009; Maycock & Jayaswal, 2016), each genotype exhibits 5% or more sequence divergence between different genotypes (Siddiqui & Khan, 2012a). *A. polyphaga* and *A. castellanii* belong to group III (small cysts less than 18µm with polygonal or stellate endocysts and irregular or wrinkled ectocysts) and the genotype T4 (Kong, 2009). There is a strong resemblance among these species with the major differences being related with their length while moving and in their cyst structure (Page, 1967). *A. castellanii* has a median locomotion length of approximately 26.2µm, whilst *A. polyphaga* has a length of approximately 20.7µm. *A. castellanii* usually moves faster than *A. polyphaga*, but both species have

only one contractile vacuole. The cysts from *A. polyphaga* are usually smaller (12.6μm median length) than the cysts from *A. castellanii* (17.1μm median length) (Page, 1967). Lastly, both species are capable of causing the same diseases, which will be described in more detail in Section 1.10.

1.10. *Acanthamoebae* pathogenesis and clinical features

The majority of human infections due to *Acanthamoebae* have been associated with the isolates from the genotype T4, most likely associated with their greater virulence and properties that enhance their transmissibility (Siddiqui & Khan, 2012a). Eight species of *Acanthamoebae* have been reported to cause human infection (Maycock & Jayaswal, 2016). *Acanthamoebae* cause two well-known diseases that are a major concern to human health: *Acanthamoebae* keratitis (AK) and *Acanthamoebae* granulomatous encephalitis (AGE) (Siddiqui & Khan, 2012a). Although the trophozoites are the infective forms, both cysts and trophozoites may gain entry into the human body (Fig. 1.8) (CDC, 2012).

AK is a potentially sight-threatening infection of the ocular surface that may lead to blindness and it is caused by different species of *Acanthamoebae*, the most common of which are *A. castellanii* and *A. polyphaga* (Maycock & Jayaswal, 2016). Although AK may occur in non-contact lens wearers, this disease is most common in individuals who use contact lenses (CL) that were exposed to contaminated water (Maycock & Jayaswal, 2016). AK has been increasing in prevalence in recent years, with reporting rates of 1 to 33 cases per million CL wearers (Maycock & Jayaswal, 2016). The main reasons why AK occurs are due to extensive time or re-use of CL, inappropriate cleaning of CL, lack of personal hygiene and exposure of the CL to contaminated water (e.g. swimming-pools) (Khan, 2006; Maycock & Jayaswal, 2016). The sequence of events of AK

involves adhesion of *Acanthamoeba* to the corneal epithelial cells through cell surface proteins, such as the mannose binding protein MBP, followed by invasion with secretion of toxins, resulting in stromal degradation and deep penetration into the cornea, contributing to eye damage (Maycock & Jayaswal, 2016). Symptoms include considerable production of tears, photophobia, inflammation and redness of the eye, stromal opacity, stromal abscess formation with vision-threatening consequences and excruciating pain (Khan, 2006). AK is a difficult infection to treat, requiring early diagnosis and an aggressive treatment (Khan, 2006). Although amoebic trophozoites are sensitive to a large number of available antibiotics (e.g. metronidazol), the cysts may lead to a prolonged or resistant infection, as most of the treatment is ineffective. The most effective cysticidal antiamoebics are diamines and biguanines (Maycock & Jayaswal, 2016).

AGE is a rare central nervous system (CNS) infection that occurs mainly in immunocompromised patients and almost always leads to death (Siddiqui & Khan, 2012a). *Acanthamoebae* enter the human host by inhalation through the lower respiratory tract or through skin lesions, leading to invasion of the blood vessels followed by spread from the lungs or skin and cross of the blood-brain-barrier further entering in the CNS and resulting in neuronal damage and brain dysfunction (Marciano-Cabral & Cabral, 2003). The symptoms include headache, fever, stiff neck, aphasia, lethargy, vomiting, behavioural changes, increased intra-cranial pressure, seizures and coma (Siddiqui & Khan, 2012a). These symptoms are due to necrotising lesions with severe encephalitis (Khan, 2006). There is no recommended treatment and the majority of cases are diagnosed post-mortem (Siddiqui & Khan, 2012a). The current treatment regimen for AGE involves a mixture of drugs to provide additive/synergistic effects and even so the mortality remains more than 90% (Siddiqui & Khan, 2012a). However, the existing drugs have limitations due to a high degree of toxicity associated with

deleterious side effects (Kulsoom *et al*., 2014). Combinations of drugs are being tested and have been shown to have effective amoebicidal effects. These include (i) prochlorperazine plus loperamide; (ii) prochlorperazine plus apomorphine; and (iii) procyclidine plus loperamide (Kulsoom *et al*., 2014).

Other common infections due to *Acanthamoebae* include cutaneous infections, which are characterised by nodules and skin ulcerations. In healthy individuals, this is self-limiting, but, in immunocompromised individuals, it may lead to severe and/or fatal consequences since *Acanthamoebae* may gain entry into the blood stream and spread to different tissues (Khan, 2006). Treatment includes different antifungal drugs, such asitraconazol (Khan, 2006).

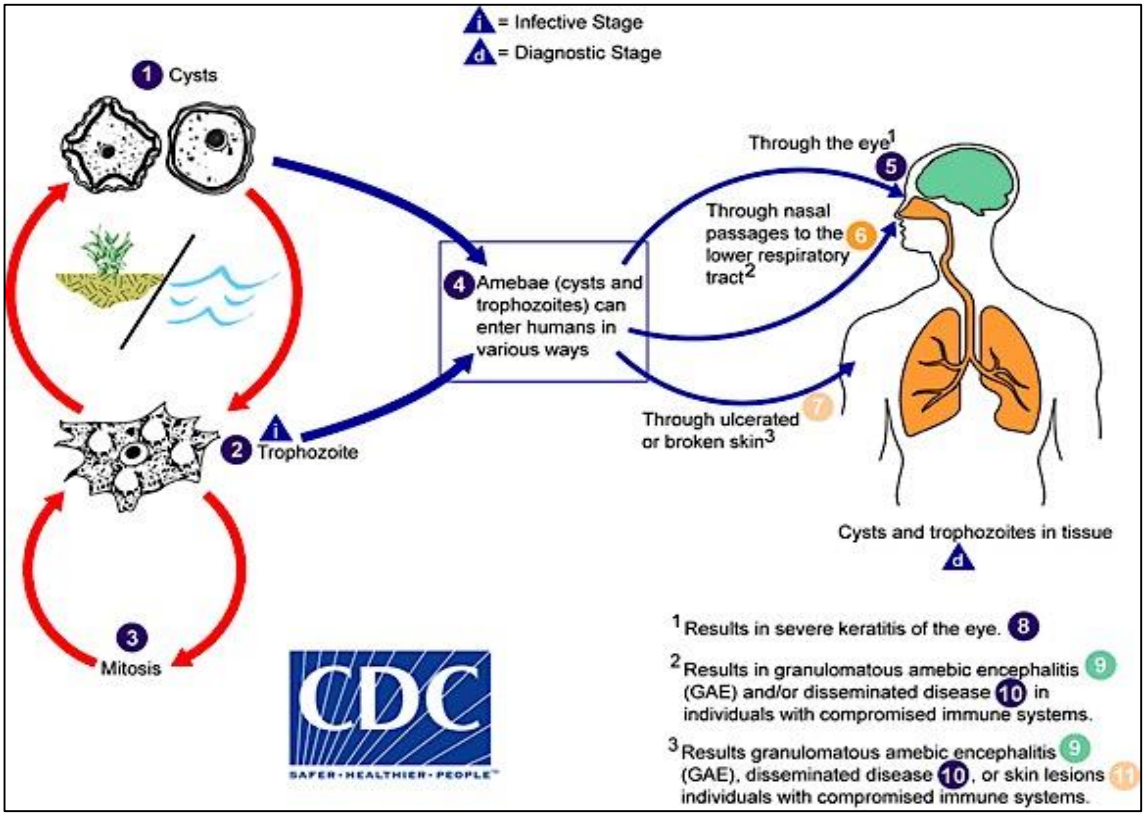


Figure 1.8. The life cycle of *Acanthamoebae* spp. in humans. *Acanthamoeba* presents two stages, cysts (1) and trophozoites (2), in its life cycle. The trophozoites replicate by mitosis (3). The trophozoites and cysts enter into the body (4) through various means. Entry can occur through the eye (5), the nasal passages to the lower respiratory tract (6), or ulcerated or broken skin (7). When *Acanthamoeba* spp. enters the eye it can cause severe AK (8). When it enters the respiratory system or through the skin, it can invade the CNS causing AGE (9), disseminated disease (10), or skin lesions (11) in individuals with compromised immune system (CDC, 2012).

1.11. *Acanthamoebae* interaction with microbial species

Historically, amoebae were known to consume microbes and to regulate bacterial populations in the environment, contributing to the functioning of ecosystems (Siddiqui & Khan, 2012a). Nowadays, an increasing number of microorganisms, such as bacteria and viruses, have been shown to benefit from the interaction with these free-living pathogens, as they play a role as a reservoir, allowing them to escape predation and potentially enabling them to survive or multiply inside their host (Thomas *et al*., 2010). Since they are highly resistant to physical and chemical stresses and may serve bacteria as their hosts, amoebae enable pathogenic bacteria to survive under conditions that would normally kill them (Thomas *et al*., 2010). *Acanthamoebae* can also act as "Trojan horses" for bacteria by facilitating their transmission and providing protection against the human immune system (Siddiqui & Khan, 2012a). Pathogenic microorganisms residing inside amoebae become more resistant to disinfectants, making it difficult to eradicate them from public water supplies (Winiecka-Krusnell & Linder, 2001). Moreover, interaction with *Acanthamoebae* can increase bacterial virulence and their resistance to antibiotics (Cirillo *et al*., 1994; Barker & Brown, 1995; Cirillo *et al*., 1997; Cirillo *et al*., 1999).

Different types of interaction between bacteria and protozoa have been described, with possible outcomes including intracellular survival, with or without multiplication, (leading to amoebic lysis in case of bacterial multiplication) or intracellular lysis of bacteria, followed by their digestion by amoebae (Thomas *et al*., 2010). The outcome of amoebal-bacteria interactions is dependent on the virulence properties of the amoebae and the bacteria, or the environmental conditions they encounter (Khan & Siddiqui, 2014). Because 22% of the *Acanthamoeba* isolates contain endosymbiont pathogenic microorganisms (Guimaraes *et al*., 2016), these associations are thus of great concern to human, animal and ecosystem health, especially since *Acanthamoeba* can co-exist in the

same environments as bacteria, e.g. in the water of industrial poultry houses, despite the stringent biosecurity measures (Bare *et al*., 2009; Vaerewijck *et al*., 2014).

Amoebae are able to harbour a wide variety of microorganisms, share remarkable similarities with macrophages (in cellular structure, molecular motility, biochemical physiology, ability to capture their prey by phagocytosis, and in the way they interact with microbial pathogens), and are easy to handle experimentally (Sandstrom *et al*., 2011; Ruqaiyyah & Naveed, 2011; Siddiqui & Khan 2012b; Tosetti *et al*., 2014; Guimaraes *et al*., 2016). Thus, this eukaryotic organism is an attractive and simple model of infection to study host-pathogen interactions *in vitro,* allowing the discovery of new bacterial virulence factors, which may assist in the development of new antibacterial therapeutic agents. In addition, through their capacity to resist digestion by amoebae, potential intracellular bacterial species are also likely to resist digestion by macrophages and thus represent new pathogenic species (Greub & Raoult, 2004).

1.11.1. Interaction with viruses, fungi and parasitic protozoa

The number of viruses known to survive and reside inside *Acanthamoebae* is increasing (Aherfi *et al*., 2016; Colson *et al*., 2017). For instance the human pathogenic enterovirus Coxsackie virus b3 (small RNA virus) was shown to survive intracellularly (IC) in *A. castelannii* and that the latter plays the role of a host and as a vehicle of transmission of this virus to humans (Mattana *et al*., 2006). The human pathogens adenoviruses, polioviruses and echoviruses were also shown to survive within *Acanthamoeba* and be transmitted by this host (Danes & Cerva, 1981; Scheid & Schwarzenberger, 2012). Recently identified genera of giant viruses (nucleocytoplasmic large DNA virus) that infect amoebae are *Mimiviruses* (mimicking microbes) and *Marseilleviruses*. These genera of viruses are able to survive and multiply within amoebae and the latter is considered to be its natural host (La Scola *et al*., 2003; Colson *et al.*, 2013). As giant

viruses were discovered using amoebal co-cultures, these have become the method of choice to hunt for these microorganisms (Colson *et al*., 2017). Mimivirus isolations also led to the discovery of a new type of virus, named virophages that cannot replicate alone in *Acanthamoeba* spp., but replicates in the presence of a mimiviral host (La Scola *et al*., 2008). To date, there are very little data available in regards to human infection by giant viruses, but Mimiviruses were associated with pneumonia and Marseille viruses were associated with adenitis and lymphoma, posing a great clinical concern (Colson *et al*., 2017).

Different types of fungi have also been reported to survive and multiply inside *Acanthamoebae* (Thomas *et al*., 2010). For instance, *Cryptococcus neoformans*, a soil fungus that causes life-threatening meningitis in immunocompromised patients, has been shown to multiply within *A. castellanii* leading to its lysis (Steenbergen *et al*., 2001). Other pathogenic fungi *Blastomyces dermatitidis*, *Sporothrix schenckii*, and *Histoplasma capsulatum* are also able to multiply within *A. castellanii* leading to its lysis (Steenbergen *et al*., 2004).

The obligate intracellular parasitic alveolate *Toxoplasma gondii*, that causes the disease toxoplasmosis, was shown to survive within *A. castellanii* and to be transmitted by this host in water environments (Winiecka-Krusnell *et al*., 2009).

1.11.2. Interaction with bacteria

After *Legionella pneumophila* was found to be able to survive and grow within amoebae (Rowbotham, 1980), an increasing interest in studying the interaction between pathogenic bacteria and amoebae was raised (Thomas *et al*., 2010). Although *Legionella* are the most established pathogenic bacteria that are able to survive and multiply intracellularly within *Acanthamoeba*e, many other pathogens have been identified as able to grow within this host (Thomas *et al*., 2010). An extensive review of

the literature listed 102 bacterial species capable of interacting with FLA, where the majority of the studies have used *A. polyphaga* and *A. castellanii* as a host (Thomas *et al*., 2010). In addition, it has also been reported that amoebae can harbour two different bacterial species and co-exist within separate IC compartments (Heinz *et al*., 2007). Table 1.1 presents a non-exhaustive list of some *Acanthamoebae*-bacteria interactions described. Importantly, it has been reported that some pathogenic bacteria are capable of survival and persistence within amoebic cysts, thus, enabling foodborne pathogens to survive physical and chemical cleaning and disinfection methods in food-related environments, increasing the risk of infection (Thomas *et al*., 2010; Lambrecht *et al*., 2015).

Several bacterial factors, especially in *Legionella pneumophila*, have been reported as crucial for the interaction with amoebae. Examples are the flagellin structural protein FliC and the secretion system apparatus protein SsaU that are required for invasion and survival of *Burkholderia pseudomallei* and *Salmonella typhimurium* within *Acanthamoeba*, respectively (Inglis *et al*., 2003; Bleasdale *et al*., 2009). The *Escherichia coli* and *Klebsiella pneumoniae* outer membrane protein OmpA was shown be required for survival of these pathogens within amoebae (Alsam *et al.*, 2006; March *et al*., 2013). The transcriptional regulators PhoB, PhoP and ToxR were shown to have secondary roles in the survival of *E. coli*, *S. typhimurium* and *Vibrio cholerae* within *Acanthamoebae*, respectively (Bleasdale *et al.*, 2009; Chekabab *et al.*, 2012; Valeru *et al*., 2012). The type III system from *E. coli* and *Pseudomonas aeruginosa* was also shown to be involved in this interaction (Abd *et al.*, 2008; Siddiqui *et al.* 2011). Capsule form *E. coli*, the intracellular protease Lon and the ribosome-binding GTPase TypA from *P. aeruginosa* are required for survival of both these pathogens within amoebae (Jung *et al*., 2007; Breidenstein *et al*., 2012; Neidig *et al.*, 2013). Flagellin FlaA, 3-dehydroquinated synthase AroB, ferrous iron transport FeoB, ankyrin AnkB, the outer

membrane efflux TolC, two-component regulator system signal sensor kinase PmrB, protease ClpP and the outer membrane OmpA are a few examples of *L. pneumophila* bacterial factors shown to be required for the interaction of this pathogen with amoebae (Dietrich *et al*., 2001; Polesky *et al*., 2001; Robey & Cianciotto, 2002; Al-Khodor *et al*., 2008; Ferhat *et al.,* 2009; Al-Khodor *et al.,* 2009; Li *et al*. 2010; Goodwin *et al.,* 2016). Despite great advances in the exploration of bacteria-amoebae interactions, other bacterial determinants involved in this interaction remain to be elucidated.

Table 1.1. Interactions described for different bacteria and *Acanthamoebae* species

Bacterial species	Interaction with *Acanthamoebae*	References
Acinetobacter baumannii	ICS; ICM; ICCS	Cateau *et al*., 2011
Aeromonas hydrophila	ICS; ICCS (not able to multiply IC)	Rahman *et al.*, 2008; Anacarso *et al*., 2012; Yousuf *et al*., 2013
Burkholderia cepacia	ICS; ICM*	Lamothe *et al*., 2004; Landers *et al.*, 2000
Burkholderia pseudomallei	ICS	Inglis *et al.*, 2000; 2003
Citrobacter freundii	ICS	King *et al*., 1988
Coxiella burnetii	ICS	La Scola & Raoult, 2001
Enterococcus faecalis	ICS; ICM	Anacarso *et al*., 2012
Enterobacter aerogenes	ICS; ICCS	Yousuf, *et al*., 2013)
Escherichia coli	ICS; ICCS: ICM	Alsam *et al.*, 2006; Jung *et al.*, 2007; Siddiqui *et al*., 2011; Matin & Jung, 2011; Chekabab *et al*., 2012; Lambrecht *et al*., 2015
Francisella tularensis	ICS; ICM; ICCS	Abd *et al*., 2003; El-Etr *et al.*, 2009
Helicobacter pylori	ICS	Winiecka-Krusnell *et al.*, 2002
Klebsiella pneumoniae	ICS	King *et al.*, 1988
Legionella pneumophila	ICS; ICM; ICCS	Thomas *et al.*, 2010; Tosetti *et al*.,2014; Gunderson *et al.*, 2015; Goodwin *et al*., 2016; Mengue *et al*., 2016
Listeria monocytogenes	Not able to ICS* and ICM*	Akya *et al*., 2009a; 2009b; Akya *et al*., 2010; Anacarso *et al.*, 2012; Doyscher *et al.*, 2013; Fieseler *et al.*, 2014
Mycobacterium avium	ICS; ICM; ICSS	Cirillo *et al*., 1997; Steinert *et al.*, 1998; Miltner & Bermudez, 2000; Mura *et al.*, 2006; Chan *et al.*, 2015
Mycobacterium smegmatis	ICS; ICCS	Taylor *et al*., 2003; Sharbati-Tehrani *et al*., 2005; Lamrabet *et al*., 2012
Mycobacterium bovis/marinum/tuberculosis	ICS; ICCS	Kennedy *et al*., 2012; Medie *et al.*, 2011
Pasteurella multocida	ICS; ICM	Hundt & Ruffolo, 2005
Pseudomonas aeruginosa	ICS; ICCS	Maschio *et al*., 2015; Siddiqui *et al*., 2015)
Salmonella typhimurium/enterica	ICS; ICM; ICCS	Gaze *et al.*, 2003; Tezcan-Merdol *et al*., 2004; Bleasdale *et al.*, 2009; Douesnard-Malo & Daigle, 2011; Lambrecht *et al.*, 2015
Shigella dysenteriae/sonnei	ICS; ICM	Jeong *et al*., 2007; Saeed *et al*., 2009
Staphylococcus aureus	ICS; ICM; ICCS	Anacarso *et al*., 2012; Cardas *et al.*, 2012
Streptococcus pneumoniae	ICS; ICM	Evstigneeva *et al*., 2009)
Vibrio cholerae	ICS; ICM;ICCS	Abd *et al.*, 2007; Abd *et al.*, 2009; Sandstrom *et al.*, 2010; van der Henst *et al*., 2016)
Yersinia pestis/enterocolitica/pseudo tuberculosis	ICS; ICM: ICCS	King *et al.*, 1988; Anacarso *et al.*, 2012; Lambrecht *et al.*, 2015; Santos-Montanez *et al.*, 2015

ICS, intracellular survival; ICM, intracellular multiplication; ICCS, intracellular cyst survival; *contradictory results; If ICM is not referred to this means it was not studied.

1.11.2.1. Interaction with *Campylobacter jejuni*

The results from some publications describing the interaction between *Acanthamoeba* and *C. jejuni* are contradictory. Whilst some of them suggest the ability of *C. jejuni* to survive and/or multiply within amoebae (Axelsson-Olsson *et al.,* 2005; Snelling *et al.,* 2005; Olsson *et al.,* 2007; Snelling *et al*., 2008; Axelsson-Olsson *et al.,* 2010a; 2010b; Griekspoor *et al.,* 2013; Olofsson *et al.,* 2013) others support only an extracellular mode of survival (Bare *et al.,* 2010; Bui *et al.,* 2012a; 2012b; Dirks & Quinlan, 2014). These conflicting results may be explained by variation in experimental strains and conditions (Backert & Hofreuter, 2013). For instance, invasiveness of *C. jejuni* is both bacterial strain (due to variability of the structures of lipooligosaccharides and capsular polysaccharides) and host cell line dependent (Poly *et al.,* 2007; Backert & Hofreuter, 2013). The bacterial multiplicity of infection (MOI) is another factor that may influence the efficiency of infection (Backert & Hofreuter, 2013). The absence or presence of gentamicin to kill the extracellular bacteria is also a crucial factor for the outcome of the *Acanthamoebae* infection by bacteria (Backert & Hofreuter, 2013). Recently, a critical analysis of these data was carried out by the author and colleagues, which discussed possible reasons for the conflicting results. In this review, the *Campylobacter* factors, which may be involved in the interaction of this pathogen with amoebae and other host cells were explored (Vieira *et al*., 2015). Although *Campylobacter-Acanthamoebae* interactions are described in detail in the review written by Vieira *et al*., 2015 that is attached to the Appendix section, a brief description of the studies about this interaction is given below.

The survival of *C. jejuni* within both *A. polyphaga* and *A. castellanii* species was demonstrated in some studies, but, despite the fact that they did not use gentamicin to kill extracellular bacteria, the experimental data were supported by the use of microscopy where *C. jejuni* cells could be seen inside amoebae (Snelling *et al.,* 2005; Axelsson-

Olsson *et al.*, 2005; Griekspoor *et al.*, 2013; Olofsson *et al*., 2013). Massive invasion of *A. polyphaga* by *C. jejuni* was reported, a process shown to be dependent on bacterial viability, and that the bacteria may escape degradation by avoiding localisation in amoebae lysosomal vacuoles and instead, reside within non-digestive vacuoles (Olofsson *et al*., 2013). Unfortunately, some of the studies that reported IC survival not only did not use a gentamicin stage, but, also, did not provide any supporting visual evidence (Olsson *et al.*, 2007; Axelsson-Olsson *et al.*, 2010a). Importantly, amoebae were shown to protect *C. jejuni* against harsh environmental conditions, such as low pH, a condition encountered in the human or chicken stomach, which may possibly help *C. jejuni* survival and passage through the gastrointestinal tract (Axelsson-Olsson *et al*., 2010b). In addition, *C. jejuni* within *A. castellanii* was able to colonise broilers and was resistant to chlorination of the drinking water of chickens (King *et al.* 1988; Snelling *et al*., 2008). This is important as *C. jejuni* and protozoa co-exist in the broilers drinking water and thus, increase the risk of infection of this host (Snelling *et al*., 2008). Although one study reported on the extracellular (EC) mode of survival only, it was observed that *C. jejuni* cells started to lose their viability inside *A. castellanii* after five hours (Bare *et al*., 2010). However, even if *Campylobacter* was able to reside within amoeba for a short time period, this may be of epidemiological importance as this could still represent a sufficient period of time for this eukaryotic organism to be a source of transmission of this pathogen.

The first reports that used gentamicin in their experiments to elucidate this interaction observed a decrease in the viability of *C. jejuni* inside *A. castellanii* after a few hours and that multiplication of this foodborne pathogen occurs EC only (Bui *et al.*, 2012a; Bui *et al.*, 2012b; Dirks & Quinlan, 2014).

In general, it is clear that *Campylobacter* survival is increased in the presence of *Acanthamoebae*, but that there is a disagreement on whether it occurs intra- or

extracellularly. The enhanced survival of *C. jejuni* in the presence of *Acanthamoeba* was due to the uptake of oxygen by amoebae, creating the necessary microaerobic conditions necessary to support *C. jejuni* growth (Bui *et al*., 2012b). The type of interaction between *C. jejuni* and *A. polyphaga* and the molecular mechanisms (e.g. bacterial factors) involved in this interaction still need to be elucidated.

1.12. Aims and objectives

The association between foodborne pathogens and protozoa leads to serious consequences in food safety, increasing the risk of infection (Vaerewijck *et al*., 2014). It is suggested, therefore, that the deciphering of the molecular mechanisms of *Campylobacter*-amoebae interaction will assist in a better understanding of *Campylobacter* lifestyle aiding in the development of novel intervention strategies.

The main aim of this study was to explore the type of interaction between *C. jejuni* and *A. polyphaga* and to identify the bacterial factors involved in this interaction. A second aim of this study was to study antibiotic resistance mechanisms in *C. jejuni*.

Therefore the objectives of this study were to (i) elucidate the type of interaction between *Campylobacter jejuni* and *Acanthamoeba polyphaga;* (ii) investigate if the CmeB multidrug efflux transporter and capsule production were involved in the interaction between *A. polyphaga* and *C. jejuni*; (iii) investigate if CmeB was involved in biofilm formation, motility and oxidative stress; and (iv) investigate the contribution of amino acid sequence variation of the *cmeB t*o tetracycline resistance of *C. jejuni*.

CHAPTER 2: Material and Methods

2.1. General methods

2.1.1. Bacterial strains and plasmids

Four main strains of *C. jejuni* were used in this study: 81-176 a highly virulent strain isolated from a girl infected in a raw milk outbreak (Hofreuter *et al*., 2006); 11168H, a hyper motile variant of the reference strain NTCT11168 (Karlyshev *et al*., 2002); G1, a strain isolated from a Guillan-Barré syndrome patient (Gregson *et al*., 1997) and X, a strain isolated from a patient with enteritis (Karlyshev & Wren, 2001). A chimeric *C. jejuni* strain B7 was created naturally by growing *C. jejuni* 81-176 and G1 strains together. *C. jejuni* 81-176-pCPE107/28/GFP strain was kindly provided by Dr. Patricia Guerry from the Naval Medical Research Centre. The plasmids used in this study are listed in Table 2.1.

Table 2.1. Plasmids used for the generation of *C. jejuni* mutant and derivative strains

Plasmids	Description	Source/Reference
pGEM-T easy	Cloning vector to construct *C. jejuni* mutants	Promega
pJMK30	Source of *kan*^{r} cassette to construct *C. jejuni* mutants	(van Vliet *et al.*, 1998)
pRRC	Vector with a fragment of rRNA gene cluster and a *cam*^{r} cassette used as a control to check efficiency of competent cells	(Karlyshev & Wren, 2005)
pRED1	pRRC vector with *egfp* gene used for complementation of *C. jejuni* mutants via insertion of a *cam*^{r} cassette into rRNA gene cluster	(Karlyshev & Wren, 2005)
pCPE107/28/GFP	GFP plasmid kindly provided by Dr. Guerry	(Ewing *et al*., 2009)

2.1.2. Bacterial media and growth conditions

Laboratory stocks were stored at -80°C in Mueller-Hinton (MH, Fluka) supplemented with 15% glycerol, prepared originally from two day culture. *Campylobacter jejuni* strains were recovered from -80°C by incubation in microaerobic conditions (5% O_2, 10% CO_2 in N_2) within a controlled atmosphere incubator (Don Whitley) on Columbia Blood Agar (CBA, Oxoid) supplemented with 5% defibrinated horse blood and *Campylobacter* selective supplement (Oxoid) at 37°C for 24 hours.

E. coli cultures were grown at 37°C on Luria Bertani (LB) media (Oxoid). Stocks of *E. coli* were stored at -80°C in LB broth with 15% glycerol.

When necessary, the media were supplemented with the appropriate antibiotics, ampicillin (100μg/ml), kanamycin (50μg/ml), tetracycline (10μg/ml) or chloramphenicol (10μg/ml).

For liquid cultures, *C. jejuni* was suspended in Brain Heart Infusion (BHI, Oxoid) broth and adjusted to an OD_{600nm} of 1. The bacterial suspension was diluted 100-fold in BHI broth in sterile conical flasks and incubated with shaking (200 rpm) at 37°C microaerobically for two days. One ml samples of each bacterial culture were taken at each time-point (0, 6, 24, 30 and 48h) and the OD_{600nm} was measured.

All culture media made in the laboratory were autoclaved at 121°C for 15 minutes prior to use.

2.1.3. Cell morphology and motility

For Gram staining, *C. jejuni* samples grown on CBA agar for 24h hours at 37°C were heated and fixed onto glass slides and stained using crystal violet (CV), iodine, 90% ethanol and carbol fuchsin. Slides were visualised using light microscopy with 100x oil immersion objective lens to check the morphology of the bacterial cells (Nikon Eclipse 80i).

Motility of *C. jejuni* was determined as previously described with a few modifications (Baldvinsson *et al*., 2014). *C. jejuni* bacteria were grown on CBA blood agar plates at 37°C for 24 hours, suspended in BHI broth and adjusted to an OD_{600nm} of 0.5, after which 1µl aliquots of the bacterial suspension were spotted onto 0.4% BHI soft-agar plates. The low density of the agar allows the bacteria to move within the agar, forming a halo of growth around the point of inoculation. Plates were incubated for three days at 37°C in microaerobic conditions.

2.2. Molecular and cloning techniques

2.2.1. DNA isolation

Plasmid DNA was isolated using the Qiagen plasmid purification Kit (Qiagen) and chromosomal DNA were isolated by the Gentra Puregene Yeast/Bacteria Kit (Qiagen). The kits were used according to the manufacturer's protocol.

DNA samples were purified using two different methods: the QIAquick PCR purification Kit (Qiagen) used to clean DNA samples after restriction digestion and PCR products, and the QIAquick Gel Extraction Kit (Qiagen) used for the isolation of specific fragments of DNA derived from restriction digested reactions. The 1% (w/v) agarose gel segment with the fragment of interest was cut using a scalpel and a long wavelength UV source.

2.2.2. Polymerase chain reaction (PCR) and agarose gel electrophoresis

Each of the PCR reactions contained the following constituents: GoTaq Hot Start G2 Green Master Mix (Promega); appropriate reverse and forward primers (10µM); nuclease-free water and the DNA sample. For the High Fidelity PCRs, used to amplify highly accurate DNA fragments, the following constituents were used: Q5 reaction buffer; Q5 DNA polymerase; appropriate primers; dNTPs (10mM); nuclease-free water

and the DNA sample. DNA samples were either bacterial genomic DNA or bacterial lysates. Bacteria lysates were obtained by suspending bacteria in 10µl of lysis buffer (1mg/ml lysozyme; 10mM EDTA; 0.02% Triton and 10mM Tris pH 8.0), followed by heating at 95ºC for 10 sec. Lysates were then diluted in 50µl of Tris-EDTA (TE). The cycler conditions were performed in accordance to the manufacturer's protocol. Thermal cycler conditions for the DNA polymerases used are listed in Tables 2.2 and 2.3. Primers used to check identity of the *C. jejuni* strains are listed in Table 2.4.

To visualise PCR products, plasmids and DNA fragments, the samples were run on 1% (w/v) agarose gels, containing 1g of agarose (Sigma), 100ml 1x Tris-Borate EDTA Buffer (TBE) (Fisher) and 0.1µg/µl ethidium bromide (Fisher) for visualisation. Samples were directly loaded into the gel when amplified with Go Taq G2 Green Master Mix or supplemented with bromophenol blue based loading buffer (NEB) when amplified with Q5 Reaction PCR mix. Quick-Load 2-Log DNA Ladder (NEB) was used as a standard to estimate the DNA fragment sizes. Gel electrophoresis was conducted in a horizontal gel tank (Fisher) containing 1x TBE buffer. The gel was run at 150V for one hour and visualised with the trans illuminator setting in the G:Box (Syngene) using GeneSnap software (Syngene).

Table 2.2. Thermal cycler conditions for GoTaq Hot Start G2 DNA Polymerase

PCR step	Temperature (ºC)	Duration of step	Number of cycles
Initial denaturation	95ºC	2 min	1
Denaturation	95ºC	30 sec	
Annealing	55ºC	30 sec	25
Extension	72ºC	1 min per kb	
Final extension	72ºC	5 min	1

Table 2.3. Thermal cycler conditions for Q5 High-Fidelity DNA Polymerase

PCR step	Temperature (ºC)	Duration of step	Number of cycles
Initial denaturation	98ºC	30 sec	1
Denaturation	98ºC	10 sec	
Annealing	60ºC	30 sec	35
Extension	72ºC	30 sec per kb	
Final extension	72ºC	2 min	1

Table 2.4. Primers used for identification of *C. jejuni* strains

Gene Name	Primer Name	Primer sequence (5' to 3')
dmhA	DmhA_for	ATGAAAAAAACAGCGTTAATTACAGGATTTACAG
	DmhA_rev	GAATTCTGCCTCTCTTAATTTCATTTCTCCAATG
tag	Tag_for	GCTCTTGAAAACATGAGAAGATATCAAGAAGCGG
	Tag_rev	AAGATTTTTATCCTTTAGAATATCTTCAGATAAC
cj1435	Cj1435_for	GCCTTATTTGATTTTTGTGAAACTTTAAC
	Cj1435_rev	GAGCTAATTTGTAAAGTTTTCTTTCTTGC
moaA	MoaA_for	GACTAGCGAATTGAAAAGAAATATAACTTCATTTAC
	MoaA_rev	CTAATAATTTATATGGCTGAGAACATTGAAATTTC
tet(O)	pTet_for	GGCGTTTTGTTTATGTGCG
	pTet_rev	ATGGACAACCCGACAGAAGC
virB11	pVir_for	GAACAGGAAGTGGAAAAACTAGC
	pVir_rev	TTCCGCATTGGGCTATATG
	LuxS-81-176	GAAAACACCTAAGGGTGATGATATTAGT
luxS	LuxS-G1	TAAGGGTGATGATATTAGCGTG
	LuxS-Rev	GTAAATCAAGTATAGGTAAGTTCATTTTTG

2.2.3. Restriction digestion and other enzymatic reactions

The restriction mixtures were prepared according to the manufacturer's protocol, which included an appropriate NEB buffer at 1x concentration, a respective restriction enzyme, a DNA sample and nuclease-free water. The digestion reactions were incubated for one hour and the temperature was dependent on the type of restriction enzyme used.

Antarctic phosphatase (NEB) was used for dephosphorylating vectors after restriction digestion, by the removal of the 5' phosphate from DNA to prevent re-circularisation, which creates background during cloning. Antarctic phosphatese buffer at 1x

concentration and Antarctic phosphatase enzyme were added to the digestion reaction and incubated at 37ºC for 30 minutes.

T4 DNA Ligase (Promega) was used to ligate the fragment of interest to a vector in order to create a recombinant vector. The ligation reaction contained T4 DNA ligase buffer (1X), T4 DNA ligase, nuclease-free water and a DNA sample and was incubated at 4ºC overnight.

T4 DNA polymerase (NEB) catalyses the synthesis of DNA in the 5' – 3' direction and removes 3' overhangs to form blunt ends with dNTPs. The T4 polymerase, T4 DNA polymerase buffer (NEB) and dNTPs were added to the DNA sample and incubated for 30 minutes at 37ºC.

CloneChecker kit (Invitrogen) was used to rapidly analyse and screen recombinant bacterial cultures for target plasmids, according to the manufacturer's protocol.

2.2.4. Transformation of *E. coli* and *C. jejuni* cells

NEB Express Competent *E. coli* (High Efficiency) (NEB) and *E. coli* XL1 Blue Super competent cells (Stratagene) were used according to the manufacturer's protocol. For transformation, 50µl aliquots of *E. coli* competent cells were inoculated with 5µl of the ligation mixture, followed by heat shock at 42ºC. Recovery of the cells in Super Optimal Broth (SOC, Fisher) was performed for one hour at 37ºC with shaking. *E. coli* transformation mixtures were plated in LB agar supplemented with an appropriate antibiotic.

To prepare *C. jejuni* competent cells, bacteria were grown as a lawn on CBA blood agar overnight at 37ºC under microaerobic conditions. The cells were harvested in 1ml of MH broth and pelleted by centrifugation at 10000xg for five minutes at 4ºC. The pellet was suspended in 1ml of cold wash buffer (272mM sucrose, 15% glycerol) and centrifuged in the same conditions. This procedure was repeated three times before cells were

suspended in 500µl of wash buffer. The competent cells were immediately stored at -80ºC in 50µl aliquots.

For electroporation, 2µg of DNA was added to 50µl competent cells and then transferred to 0.2cm electroporation cuvettes (Thermo Scientific). Electroporation was conducted at 2.5kV, 200Ω and 25µF, using a BioRad MicroPulser electroporation apparatus (BioRad). After electroporation, cells were flushed with 100µl of SOC, spread onto a CBA blood agar plate and incubated overnight at 37ºC in microaerobic conditions for recovery. The following day, cells were plated onto a CBA blood agar plate with the respective selective antibiotic for three days at 37ºC microaerobic conditions.

2.2.5. Generation of *kpsM* mutants

The genomic DNA of *C. jejuni* strain 11168H/*kpsM*::*kan*^{r} (Karlyshev *et al*., 2000), available in the laboratory -80ºC collection, was isolated using the Gentra Puregene Yeast/Bacteria Kit and electroporated into *C. jejuni* 81-176 or G1. The electroporation mixture was plated onto CBA agar supplemented with kanamycin (50µg/mL) and allelic replacement of the clonal isolates was confirmed by PCR using gene-specific primers (Table 2.5).

Table 2.5. Primers used for confirmation of *C. jejuni kpsM* mutants

Primer name	Sequence (5' – 3')
ak55-Fw	CCCCATCAAACCTATGCTAC
ak59-Rev	GCCTATAAACCTGTAAAGCCTATAC

2.2.6. Generation of *cmeB* mutants

Inactivation of the *C. jejuni* 81-176 gene *cmeB* was achieved by an insertional mutagenesis approach (Karlyshev & Wren, 2001) where *cmeB* was disrupted by insertion of a kanamycin resistance cassette (*kan*^{r}). Primers CmeB-Fw and CmeB-Rev (Table 2.6) were used to amplify the 3kb fragments containing the *cmeB* gene from *C. jejuni* strains 81-176, 11168H and G1 chromosomal DNA. GoTaq Hot Start G2 DNA Polymerase Master Mix (one cycle) was used for the creation of A' overhands in the HF Q5 PCR products, which were then purified with the PCR purification Kit and subsequently cloned into pGEM-T Easy vector (Promega). The ligation mixture was transformed into NEB Express *E. coli* and recombinant plasmids were selected using the CloneChecker kit The kanamycin cassette was isolated from vector pJMK30 by digestion with *Sma*I enzyme (1.5 kb), followed by gel extraction of the 1.5 kb fragment containing the *kan*^{r} cassette. To create blunt ends the plasmid pGEM-T Easy/*cmeB* was digested with *Sma*I and then it was ligated with the *kan*^{r} fragment using T4 DNA ligase. After transformation of *E. coli* with the ligation mixture, the pGEM-T Easy/*cmeB*::*kan*^{r} plasmid containing the insert of interest in the correct orientation was isolated. The pGEM-T Easy/*cmeB*::*kan*^{r} recombinant plasmid was transformed into *C. jejuni* via electroporation and transformants were selected on CBA agar supplemented with kanamycin (50μg/mL). The *C. jejuni cmeB*::*kan*^{r} mutants were confirmed by PCR analysis.

Table 2.6. Primers used for generation of *C. jejuni cmeB* mutants

Primer name	Sequence (5' – 3')
CmeB-Fw	AAGGAGATATACCATGTTTTCTAAATTTTTTATAGAAAGACCTATTTTTG
CmeB-Rev	TCATTCATGAATCTTACCTCTTTTTTTATCTAGC

2.2.7. Complementation of the *cmeB* mutants

Initially complementation of *cmeB* mutant strain was attempted by an insertional system for gene delivery and expression in *Campylobacter jejuni* (Karlyshev & Wren, 2005). Briefly, this consists of a pRRC vector containing a *cam*^{r} cassette that has complementary flanking regions with three rRNA gene clusters, allowing the insertion of the construct into the chromosome by homologous recombination, where the gene of interest would be under the control of *cam*^{r} (Karlyshev & Wren, 2005). The *cmeB* fragment was amplified by a high fidelity PCR using the complementation primers (Table 2.7, 1) and digested with *Xba*I enzyme for further ligation to the pRRC plasmid. The vector was also digested with *Xba*I and dephosphorylated after digestion. The ligation mixture was then transformed into *E. coli* and CloneChecker using *ClaI* enzyme was conducted to check the orientation of the *cmeB* fragment into the pRRC plasmid, for further extraction and purification of the recombinant plasmid and transformation into *C. jejuni cmeB* mutant. Due to failure in cloning the *cmeB* fragment into pRRC in the correct orientation, the pRED1 plasmid (pRRC containing an *egfp* gene) was used for selection of the clones lacking fluorescence, following a replacement of the *egfp* gene (Karlyshev & Wren, 2005). The *cmeB* fragment was amplified by a high fidelity polymerase using the new complementation primers (Table 2.7, 2) and double digested with *Eco53*KI/*Xba*I enzymes. The pRED1 plasmid was double digested with *Swa*I/*Xba*I enzymes (that allowed for the *egfp* replacement for further cloning of *cmeB* in the plasmid), and dephosphorylated. The digested *cmeB* and pRED1 products were ligated with T4 DNA ligase and the mixture was then transformed into *E. coli*, for further selection of non-fluorescent clones. CloneChecker using *Cla*I enzyme was used to check the orientation of the *cmeB*-containing fragment in the pRRC plasmid, for further extraction and purification of this recombinant plasmid and transformation into *C. jejuni cmeB* mutant.

The insertion of the *cam*^{r} cassette into the rRNA clusters was confirmed by PCR using Ak233/234/235/237 primers (Table 2.7).

Table 2.7. Primers used for generation of *C. jejuni* complementation derivatives

Primer name	Sequence (5' – 3')
CmeB-compl-Fw-1	AATATCTAGAAGGAGAATTCTCATGTTTTCTAAATTTTTTATAGAAAGACC
CmeB-compl-Rv-1	TATTTCTAGATTATTCATGAATCTTACCTCTTTTTTTATCTAGCCATTC
CmeB-compl-Fw-2	GAGTAAATAGAGCTCATGTTTTCTAAATTTTTTATAGAAAGACC
CmeB-compl-Rv-2	GAAATTATTTCTAGATTATTCATGAATCTTACCTCTTTTTTTATC
AK233	GCAAGAGTTTTGCTTATGTTAGCAC
AK234	GAAATGGGCAGAGTGTATTCTCCG
AK235	GTGCGGATAATGTTGTTTCTG
AK237	TCCTGAACTCTTCATGTCGATTG

2.2.8. Generation of G1/pTet derivatives

To generate the *C. jejuni* G1/pTet strain, the pTet plasmid from *C. jejuni* 81-176 was transferred to *C. jejuni* G1 by conjugation. *C. jejuni* 81-176 and G1 bacteria were grown on CBA blood agar plates at 37°C for 24 hours suspended in MH broth and adjusted to an OD_{600nm} of 1. A mixture of 81-176 donor strain with G1 recipient strain was plated on top of a 0.22μm sterile filter membrane (Millipore), placed on the surface of a CBA agar plate, and incubated overnight at 37ºC under microaerobic conditions. The bacterial culture was collected from the membrane and plated onto CBA agar supplemented with tetracycline (15μg/mL) and ampicillin (5μg/mL), the latter to prevent the growth of *C. jejuni* 81-176 strain. PCR was conducted to confirm the derivative strain using the pTet primers described in Table 2.4.

2.3. Bioinformatics tools

To verify that there were no mutations in the target genes and recombinant plasmids, these were sent for sequencing to Genewiz, the former Beckman Coulter Genomics. Nucleotide sequencing results from Sanger DNA Sequencing (Genewiz) were analysed as chromatograms and in FASTA format using the Chromas Lite program. Sequences of genes and genomes were obtained from the National Centre for Biotechnology (NCBI) database or from the CLC Genomics Workbench software (Qiagen bioinformatics). Gene and genome region analysis was conducted using BLAST and the cladogram was constructed using multiple sequence alignment in the Clustal Omega program (EMBL-EBI). NEBcutter V2.0 tool was used for restriction mapping in order to ensure the correct orientation of the gene fragment in the vector. The NEB digest finder online tool was used to determine restriction digest reactions with two different enzymes that required different reaction conditions. Primers were designed using the OligoCalc online tool and were synthesised by Sigma Genosys, UK. It should be noted that, although I was involved in the project, Professor Karlyshev and his co-workers conducted the genome sequencing of the *C. jejuni* G1 strain (Lehri *et al.,* 2015) and that is the reason why the genome-sequencing methodology is not referred to in this thesis. Professor Karlyshev also conducted the genome sequencing of the chimeric *C. jejuni* strain B7.

2.4. Biofilm assays

2.4.1. Non-attached aggregates in culture flasks

The ability of *C. jejuni* to form non-attached aggregates in liquid culture (flocs) was performed as described previously (Joshua *et al*., 2006). Briefly, *C. jejuni* was grown in CBA blood agar plates at 37°C for 24h and suspended in MH broth at an OD_{600nm} = 1. In 25cm^2 culture flasks, 100µl of the bacterial suspension was added to 5mL of MH broth and the flasks were incubated at 37°C under microaerobic conditions with 50 rpm

shaking for three days. Flasks were visualised using the upper light setting in a G:Box machine (Syngene).

2.4.2. Attached biofilms in glass tubes

Ability of *C. jejuni* to form attached biofilms was performed as described previously (Joshua *et al*., 2006). *C. jejuni* was grown in CBA blood agar plates at 37°C for 24h, suspended in BHI broth and adjusted to an OD_{600nm} of 0.5. The bacterial suspension (1ml) was transferred to borosilicate glass tubes and incubated statically at 37°C for four days in microaerobic conditions, after which an attached pellicle in the gas-liquid interface was observed. For crystal violet staining, the glass tubes were washed twice with distilled water and let dry at 85°C for 30 minutes. Then, 1ml of 0.5% crystal-violet solution was added to the tubes and incubated at room temperature for 30 minutes with gentle shaking. Tubes were washed with distilled water and let dry at 85°C for 30 minutes. Lastly, 1ml of 80% ethanol/20% acetone mixture was added for 15 minutes to dissolve the CV stain. Samples were transferred to 96-well plates in triplicate and the OD_{595nm} was measured using an Infinite 200 PRO plate reader (Tecan).

2.5. Oxidative stress resistance assay

C. jejuni was grown in CBA blood agar plates at 37°C for 24h, suspended in MH broth and adjusted to an OD_{600nm} of 1. Aliquots of the bacterial suspension (100µl) were spread onto MH agar plates and a sterilised blank paper disc was placed at the centre of the plate. A 10µl aliquot of 100mM hydrogen peroxidase 30% solution (Sigma) was dropped onto the disc. The plates were incubated for 48h at 37°C microaerobically and the inhibition zone halos were posteriorly measured (mm).

2.6. Antibiotic susceptibility assays

2.6.1. Antibiotic disc diffusion assay

Antibiotic disc susceptibility of *C. jejuni* strains was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (Sifre *et al*., 2015). *C. jejuni* was grown in CBA blood agar plates at 37°C for 24h and suspended in 1ml of MH at an OD_{600nm} of 0.5. The suspension (100µl) was spread onto MH agar supplemented with 5% lysed horse blood plates using a spreader. Antibiotic discs (Oxoid) were placed at the surface of the agar plates using sterile tweezers and plates were incubated for two days at 37°C under microaerobic conditions. Inhibition zone diameters were measured and interpreted accordingly to EUCAST zone diameter breakpoint (mm). For tetracycline 30µg disc sensitive (S) ≥30 and resistant (R) <30 (Sifre *et al*., 2015).

2.6.2. Microdilution broth assay

The minimal inhibitory concentrations (MIC) were determined according to EUCAST recommendations (Sifre *et al*., 2015). The MIC of tetracycline (Sigma) for *C. jejuni* 81-176 strains was determined by a micro dilution broth method, using MH-F broth (cation-adjusted MH broth, 5% lysed blood, 20mg/L β-NAD). Briefly, 10µl of *C. jejuni* suspension in MH-F (OD_{600nm}=0.5) was added to 90µl of two-fold dilutions of tetracycline in MH-F. Suspensions were transferred to a 96-well flat-bottomed microtiter plate (Corning) and incubated for two days at 37ºC under microaerobic conditions with shaking at 100rpm. The tetracycline concentration range tested was from 0.03µg/ml to 500µg/ml, and control wells with no tetracycline were included. MICs were measured at OD_{600nm} using an Infinite 200 PRO plate reader (Tecan). According to EUCAST, the tetracycline MIC breakpoint for *C. jejuni* is (S) for ≤2 and (R) >2 (Sifre *et al*., 2015).

2.7. *In vitro* co-culture assays

2.7.1. *Acanthamoeba polyphaga* strain and culture conditions

Acanthamoeba polyphaga (Linc Ap-1) was used in all experiments and was kindly provided by Dr. Bernard de La Scola, University de La Mediterranee in France. *A. polyphaga* was maintained in peptone yeast glucose (PYG) medium (1L: 20g protease peptone, 18g glucose, 1g yeast extract, 1g $MgSO_4$ x $7H_2O$, 1g Na Citrate x $2H_2O$, 0.02g $Fe(NH4)_2(SO4)_2$ x $6H_2O$, 0.06g $CaCl_2$, 0.14g H_2PO_4, 0.35g Na_2HPO_4 x 7H2O; 0.22μm filter sterilised; pH 6.8), aerobically at 25ºC in $75cm^2$ treated culture flasks (Thermo Scientific™). The concentration and viability of amoebae were determined by Trypan blue exclusion assay using 0.2% Trypan Blue (GE Hyclone) and haemocytometers (Immune Systems). To determine the viability of *Acanthamoeba* at different temperatures (25°C, 37°C and 42°C), *Acanthamoeba* was incubated in $50cm^2$ cell culture flasks (Thermo Scientific) in PYG medium for two, four and 24 hours. Amoebae cells were visualised by phase contrast microscopy with a 40x objective in an inverted cell culture microscope (Motic AE31).

2.7.2. Intracellular survival and multiplication assay

Co-culturing of *C. jejuni* with attached monolayers of *A. polyphaga* cells was conducted in 24-well plates. *A. polyphaga* cells were seeded at a density of 10^6 amoebae per ml in PYG medium and incubated at 25°C for two hours to allow the cells to settle and form a monolayer at the bottom of the well. Bacterial cells were harvested from overnight CBA blood agar plates, suspended in PYG medium and adjusted to OD_{600nm}= 1. Then, 100μl of bacterial suspension was added to the wells with *A. polyphaga* achieving an MOI ranging from 100 to 400 bacteria per well. To allow for invasion to occur, co-cultures were incubated under aerobic conditions for two hours at 25ºC and 37ºC. Following co-

incubation, wells were washed once with PYG medium and incubated aerobically with 100μg/mL of gentamicin (Gibco) (this concentration was shown to be effective at fully eliminating extracellular bacterial cells in one hour). Following gentamicin treatment, the wells were washed three times with PBS and the amoebal cells were lysed with 0.1% (v/v) Triton X-100 (Sigma) for 10 minutes at room temperature, releasing the intracellular *C. jejuni*. Samples were serially diluted in PBS 1X (Fisher BioReagent™) and plated onto CBA blood agar plates in duplicate, followed by two days incubation at 37ºC in microaerobic conditions. For the longer incubation time-points (24, 48 and 72h), the wells were incubated with PYG medium without gentamicin. In the designated time-points, prior to the addition of Triton 0.1%, the respective wells were re-incubated with gentamicin (100μg/ml) for one hour (modification of the standard gentamicin protection assay). Cells were washed three additional times with PBS 1X, lysed, diluted and plated in the same conditions as described above. To calculate the number of intracellular bacteria, the following formula was used: recovered *C. jejuni* (c.f.u) / total *C. jejuni* (c.f.u) x 100 = % of intracellular *C. jejuni* in *A. polyphaga*.

2.7.3. Triton X-100 0.1% (v/v) resistance assay

To test the sensitivity of *C. jejuni* 81-176 *cmeB* mutant strain to Triton X-100 0.1% (v/v), bacterial cells were harvested from overnight CBA blood agar plates suspended in BHI medium and adjusted to OD_{600nm}=1 (initial inoculum). A 100μl aliquot of the initial suspensions of *C. jejuni* 81-176 wt, *cmeB* mutant and complement strains were serial diluted in PBS 1X and plated in CBA agar plates for the control c.f.u counts. The remaining initial suspension was then centrifuged for two minutes at 10.000xg speed and 1ml of Triton X-100 0.1% (v/v) was added and cells were incubated for 15 minutes at RT. Bacterial suspensions in Triton detergent were serial diluted in PBS 1X and plated in

CBA blood agar plates. The c.f.u obtained after the detergent treatment were normalised with the c.f.u obtained in the initial inoculum plating. This assay was kindly conducted by my co-worker, Amiritha Ramesh.

2.7.4. Extracellular survival assay

To test extracellular survival of *C. jejuni* in the presence of amoebae, 100µl of bacterial inoculum prepared as described above was added to each of the 24 wells containing 1ml of 10^6 amoebae cells or 1ml of PYG medium only. The wells were incubated over a period of six days aerobically at 25°C. At each time-point, the culture medium was diluted in PBS, plated in CBA blood agar plates and incubated for 48 hours at 37ºC under microaerobic conditions for further bacterial c.f.u counts.

2.8. Statistical analysis

All experiments were repeated three times (biological replicates) with three technical replicates in each experiment, except where otherwise indicated, and the data were expressed as mean ± SD. Comparison of two groups was made with an unpaired, two-tailed Student's t-test. Mean differences were considered statistically non-significant (ns) when p values were above 0.05. For statistically significant differences: * for $0.01<p\leq0.05$, ** for $0.001<p\leq0.01$ and *** for $p\leq0.001$. ND stands for not detected.

CHAPTER 3: Results

Part I

3.1. Identification and characterisation of *Campylobacter jejuni* strains

3.1.1. Overview

As noted earlier, different strains of *Campylobacter jejuni* display distinct structures of lipooligosaccharides (LOS), capsule and flagellum or the presence/absence of the virulence plasmid pVir, a result of high genetic variation, which leads to different capabilities to adhere/invade, survive or colonise various hosts (Young *et al.*, 2007). In this part I of the results chapter the different *C. jejuni* strains used in this study are described along with how a chimeric strain of *C. jejuni* was created.

3.1.2. Identification of *C. jejuni* strains

The initial step in this study was to review the range of *C. jejuni* wild-type (wt) strains present in the laboratory -80°C bacterial collection to select the *C. jejuni* strain that was going to be used for the *in vitro* assays using *Acanthamoeba polyphaga*. *C. jejuni* strains 11168H, 81-176, G1 and X from the lab collection (as described in the methods section) were used throughout this study. The Gram staining technique was used to check the purity of the different *Campylobacter* strains after 24h and 72h growth at 37°C in microaerobic conditions. The four wt strains were confirmed to be pure Gram-negative cells presenting a spiral shape at 24h (Fig. 3.1, A) and a coccoid form at 72h (Fig. 3.1, B), which is characteristic of *Campylobacter* bacteria.

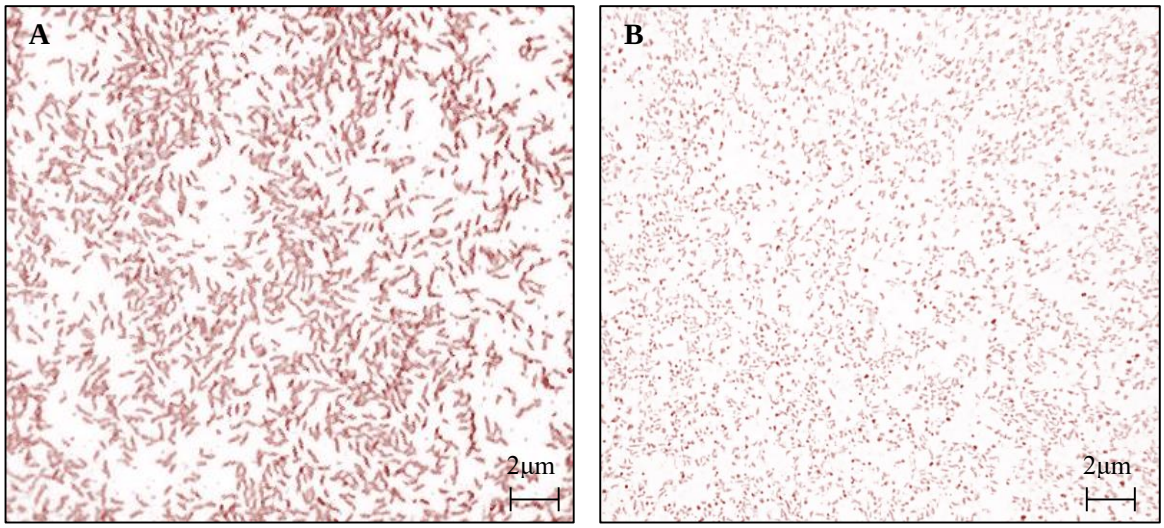


Figure 3.1. Image of *C. jejuni* cells visualised using light microscopy. Spiral shaped cells can be seen at 24h (A) and coccoid cells at 72h (B). This figure is representative of the *C. jejuni* 11168H, 81-176, G1 and X strains tested. The strains were grown micro-aerobically at 37°C for 24h and 72h and were prepared as described in Section 2.1.3. The samples were visualised under x100 magnification and images were taken with a Nikon 80i microscope.

To confirm the identity of the *C. jejuni* strains, PCR was conducted to amplify a unique gene present in each strain. To confirm the identity of the strains 11168H, 81-176, G1 and X, the genes *cj1435c*, *dmhA*, *tagF* and *moaA* that encoded a putative phosphatase, a GDP-mannose 4, 6-dehydratase, a CDP-glycerol glycerophosphotransferase and a molybdenum cofactor biosynthesis protein A, respectively, were amplified by multiplex PCR using the respective primers described in Table 2.4. To detect the virulence and the tetracycline resistance plasmids pVir and pTet, respectively, in these strains a PCR with VirB11 and TetO primers was conducted in parallel. It was possible to distinguish different *C. jejuni* strains since each strain produced different size bands. Amplification of *cj1435c*, *dmhA*, *tagF* and *moaA* fragments were observed for strains 11168H, 81-176, G1 and X, respectively (Fig. 3.2, lanes 1-4). The presence of plasmids pVir and pTet was observed for strains 81-176 and X, while they were absent in strains 11168H and G1 (Fig.

3.2, lanes 5-8 and lanes 9-12, respectively). Glycerol stocks of these *C. jejuni* strains in MH broth were stored in my personal -80°C bacterial collection.

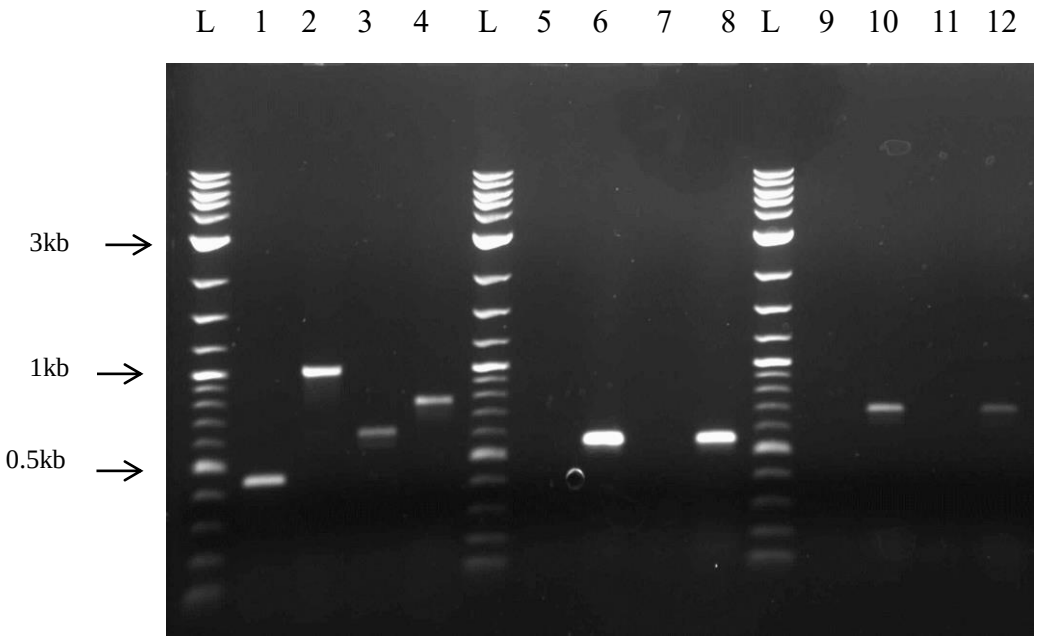


Figure 3.2. Multiplex PCR for *C. jejuni* strain confirmation and for the detection of virulence and tetracycline resistance plasmids. PCR was conducted using DmhA, TagF, Cj1435c, TetO and VirB11 primers Lane L: 2-log DNA ladder; lane 1: 11168H - 450bp; lane 2: 81-176 - 1021bp; lane 3: G1 A3 - 642bp; lane 4: X - 803bp; lane 5: 11168H - no amplification; lane 6: 81-176 - 559bp; lane 7: G1 - no amplification; lane 8: X - 559bp; lane 9: 11168H - no amplification; lane 10: 81-176 - 708bp; lane 11: G1 - no amplification; lane 12: X - 708bp.

3.1.3. Growth characteristics of *C. jejuni* strains

Growth of four *C. jejuni* strains was assayed at 37°C in BHI broth for a period of 48 hours microaerobically (Fig. 3.3). The strains tested were able to grow in BHI medium, but have different growth rates. Strains 81-176 and X were shown to have higher growth rates compared with both 11168H and G1 strains (Fig. 3.3). Strain G1 had a statistically significant lower growth rate than the other strains (24h: $p=0.007$; 30h: $p=0.03$ and 48h: $p=0.01$, in comparison with 11168H and p values<0.01 for these time-points in comparison with both 81-176 and X strains). *C. jejuni* strains 81-176 and X shared

similar growth rates with no statistically significant differences between these strains (24h, p=0.9; 30h; p=0.5 and 48h, p=0.09) (Fig. 3.3). Statistically significant differences of 81-176 and X strains in comparison with the reference strain 11168H were obtained for time-points 24h to 48h (81-176: 24h, p=0.01; 30h; p=0.007; 48h, p=0.01 and X: 24h, p=0.01; 30h; p=0.004; 48h, p=0.08).

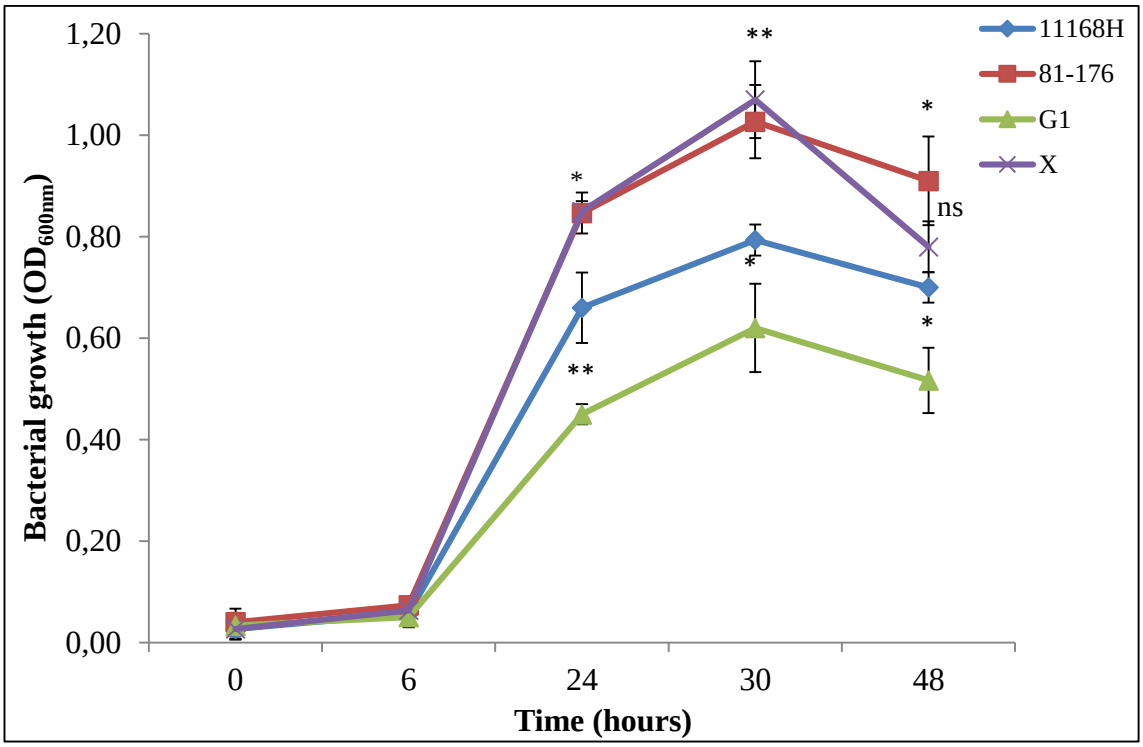


Figure 3.3. Growth of *C. jejuni* strains in BHI broth. *C. jejuni* strains 11168H, 81-176, G1 and X were grown in BHI broth at 37°C for 48h and are represented in blue, red, green and purple colours, respectively. Statistical analysis was conducted in relation to 11168H strain. No statistically significant differences were observed for time-points 0 and 6h among all strains tested. Values are mean ± SD from three independent experiments with one technical repeat each.

As already mentioned, *C. jejuni* motility plays an important role in invasion of host cells (Lugert *et al*., 2015). In order to check the motility of the different *C. jejuni* wt strains, suspensions of these bacteria were transferred onto the surface of BHI soft-agar plates and incubated microaerobically for three days at 37°C. The average diameters of bacterial growth were compared visually (Fig. 3.4). It was observed that *C. jejuni* strains 11168H

and G1 shared similar growth in the motility plates (Fig. 3.4. A, C) whilst X was the least motile strain tested (Fig. 3.4, D). *C. jejuni* strain 81-176 was observed to be the most motile among these wt strains (Fig. 3.4, B).

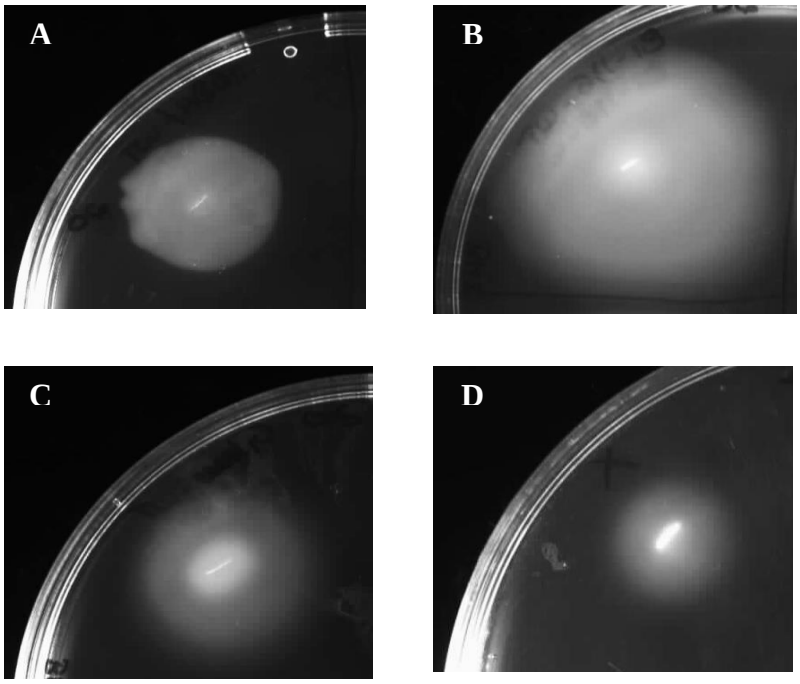


Figure 3.4. Motility of *C. jejuni* strains in soft agar plates. BHI 0.4% soft agar plates were inoculated with the same amount of bacteria (A) 11168H, (B) 81-176, (C) G1 and (D) X for three days at 37°C. This figure is representative of three different assays with one technical repeat each and pictures were taken with the same magnification.

As noted earlier, the ability to form biofilms is considered to be an important factor in the pathogenesis of *C. jejuni,* which helps in its survival in the environment (Bronowski *et al*., 2014). Biofilms are defined as matrix-enclosed bacterial population's adherent to each other and/or to surfaces or interfaces (Costerton *et al*., 1995). *C. jejuni* is shown to produce three forms of biofilm in liquid culture: it may attach to a glass surface; form a pellicle at the liquid-gas interface in glass tubes and form non-attached and floating aggregates designated as flocs (Joshua *et al*., 2006).

To check biofilm formation, the ability of the different *C. jejuni* wt strains to form flocs was tested using 25 cm^2 tissue culture flasks containing bacterial suspensions in MH

broth which were incubated for three days at 37°C with slow speed shaking. Flocs were then visually observed and quantified according to those previously defined by Joshua *et al.* (2006) (Fig. 3.5). All strains were able to form aggregates and, whilst *C. jejuni* strain 11168H formed a large number of aggregates (Fig. 3.5, A), X strain exhibited one giant aggregate (Fig. 3.5, D). The G1 strain formed small sized aggregates (Fig. 3.5, C), whilst *C. jejuni* strain 81-176 presented normal size aggregates, but in a smaller amount than strain 11168H (Fig. 3.5, B).

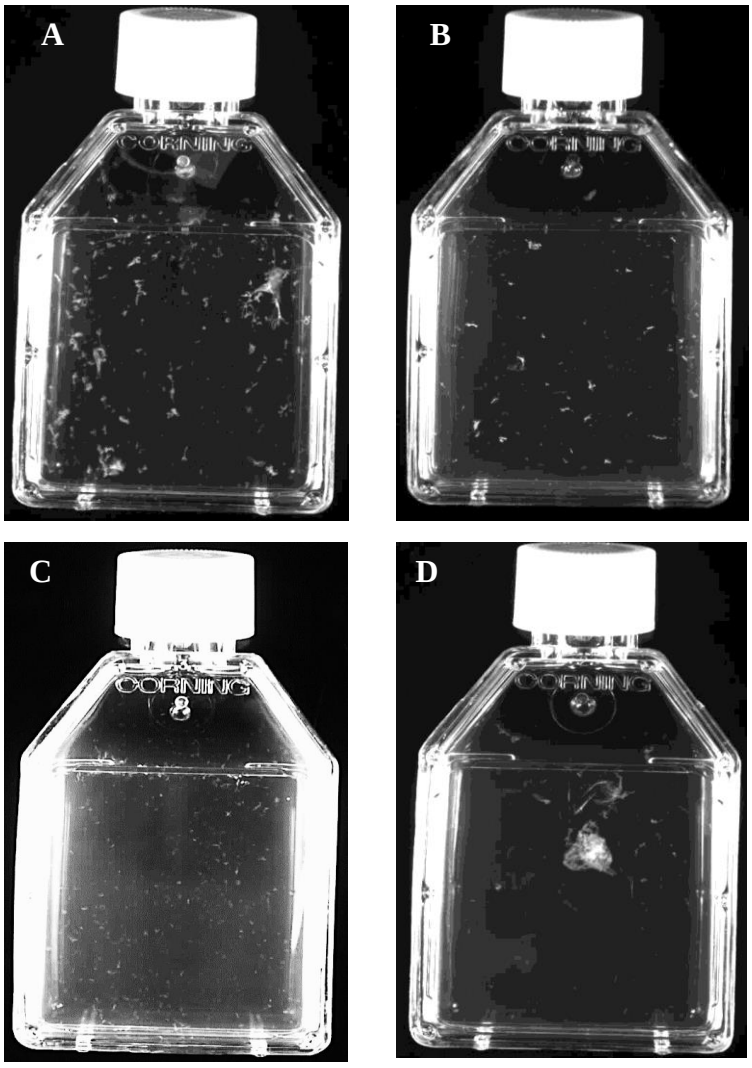


Figure 3.5. Flocs formation in *C. jejuni* strains. Quantification of non-attached aggregate formation (flocs) in BHI broth for *C. jejuni* strains was (A) 11168H, +++ (B) 81-176, +++ (C) G1, + and (D) X, ++. Quantification of the aggregates was determined as previously reported, where +++ (extensive), ++ (intermediate) and + (small, just visible) (Joshua *et al.*, 2006). This figure is representative of three different assays with one technical repeat.

One of the initial objectives was to infect amoebae cells with a *C. jejuni* GFP strain and visualise it using confocal microscopy to monitor the survival of *C. jejuni* within *A. polyphaga*. However, I failed to electroporate the GFP plasmid pMEK91 (Mixter *et al.*, 2003) into the *C. jejuni* strain 81-176 in our laboratory. Fortunately, Dr. Patricia Guerry (from the Naval Medical Research Centre) kindly provided us with a sample of the *C. jejuni* strain 81-146/GFP containing the plasmid pCPE111/28/GFP (Hickey *et al*., 2005). This *C. jejuni* strain 81-146/pCPE111/28/GFP was observed in a fluorescence microscope, and it showed a high level of fluorescence as expected (Fig. 3.6).

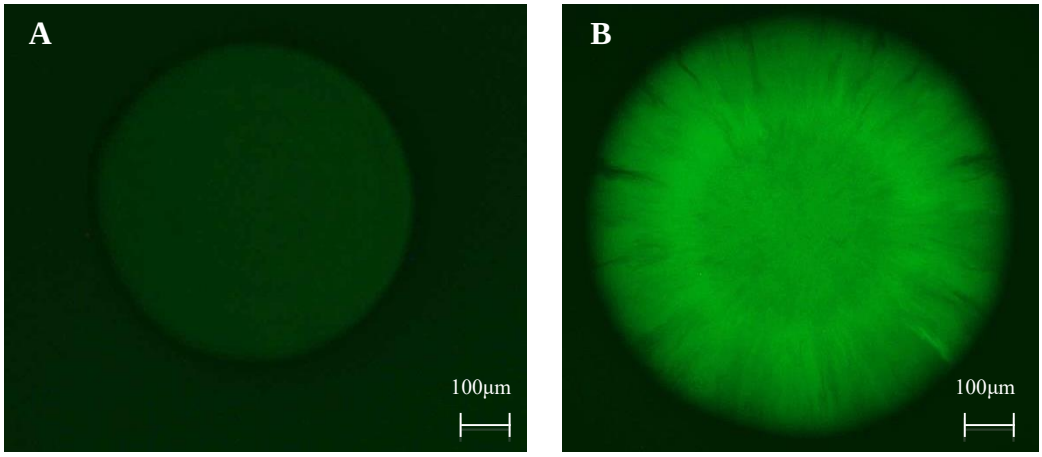


Figure 3.6. Fluorescence microscopy of *C. jejuni* strains 81-176 and 81-176/ pCPE111/28/GFP. (A) 81-176 wt strain is non-fluorescent as opposite to the (B) 81-176/ pCPE111/28/GFP strain (provided by Dr, Patricia Guerry) which exhibits high fluorescence. PCR was conducted to confirm the presence of both pTet and pVir plasmids in the fluorescent GFP strain (data not shown). Images were observed with 40x objective in a fluorescence microscope (Nikon Eclipse 80i).

In summary, *C. jejuni* strain 81-176 contained both virulence and tetracycline plasmids, had a higher growth rate in BHI broth, was the most motile strain and was able to form biofilms. Furthermore, a fluorescent GFP derivative of this strain was available in the lab collection. Thus, *C. jejuni* 81-176 was selected as the best candidate strain for the amoebae experiments in this study.

3.1.4. Generation of the chimeric *C. jejuni* B7 strain

A draft genome sequence of *C. jejuni* strain G1 was determined in our laboratory (Lehri *et al.,* 2015). The gDNA from a strain located at the D6 position of the -80°C laboratory stock culture was collected after growth on a CBA plate supplemented with tetracycline (10μg/ml) at -80°C stock was used for genome sequencing. CLC genomics workbench software was used to assembly the genome of the assumed G1 strain, however, after analysis it was noticed that we actually sequenced the genome of strain 81-176 (Table 2.4). Two distinct *C. jejuni* G1 bacterial stocks were used as controls (one located at position D6 and another located at A3 position in the laboratory collection) (Fig. 3.7). It was observed that the strain initially sequenced by my co-workers and myself was solely 81-176 and not the intended G1 strain (Fig. 3.7, lane 1). In addition, it was observed that the strain G1 (D6) grown in Tet for further genome sequencing was contaminated with a high fraction of the 81-176 strain as it amplified both *dmhA* (1021bp) and *tagF* (642bp) size bands (Fig. 3.7, lane 2). The strain G1 (A3) was a pure culture (Fig. 3.7, lane 3). Because the initial G1 stock was contaminated with 81-176 and 10μg/ml of tetracycline was too high to allow for the former strain to grow, the strain that was initially sequenced was in fact pure 81-176 and not the intended G1 strain. The bacterial culture grown in tetracycline was kept in glycerol stock and was named B3. Eventually, my co-workers were able to use a pure stock of the G1 strain located in position A3 in the lab -80°C collection for genome sequencing (Lehri *et al.,* 2015).

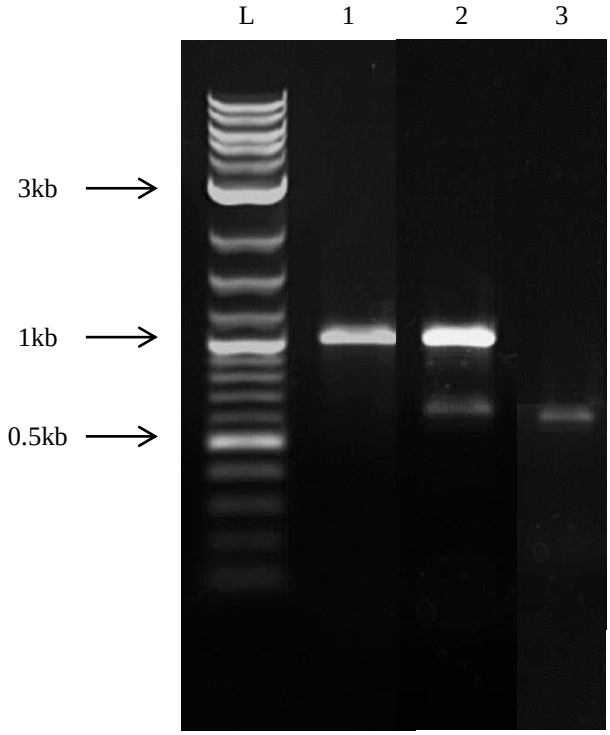


Figure 3.7. Duplex PCR for confirmation of the *C. jejuni* G1 bacterial stock used for genome sequencing. PCR was conducted using DmhA and TagF primers. Lane L: 2-log DNA ladder; lane 1: strain originally sequenced – 1021bp; lane 2: G1 D6 – 1021bp and 642bp; lane 3: G1 A3 – 642bp.

It should be noted that the -80°C stock of G1 from my personal bacterial collection was created from the G1 strain located at position A3 from the -80°C laboratory bacterial collection, which was shown to be pure G1 and was not contaminated with strain 81-176 strain (Fig. 3.2, lane 3; Fig. 3.7, lane 3) and was used for the experimental work.

As previously mentioned, the concentration of Tet used was too high to support the growth of strain G1, which was, therefore, killed so that only strain 81-176 survived. As already noted, *C. jejuni* can easily acquire DNA from other strains (Parkhill *et al.,* 2000). Since G1 and 81-176 strains were mixed-up in the G1 D6 bacterial stock and plated together on a tetracycline plate, which gave rise to the B3 culture. There was an interest in investigating if these strains exchanged genomic material during this stage. So, B3 bacterial culture was plated onto CBA agar and a single clonal isolate was kept in glycerol stock. The latter was found to be a chimeric *C. jejuni* strain and named strain B7.

3.1.4.1. Comparison of the genome of B7 strain with other genomes

The gDNA from strain B7 was isolated and Professor Karlyshev sequenced its genome. When the genome of *C. jejuni* strain B7 was compared with the reference strain 81-176 using CLC genomic workbench software, a large number of mutations were observed in two specific parts of its genome, although there was still high similarity with strain 81-176. From genome regions between CJJ81176_1102 to CJJ81-176_1117 and CJJ81176_1167 to CJJ81-176_1222 a higher number of mismatches was observed in the B7 strain in comparison with strain 81-176. The remaining part of the genome (from CJJ81176_0026 to CJJ81176_1223 and CJJ81176_1118 to CJJ81176_1166) was identical to strain 81-176, with the exception of the gene *cjj81176_1449* (Fig. 3.8). The genome regions presenting variation between the *C. jejuni* strains 81-176 and B7 (Fig. 3.8) were identical between the latter strain and G1. Exceptionally, the B7 *cjj81-176_1449* gene presented a high number of mismatches when compared with both 81-176 and G1 strains. Using NCBI nucleotide blast, the latter gene was shown to be 100% identical (100% query cover) to the *cjj81176_pVir0048* gene from the *C. jejuni* strain 81-176 virulence plasmid pVir (data not shown), possibly demonstrating that a plasmid gene was able to integrate itself into the chromosome of the respective bacterial strain. However, further evidence for this claim is still required. In summary, comparison of the genome of strain B7 with that of the strain G1 showed that the entire genome regions that were different between B7 and 81-176 strains were 100% identical with the G1 strain. Thus, it was concluded that strain B7 was a hybrid between strains G1 and 81-176 and that by growing together they were able to exchange part of their genome, confirming the ability of *C. jejuni* to acquire exogenous DNA. Examples of proteins in strain G1 that were encoded by the genes that were integrated intothe chromosome of the strain 81-176 were the transcription-repair coupling factor Mfd and the S-ribosylhomocysteinase LuxS.

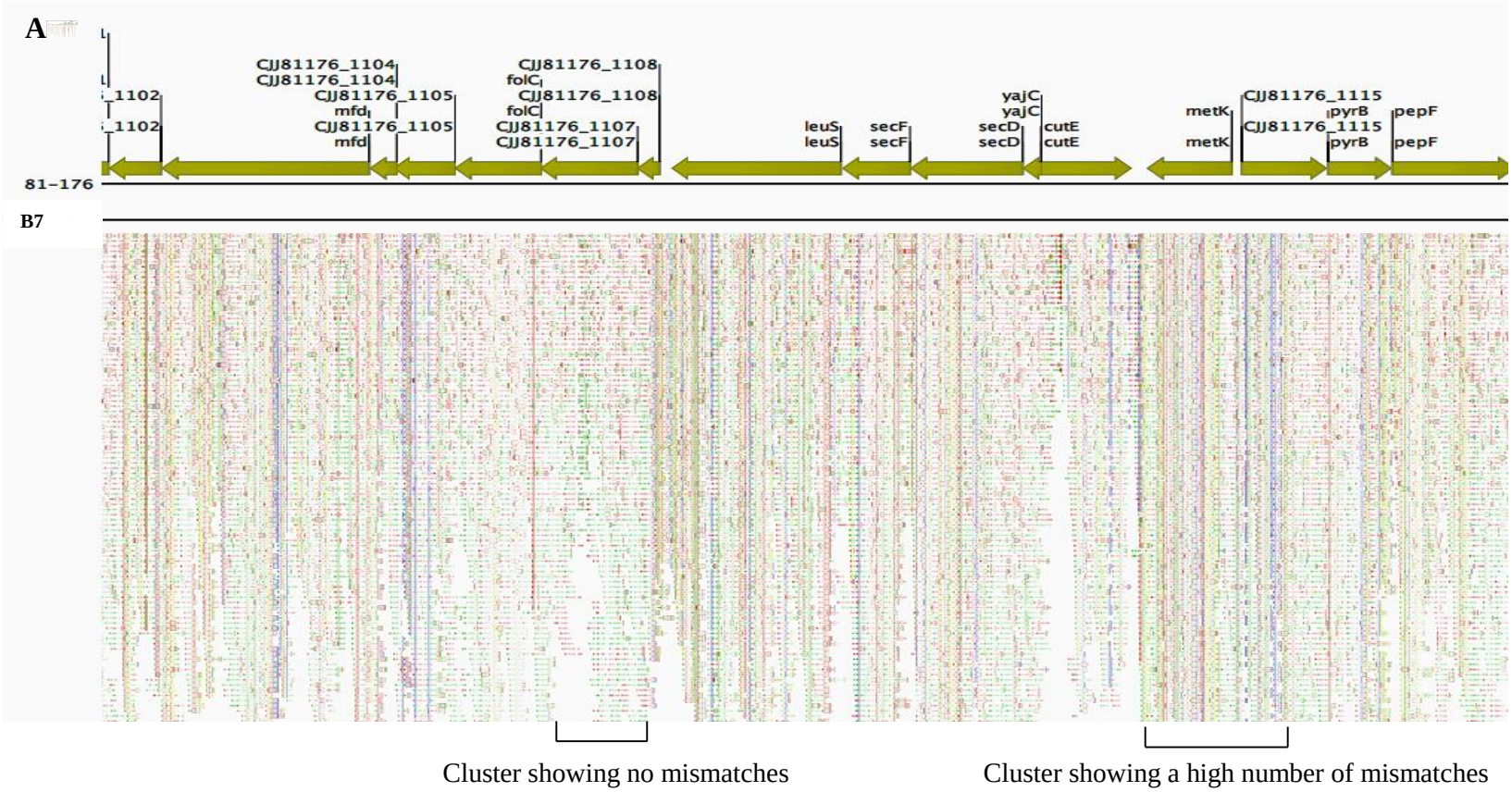


Figure 3.8. Comparative analysis of the genome sequences of *C. jejuni* strains B7, 81-176 and G1. B7 sequencing reads were mapped onto the genome of 81-176 using CLC Genomics Workbench software. The yellow, red, blue or green vertical lines highlighted represent the mismatches between the G, A, C and T nucleotide bases, respectively, of a sequence region in B7 strain in comparison with strain 81-176.

3.1.4.2. Origin and identification of strain B7

LuxS is an enzyme that catalyses the production of the auto inducer signalling molecule AL-2 (Plummer *et al*., 2012). To distinguish between *C. jejuni* strains B7, G1 and 81-176, primers for *luxS* gene amplification were designed using the OligoCalc online tool (Table 2.4). The *luxS* gene was selected since it is identical to that in the G1 strain and is found in the chromosome of strain B7. PCR using the LuxS and the identification primers DmhA and TagF was conducted to distinguish between these strains. The primers were designed to identify the two versions of the *luxS* gene where LuxS-81-176, a forward primer that amplified a region for the *C. jejuni* 81-176 *luxS* gene and LuxS-G1, a forward primer that amplified a region of *C. jejuni* G1 *luxS* gene (Fig. 3.9). The reverse primer LuxS-rev amplified an identical downstream region identical in both *C. jejuni* strains G1 and 81-176.

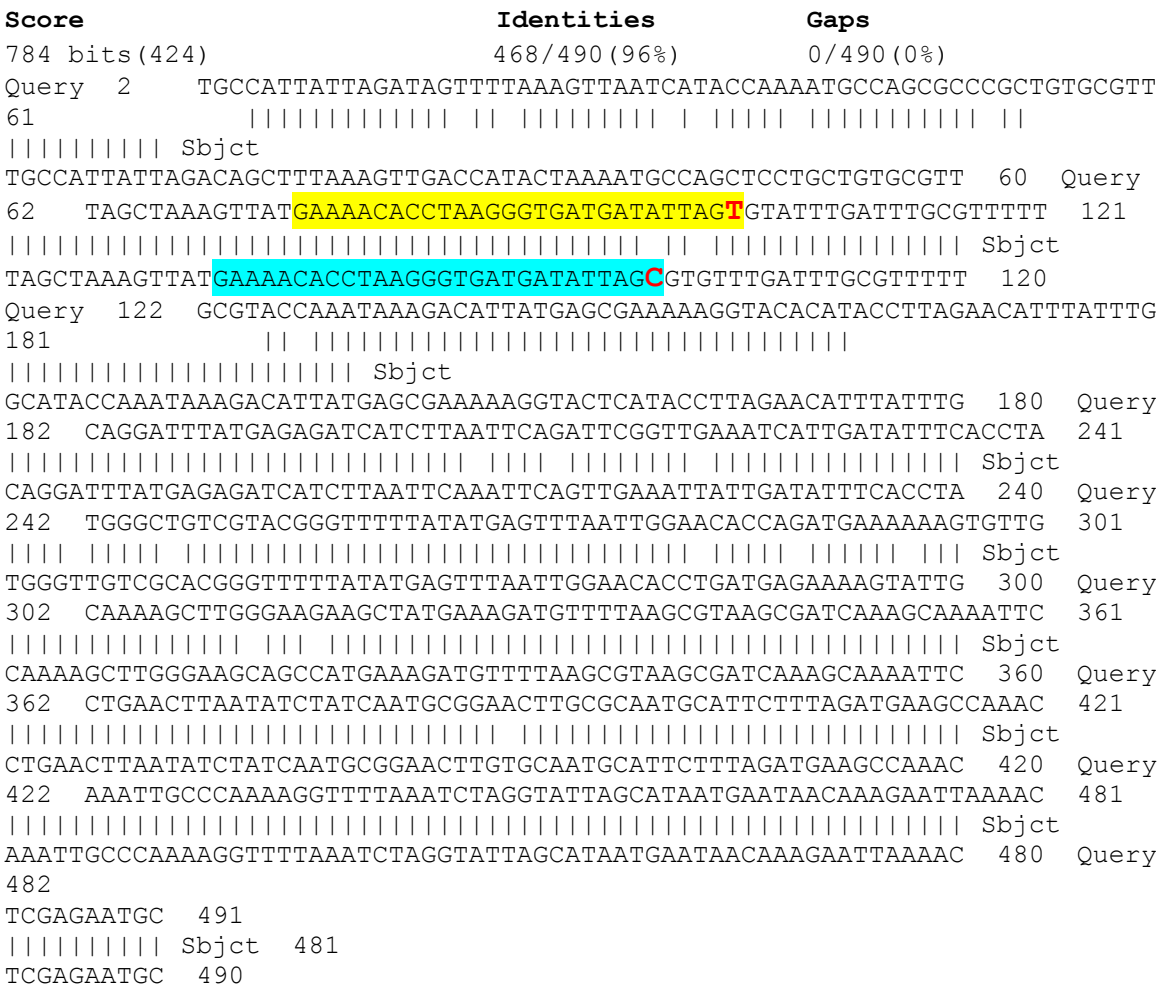


Figure 3.9. BlastN result of the *luxS* gene from *C. jejuni* strains 81-176 and G1. To distinguish between the three *C. jejuni* strains B7, 81-176 and G1, primers for *luxS* gene amplification were designed. The forward primer LuxS-81-176 was used for amplification of the wt *luxS* version that is found in strain 81-176 (yellow). The primer LuxS-G1 was used for amplification of the allele version of the *luxS* gene found in both G1 and B7 strains (blue). The difference between both these forward primers is highlighted in red (T replaced by C).

For the PCRs with the LuxS primers specific conditions were used: the gDNA from the samples was diluted 10 times, the denaturation step was performed with 20 cycles only and the annealing temperature was 60°C. As expected, in *C. jejuni* G1 strain there was amplification of a 642bp size band corresponding to *tagF* gene (Fig. 3.10, lane 1) and in both B7 and 81-176 strains amplification of a 1021bp corresponding to *dmhA* gene was observed (Fig. 3.10, lanes 2,3). Moreover, as expected, there was no amplification of the *tetO* and *virB11* genes in this strain (Fig. 3.10, lane 4). On the contrary, in both B7 and

81-176 strains amplification of a 659bp and 748bp bands corresponding to the latter genes was observed, confirming the presence of the tetracycline and virulence plasmids (Fig. 3.10, lanes 5, 6). With the LuxS-G1 primer amplification of a 760bp size band in both 81-176 and B7 strains, corresponding to the allele version of the *luxS* gene was observed (Fig. 3.10, lanes 7, 8). As expected, no amplification of this *luxS* version was observed for 81-176 wt (Fig. 3.10, lane 9). However, it was expected to be the other way around when using the primer LuxS-81-176. Whilst in *C. jejuni* strain 81-176 the expected band for the *luxS* wt version was seen (Fig. 3.10, lane 12), in both strains B7 and G1 there was also a 760bp band (Fig. 3.10, lanes 10-11). As this 760bp band was always observed in different *C. jejuni* strains with the *luxS* allele version when using the LuxS-81-176 primer (data not shown), it was assumed that the reason for the appearance of this unexpected band was likely caused by mis-priming during PCR.

In summary, strain B7 could be distinguished from G1 and 81-176 wt strains by PCR if it amplifies a 1021bp size band using the DmhA primers and at the same time no amplification should be observed when using the LuxS-G1/LuxS-Rev primers.

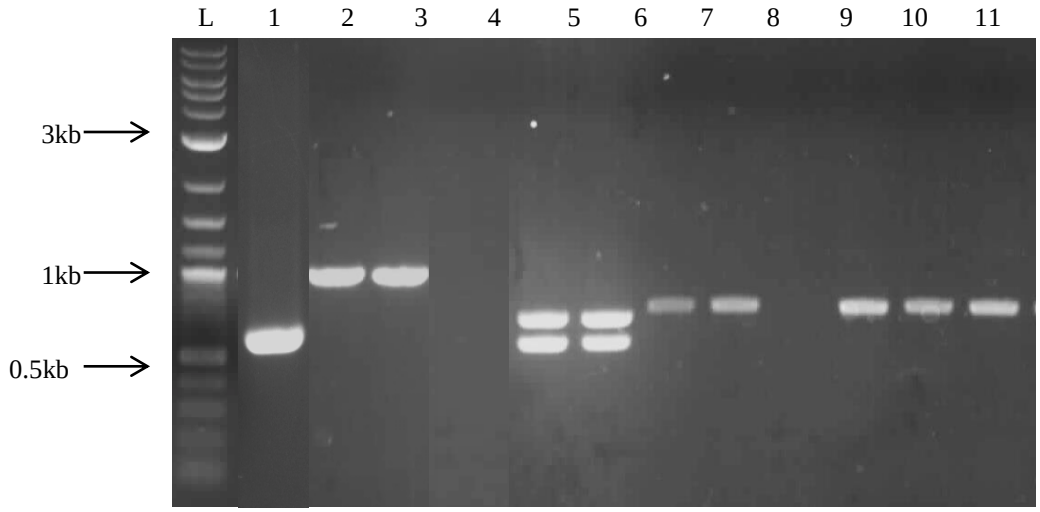


Figure 3.10. PCR analysis of *C. jejuni* strains G1, B7 and 81-176. PCR was conducted using gDNA sample diluted 10 times; with 60°C annealing temperature; 20 denaturation cycles and the primers DmhA, TagF, pTet, pVir, LuxS-81-176 and LuxS-G1**.** lane L: 2-log DNA ladder; lane 1: G1 – 642bp; lane 2: B7 – 1021bp; lane 3: 81-176 – 1021bp; lane 4: G1 – no amplification; lane 5: B7 – 559bp and 748bp; lane 6: 81-176– 559bp and 748bp; lane 7: G1- 760bp; lane 8: B7 – 760bp; lane 9: 81-176 – no amplification; lane 10: G1 – 760bp; lane 11: B7 – 760bp; lane 12: 81-176 – 760bp.

To determine the origin of the *C. jejuni* chimeric B7 present the B3 culture, a PCR with the LuxS primers and identification primers was conducted with twelve single clonal isolates of B3 strain growing on CBA agar with no antibiotic supplementation. Control strains G1, B3, 81-176 and B7 were included (Fig. 3.11). Amplification of a 642bp size band corresponding to *tagF* was observed for strain G1, whilst a 1021bp size band correspondingto the *dmhA* gene was observed for the three latter strains (Fig. 3.11, A). To distinguish between the chimeric strain B7 and the 81-176 wt, a PCR was conducted using the LuxS-G1 primers (Fig. 3.11, B).

As expected, in the control strains G1, B3 and B7 there was amplification of a 760bp band corresponding to the allele version of the *luxS* gene (Fig. 3.11B, lanes 17, 18, 20) and in the 81-176 wt strain no PCR product was observed (Fig. 3.11B, lane 19). Only in three B3 clonal isolates there was a 760bp band corresponding to the allele version of the *luxS* gene (Fig. 3.11B, lanes 22, 25 and 27), whilst in the other nine clones there was no PCR product (Fig. 3.11B, lanes 21, 23, 24, 26, 28-32). This shows that the fraction of *C. jejuni* strain B7 in the B3 stock culture was 25%. The same PCR analysis was conducted with some clonal isolates of G1 D6 original bacterial stock that was contaminated with 81-176 and no chimeric strains were detected (data not shown). This suggests that the chimeric strains were created after plating G1 D6 culture (mixture of G1 + 81-176 strains) onto CBA medium containing a high tetracycline concentration, which gave rise to the B3 heterogeneous culture (81-176 + B7 strains). The pure chimeric strain, named B7, was obtained by plating the B3 culture to single colonies onto CBA agar plates. In summary, the chimeric B7 strain was strain 81-176 containing parts of the genome from G1 (Fig. 3.12).

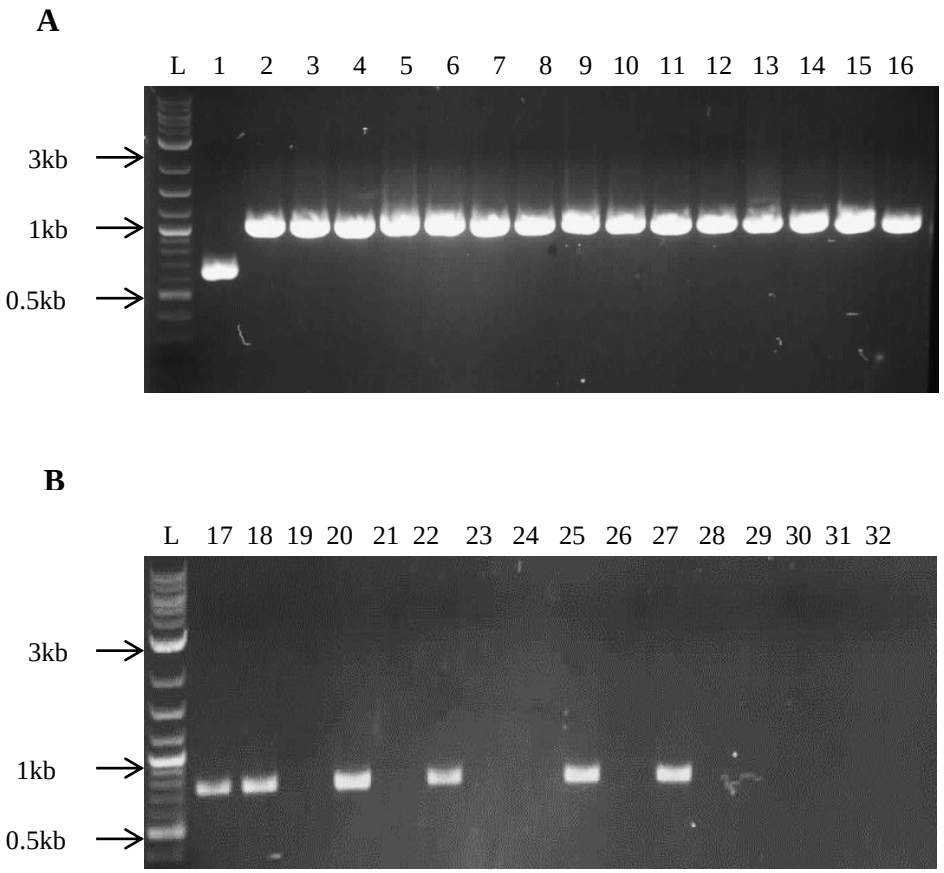


Figure 3.11. PCR analysis of *C. jejuni* B3 clonal isolates. The primers DmhA, TagF and LuxS-G1 primers were used and the G1, B7 and 81-176 strains were included as controls. (A) lane L: 2-log DNA ladder; lane 1: G1 – 642bp; lane 2: B3 – 1021bp; lane 3: 81-176 – 1021bp; lane 4: B7 – 1021bp; lanes 5-16: B3 clonal isolates – 1021bp; (B) lane L: 2-log DNA ladder; lane 17: G1 – 760bp; lane 18: B3 – 760bp; lane 19: 81-176 – no amplification; lane 20: B7 – 760bp; lanes 22, 25, 27: B3 clonal isolates – 760bp; lanes 21, 23, 24, 26, 28-32: B3 clonal isolates – no amplification.

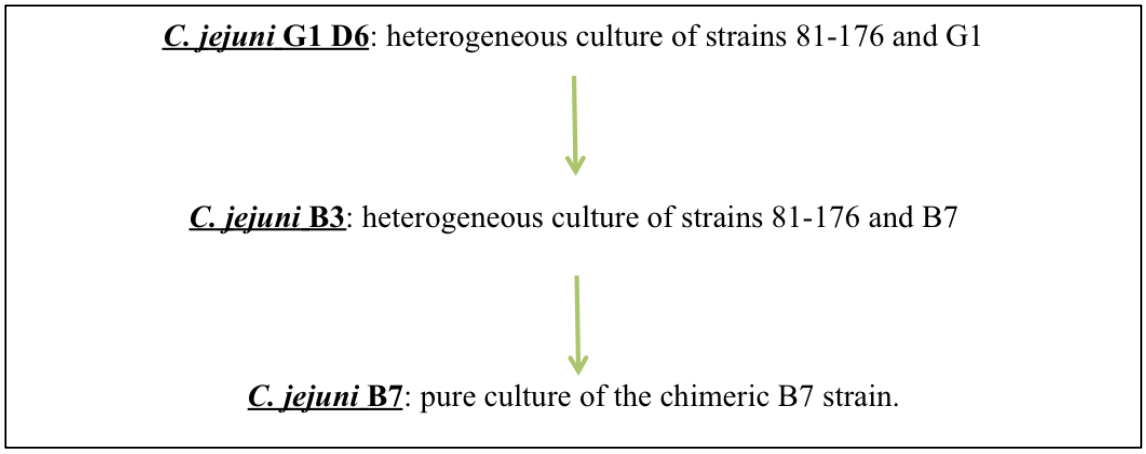


Figure 3.12. Schematic representation of how the chimeric *C. jejuni* strain B7 was created.

The growth of *C. jejuni* B7 strain was compared with that of *C. jejuni* 81-176 wt strain in BHI broth at 37°C for 48h, where the former strain was shown to grow somewhat better than the latter (Fig. 3.13). From time-point 24h onwards the growth differences among these strains were statistically significant (24h: p=0.0317; 48h: p=0.0008; p=0.0009) (Fig. 3.13).

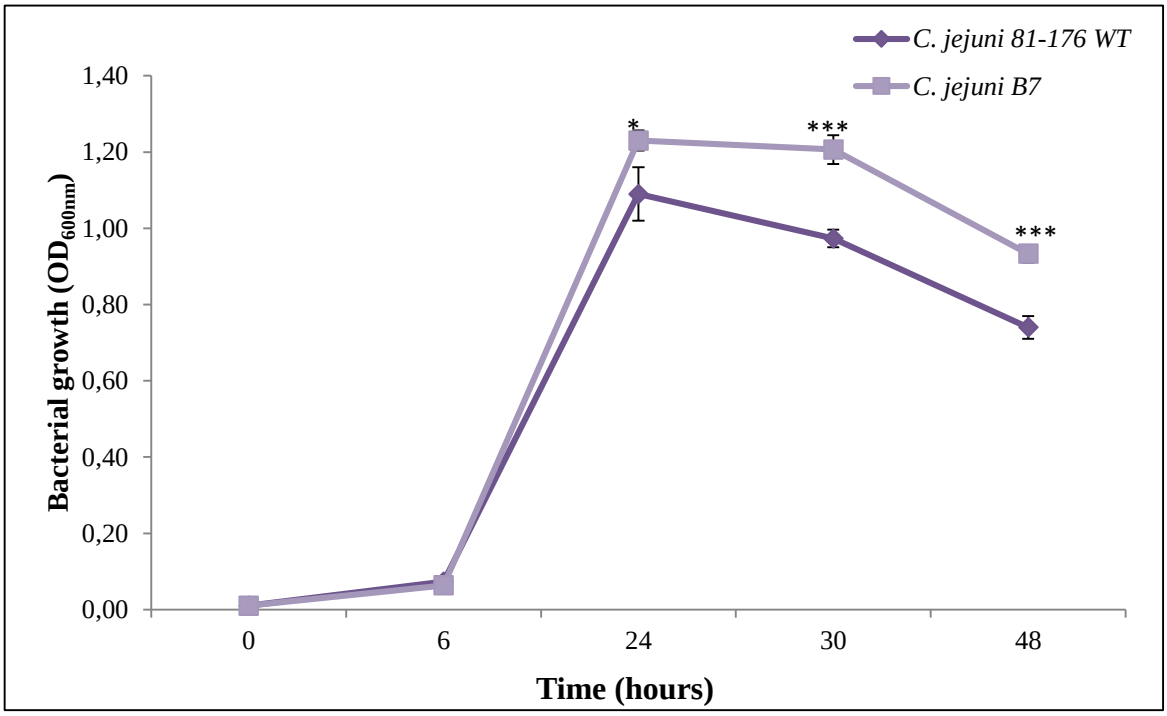


Figure 3.13. Growth of *C. jejuni* strains 81-176 and B7. The growth of *C. jejuni* strains 81-176 (dark purple) and B7 (light purple) was assessed in BHI broth for two days and OD_{600nm} was measured in different time-points. Values are mean ± SD from three independent experiments with one technical replicate each.

3.1.4.3. Tetracycline resistance of strain B7

Since B7 strain was created after growth of B3 on a tetracycline plate, it was assumed that the chimeric strain B7 was probably generated due to selective pressure and that this strain could be more resistant to tretacycline than 81-176. One clue for this hypothesis was the fact that one of the genes that was transferred from G1 to the strain 81-176 was the mutation frequency decline *mfd* gene (Fig. 3.14). This gene encodes for a transcription-repair coupling factor involved in strand-specific DNA repair. This gene was also shown to be important for antibiotic resistance (e.g. to tetracycline) in different foodborne pathogens (e.g. *Helicobacter pylori*) (Lee *et al*., 2009) and for fluoroquinolone resistance in *C. jejuni* (Han *et al*., 2008)

It was presumed that, due to antibiotic pressure, the *mfd* gene was horizontally transferred from strain G1 to 81-176 creating a strain with a different antibiotic resistance profile.

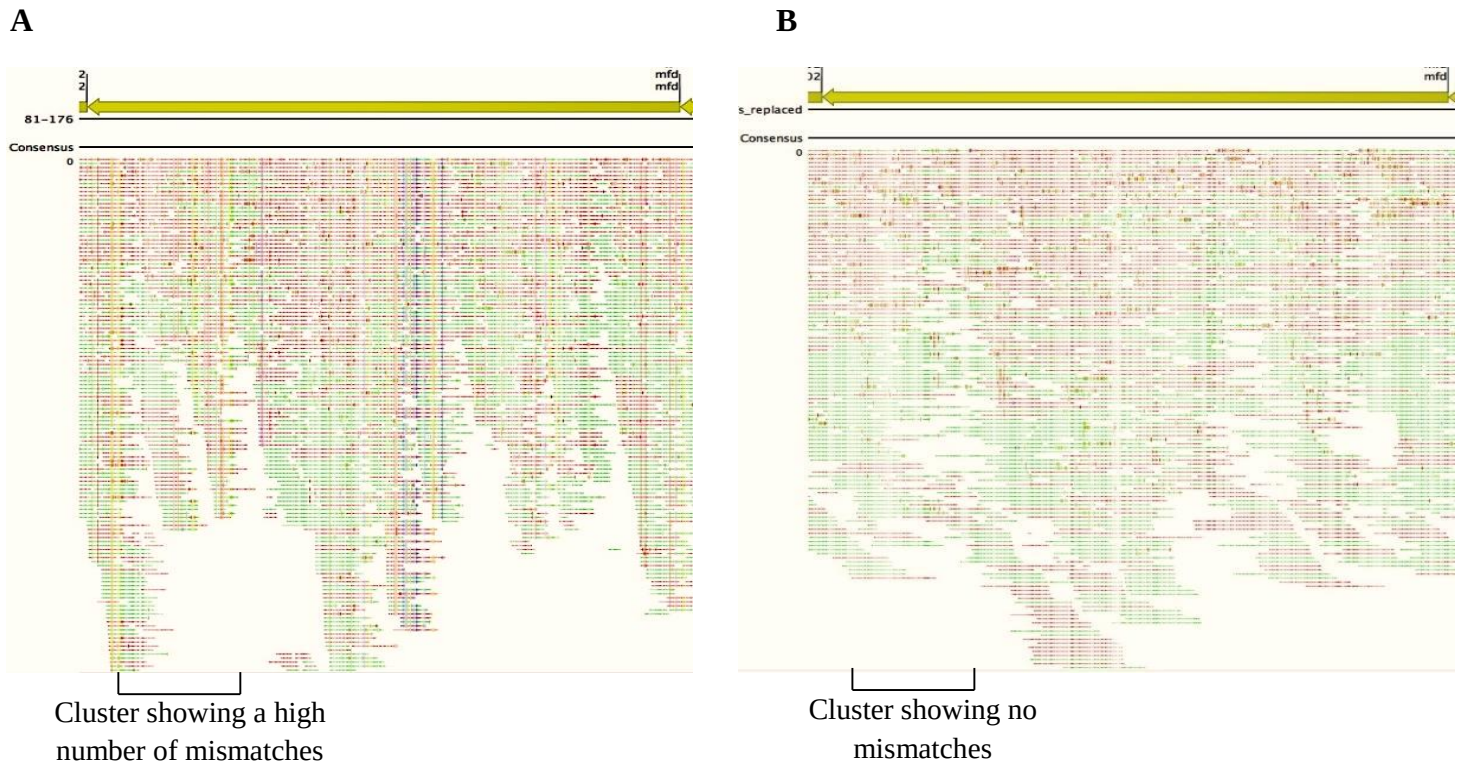


Figure 3.14. Comparative analysis of the *mfd* gene from *C. jejuni* strains G1, 81-176 and B7. Using CLC genomics workbench software, (A) B7 sequencing reads were mapped onto the genome of 81-176 and (B) G1 sequencing reads were mapped onto the genome of B7 strain. The yellow, red, blue or green vertical lines highlighted represent the mismatches between the G, A, C and T nucleotide bases, respectively, of (A) B7 *mdf* sequence in comparison with 81-176 strain and (B) B7 *mdf* sequence in comparison with G1 strain.

Tetracycline resistance of *C. jejuni* strain B7 was assessed and compared with that of wt strain 81-176 in both liquid and solid media. Antibiotic resistance was tested on CBA agar plates supplemented with increasing tetracycline concentrations (5 to 75μg/ml) and in BHI broth supplemented with doubling tetracycline concentrations (3.9 to 250μg/ml) (Fig. 3.15, A-B, respectively). No differences were observed in the growth of *C. jejuni* strains B7 and 81-176 in CBA plates supplemented with increasing concentrations of tetracycline, as both strains were able to grow in 50μg/ml of Tet, but failed to grow in agar plates supplemented with 75μg/ml of Tet (Fig. 3.15, A).

In parallel, minimal inhibitory concentrations (MIC) of Tet were determined for these strains in BHI broth supplemented with 5% lysed blood and doubling concentrations of Tet and the bacterial strains were incubated at 37°C for 2 days. According to the EUCAST recommendations, the quality control ranges for tetracycline in *Campylobacter* were established for testing at both 37°C for 48h or 42°C for 24h (Ge *et al*., 2013; Sifré *et al*., 2015). In addition, it is recommended that if there is insufficient bacterial growth after 24h incubation period at 37°C, as observed in the experiments of this study, *Campylobacter* should be incubated for an extra 24 hours (Ge *et al*., 2013; Sifré *et al*., 2015). It was observed that the Tet MIC for both 81-176 and B7 strain was 62.5μg/ml, showing that neither of these strains were able to grow above this Tet concentration and shared the same Tet resistance level (Fig. 3.15, B). Although the same starting OD_{600nm} for both strains was used, B7 strain grew better than 81-176 when no antibiotic was present in the medium. This is in accordance with the results obtained in Fig. 3.13. This indicates that strain B7 is able to grow faster than the wt strain 81-176.

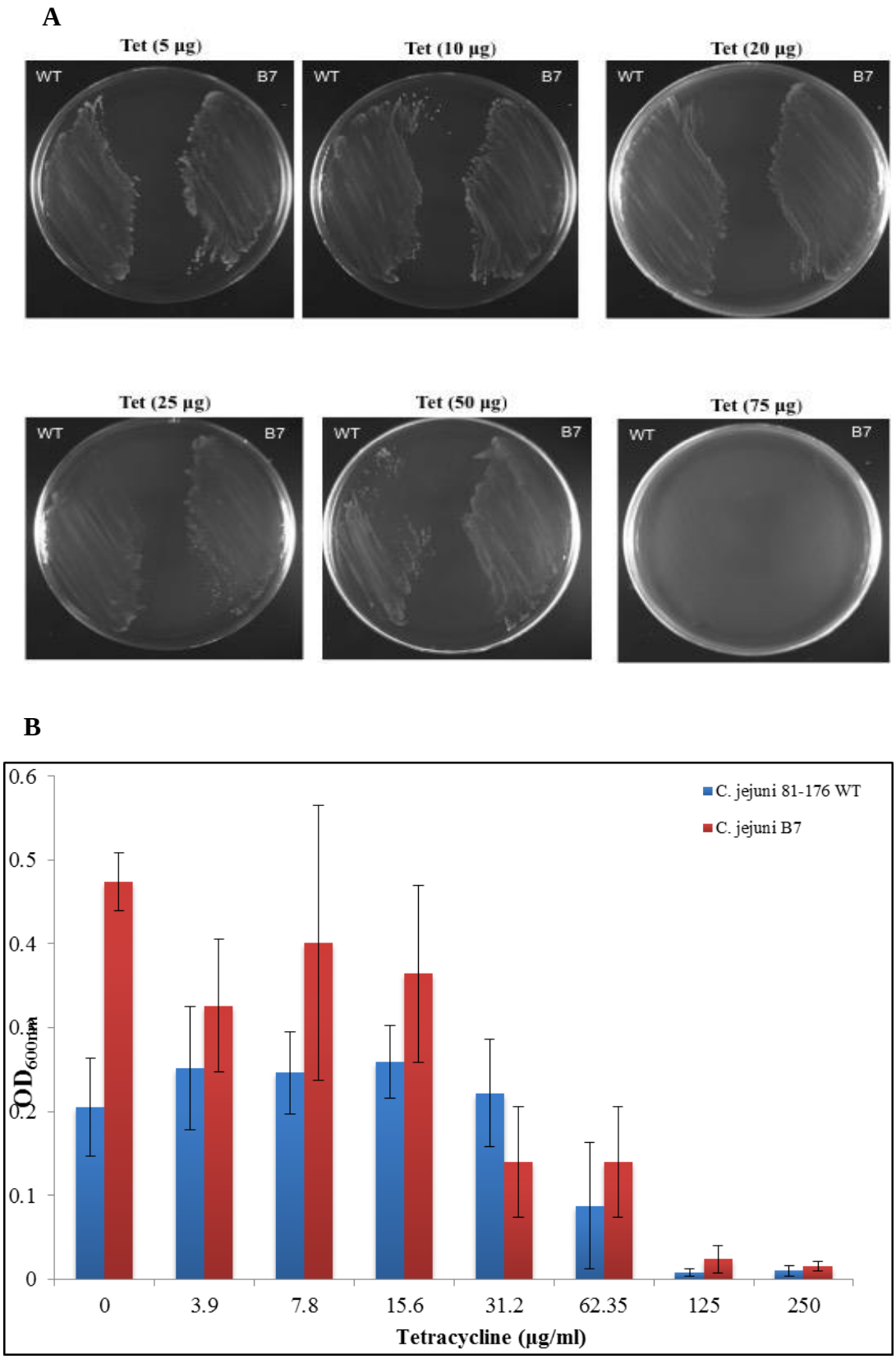


Figure 3.15. Tetracycline susceptibility of the chimeric *C. jejuni* strain B7. Tetracycline resistance of B7 strain (red) was compared with that of 81-176 strain (blue) and determined by (A) growth of these strains in CBA agar plates supplemented with increasing concentrations of Tet, ranging from 5 to 75µg/ml and (B) by the micro dilution broth method. The bacterial strains were incubated for 2 days at 37°C. Values are mean ± SD from three independent experiments.

Part II

3.2. Molecular mechanisms of *Campylobacter jejuni* resistance to antibiotics

3.2.1. Overview

Knowledge of the association between virulence and antibiotic resistance is increasing, leading to prolonged survival of pathogenic bacteria (Beceiro *et al*., 2013). Bacterial virulence and antibiotic resistance share common characteristics, such as both processes are required for bacteria to survive under harsh conditions (virulence mechanisms are necessary to overcome host defence and development of AR is essential to enable pathogenic bacteria to surpass antimicrobial therapies and to adapt to and survive in competitive and demanding environments). In addition, bacterial factors involved in virulence are often involved in AR (e.g. multidrug efflux pumps) and AR is greatly associated with infections and is, therefore, related to virulence (Beceiro *et al*., 2013). In this section of the results, antibiotic resistance mechanisms of different *C. jejuni* strains were explored. Experimental work covered in the following section includes mainly different Tet resistance assays and construction of *C. jejuni* G1 *cmeB* mutant and derivative strains.

3.2.2. Genome sequencing of *C. jejuni* strain G1

As previously mentioned, *C. jejuni* G1 and 11168H strains do not contain pTet and pVir plasmids (Fig. 3.2). Despite the absence of the tetracycline resistance plasmid pTet in G1, it was previously observed that this strain was able to grow in the presence of tetracycline whilst strain 11168H could not, suggesting that other mechanisms may be associated with Tet resistance in the former strain (Lehri *et al.*, 2015).

The genome of *C. jejuni* strain G1 (A3) was sequenced by co-workers in our laboratory and its genome was then compared with that of a reference strain NTCT 11168 using CLC genomics software. A remarkable difference between the CmeABC operons of these two strains, especially in the *cmeB* gene was revealed (Fig. 3.16), whilst the surrounding areas showed considerable conservation (Lehri *et al*., 2015). Protein blast showed 81% identity between the CmeB amino acid sequences of these two strains, whilst there was 99% similarity between the amino acid sequences of 11168H and 81-176 strains.

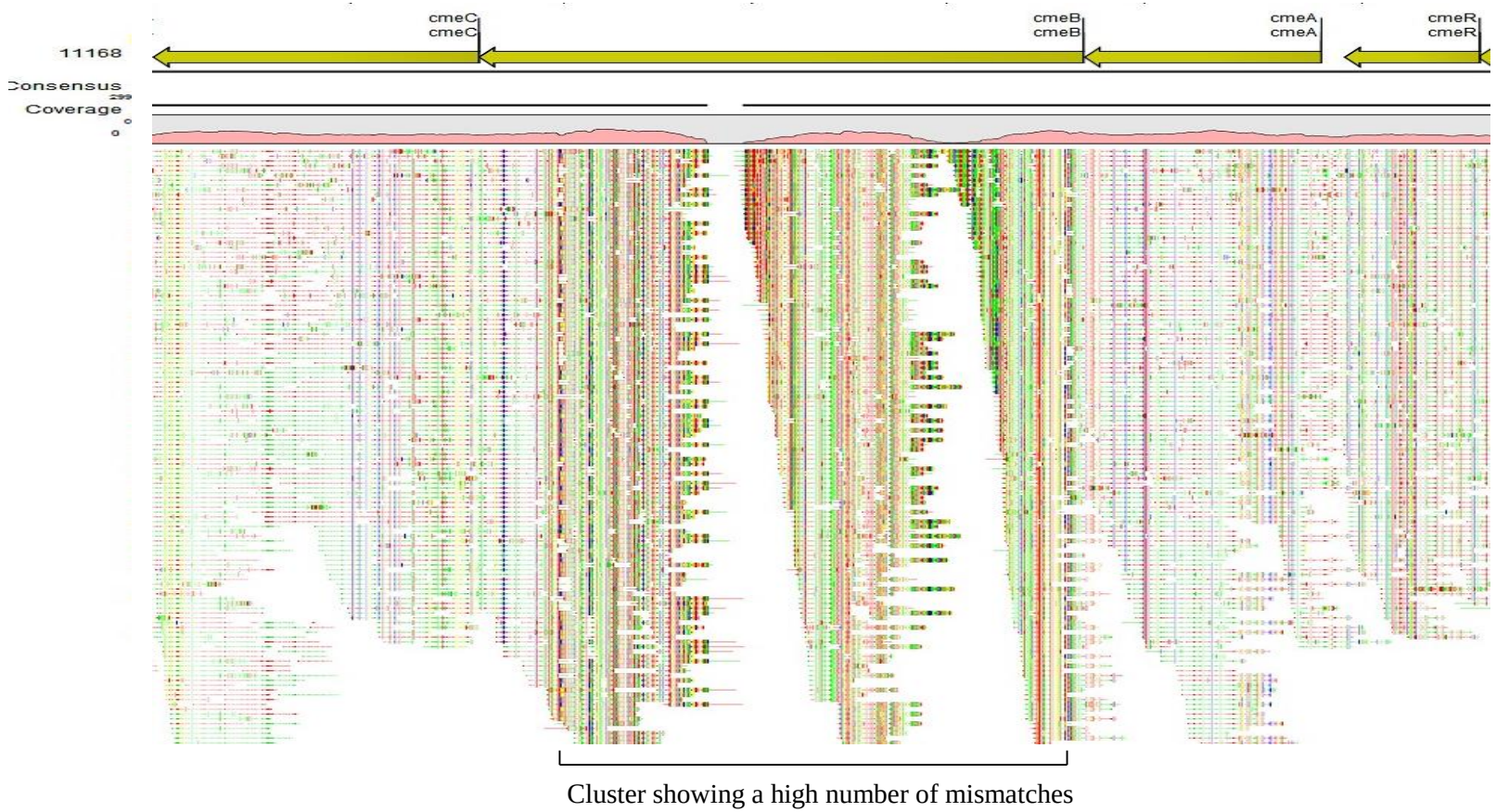


Figure 3.16. Comparative analysis of the *cmeABC* operons of *C. jejuni* strains G1 and NTCT 11168. G1 sequencing reads were mapped onto the *cmeABC* operon of 11168 using CLC genomics Workbench software. The yellow, red, blue or green vertical lines highlighted represent the mismatches between the G, A, C and T nucleotide bases, respectively, of G1 *cmeB* in comparison with the *cmeB* from strain 11168. The white areas represent areas without similarity between the G1 *cmeB* and the *cmeB* from 11168 strain (Lehri *et al*., 2015).

CmeB is already known to be involved in *C. jejuni* resistance to antibiotics and to act synergistically with other resistance mechanisms to increase AR (Iovine, 2013). This supports the observed increased resistance of *C. jejuni* strain G1 to tetracycline in comparison with 11168H, which was probably related with the *cmeABC* operon. Tetracycline resistance was tested for *C. jejuni* G1 wt and *cmeB* mutants in order to check if the contribution of this efflux pump to AR was dependent on the *cmeB* sequence variation.

3.2.3. Generation of *C. jejuni cmeB* mutant and derivative strains

To test sequence variation contribution of *cmeB* to tetracycline resistance, 11168H, G1 and 81-176 *cmeB* mutants and complemented strains were generated. In this section, it is shown in detail how the G1 *cmeB* mutant strain was constructed. In addition, a derivative of G1 containing the pTet plasmid from strain 81-176 was generated. These constructs and the respective wt strains were further assayed to detect variation in antibiotic resistance.

3.2.3.1. Generation of G1/*cmeB::kan*^{r} mutant

Construction of the G1/*cmeB*::*kan*^{r} mutant strain was achieved by inactivation of the *C. jejuni* G1 gene *cmeB* through insertion of a kanamycin resistance (*kan*^{r}) cassette, as described in the literature (Vieira *et al*., 2017). Briefly, the *cmeB* PCR product was purified and cloned into pGEM-T easy vector by ligation and transformed into *E. coli* cells. After transformation *E. coli* colonies were verified for the presence of *cmeB* gene by PCR and the orientation of the *cmeB* gene in the pGEM-T/*cmeB* plasmid was checked in several clones by restriction analysis, by which the clone in the forward orientation was used for the insertion of the *kan*^{r} gene.

The pJMK30 plasmid was digested with *Sma*I and the 1.5kb *kan*^{r} fragment was extracted and purified before ligation to the pGEM-T/*cmeB* plasmid. The *kan*^{r} was then ligated to the pGEM-T/*cmeB* recombinant plasmid and transformed into *E. coli* cells. To confirm the orientation of the *kan*^{r} cassette in the recombinant plasmid, restriction maps were constructed using the *Sal*I enzyme (Fig. 3.17). In the forward orientation of *kan*^{r} cassette, the sizes of fragments were 6653bp and 1000bp (Fig. 3.17, A), whereas, in the reverse orientation of this cassette, the sizes of fragments were 5188bp and 2465bp (Fig. 3.17, B). The ligation mixture was transformed into *E. coli* cells producing colonies on LB supplemented with kanamycin (50µg/ml). The CloneChecker kit was used to confirm the orientation of the *kan*^{r} in the clones transformed with pGEM-T/*cmeB*/*kan*^{r} plasmid. The clone with the cassette in the forward orientation was confirmed by restriction digestion with *Sal*I (Fig. 3.17, C) and was then selected for further transformation of *C. jejuni* G1 wt via electroporation. It is important to select the clone in the forward orientation to avoid a polar mutation that would affect the expression of the downstream genes in the operon (*cmeA* and *cmeC*).

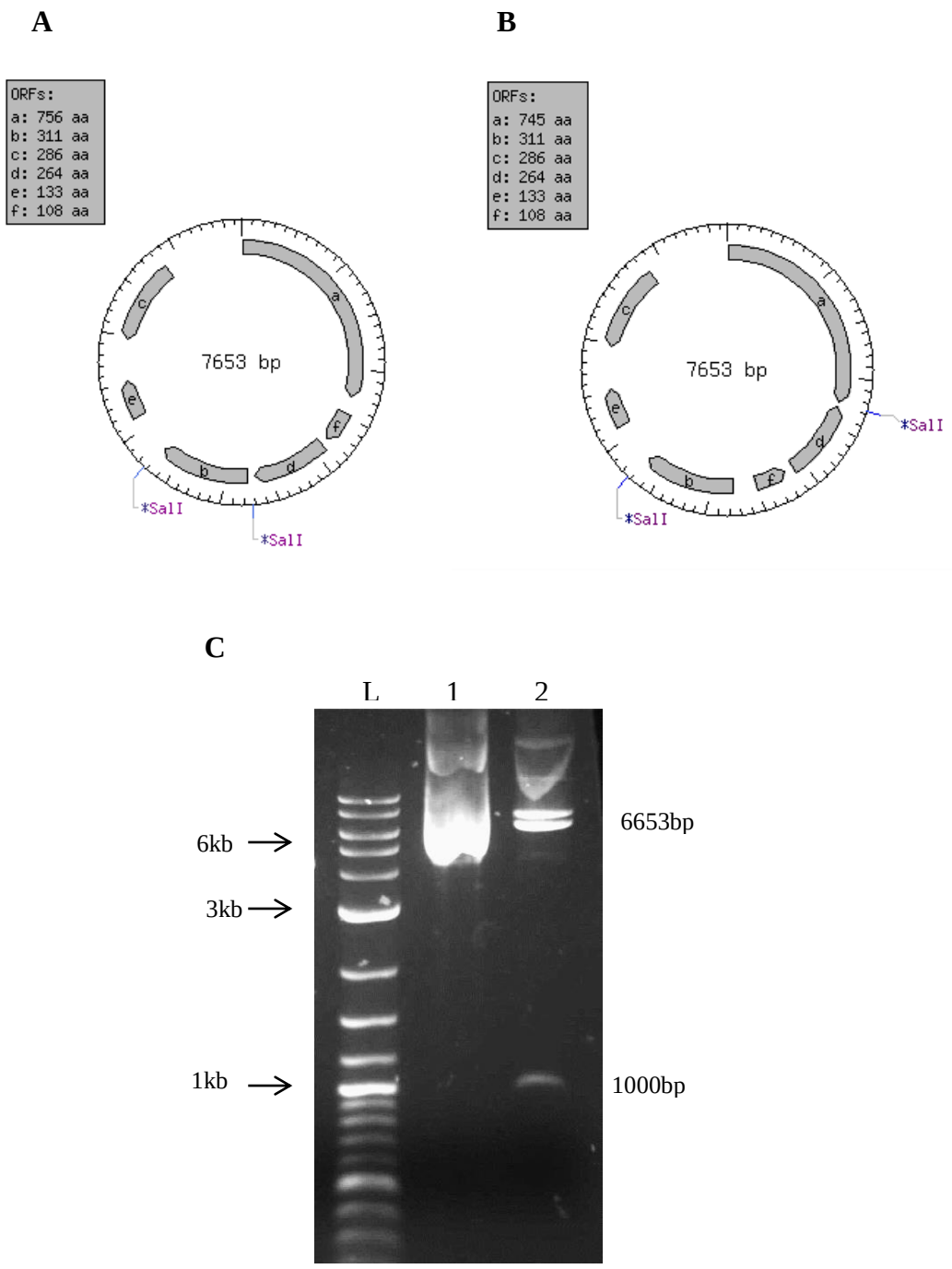


Figure 3.17. Restriction analysis of pGEM-T/*cmeB/kan*^{r} recombinant plasmid with *Sal*I. Restriction digestion maps using NEBcutter online tool of pGEM-T/*cmeB*/*kan*^{r} digested with *Sal*I, showing the *kan*^{r} in the (A) forward orientation and (B) reverse orientation. (C) Restriction digestion analyse with *Sal*I. Lane L: 2-log DNA ladder; lane 1: uncut pGEM-T/*cmeB*/*kan*^{r}; lane 2: pGEM-T/*cmeB*/*kan*^{r} digested with *Sal*I – 6653bp + 1000bp (forward orientation).

Lysates of three *C. jejuni* clones were tested to confirm the presence of *kan*^{r} by PCR using the CmeB primers. The expected size of the PCR product in the G1/*cmeB::kan*^{r} should be 4.6kb (3.1kb size of *cmeB* + 1.5kb size of the *kan*^{r} cassette). The difference of the 1.5kb among the *cmeB* gene bands of wt and mutants indicates the presence of *kan*^{r} in the mutant strains. Confirmation that the mutants were the G1 strain was conducted by PCR using the TagF primers (Fig. 3.18).

As mentioned previously, a similar procedure was used for construction of the *cmeB* mutant strains in *C. jejuni* strains 11168H and 81-176.

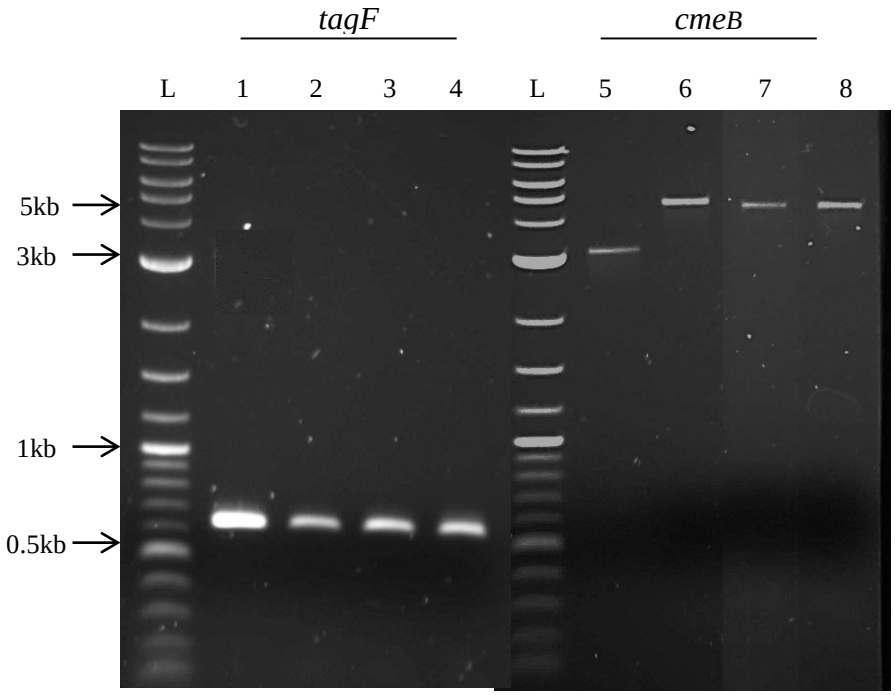


Figure 3.18. Confirmation of *C. jejuni* G1/*cmeB::kan*^{r} mutant. Gel electrophoresis analysis of the lysates of three *C. jejuni* G1/c*meB*::*kan*^{r} clonal isolates. Lane L: 2-log DNA ladder; lane 1: G1 – 642bp; lanes 2-4: G1/c*meB*::*kan*^{r}– 642bp; lane 5: G1 – 3.1kb; lanes 6-8: G1/c*meB*::*kan*^{r}– 4.6kb.

3.2.3.2. Generation of G1/*cmeB::kan*^{r}/*cmeB* derivative

Complementation of *C. jejuni* G1/c*meB*::*kan*^{r} was attempted using pRRC plasmid as the gene delivery vector, a technique that has previously been shown to be successful

(Karlyshev & Wren 2005). Complementation CmeB primers were designed (Table 2.7, 1) and the *cmeB* gene was amplified by a high fidelity PCR, digested with *Xba*I and cloned into pRRC, a plasmid containing a *cam*^{r} cassette. After transformation of the recombinant plasmid pRRC/*cmeB*/*cam*^{r} into *C. jejuni* G1/c*meB*::*kan*^{r} cells for further integration of the *cam*^{r} cassette into one of the three rRNA clusters where *cmeB* would be under the control of the constitutive *cam*^{r} gene promoter. After transformation of the recombinant plasmid in *E. coli* cells, the CloneChecker kit was used for restriction analysis with *Cla*I enzyme to check the orientation of the *cmeB* gene in the pRRC plasmid. It was considered important that the *cmeB* was cloned in the forward orientation so that the *cam*^{r} promoter could express this gene. Unfortunately, all clones tested either had the *cmeB* fragment cloned in the reverse (incorrect) orientation (Fig. 3.19, lanes 2, 4, 7) or did not have this fragment cloned in the pRRC (Fig. 3.19, lanes 1, 3, 5, 6), which could be due to inefficient ligation reaction.

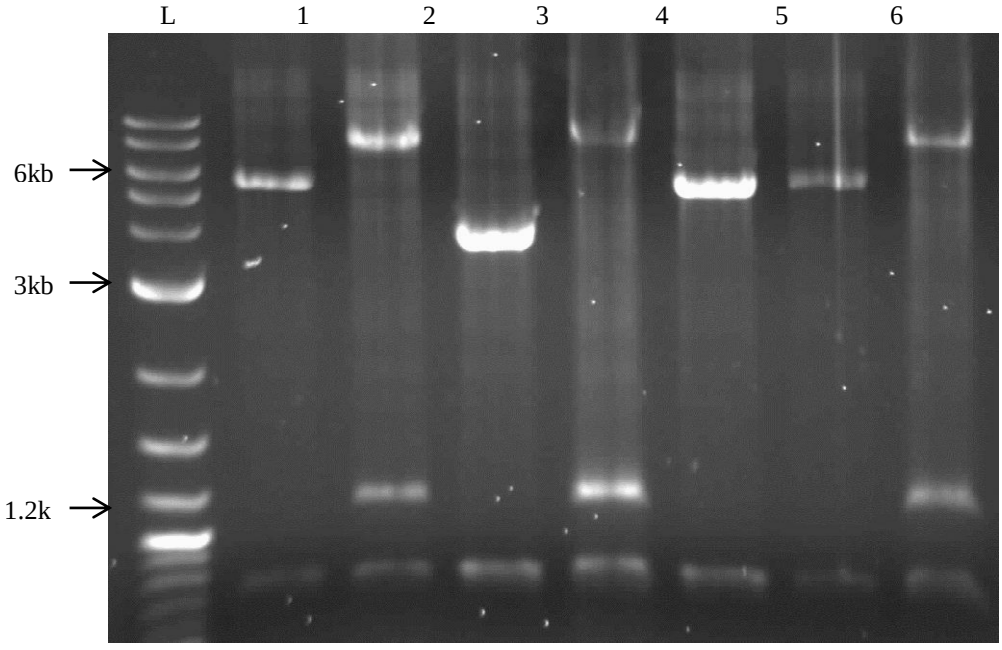


Figure 3.19. Using CloneChecker for selection of a pRRC/*cmeB* plasmid with *cam*^{r} cassette insert**.** Lane L: 2-log DNA ladder; lane 1: pRRC – 5115bp and 730bp; lanes 2, 4, 7: pRRC/*cmeB*/*cam*^{r} in the reverse orientation – 7156bp + 730bp + 1136bp; lanes 3, 5, 6: empty pRRC – 5115bp and 730bp.

Further to the failure using the pRRC plasmid, complementation via the same gene delivery system technique was attempted, but this time using another plasmid. The plasmid pRED1 containing the *egfp* gene was used to select recombinant clones via fluorescence. New complementation primers were used (Table 2.7, 2) to amplify the *cmeB* gene from *C. jejuni* G1. The PCR product was then purified and digested with *Eco53*KI and *Xba*I enzymes and ligated to the pRED1 plasmid digested with *Swa*I and *Xba*I and dephosphorylated. The ligation mixture was then transformed into *E. coli* cells and the CloneChecker kit was used for restriction analysis using *Cla*I to check for the clones lacking fluorescence (*egfp* was cut out after digestion and replaced by *cmeB* fragment, so these clones were no longer fluorescent). The clones with the *cmeB* in the correct orientation (Fig. 3.20) were further sent for sequencing to check for the presence of mutations.

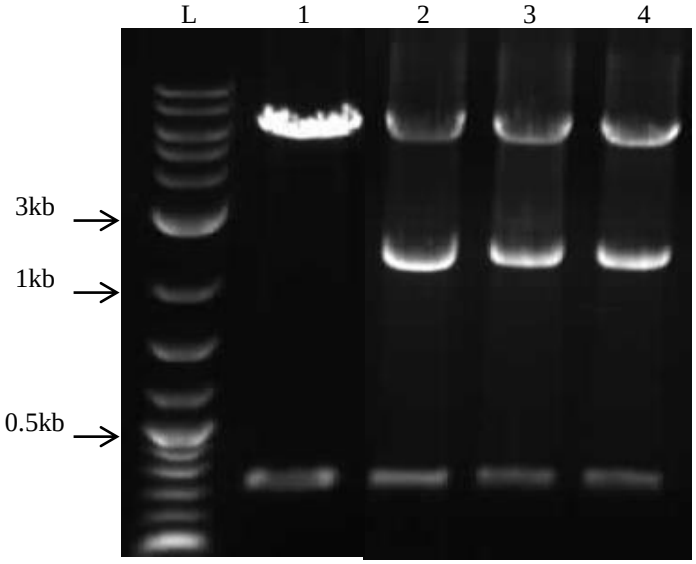


Figure 3.20. Using CloneChecker for selection of a pRED1/*cmeB* plasmid with *cam*^{r} cassette insert**.** Lane L: 2-log DNA ladder; lane 1: pRED1 – 5901bp + 730bp; lanes 2-4: *E. coli* clones with pRED1/*cmeB*/*cam*^{r} in the forward orientation – 5901bp + 730bp + 2384bp.

The recombinant plasmid pRED1/*cmeB*/*cam*^{r} was electroporated into *C. jejuni* G1/*cmeB*::*kan*^{r} strain and plated onto CBA supplemented with Cm (10μg/ml). To confirm the insertion of the *cam*^{r} cassette into the three rRNA clusters, primers Ak233/234/235/237 were used (Table 2.7). PCR using these latter primers and the TagF and CmeB primers was conducted with transformants of G1/c*meB*::*kan*^{r} with the recombinant plasmid pRED1/*cmeB*/*cam*^{r} (Fig. 3.21). The control pRRC plasmid was successfully transformed in the G1/c*meB*::*kan*^{r} as demonstrated by the 2.8kb size band when using the primer pair Ak233/Ak237 insertion primers for the *cam*^{r} cassette (Fig. 3.21, lane 8). Another control condition was the successful transformation of the recombinant plasmid pRED1/*cmeB*/*cam*^{r} in the G1 wt, which was demonstrated by the 2.8kb band using the Ak233/Ak237 primer pair (Fig. 3.21, lane 3). However, no transformants were obtained when the recombinant pRED1/*cmeB*/*cam*^{r} was electroporated into the *C. jejuni* G1/c*meB*::*kan*^{r}, demonstrated by the absence of a band when using the Ak primers (Fig. 3.21, lanes 13-15), which could be due to low transformation efficiency of the G1/c*meB*::*kan*^{r} competent cells. In addition, for an unexplained reason, the G1/c*meB*::*kan*^{r} strain might not accept the foreign DNA used, which might be degraded by the nucleases inside the bacterial cells. Different stocks of *C. jejuni cmeB* mutant strain competent cells were prepared; however, no positive results for transformation with the recombinant plasmid were observed. This complementation strategy was attempted with another *C. jejuni* strain, 11168H, but, again, without success.

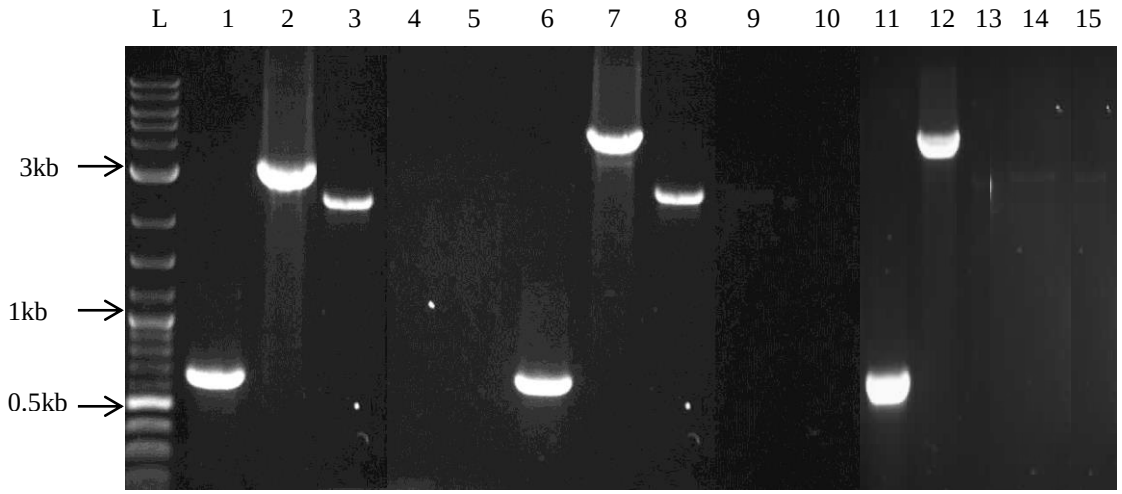


Figure 3.21. PCR analysis of *C. jejuni* G1/*cmeB::kan*^{r}/*cmeB* insertion derivatives. PCR was conducted with TagF, CmeB and Ak233/234/235/237 primers Lane L: 2-log DNA ladder; lane 1: G1+pRED1/*cmeB*/*cam*^{r} – 642bp; lane 2: G1+pRED1/*cmeB*/*cam*^{r} – 3.1kb; lanes3-5: G1+pRED1/*cmeB*/*cam*^{r} – no amplification; lane 6: G1/c*meB*::*kan*^{r}+pRRC – 642bp; lane 7: G1/c*meB*::*kan*^{r}+ pRRC – 4.6kb; lane 8: G1/c*meB*::*kan*^{r}+pRRC – 2.8kb; lanes 9-10: G1/c*meB*::*kan*^{r}+ pRRC no amplification; lane 11: G1/c*meB*::*kan*^{r} +pRED1/*cmeB*/*cam*^{r} – 642bp; lane 12: G1/c*meB*::*kan*^{r}+pRED1/*cmeB*/*cam*^{r} – 4.6kb; lanes 13-15: G1/c*meB*::*kan*^{r}+pRED1/*cmeB*/*cam*^{r} no amplification.

Since complementation of the *C. jejuni* G1/c*meB*::*kan*^{r} mutant using the pRRC gene delivery system failed, another approach was attempted.

The *cmeB* gene is required for AR in *C. jejuni* and thus, it could act as an antibiotic resistance marker. The strain *C. jejuni* G1/*cmeB*::*kan*^{r}/*cmeB* was constructed via homologous recombination and considered a repair strain as this is a strain in which the mutant allele was replaced by the wt allele. The genomic DNA from the *C. jejuni* G1 wt strain was transformed into G1/*cmeB*::*kan*^{r}/*cmeB* mutant via electroporation and transformants were selected in CBA agar supplemented with 10μg/mL of chloramphenicol, a concentration which did not support the growth of G1/*cmeB*::*kan*^{r}, but allowed the growth of G1 wt strain. Due to the selective pressure, this bacterial strain was forced to replace the *kan*^{r} cassette from its genome so that it would re-integrate the *cmeB* gene by homologous recombination and restore the AR required for its survival. A similar procedure was used for construction of the derivative of *C. jejuni* 81-176 strain, but these transformants were selected on CBA agar supplemented with 10μg/mL of

tetracycline, a concentration which did not support the growth of the 81-176/*cmeB*::*kan*^{r} mutant strain, but allowed for the 81-176 wt strain to grow. PCR using the respective TagF and CmeB primers was conducted to confirm the complementation derivatives of *C. jejuni* G1/*cmeB*::*kan*^{r} (Fig. 3.22). The repaired strain derivatives were confirmed by amplification of a 3.1kb size band corresponding to the wt version of the *cmeB* gene (Fig. 3.22, lane 5). The G1 wt and G1/c*meB*::*kan*^{r} mutants were used as positive and negative controls, respectively (Fig. 3.22, lanes 2-3). The repaired strains were designated *C. jejuni* G1/c*meB*::*kan*^{r}*/cmeB* and *C. jejuni* 81-176/c*meB*::*kan*^{r}/*cmeB*.

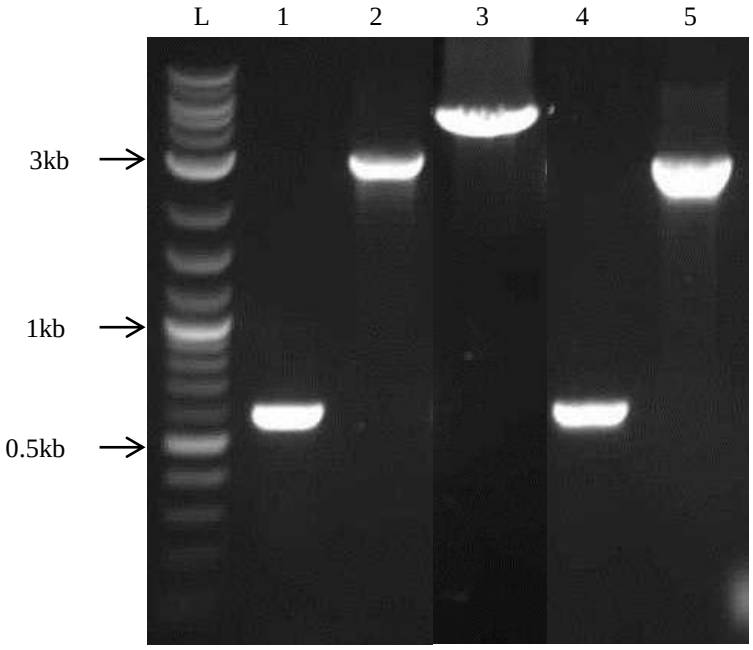


Figure 3.22. PCR analysis of *C. jejuni* G1/*cmeB::kan*^{r}*/cmeB* repair strain obtained by homologous recombination. PCR was conducted using TagF and CmeB primers. Lane L: 2-log DNA ladder; lane 1: G1 – 642bp; lane 2: G1 – 3.1kb; lane 3: G1/c*meB*::*kan*^{r} – 4.6kb; lane 4: G1/c*meB*::*kan*^{r}*/cmeB* – 642bp; lane 5: G1/c*meB*::*kan*^{r}*/cmeB* – 3.1kb.

The objective of this part of the work was to construct a complementation derivative of *C. jejuni* G1/*cmeB::kan*^{r} mutant with the *cmeB* genes from both G1 and 11168H strains. These strains were selected as the pTet plasmid was absent and they presented different

tetracycline resistance levels, a possible result of a large difference in their *cmeB* nucleotide sequences. The idea was to complement the *C. jejuni* G1/*cmeB::kan*^{r} mutant with both G1 and 11168H *cmeB* genes using the same method so that the two different *cmeB*s would be under the control of the same promoter and expressed at the same level in *C. jejuni* strain G1. Thus, if the tetracycline resistance levels were different between *C. jejuni* G1 strains with the different *cmeB*s, this would confirm that nucleotide sequence of the *cmeB* gene was very important in determining the AR levels in *C. jejuni*. However, it was not possible to ligate the 11168H *cmeB* gene in the pRED1 plasmid, as the *E. coli* transformants tested by restriction analysis were empty pRED1 vectors and this was most likely due to technical errors (data not shown).

3.2.3.3. Generation of *C. jejuni* G1/pTet derivative

C. jejuni strains G1 and 81-176 present large differences in their *cmeB* nucleotide sequences. As already mentioned, CmeB is known to act together with pTet to confer high antibiotic resistance (Iovine, 2013). It was hypothesised that if *C. jejuni* strain G1 received the pTet plasmid from *C. jejuni* strain 81-176, G1 would probably become more resistant to Tet than the latter strain. To test this hypothesis, conjugation was conducted between strains 81-176 and G1. It was first necessary to find an antibiotic that allowed growth of *C. jejuni* strain G1, but not strain 81-176. An antibiotic disc assay was conducted where bacterial suspensions of these strains were plated onto MH blood agar plates topped with several different random antibiotics and plates were incubated for three days to measure the zones of inhibition (Fig. 3.23). The higher the inhibition zone, the more susceptible was the strain to the antibiotic. It was observed that both *C. jejuni* strains G1 and 81-176 were fully resistant to ceftazidime, cefoxitin, linezolid, methicillin, penicillin G, rifampicin and trimethoprim antibiotics in the concentrations used (Fig. 3.23). Although *C. jejuni* strain 81-76 was slightly more resistant to amikacin,

erythromycin and gentamicin than G1, these differences were not statistically significant. Overall, *C. jejuni* strain G1 was significantly more resistant to the vast majority of the antibiotics tested (amoxicillin, ampicillin, chloramphenicol, cefquinome, clarithromycin, clindamycin and mupirocin) than strain 81-176.

Since G1 was fully resistant to ampicillin (10μg/ml) as opposed to strain 81-176, this antibiotic was selected for the conjugation experiment (Fig. 3.23).

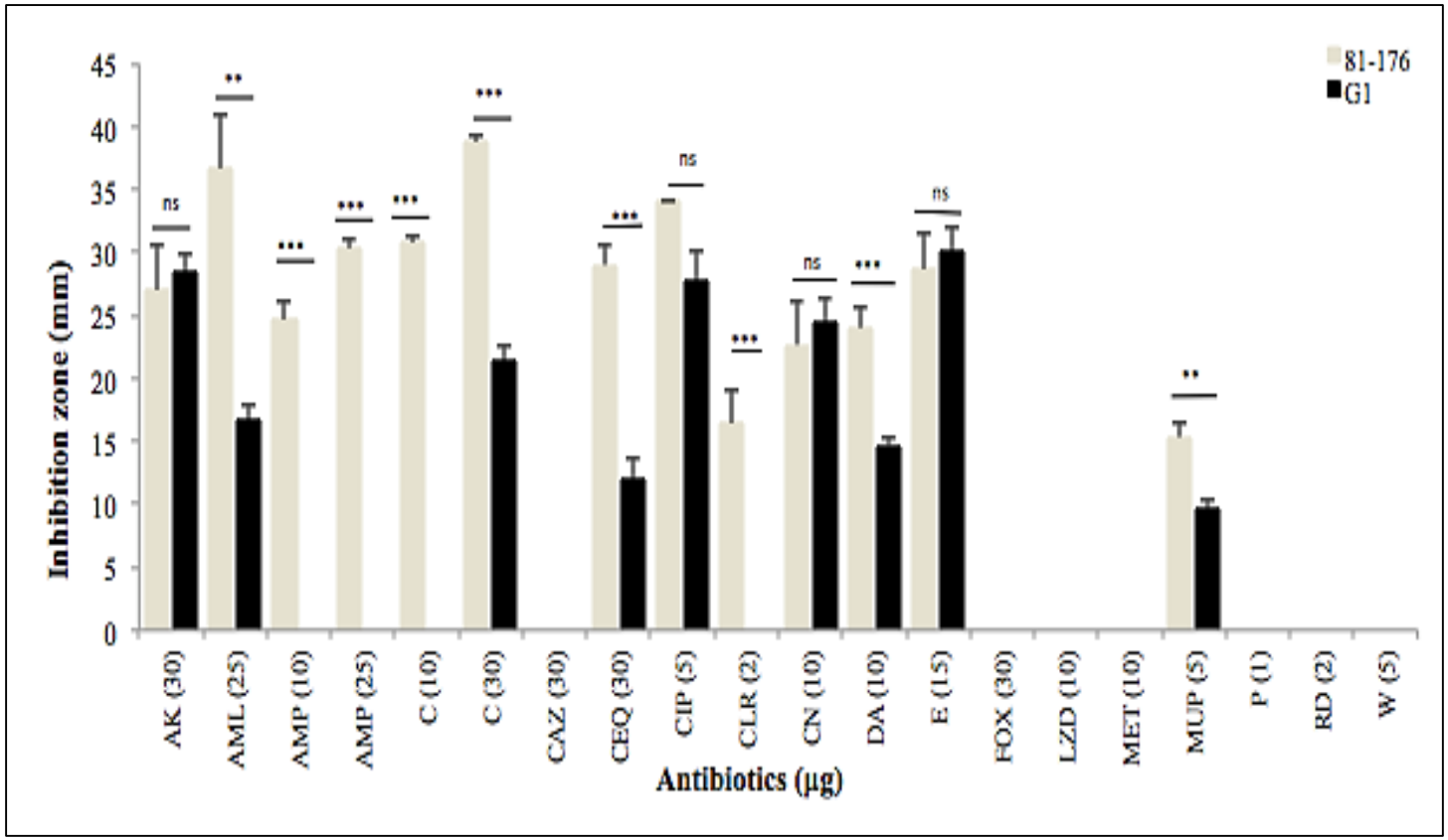


Figure 3.23. Antibiotic susceptibility of *C. jejuni* strains G1 and 81-176. The susceptibility of *C. jejuni* strains G1 (black bars) and 81-176 (beige bars) to different antibiotics was tested by disc diffusion assay over a 48h incubation period at 37°C, according to EUCAST recommendations. Absence of bars indicates full resistance of the *C. jejuni* strains. Amikacyn (AK), amoxicillin (AML), ampicillin (AMP), chloramphenicol (C), ceftazidime (CAZ), cefquinome (CEQ), ciprofloxacin (CIP); clarithromycin (CLR), gentamycin (CN), clindamycin (DA), erythromycin (E), cefoxitin (FOX), linezolid (LZD), methicillin (MET), mupirocin (MUP), penicillin G (P), rifampicin (RD) and trimethoprim (W). The antibiotic concentration used (µg/ml) is presented in brackets. Values are mean ± SD from three independent experiments with three technical replicates each.

For conjugation, the bacterial mixture was then plated onto CBA supplemented with both 15μg/mL of tetracycline that forced G1 strain to receive the pTet plasmid as without it, it would not be able to survive in this high Tet concentration and 10μg/mL of ampicillin, the latter did not support the growth of *C. jejuni* strain 81-176, but allowed strain G1 to grow (Fig. 3.23). PCR of the conjugation transformants with the primers TagF, DmhA, pTet and pVir was conducted to confirm the G1/pTet derivatives (Fig. 3.24). *C. jejuni* G1 and 81-176 wt strains were used as controls (Fig. 3.24, lanes 1-2, 11-12). Eight G1/pTet transformants were shown to be G1, as expected, since a 642bp size band corresponding to G1 *tagF* fragment was obtained (Fig. 3.24, lanes 3-10). Except for one of the transformants (Fig. 3. 24, lane 14) the other seven amplified the *tetO* gene (Fig. 3.24, lanes 12, 13, 15-20), confirming that pTet plasmid was successfully transferred from *C. jejuni* 81-176 to G1 strain. In addition, and as expected, no pVir plasmid was present in the G1/pTet derivatives (Fig. 3.24, lanes 13-20).

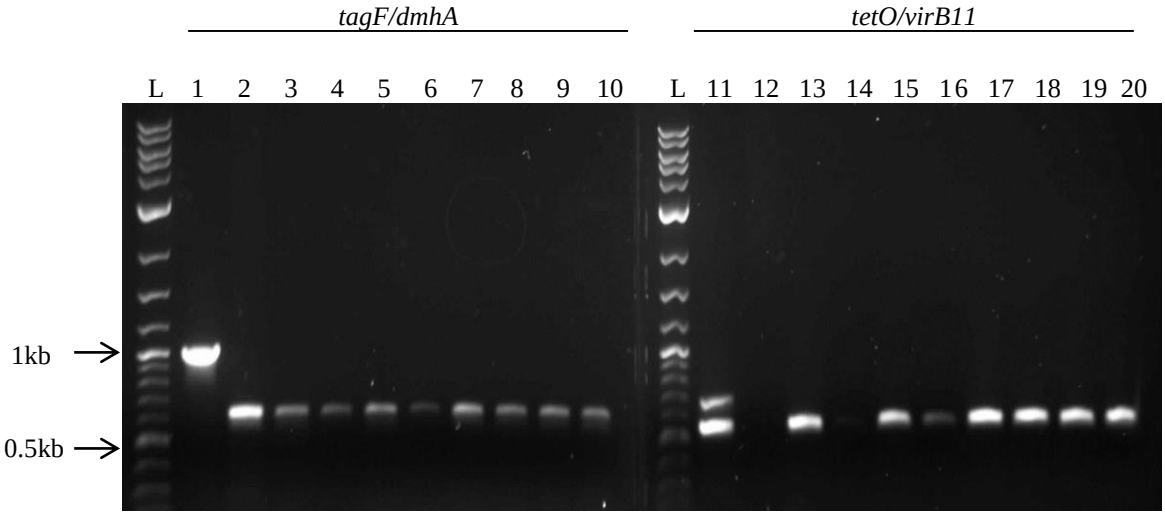


Figure 3.24. Duplex PCR analysis for confirmation of the *C. jejuni* G1/pTet derivatives. PCR was conducted with lysates of the conjugation transformants**.** Lane L: 2-log DNA ladder; lane 1: 81-176 – 1021bp; lane 2: G1 – 642bp; lanes 3-10: G1/pTet isolates - 642bp; lane 11: 81-176 – 708bp + 559bp; lane 12: G1 – no amplification; lanes 13, 15-20: G1/pTet transformants - 559bp; lanelane 14: negative G1/pTet transformants – no amplification.

3.2.4. Tetracycline resistance levels of *C. jejuni* wt, mutant and derivative strains

The minimal inhibitory concentrations to Tet of *C. jejuni* strains 81-176, G1 and 11168H and the respective *cmeB* mutants as well as the derivative strain G1/pTet were determined by the micro dilution broth method in MH broth supplemented with 5% lysed blood over a 48h incubation period at 37°C, according to the EUCAST recommendations (Sifre *et al*., 2015). The MIC values obtained for the different *C. jejuni* strains, as well as the fold-change difference in Tet resistance between the wt strains and the respective *cmeB* mutants, (three clonal isolates were tested for each strain) are shown in Table 3.1. Complementation of *C. jejuni* G1 and 81-176 *cmeB* mutants restored the level of Tet resistance (data not shown). As expected, in comparison with the other strains tested 81-176 exhibited a higher level of Tet resistance due to the presence of the pTet plasmid. Mutation of the *cmeB* gene in this strain, led to a 8-fold decrease in the Tet resistance levels. Importantly, a 16-fold difference in the Tet resistance levels was observed between G1 and 11168H with G1 strain having higher resistance to this antibiotic. In addition mutation of *cmeB* in 11168H and G1 strains resulted in 2-fold and 32.5-fold reduction in tetracycline resistance, respectively. The latter results were confirmed by growth of *C. jejuni* 81-176, G1 and 11168H wt strains and three clonal isolates of the G1/*cmeB::kan*^{r} in CBA plates supplemented with 2μg/ml of tetracycline. It was observed that strains 81-176 and G1 were able to grow at this Tet concentration, whilst 11168H and G1/*cmeB::kan*^{r} clonal isolates could not (Fig. 3.25, A). Strikingly, transfer of pTet plasmid from *C. jejuni* 81-176 to G1 strain by conjugation made the latter eight times more resistant to Tet than the donor *C. jejuni* strain 81-176 carrying this plasmid (Table 3.1). Due to the large 250-fold increase in the Tet resistance of G1/pTet compared with the wt strain, three clonal isolates of this derivative and the control 81-176 and G1 strains were plated onto CBA medium supplemented with 250μg/ml of tetracycline to confirm

these results. It was observed that indeed the three G1/pTet derivatives were able to grow in a high Tet concentration, whilst the wt strains could not (Fig. 3.25, B).

These findings possibly suggest that the CmeB of *C. jejuni* strain G1 has a higher activity to excrete this drug than its homologues in *C. jejuni* strains 11168H and 81-176.

Table 3.1. Tetracycline susceptibility of *C. jejuni* wt, mutant and derivative strains

Campylobacter jejuni strains	Tetracycline MIC (µg/ul)	St Deviation	Fold-change (WT vs mutant)
81-176/*cmeB*$^{+}$/pTet^{+} (WT)	62.5	0.010	--
81-176/*cmeB*$^{-}$/pTet^{+}	7.8	0.020	8
11168H/*cmeB*$^{+}$/pTet^{-} (WT)	0.12	0.017	--
11168/*cmeB*$^{-}$/pTet^{-}	0.06	0.017	2
G1/*cmeB*$^{+}$/pTet^{-} (WT)	1.95	0.002	--
G1/*cmeB*$^{-}$/pTet^{-}	0.06	0.011	32.5
G1/*cmeB*$^{-}$/pTet^{+}	500	0.031	256

-, absence or disruption of the gene; +, presence or integrity of the gene. MIC values were determined by the microdilution broth method over an incubation period of 48h at 37°C. Values are mean ± SD from three independent experiments with three technical replicates each

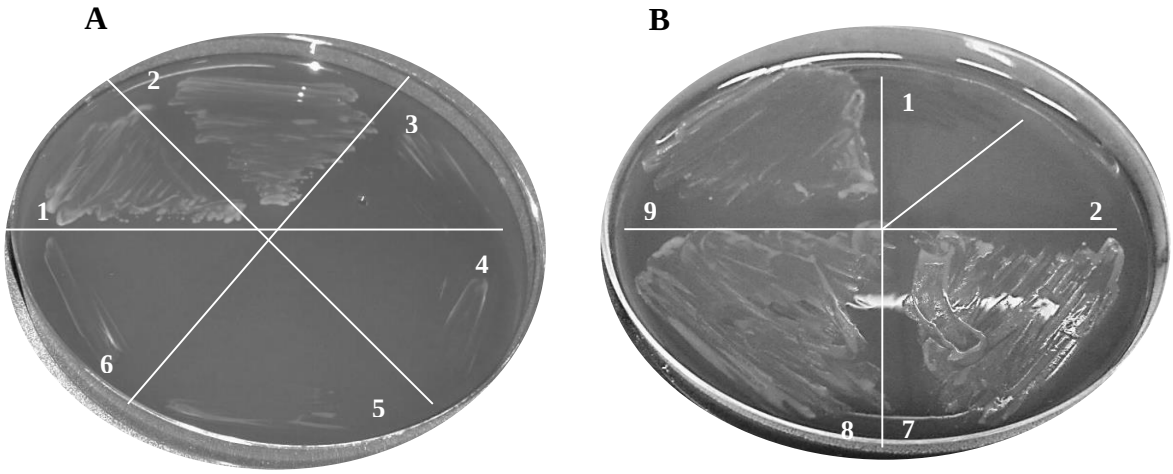


Fig. 3.25. Growth of *C. jejuni* wt, mutant and derivative strains on CBA plates supplemented with different tetracycline concentrations. (A) *C. jejuni* strains (1) 81-176; (2) G1; (3) 11168H; (4) G1/*cmeB*::*kan*^{r} clone 1; (5) G1/*cmeB*::*kan*^{r} clone 2; (6) G1/*cmeB*::*kan*^{r} clone 3 plated onto CBA supplemented with 2μg/ml of tetracycline. (B) *C. jejuni* strains (1) 81-176; (2) G1; (7) G1/pTet clone 1; (8) G1/pTet clone 2; (9) G1/pTet clone 3 plated onto CBA supplemented with 250μg/ml of tetracycline.

3.2.5. Antibiotic susceptibility of different *C. jejuni* strains

Resistance to other antibiotics, such as aminoglicosides, amphenicols, β-lactams, fluoroquinolones, lincosamides, macrolides and rifamycins of different *C. jejuni* strains was also tested. As previously shown in Figure 3.23, *C. jejuni* strain G1 was significantly more resistant to the majority of the antibiotics tested than strain 81-176. An antibiotic disc diffusion assay was also conducted to test resistance of *C. jejuni* strains G1 and 11168H strains (Fig. 3.26, A). It was observed that strain G1 was significantly more resistant to amoxicillin, ampicillin, chloramphenicol, cefquinome, ciprofloxacin, clarithromycin, clindamycin, mupirocin, oxytretracycline and tetracycline antibiotics. No statistically significant differences between these two strains were observed in AR to the other antibiotics tested (amikacyn, gentamicin and erythromicin) (Fig. 3.26, A). Interestingly, AR resistance levels of *C. jejuni* strains 11168H and 81-176 were very similar, except for tetracycline due to the presence of the pTet plasmid in strain 81-176 (Fig. 3.26, B). In addition, strain 11168H was significantly more resistant to the β-lactams, amoxicillin and ampicillin (Fig. 3.26, B).

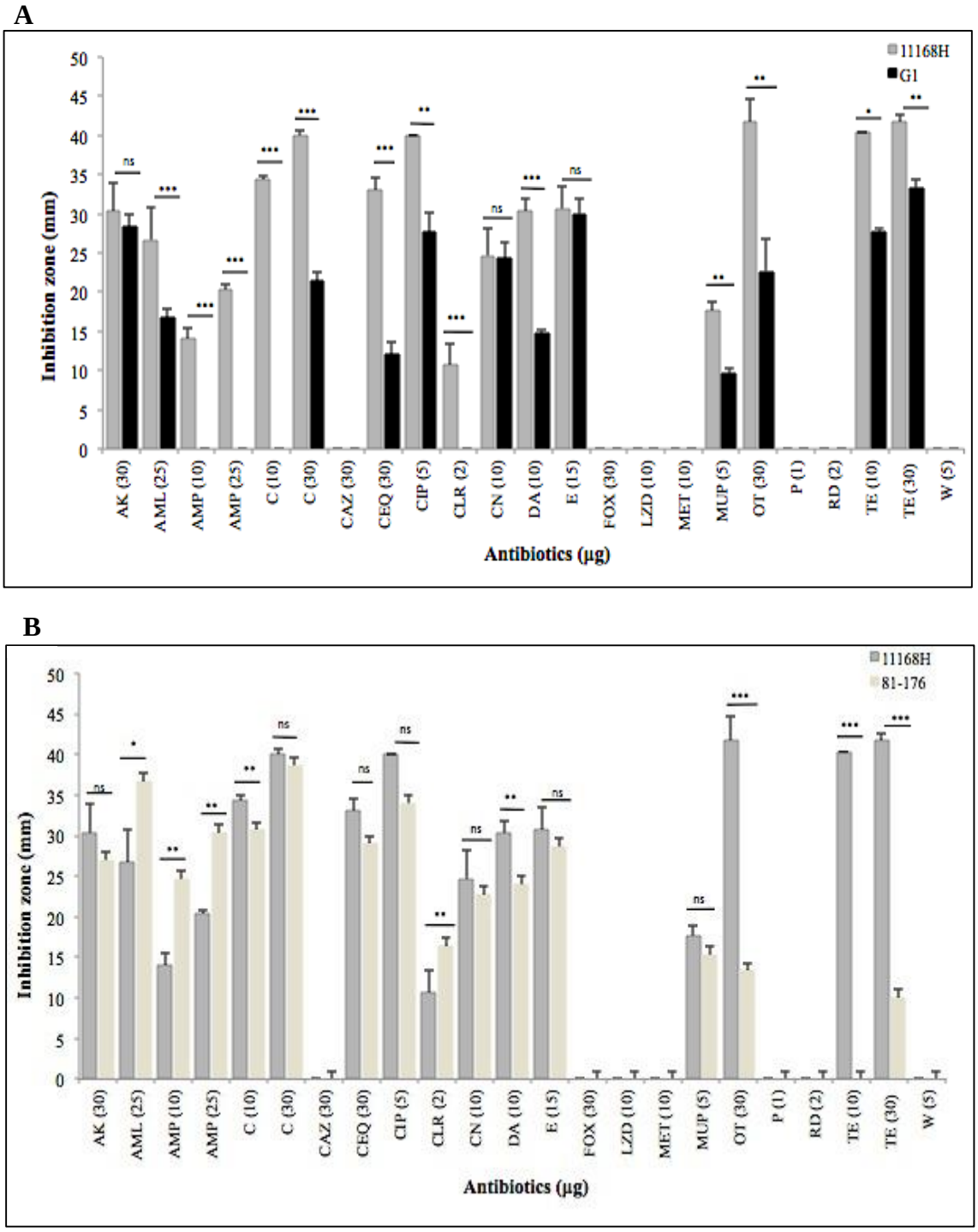


Figure 3.26. Antibiotic susceptibility of *C. jejuni* G1, 11168H and 81-176 strains. (A) The susceptibility of *C. jejuni* strains G1 (black bars) and 11168H (grey bars) and (B) *C. jejuni* strains 11168H (grey bars) and 81-176 (beige bars) to different antibiotics was tested by disc diffusion assay over a 48h incubation period at 37°C, according to EUCAST recommendations. Absence of bars indicates full resistance of the *C. jejuni* strains. Amikacyn (AK), amoxicillin (AML), ampicillin (AMP), chloramphenicol (C), ceftazidime (CAZ), cefquinome (CEQ), ciprofloxacin (CIP); clarithromycin (CLR), gentamycin (CN), clindamycin (DA), erythromycin (E), cefoxitin (FOX), linezolid (LZD), methicillin (MET), mupirocin (MUP), oxytetracycline (OT), penicillin G (P), rifampicin (RD), tetracycline (TE) and trimethoprim (W). The antibiotic concentration used (µg/ml) is presented in brackets. Values are mean ± SD from three independent experiments with three technical replicates each.

In summary, these findings show that strain G1 is much more resistant to various categories of antibiotics than the two other wt strains 11168H and 81-176.

To determine if the *cmeB* sequence variation in G1 was also responsible for the resistance to other antibiotics rather than only Tet, an antibiotic disc diffusion assay was conducted on *C. jejuni* G1 wt and *cmeB* mutants (three clonal isolates) (Fig. 3.27). It was observed that the G1 wt and the three G1/*cmeB/kan*^{r} mutants were fully resistant to the antibiotics penicillin, rifampicin and trimethoprim most likely because the concentrations of these antibiotics tested (1, 2 and 5μg/ml, respectively) were too low (Fig. 3.27). On the other hand, when *cmeB* was mutated in G1 strain, resistance to the other antibiotics tested was greatly decreased. The antibiotic resistance differences obtained between the wt and G1/*cmeB/kan*^{r} clonal isolates were statistically significant ($p<0.01$) (Fig. 3.27). *C. jejuni/cmeB::kan*^{r} mutants were highly susceptible to the antibiotics tested, confirming what was described in the literature that *cmeB* is required for AR. In addition, it suggests that the difference in the nucleotide sequence of the *cmeB* gene in these *C. jejuni* wt strains may be the cause of the different levels of AR observed.

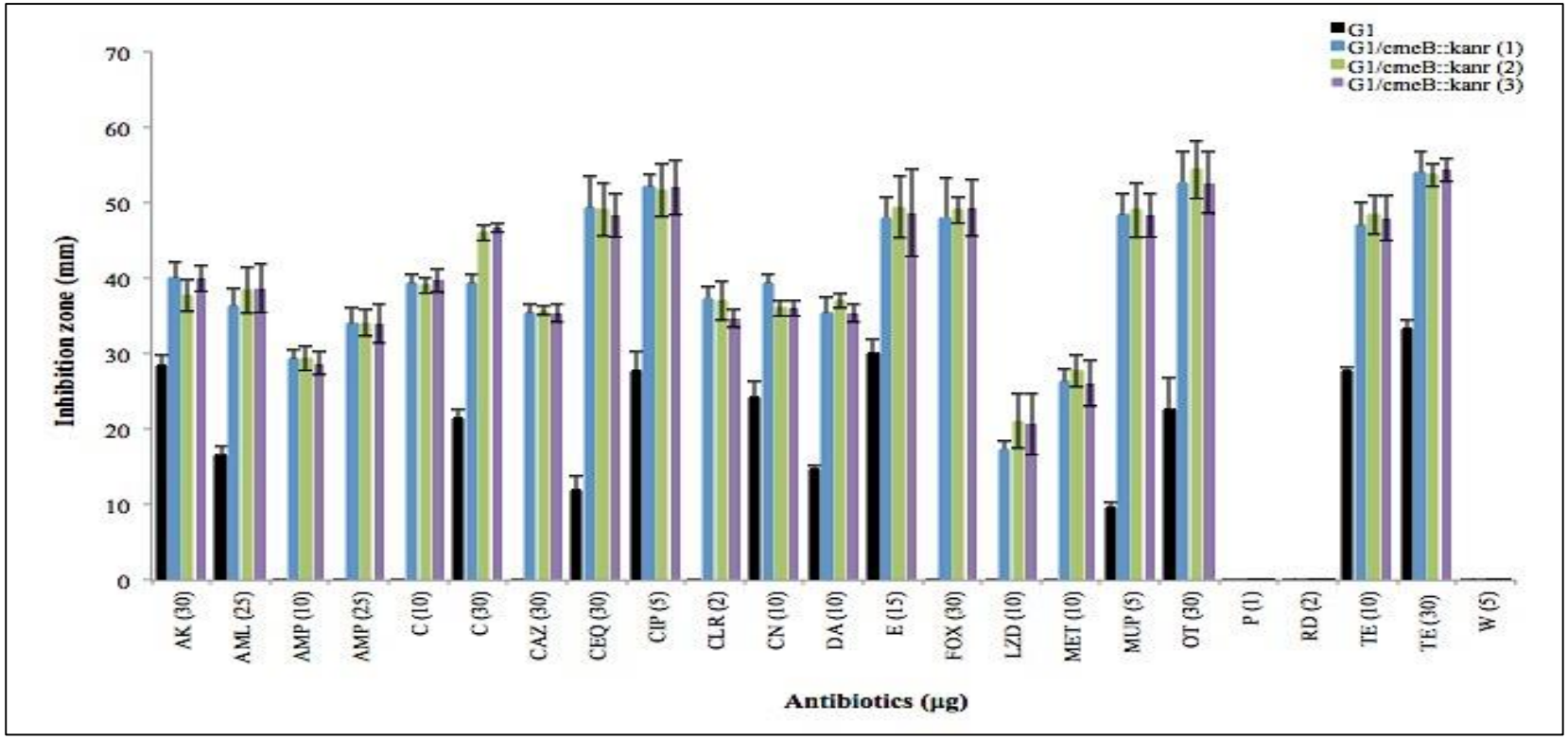


Figure 3.27. Antibiotic susceptibility of *C. jejuni* strains G1 and G1/*cmeB/kan*^{r}. The susceptibility of *C. jejuni* strains G1 (black bars) and G1/*cmeB*::*kan*^{r} mutants (clonal isolate 1, blue bars; clonal isolate 2, green bars; clonal isolate 3, purple bars) to different antibiotics was tested by disc diffusion assay over a 48h incubation period at 37°C, according to EUCAST recommendations. Absence of bars indicates full resistant of the *C. jejuni* strains. Amikacyn (AK), amoxicillin (AML), ampicillin (AMP), chloramphenicol (C), ceftazidime (CAZ), cefquinome (CEQ), ciprofloxacin (CIP); clarithromycin (CLR), gentamycin (CN), clindamycin (DA), erythromycin (E), cefoxitin (FOX), linezolid (LZD), methicillin (MET), mupirocin (MUP), oxytetracycline (OT), penicillin G (P), rifampicin (RD), tetracycline (TE) and trimethoprim (W). The antibiotic concentration used (µg/ml) is in brackets. Except for P, RN and W antibiotics differences of $p<0.01$ were obtained between *C. jejuni* G1 wt strain and the three G1/*cmeB/kan*^{r} mutants. Values are mean ± SD from three independent experiments with three technical replicates each.

Additionally, to check if the *C. jejuni cmeB* could work with other efflux systems as a membrane component and confer resistance to tetracycline in other bacterial species, an *E. coli* strain susceptible to Tet was used. As previousely mentioned, *cmeA*, *cmeB* and *cmeC* are homologues of the *E. coli acrA*, *acrB* and *tolC* genes, respectively. According to the manufacturer (NEB) of this *E. coli* strain there is no evidence that the AcrAB-TolC efflux system was deleted. MIC values were determined for *E. coli* NEB transformed with pRED1/*cmeB* plasmid (*cmeB* from *C. jejuni* G1) (Fig. 3.28). *E. coli* wt and *E. coli* transformed with an empty pRED1 plasmid were used as controls. The plasmid pRED1 was selected since it does not require an inducer to express the gene and the former is constitutively expressed. It was observed that expression of *cmeB* in *E. coli* did not alter the tetracycline resistance levels of this strain since the Tet MIC values for *E. coli* wt, *E. coli*/pGEM-T and *E. coli*/pGEM-T/*cmeB* was the same (0.5µg/ml) (Fig. 3.28).

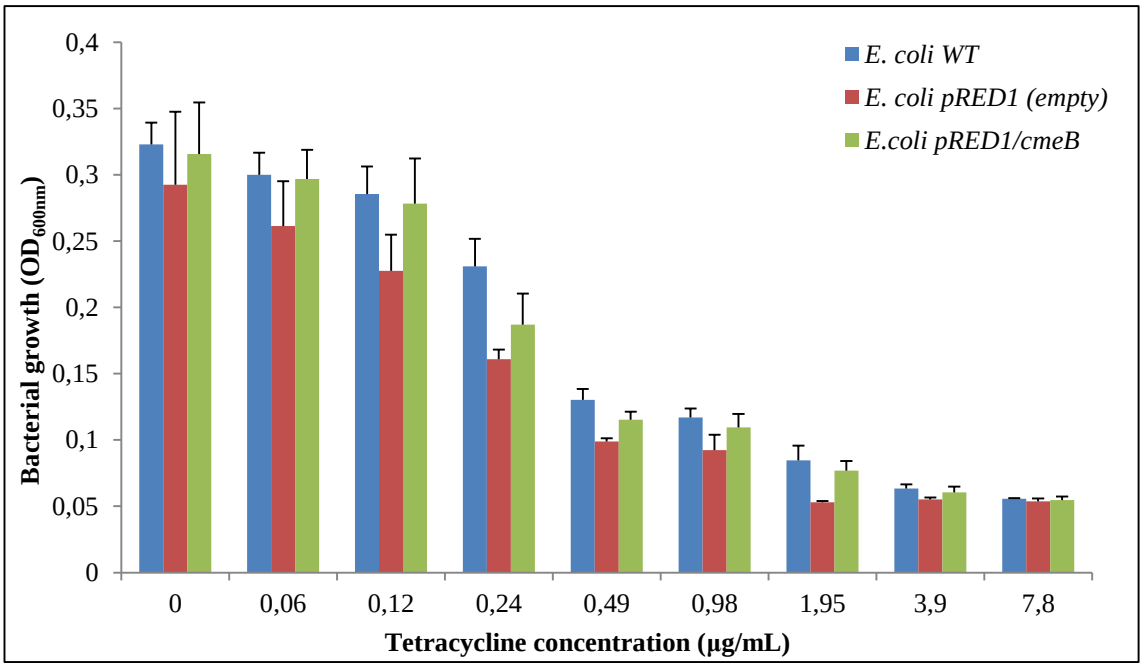


Figure 3.28. Tetracycline susceptibility of *E. coli* expressing the *C. jejuni* G1 *cmeB* gene. Serial dilutions of tetracycline were tested for *E. coli* NEB wt strain (blue bars), *E. coli*/pRED1 plasmid (red bars) and *E. coli*/pRED1/*cmeB* (green bars) by the microdilution broth method over a 24h incubation period at 37°C, according with the EUCAST reccommendations. The Tet MIC value for the three *E. coli* strains was 0.5µg/ml. Values are mean ± SD from three independent experiments with three technical replicates each.

Overall, it was demonstrated that the contribution of CmeABC MDR pump to antibiotic resistance was not only dependent on the different levels of regulation of this gene operon (Lin *et al*., 2005; Cagliero *et al*., 2007; Guo *et al*., 2008; Perez-Boto *et al*., 2015), but may also be due to its sequence variation observed in this study, which is in accordance with what was previously observed (Cagliero *et al*., 2006; Yao *et al*., 2016). Since nucleotide sequence differences were predominantly limited to *cmeB*, the variation in the efficiency of this pump may be primarily associated with the product of this gene, although other contributors, such as other components of the CmeABC multidrug efflux pump as well as the *cmeR* transcriptional repressor cannot be excluded (Tables 3.2-3.4). In addition, secondary efflux pumps might also be responsible for these antibiotic resistance differences. Further studies are therefore required to check the main role of CmeB.

Table 3.2. Sequence identities between *C. jejuni* G1 *cmeA, cmeB, cmeC* and *cmeR* genes and their respective proteins in comparison with those of strain 11168H.

C. jejuni G1		
***C. jejuni* 11168H CmeABC/R**	**Amino acid sequence identity**	**Nucleotide sequence identity**
CmeA	97% (100%)*	96% (100%)
CmeB	81% (100%)	80% (99%)
CmeC	99% (98%)	98% (100%)
CmeR	94% (100%)	93% (100%)

*The numbers between brackets represent the coverage %.

Table 3.3. Sequence identities between *C. jejuni* G1 *cmeA, cmeB, cmeC* and *cmeR* genes and their respective proteins in comparison with those of strain 81-176.

C. jejuni G1		
***C. jejuni* 11168H CmeABC/R**	**Amino acid sequence identity**	**Nucleotide sequence identity**
CmeA	97% (100%)*	96% (100%)
CmeB	82% (100%)	80% (100%)
CmeC	99% (98%)	98% (100%)
CmeR	95% (100%)	94% (100%)

*The numbers between brackets represent the coverage %.

Table 3.4. Sequence identities between *C. jejuni* 81-176 *cmeA, cmeB, cmeC* and *cmeR* genes and their respective proteins in comparison with those of strain 11168H.

C. jejuni 81-176		
***C. jejuni* 11168H CmeABC/R**	**Amino acid sequence identity**	**Nucleotide sequence identity**
CmeA	98% (100%)*	98% (100%)
CmeB	98% (100%)	99% (100%)
CmeC	99% (100%)	99% (100%)
CmeR	99% (100%)	99% (100%)

*The numbers between brackets represent the coverage %.

Part III

3.3. Molecular mechanisms of *Campylobacter jejuni* survival in *Acanthamoebae*

3.3.1. Overview

As already noted, in addition to a role in multidrug resistance, the *Campylobacter* CmeABC RND-type multidrug efflux pump may also be involved in virulence. The protozoan *Acanthamoeba* may act as a vector for various pathogenic bacteria and is considered to be a good model for investigation of bacterial survival in the environment. In part III of the results section the relation between *Campylobacter jejuni* and the protozoa, *Acanthamoeba polyhaga*, and two possible factors involved in this interaction, CmeB and capsule production were investigated. In addition, the role of CmeB in biofilm, oxidative stress and motility was explored.

3.3.2. Setup of the *in vitro* co-culture assays

Although *A. polyphaga* is considered to be easy to handle experimentally, a few initial trial experiments were conducted. Due to its lifestyle, *Campylobacter* is likely to encounter a wide range of environmental conditions including different temperatures (Stintzi, 2003). So, firstly *A. polyphaga* viability was tested at distinct temperatures (25, 37 and 42°C) and at different time-points (2h, 4h and 24h). The temperatures 25°C and 37°C were selected for this study to mimic environmental and human host temperatures respectively. The temperature 42°C was selected since it is the body temperature of chickens. It was observed that at both 25ºC and 37ºC, *A. polyphaga* was able to replicate since the number of throphozoite cells increased with time (Fig. 3.29). In contrast, at 42ºC *A. polyphaga* was not able to replicate, and although it was still viable, it changed to the dormant cyst form; however, this process was reversible when shifted back to 37ºC

(Fig. 3.29). Overall, these eukaryotic organisms could be maintained in PYG medium for long periods of time, especially at environmental temperatures such as 25°C.

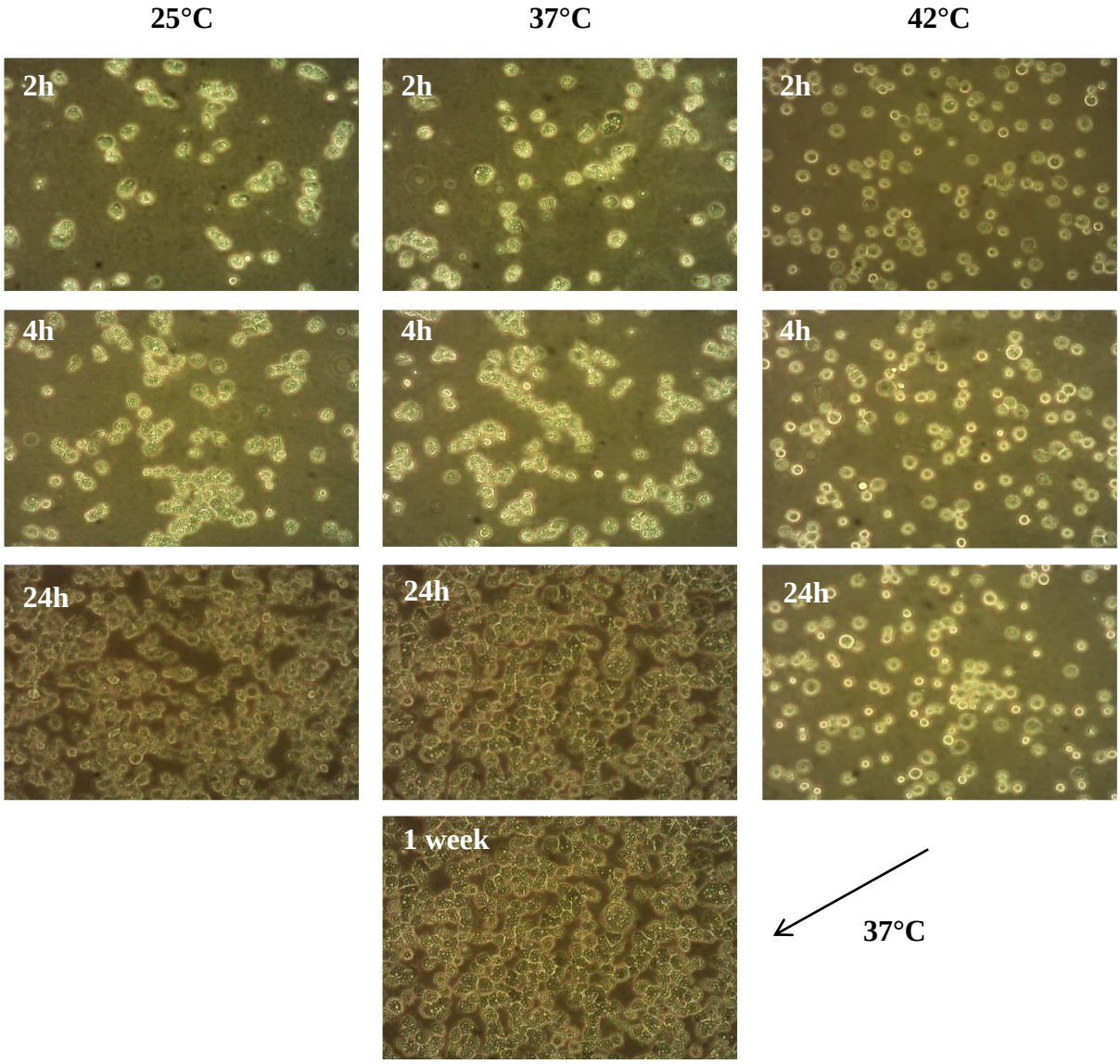


Figure 3.29. Phase-contrast microscopy of *A. polyphaga* cultured at different temperatures. These cells were grown at 25°C, 37°C and 42°C for 2, 4 and 24 hours. Cells grown at 42°C overnight were shifted back to 37°C for one week. Throphozoite form and replication of the amoebic cells were observed at both 25°C and 37°C but not at 42°C.

The multiplicity of infection (MOI) is important for the outcome of infection (Dasti *et al*., 2010). So, to determine the MOI, the bacterial numbers of *C. jejuni* cells OD_{600nm}=1 was quantified. A loop of bacterial cells was inoculated in PYG broth and adjusted to

OD_{600nm}=1. Then, the bacterial suspensions were serially diluted 10-fold in PBS and 50µl aliquots were plated in duplicates onto a CBA blood agar plate (triplicate assay). It was observed that, although the number of colonies varied slightly between the different assays, these were always seen in dilution -6 (data not shown). The c.f.u/ml was calculated by dividing the number of colonies that were multiplied by the dilution factor with the volume of culture plated. *C. jejuni* at an OD_{600nm}=1 was equivalent to 2-4 x 10^9 c.f.u/ml, as this depends on growth conditions, especially growth time.

Although 100µg/ml of gentamicin is widely used to kill extracellular bacteria in the co-culture experiments, this concentration was tested during the outset of this experimental work. For that, *C. jejuni* suspension in PYG medium was inoculated with amoebae cells and further incubated at 25ºC for two hours. Then, the co-culture was incubated for one hour with 100µg/ml of gentamicin, after which the supernatant (co-culture medium) was collected and plated onto a CBA blood plate (Fig. 3.30). As a control *C. jejuni* in co-culture medium without gentamicin was also plated in parallel (Fig. 3.30, A). As expected, it was observed that 100µg/ml of gentamicin was sufficient to kill all bacterial cells (Fig. 3.30, B). In addition, the viability of the amoebae cells incubated with gentamicin was not affected (data not shown). So, for all the co-culture experiments in this study, 100µg/ml of gentamicin was used to kill the extracellular bacteria for quantification of intracellular bacteria.

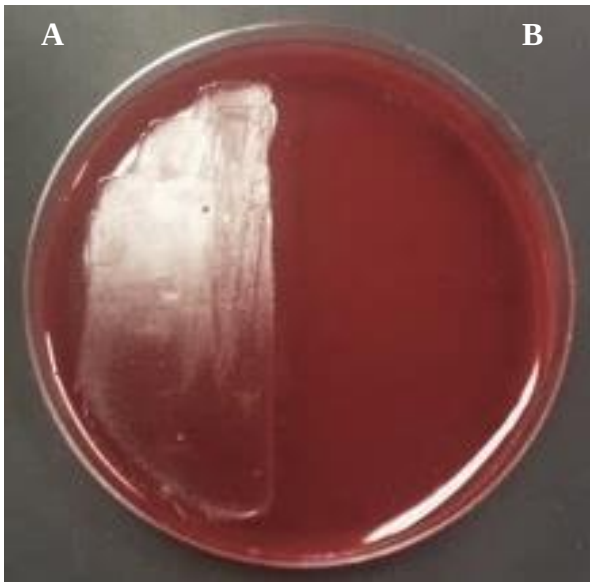


Figure 3.30. Extracellular *C. jejuni* 81-176 before and after gentamicin treatment. The co-culture medium (A) without gentamicin and (B) with 100μg/ml of gentamicin was plated onto CBA agar plates.

3.3.3. Quantification of *C. jejuni* 81-176 in co-culture assays

During co-culture of *C. jejuni* with *A. polyphaga*, the former can be quantified extracellularly or intracellularly. To quantify extracellular bacteria, the supernatant was diluted and plated onto CBA agar plates. To detect the intracellular bacteria, amoeba cells were treated with gentamicin, lysed and plated onto CBA blood agar. For bacteria quantification in medium alone, *C. jejuni* was incubated in the same conditions, but in PYG with no amoebae (Fig. 3.31). It was observed that there was 1-log decrease in the bacterial numbers of extracellular bacteria in medium alone as compared with bacteria in the initial inoculum. In addition, the number of *C. jejuni* inside the amoebae cells was significantly lower (10^5 c.f.u/ml) as compared with the initial inoculum and the extracellular bacteria (10^8 c.f.u/ml) (Fig. 3.31).

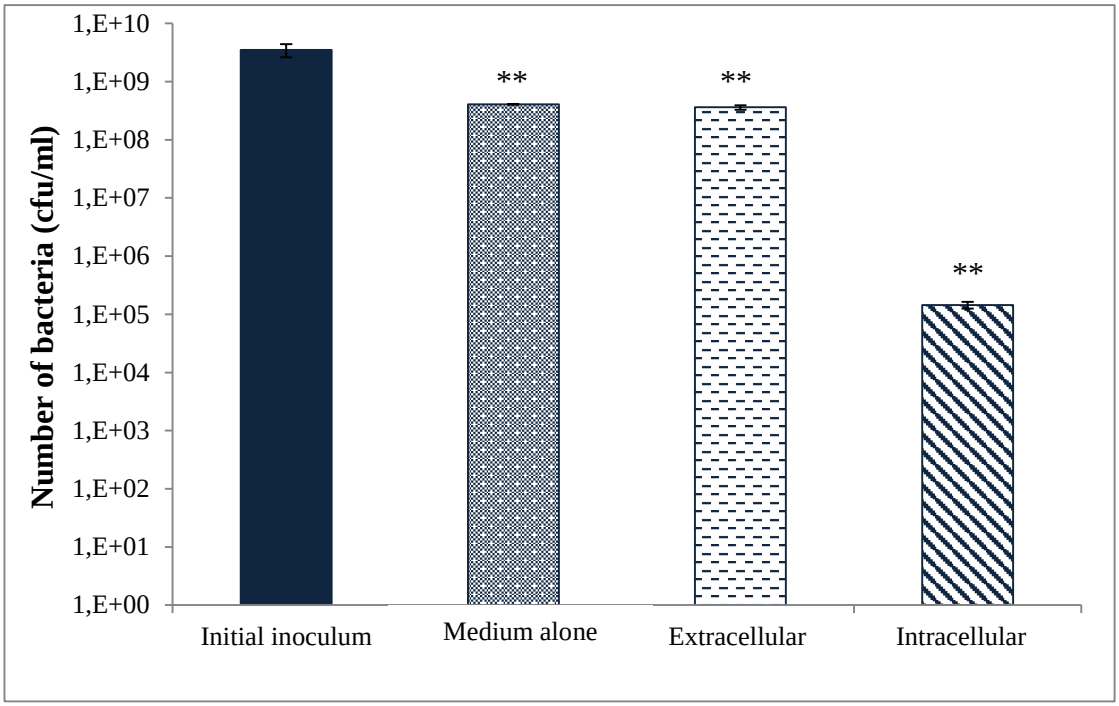


Figure 3.31. Quantification of *C. jejuni* strain 81-176 in co-culture with *A. polyphaga.* The quantity of bacterial cells in the initial inoculum (dark blue); in the medium alone (dots); extracellularly (horizontal strips) and intracellularly (diagonal stripes**)** is represented**.** Values are mean ± SD from three independent experiments with three technical replicates each.

3.3.4. Quantification of *C. jejuni* within *A. polyphaga*

To evaluate the numbers of different *C. jejuni* wt strains inside *A. polyphaga*, the MOI used ranged between 200-400 bacteria per cell depending on the initial c.f.u/ml of the bacterial inoculum. To normalise the numbers of the intracellular bacteria recovered in each assay, the initial bacterial inoculum was always plated. The intracellular bacterial numbers were determined at time-point 0h. This was defined as the time-point immediately after the first gentamicin. A statistically significant increase in the intracellular bacterial numbers for *C. jejuni* strain 81-176 in comparison with strains 11168H, G1 and X was observed (p=0.003; p=0.007; p=0.0016, respectively), indicating that the former strain invaded and/ or survived better inside this amoebic host (Fig. 3.32). Although it was expected that lower numbers of *C. jejuni* strains 11168H and G1 would be found inside amoebae due to the absence of the virulence plasmid, this was not

expected for *C. jejuni* X, a strain that contains the virulence plasmid (Fig. 3.32). One explanation could be the fact that different *C. jejuni* strains present high variability in their capsular polysaccharides structure, and that capsule was shown to be involved in bacterial invasion of host cells and virulence (Bacon *et al*., 2000).

Overall, *C. jejuni* strain 81-176 was selected for the co-culture *in vitro* experiments since this strain survived better in the amoebae host.

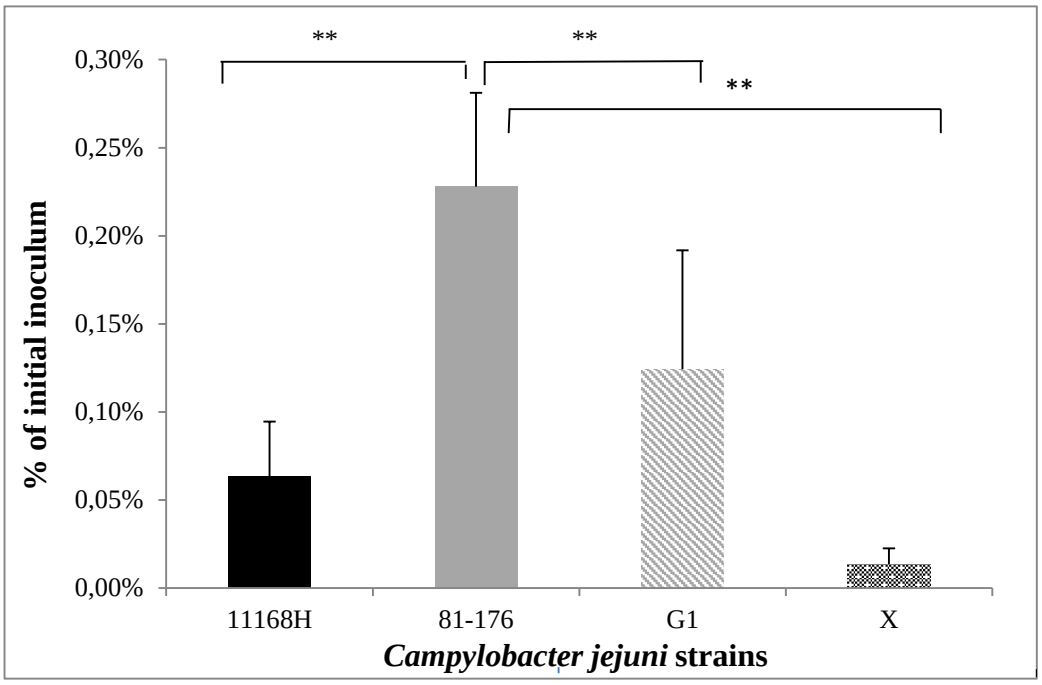


Figure 3.32. Quantification of *C. jejuni* strains within *A. polyphaga*. Quantification of intracellular bacteria was determined by viable counts at 0h post-gentamicin treatment at 25°C in aerobic conditions. Values are mean ± SD from three independent experiments with three technical replicates each.

3.3.4.1. Quantification of *C. jejuni* 81-176/pCPE111/28/GFP within *A. polyphaga*

Since one objective of this study was to perform microscopy of amoebae cells infected with *C. jejuni* strain 81-176/pCPE111/21GFP, the intracellular bacterial numbers within amoebae was determined as previously described. This was done to confirm that survival

of the GFP strain was the same as the wt strain, so that the microscopy observations could be extrapolated to the *C. jejuni* 81-176 strain. It was observed that although the 81-176/pCPE111/21GFP strain survived reasonably well compared to the wt strain in *A. polyphaga*, this difference was not statistically significant (p=0.3) and thus, 81-176/pCPE111/21GFP strain could be used in the microscopy experiments (Fig. 3.33), which unfortunatly was not performed.

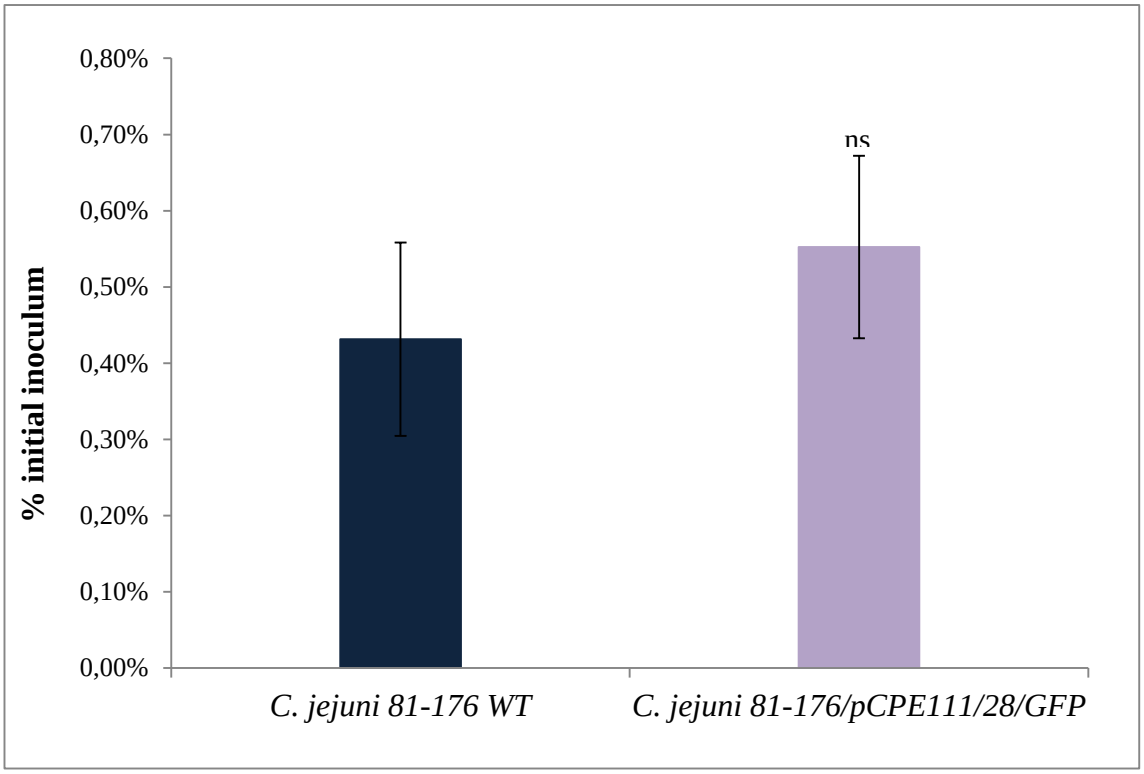


Figure 3.33. Quantification of intracellular 81-176 wt and 81-176/pCPE111/28/GFP strains within *A. polyphaga*. Quantification of intracellular bacteria was determined by viable counts at 0h post-gentamicin treatment at 25°C in aerobic conditions. Values are mean ± SD from three independent experiments with three technical replicates each.

3.3.5. Extracellular survival of *C. jejuni* in the presence of *A. polyphaga*

The number of viable extracellular bacteria in co-culture with *A. polyphaga* was monitored for a 6-day period and compared with *C. jejuni* in medium alone at 25°C. At 96h post-infection a statistically significant increase in the bacterial counts (p=0.044) was observed when compared with bacteria incubated in medium alone. Moreover, after six

days of incubation the presence of amoebae still allowed the isolation of viable bacteria whilst none could be detected in their absence (Fig. 3.34). After 72h incubation one would expect the *Campylobacter* in the absence of amoebae to be coccoid (viable but not cultural cells) however, it was observed viable bacterial counts. As it is highly doubtful that these are not *Campylobacter* cells, it could mean that *Campylobacter* cells are culturable under the conditions used in this experiment. It would be relevant to repeat this experiment to check its reproducibility and investigate further this issue. These results also indicate that amoebae can prolong survival of extracellular *C. jejuni* in the environment.

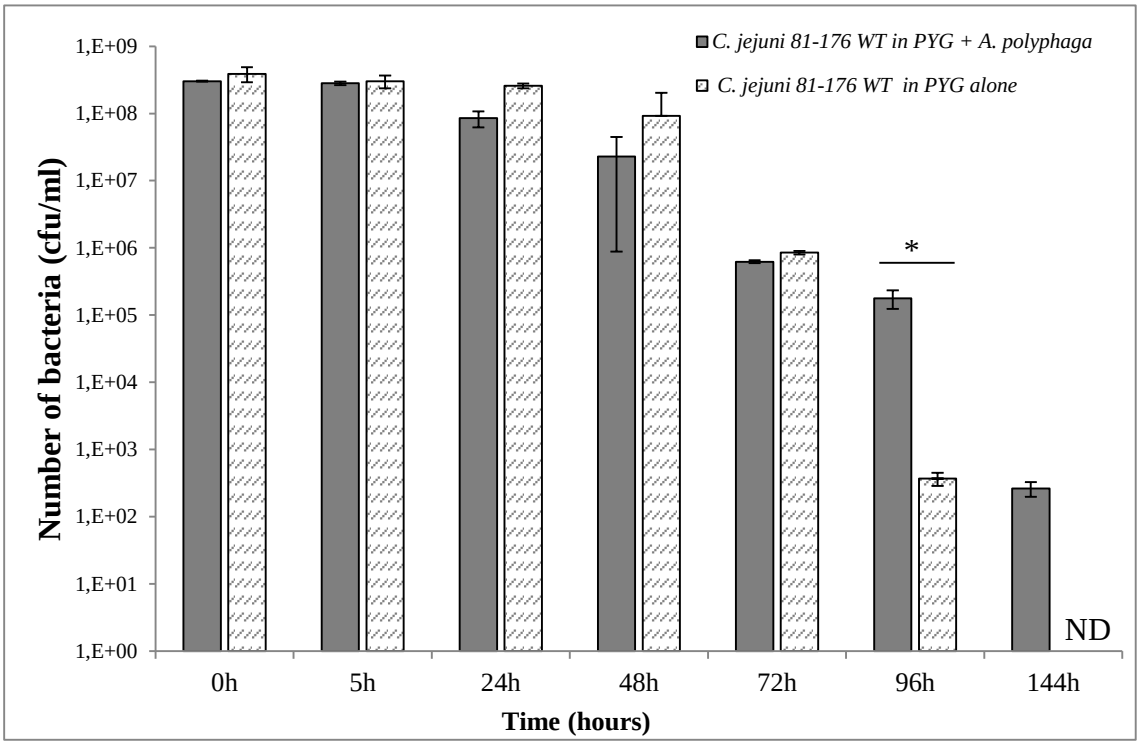


Figure 3.34. Extracellular survival of *C. jejuni* 81-176 in the presence of *A. polyphaga* at 25°C. Extracellular survival was determined by viable counts at 0, 5, 24, 48, 72, 96 and 144 hours post-infection at 25°C in aerobic conditions. Grey bars represent bacterial numbers for *C. jejuni* 81-176 in co-culture with *A. polyphaga* and white bars correspond to when in PYG medium alone. Values are mean ± SD from three independent experiments with three technical replicates each.

3.3.6. Interaction between *C. jejuni* 81-176 and *A. polyphaga*

To elucidate the interaction between *C. jejuni* and amoebae, at this stage of the project, it was decided to optimise the standard gentamicin protection method.

3.3.6.1. Strain 81-176 is able to survive and multiply within *A. polyphaga*

The standard gentamicin protection method was modified by adding an extra hour of gentamicin treatment at longer incubation time-points (from 24h after gentamicin treatment up to 72h) before lysing the cells to make sure only internal bacteria were quantified. This avoided quantification of bacteria that were attached, or which had escaped to the extracellular medium and were potentially capable of re-invasion. By using the modified version of this method a substantial difference in the numbers of intracellular bacteria when compared with the standard version at both 25°C (Fig. 3.35, A) and 37°C (Fig. 3.35, B) temperatures was observed.

As expected, at time-point 0h, no significant difference was observed in bacterial counts between both standard and modified methods. On the other hand, at 25°C a significant difference was detected at 5h and 24h time-points (90%, p=0.031 and 80%, p=0.000027, respectively) (Fig. 3.35, A). This data indicated that *C. jejuni* was able to invade and survive inside amoebae at 25°C for a certain period of time. At 37°C the decrease in intracellular bacterial numbers at 24h post-gentamicin treatment was even more pronounced when using the modified gentamicin method, and a highly significant difference was also observed for the later time-points (24h, p=0.03; 48h, p=0.0.0011; 72h, p=0.0003) (Fig. 3.35, B). With both methods, an initial reduction in the viable counts was followed by increase after prolonged incubation, suggesting bacterial multiplication (Fig. 3.35, B).

In summary, these data support both the extra- and intracellular mode of survival of *C. jejuni* when co-cultured with *A. polyphaga* under different temperatures.

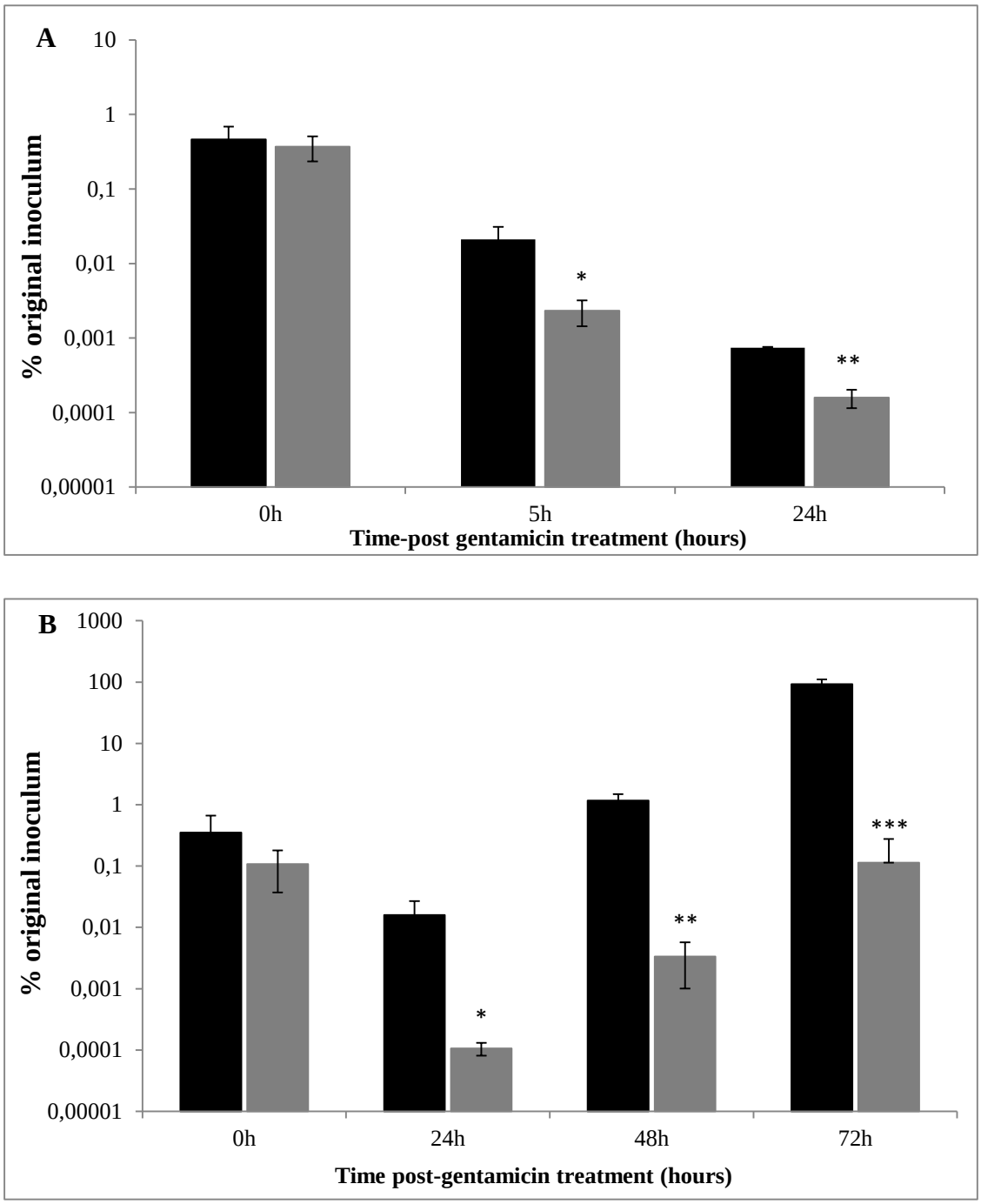


Figure 3.35. *C. jejuni* 81-176 is able to survive and multiply within *A. polyphaga.* (A) Intracellular survival of strain 81-176 was determined by viable counts at 0, 5 and 24h post-gentamicin treatment at 25°C and (B) intracellular multiplication at 0, 24, 48 and 72h post-gentamicin treatment at 37°C in aerobic conditions. Black bars represent bacterial counts for 81-176 obtained by the standard gentamicin protection assay and grey bars represents bacterial counts for 81-176 obtained by a modified version developed in this study. Values are mean ± SD from three independent experiments with three technical replicates each.

A modified gentamicin protection method, in which a lower concentration of gentamicin was constantly maintained, has been employed previously (Chu *et al*., 2010). However, using this method we observed that *C. jejuni* was not able to survive and/or multiply intracellularly and was not detected after 48h post-infection (Fig. 3.36), probably because this antibiotic was able to enter the amoebae cells during prolonged incubation periods. This observation is in accordance with the previous studies reporting the ability of gentamicin to enter the host cells during prolonged incubation and to kill intracellular bacteria (Drevets *et al*., 1994; Elsinghorst, 1994).

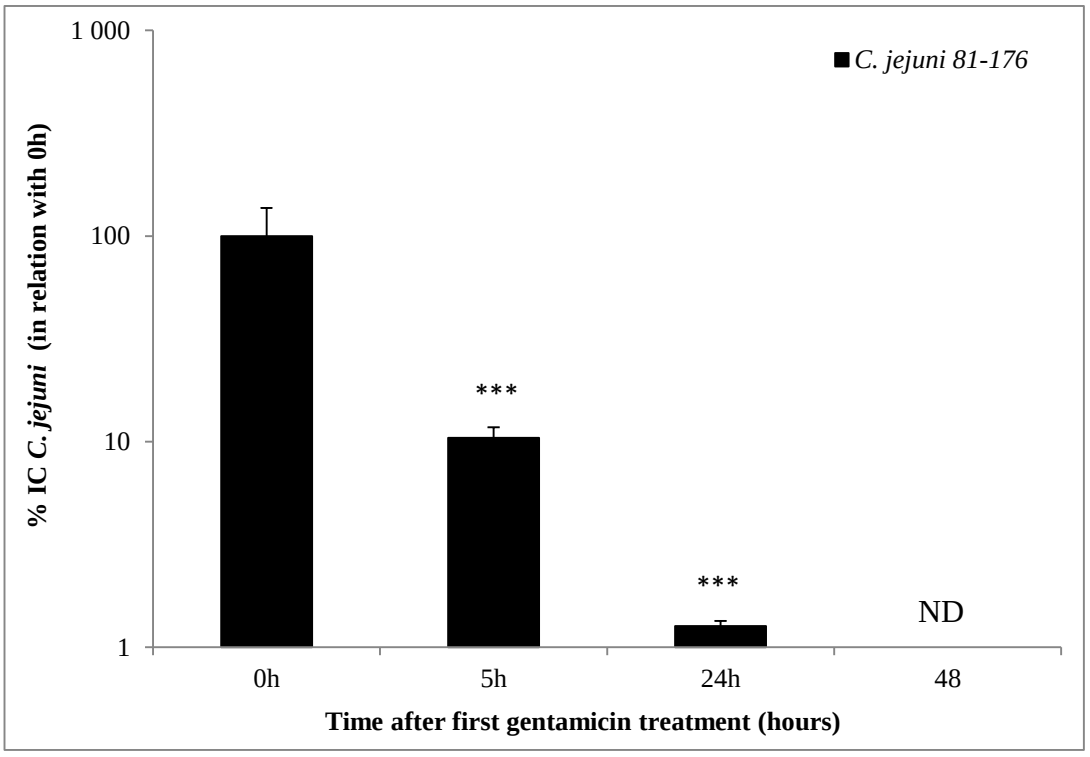


Figure 3.36. Continuous incubation with low concentrations of gentamicin kills intracellular *C. jejuni* 81-176. Intracellular multiplication of strain 81-176 (black bars) was determined by viable counts at 0, 5, 24 and 48h after the secondary gentamicin treatment at 37°C in aerobic conditions. For the time-points 5 to 48h the co-culture wells were incubated continuously with 10µg/ml gentamicin before lysis of the amoebae cells. ND (not detected). Values are mean ± SD from three independent experiments with three technical replicates each. Statistically significant differences were observed for 5h (p=0.041) and 24h (p=0.037).

3.3.7. CmeB is beneficial for survival and multiplication of *C. jejuni* within *A. polyphaga*

As previously mentioned, different CmeB homologues have been shown to be required for bacterial virulence and host cell invasion and so, it was decided to check whether CmeB was involved in the interaction between *C. jejuni* strain 81-176 and *A. polyhaga*.

3.3.7.1. Confirmation of 81-176 *cmeB* mutant and complement strains

To investigate the role of *C. jejuni* CmeB transporter in survival and multiplication within amoebae, the *cmeB* gene of *C. jejuni* strain 81-176 was inactivated by insertional mutagenesis to create the 81-176/*cmeB*::*kan*^{r} mutant, in similar way to that described previously (see Section 3.2.3.1). Complementation was achieved by replacement of the mutated gene with its wild type copy and the the repaired strain derivative was selected by using a tetracycline concentration that did not support growth of the mutant strain (Section 3.2.3.2). Construction of the mutant strain and its complementation derivative was confirmed by PCR using the DmhA and CmeB primers (Fig. 3.37, A). Increased fragment size with the latter indicates insertion of the *kan*^{r} cassette. Except for time-point 30h ($p=0.02$), the mutation had no impact on the overall bacterial growth rate (Fig. 3.37, B).

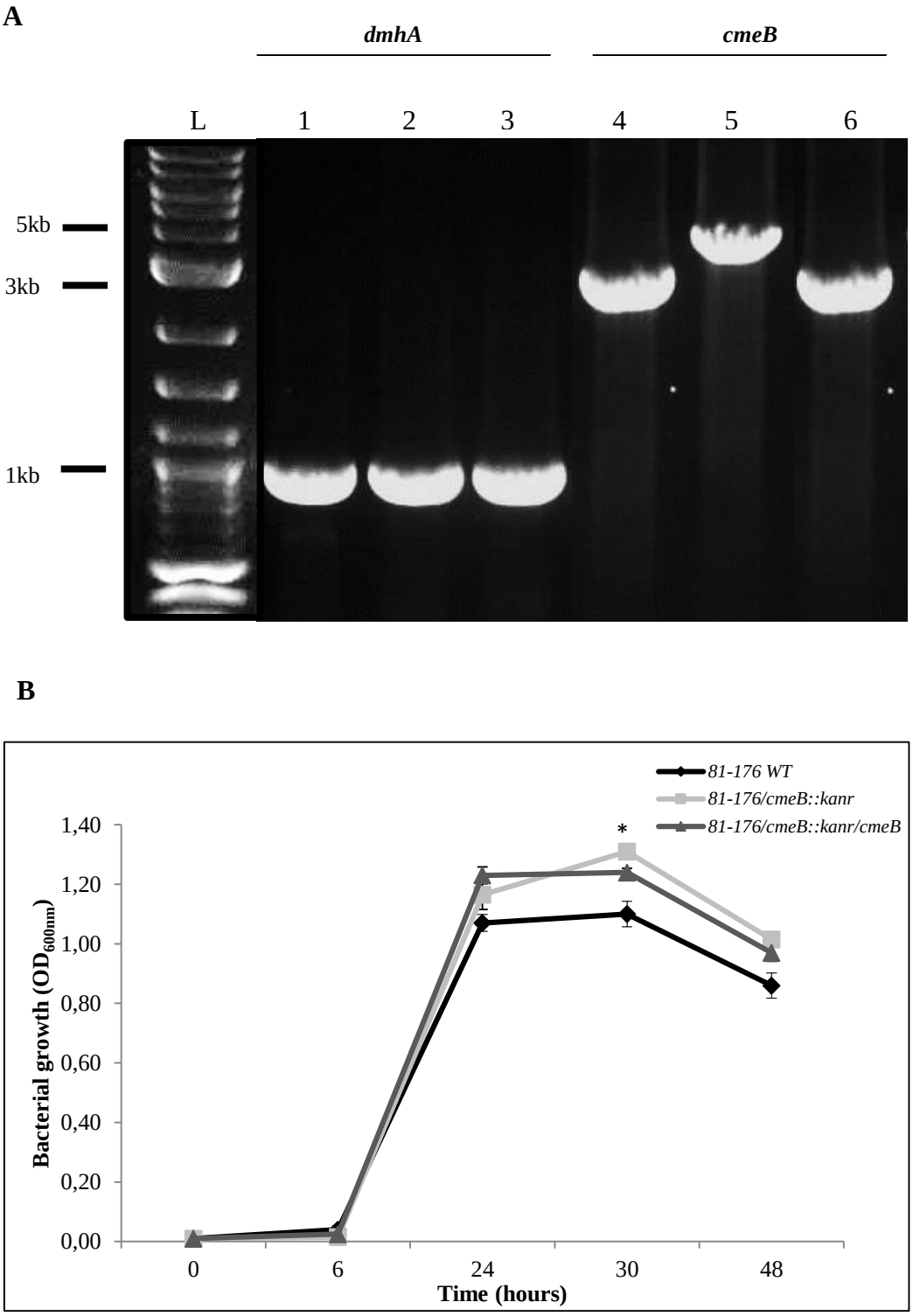


Figure 3.37. Growth of *C. jejuni* 81-176 is not affected by *cmeB* mutation (A) PCR results: Lane L: 2-log DNA ladder; lane 1: 81-176 – 1021bp; lane 2: 81-176/*cmeB::kan*^{r} – 1021bp; Lane 3: 81-176/*cmeB::kan*^{r}/*cmeB* – 1021bp; lane 4: 81-176 – 3.1kb; lane 5: 81-176/*cmeB::kan*^{r} – 4.6kb; Lane 6: 81-176/*cmeB::kan*^{r}/*cmeB* – 3.1kb. (B) Growth rates of 81-176 wt (black), 81-176/*cmeB::kan*^{r} mutant (light grey) and 81-176/*cmeB::kan*^{r}/*cmeB* derivative (dark grey). Values are mean ± SD from three independent experiments with one technical replicate each.

3.3.7.2. Involvement of CmeB in the interaction between *C. jejuni* and *A. polyphaga*

Using the modified gentamicin assay, it was observed that at 25°C the 81-176/*cmeB::kan*^{r} mutant strain was less able to survive intracellularly compared with the wt 81-176 (time-point 0h, a 10-fold reduction compared to wt, p=0.018) (Fig. 3.38, A). The difference between the wt strain and its isogenic *cmeB* mutant was even more profound at 5h and 24h (Fig. 3.38, A). At 37°C the intracellular bacterial numbers of the 81-176/*cmeB::kan*^{r} mutant strain were also significantly lower (time-point 0h, 6-fold lower than the wt, p=0.0021) (Fig. 3.38, A). At 24, 48 and 72h there was more than a 47, 36 and 1263-fold reduction (p=0.031, p=0.027 and p=0.0000039, respectively) in the intracellular numbers of the *cmeB* mutant strain compared with the wt (Fig. 3.38, B). Although there was a small increase in internal bacterial numbers for 81-176/*cmeB::kan*^{r} at 48 and 72h post-gentamicin treatment, the difference between these time-points in this strain was not statistically significant (p=0.24 and p=0.09) (Fig. 3.38, B). Complementation of the C. *jejuni* 81-176 *cmeB* mutant strain restored the phenotype in all experiments.

In summary, these results indicate that *C. jejuni* CmeB is required for the interaction between this pathogen and the host amoebae.

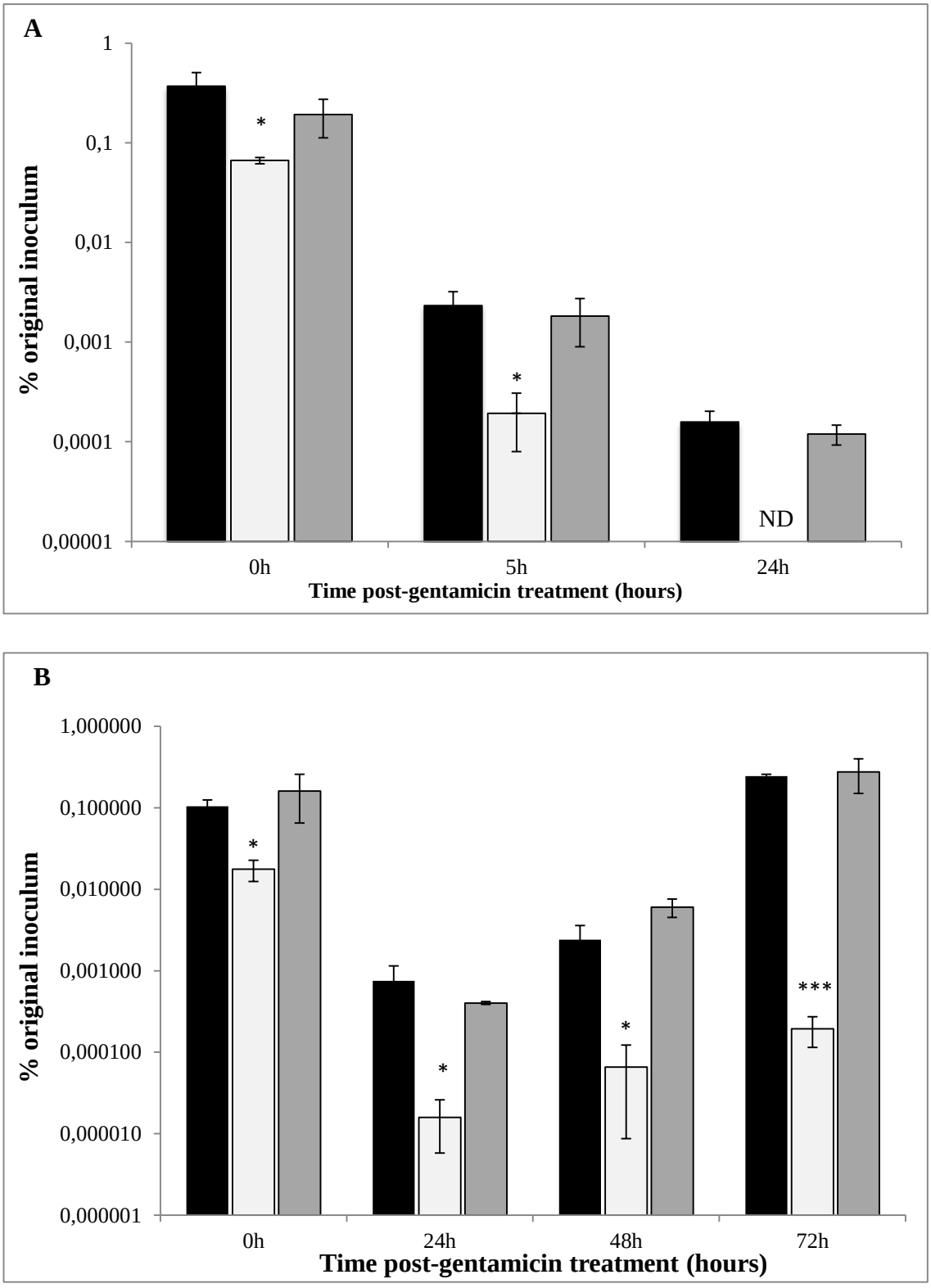


Figure 3.38. CmeB is required for survival and multiplication of *C. jejuni* 81-176 within *A. polyphaga*. (A) Intracellular survival was determined by viable counts at 0, 5 and 24h post-gentamicin treatment at 25°C and (B) intracellular multiplication at 0, 24, 48 and 72h post-gentamicin treatment at 37°C, in aerobic conditions. Colour coding: black, 81-176 wt; white, 81-176/*cmeB*::*kan*^{r} mutant; grey, 81-176/*cmeB*::*kan*^{r}/*cmeB* complementation derivative. Values are mean ± SD from three independent experiments with three technical replicates each.

The tissue culture experiments involved lysis of eukaryotic cells with 0,1% of Triton X-100 for 15 minutes at RT. As *C. jejuni cmeB* mutant strain was shown to be susceptible to this detergent by minimum inhibitory concentration testing (Lin *et al.*, 2003), experiments were performed to ensure that any data obtained were genuine and not experimental artefacts. The effect of Triton X-100 0.1% (v/v) was investigated by simulating its use in cell culture experiments, where this detergent was added to the bacteria during a short exposure time. Under these conditions bacterial counts for *C. jejuni* 81-176/*cmeB::kan*^{r} mutant were similar to the wt and complement strains after exposure to Triton X-100 0.1% (v/v) (Fig. 3.39). Also, the bacterial counts were similar before and after addition of this detergent for the three strains tested. These data indicate that Triton X-100 0.1% (v/v) had no detrimental effect on cell viability during the time-span of the experiments.

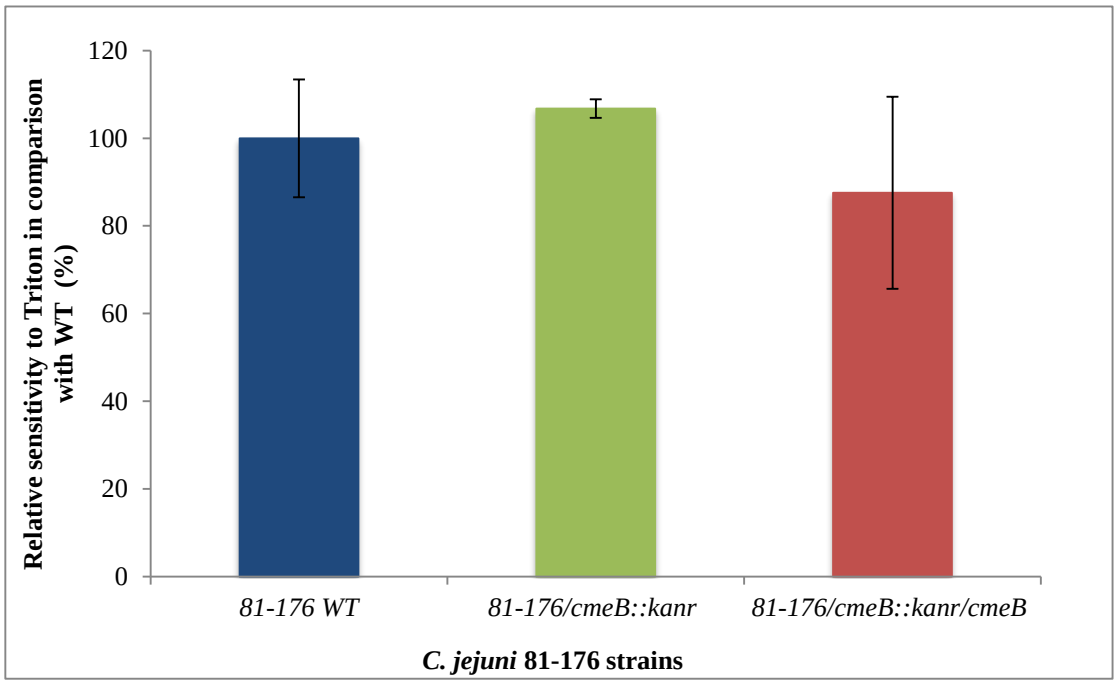


Figure 3.39. CmeB mutation had no effect on the resistance to Triton X-100 0.1% (v/v) of strain 81-176. Bacteria were in the exponential phase of growth ($OD600_{nm}$=1) for this experiment and the initial inoculum was plated as control and to normalise the viable counts after exposure to this detergent. Colour coding: blue, *C. jejuni* 81-176 wt; green, *C. jejuni* 81-176/*cmeB*::*kan*^{r} mutant; red, 81-176/*cmeB*::*kan*^{r} /*cmeB* complementation derivative. Values are mean ± SD from one independent experiment with three technical replicates.

3.3.8. Capsule production is beneficial for survival of *C. jejuni* within *A. polyphaga*

As previously mentioned, the capsular *kpsM* gene was shown to be required for *C. jejuni* virulence and invasion of INT407 (Bacon *et al*., 2000). So, it was decided to mutate the *kpsM* gene to check if capsule production was required for the intracellular survival of *C. jejuni* strains G1 and 81-176 in *A. polyphaga*.

3.3.8.1. Confirmation of G1 and 81-176 *kpsM* mutants

A non-polar *kpsM* mutant of *C. jejuni* 11168H strain (Karlyshev & Wren, 2001) present in the laboratory bacterial collection was used to create the *C. jejuni* G1 and 81-176 *kpsM* mutant strains, denominated *C. jejuni* G1/*kpsM::kan*^{r} and *C. jejuni* 81-176/*kpsM::kan*^{r}, respectively. Briefly, the gDNA of *C. jejuni* 11168H/ *kpsM::kan*^{r} was extracted using the Gentra Puregene Yeast/bacteria Kit from Qiagen and transformed individually into G1 and 81-176 via electroporation. Transformants of *C. jejuni* G1/*kpsM::kan*^{r} and *C. jejuni* 81-176/*kpsM::kan*^{r} were selected on CBA blood agar plates supplemented with kanamycin (50µg/ml) and confirmed by PCR using the ak54-fw and ak59-rev primers (Fig. 3.40 and 3.41, A, respectively). A 600bp size band corresponding to the *kpsM* gene was observed for both G1 and 81-176 strains (Fig. 3.40 and 3.41, A, lane 1). On the other hand, a 2100bp size band was observed for both G1/*kpsM::kan*^{r} and 81-176/*kpsM::kan*^{r} mutant strains. This confirmed the disruption of the *kpsM* gene by integration of the 1.5kb *kan*^{r} cassette (Fig. 3.40 and 3.41, A, lanes 2-4). *dmhA* and *tagF* identification primers were used to confirm the identity of the strains. A 642bp and 1021bp size bands were observed for wt strains G1 and 81-176 (Fig. 3.40 and 3.41, A, lane 5) and for the three clonal *kpsM* isolates of each strain (Fig. 3.40 and 3.41, A, lanes 6-8).

The growth of the G1/*kpsM::kan*^{r} and 81-176/*kpsM::kan*^{r} mutant strains (three clonal isolates) was assessed in BHI broth at 37°C for two days and compared with the G1 and

81-176 wt strains, respectively (Fig. 3.40 and 3.41, B). The *kpsM* mutation had no effect on growth of G1 (Fig. 3.40, B), but did, however, slightly affect the growth of strain 81-176 although no statistically significant differences were obtained (Fig. 3.41, B).

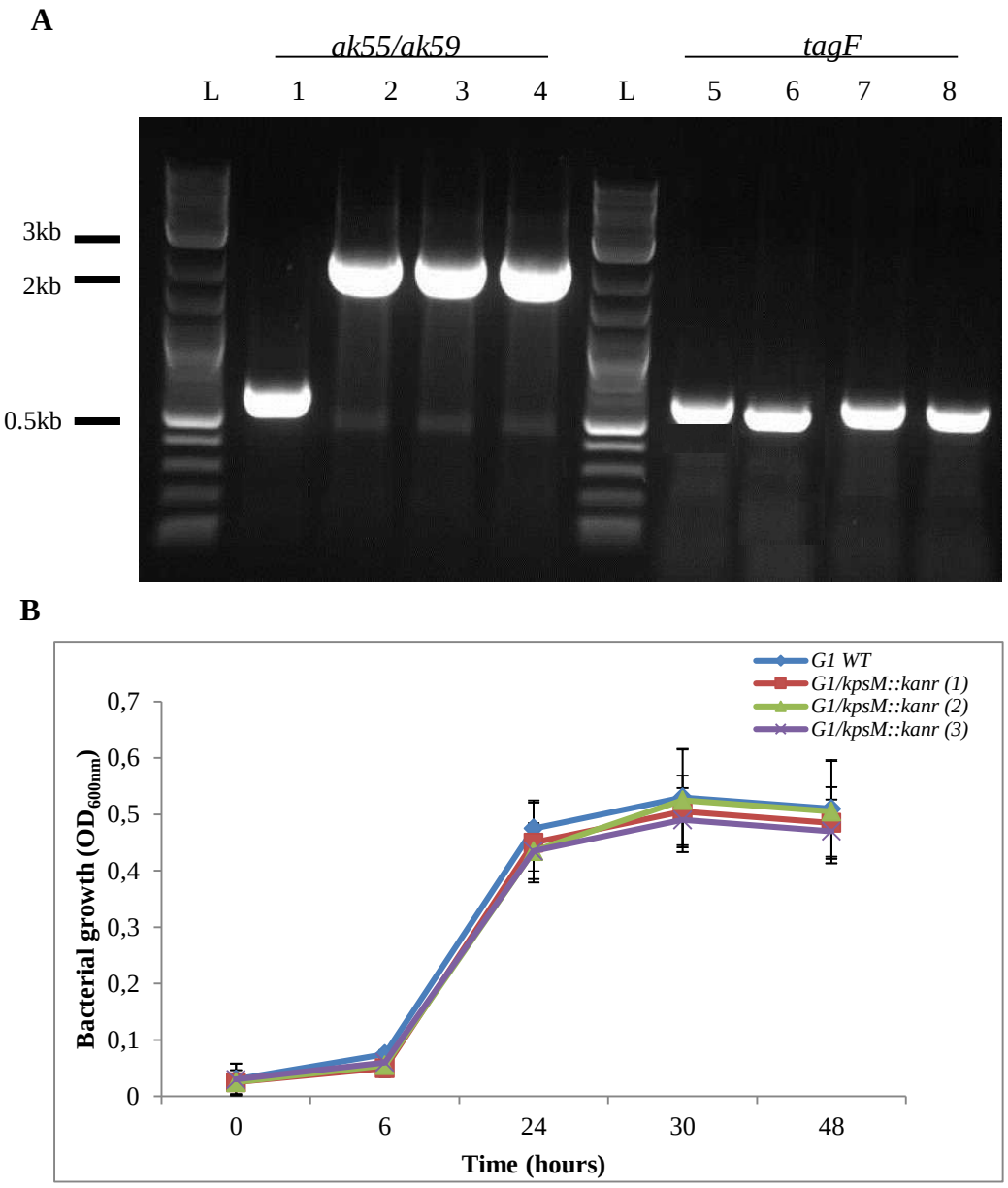


Figure 3.40. **Growth of *C. jejuni* G1 is not affected by *kpsM* mutation.** (A) PCR results: Lane L: 2-log DNA ladder; lane 1: G1 – 600bp; lanes 2-4: G1/*kpsM::kan*^{r} clonal isolates – 2100bp; lane 5: G1– 642bp; lanes 6-8: G1/*kpsM::kan*^{r} clonal isolates – 642bp. (B) Growth rates of G1 wt (blue), G1/*kpsM::kan*^{r} mutants (1, red; 2, green; 3, purple). Values are mean ± SD from three independent experiments with one technical replicate each. No statistically significant differences were obtained between the wt and the *kpsM* mutant strains.

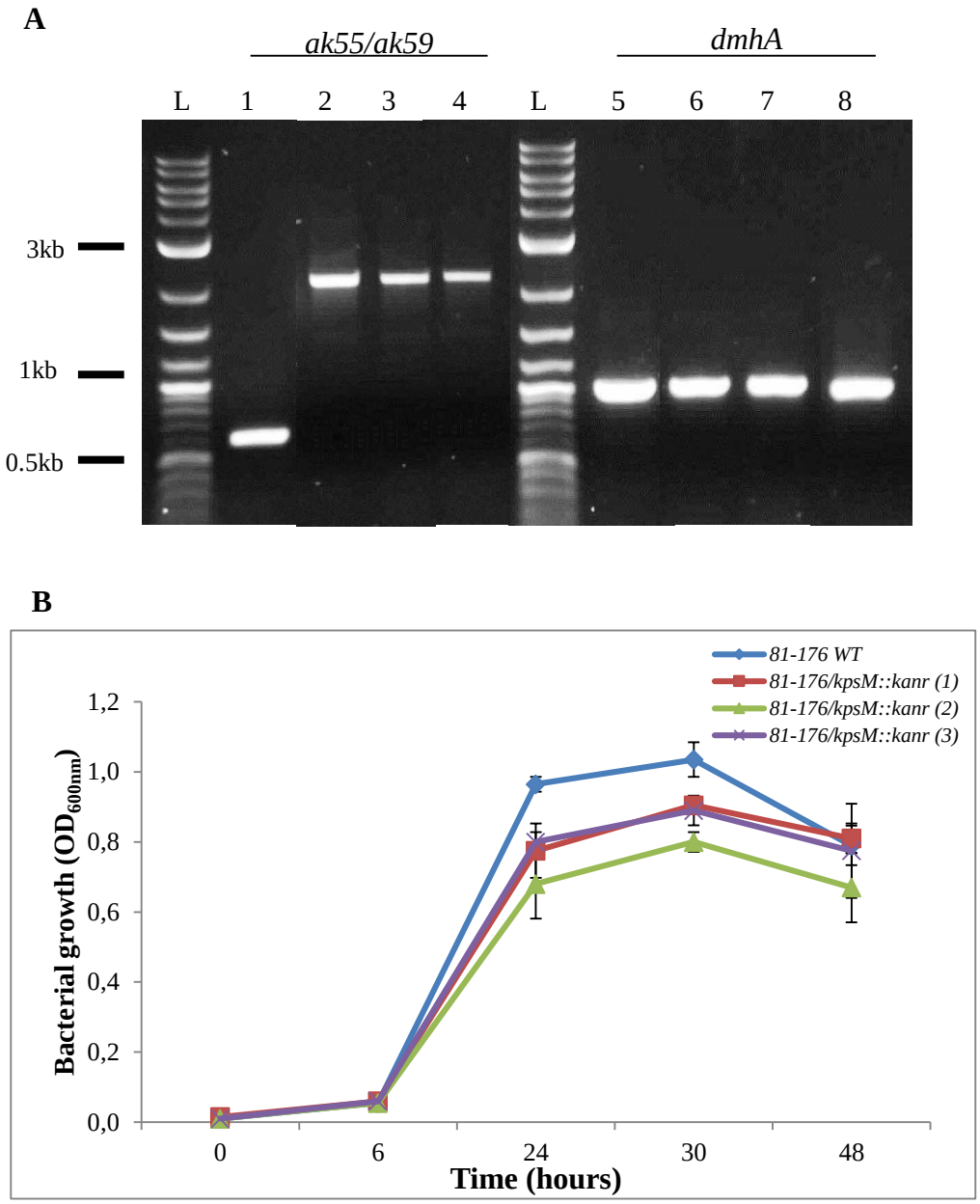


Figure 3.41. **Growth of *C. jejuni* 81-176 is slightly affected by *kpsM* mutation.** (A) PCR results: Lane L: 2-log DNA ladder; lane 1: 81-176 – 600bp; lanes 2-4: 81-176/*kpsM::kan*^{r} clonal isolates – 2100bp; lane 5: 81-176 – 1021bp; lanes 6-8: 81-176/*kpsM::kan*^{r} clonal isolates – 1021bp (B) Growth rates of 81-176 wt (blue), G1/*kpsM::kan*^{r} mutants (1, red; 2, green; 3, purple). Values are mean ± SD from three independent experiments with one technical replicate each. No statistically significant differences were obtained between the wt and the *kpsM* mutant strains.

3.3.8.2. Involvement of capsule production in the interaction between *C. jejuni* and *A. polyphaga*

To investigate if capsule production was involved in the interaction between *C. jejuni* and *A. polyphaga*, co-culture assays at 25°C were conducted for G1/*kpsM::kan*^{r} and 81-176/*kpsM::kan*^{r} mutant strains (three clonal isolates) and the respective wt strains (Fig. 3.42). Mutation of *kpsM* resulted in a reduction of intracellular bacteria in both strains (Fig. 3.42). There was a 49%, 59% and 39% reduction in the bacterial counts of the three G1/*kpsM::kan*^{r} clonal isolates as compared with the wt. These differences were statistically significant (1, p=0.0046; 2, 0.0050; 3, p=0.0038) (Fig. 3.42, A). The same results were observed for the three clonal isolates 81-176/*kpsM::kan*^{r} which showed statistically significant lower intracellular bacterial counts (42, 23 and 39%) when compared with the wt strain (1, p=0.006; 2, 0.005; 3, p=0.0046) (Fig. 3.42, B). Overall, these results suggest a possible role for the capsule in the survival of *C. jejuni* within amoebae. Whether this capsular gene is involved in *C. jejuni* multiplication inside *A. polyphaga* is still unknown and remains to be elucidated.

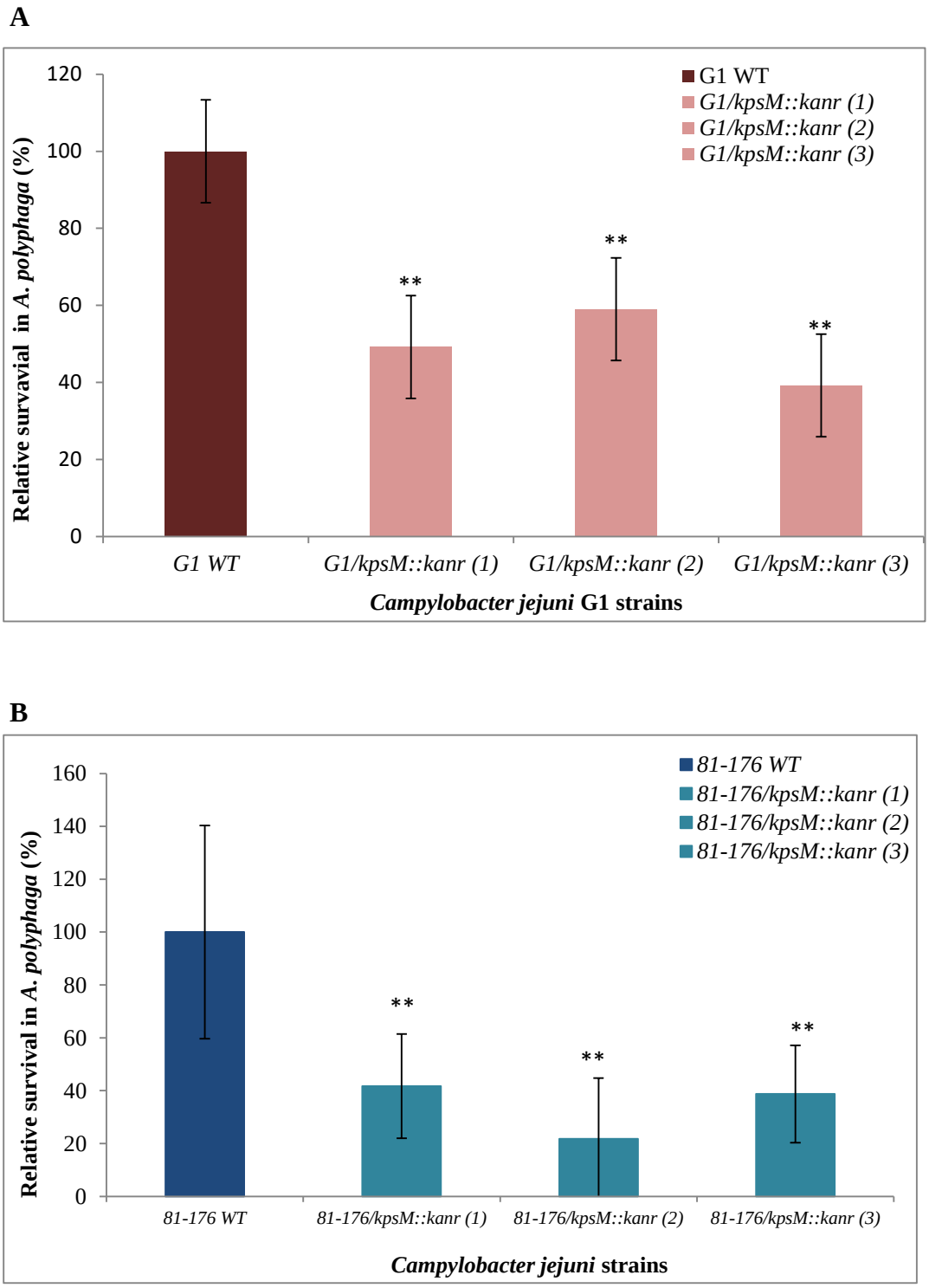


Figure 3.42. Capsule production is required for *C. jejuni* intracellular survival within *A. polyphaga*. Quantification of intracellular bacteria was determined by viable counts at 0h post-gentamicin treatment at 25°C in aerobic conditions (A) Intracellular numbers of G1 wt (dark red) and respective G1/*kpsM::kan*^{r} clonal isolates (light red); (B) Intracellular numbers of *C. jejuni* 81-176 wt strain (dark blue) and respective 81-176/*kpsM::kan*^{r} clonal isolates (light blue). Values are mean ± SD from three independent experiments with three technical replicates each.

3.3.9. CmeB is not required for *C. jejuni* strain 81-176 motility, biofilm formation and oxidative stress response

As noted earlier, *C. jejuni* motility is considerd to play an important role in the invasion of host cells (Lugert *et al*., 2015). In order to check whether the observed phenotypic changes in the *cmeB* mutant (Section 3.3.7) were attributed to defects in motility, the latter was compared among the *C. jejuni* 81-176 wt, mutant and complemented derivatives (Fig. 3.43, A). It was observed that *cmeB* mutation did not impair bacterial motility as the average diameters of bacterial growth were 34.2 ± 6.37, 26.7 ± 3.33 and 32.2 ± 4.54 for the wt, *cmeB* mutant and complement strain, respectively. No statistically significant difference in growth zones (p=0.15) was observed between the wt and *cmeB* mutant strain (Fig. 3.43, A). The ability to form biofilms is also considered to be an important factor in the pathogenesis of *C. jejuni* (Bronowski *et al*., 2014). Some studies showed that inactivation of the multidrug efflux pumps can prevent biofilm formation (Baugh *et al.*, 2014). To assess the ability of 81-176/*cmeB::kan*^{r} mutant to develop biofilms, glass test tubes were used to quantify the pellicle formation in the air-liquid interface (Joshua *et al*., 2006). The *cmeB* mutation was found to have no effect on the bacterial ability to form a biofilm (Fig. 3.43, B-C). The OD_{600nm} measured for the 81-176 wt, *cmeB* mutant and complement strains were 0.179 ± 0.06, 0.151 ± 0.01 and 0.145 ± 0.02, respectively. No statistically difference (p=0.443) in biofilm formation was observed between the wt and *cmeB* mutant strain (Fig. 3.43, B). In Figure 3.43, the biofilm halo formed in the glass tube was present in the three strains tested and, as expected, there was an absence of the halo when the glass tube was incubated with BHI broth only (Fig. 3.43, C).

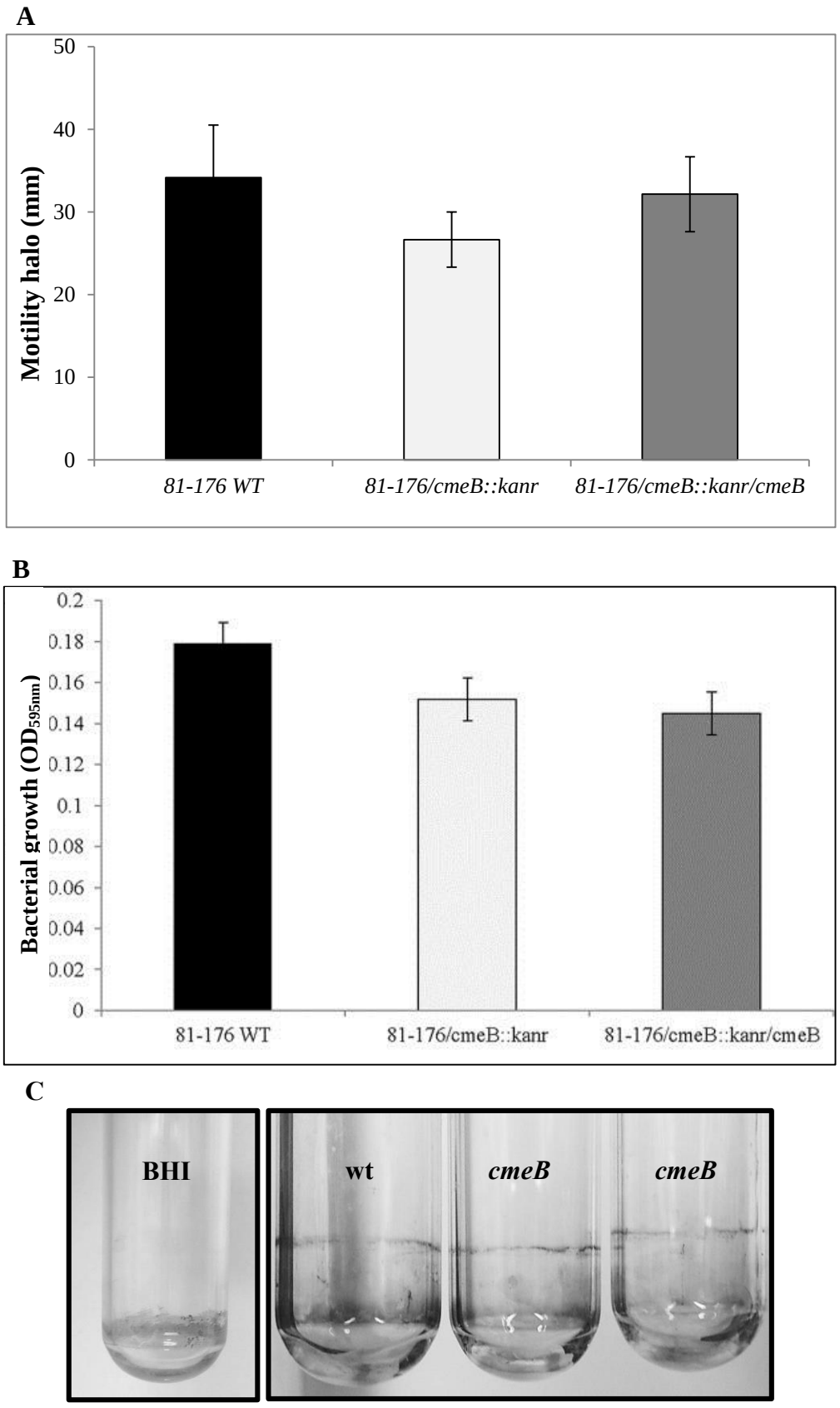
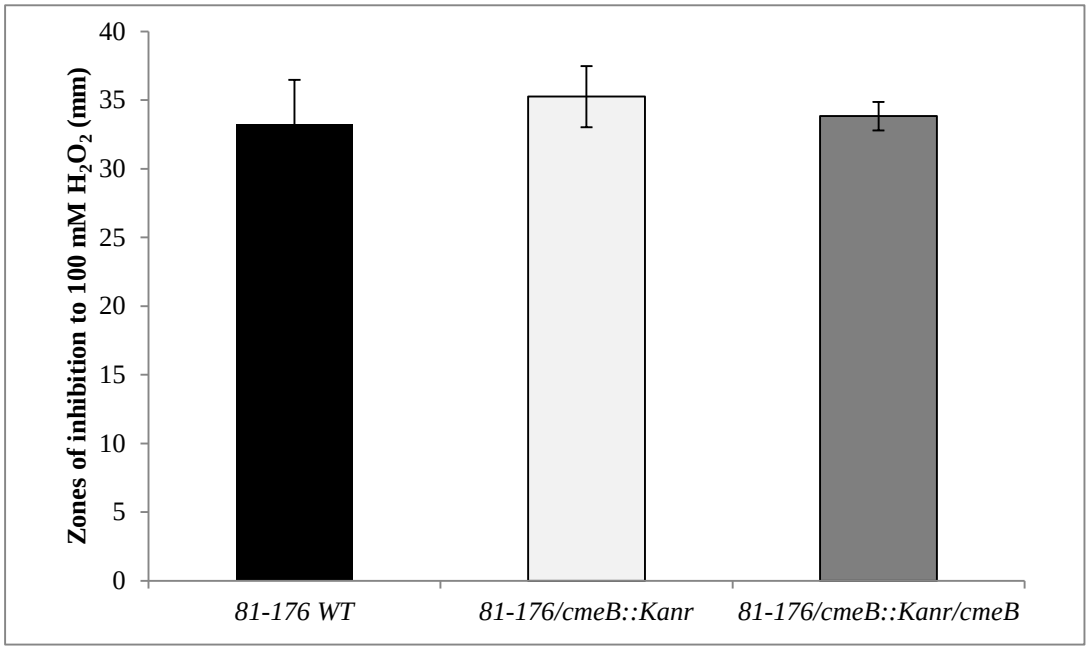


Figure 3.43. CmeB mutation has no effect on the motility and biofilm formation of strain 81-176. (A) Quantification of bacterial growth in BHI soft-agar motility plates inoculated with 81-176wt, *cmeB* mutant and complement strains. (B) Biofilm quantification in the air-liquid interface of the glass tubes and (C) visualisation of the biofilm halos in the glass tubes produced these strains. Values are mean ± SD from three independent experiments with three technical replicates each.

Increased oxygen tension in the atmosphere is the most threatening stress for *C. jejuni* viability during environmental transmission (Kim *et al*., 2015). The efflux pump component CmeG was demonstrated to be required for oxidative stress response of *C. jejun*i (Jeon *et al*., 2011). So, the role of CmeB in oxidative stress resistance to hydrogen peroxidase (H_2O_2) was investigated for *C. jejuni* strain 81-176. It was observed that *cmeB* mutation did not impair oxidative stress resistance to H_2O_2 as the average diameters of inhibition zones were 33 ± 3.21, 35 ± 2.21 and 34 ± 1.03 for the *C. jejuni* 81-176 wt, *cmeB* mutant and complement strain, respectively (Fig. 3.44, A). No statistically significant difference in the growth zones ($p=0.34$) was observed between the wt and *cmeB* mutant strain (Fig. 3.44, B).

A



B

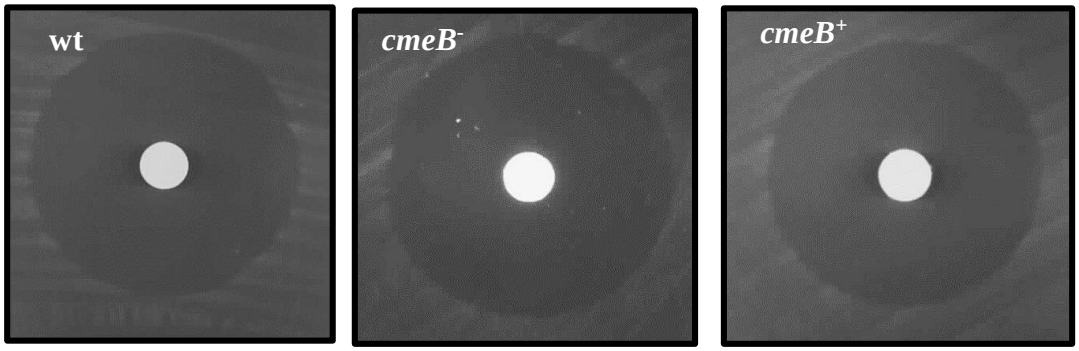


Figure 3.44. CmeB mutation has no effect on the oxidative stress response of strain 81-176. (A) Quantification of 81-176wt, *cmeB* mutant and complementation derivative inhibition zones in MH plates topped with a 100mM H_2O_2 disc; (B) Visualisation of the inhibition zones produced by these strains. Values are mean ± SD from three independent experiments with three technical replicates each.

Overall, CmeB was shown to be beneficial for intracellular survival and multiplication of *C. jejuni* 81-176 within *A. polyphaga*, but not for this pathogen´s motility, biofilm formation or oxidative stress response.

CHAPTER 4: Discussion

4.1. Selection of a strain to investigate the interaction between *C. jejuni and A. polyphaga*

As previously mentioned, *Campylobacter* possesses several pathogenicity-associated factors involved in important bacterial processes, such as motility, antibiotic resistance, adhesion/invasion, toxin production, stress response and chemotaxis (Dasti *et al.*, 2010). An essential aim of the on-going *C. jejuni* research is to clarify the precise role of these factors in bacterial pathogenesis and survival in different hosts so that possible drugs could be developed to combat infections by this pathogen (Backert & Hofreuter, 2013). In this study the *A. polyphaga in vitro* model was used to identify possible factors involved in *C. jejuni* pathogenesis. This is considered to be important because the association between free-living amoebae and pathogenic bacteria is concerning as it may have significant implications for human health (Goni *et al*., 2014). In addition, as *Campylobacter* may encounter specific antibiotics during commensal carriage in food animals or during infection in humans (Bolton, 2015), antibiotic resistance mechanisms of this pathogen were explored.

The efficiency with which *C. jejuni* interacts with cultured host cells depends on the specific properties of *C. jejuni* strains and the cultured cell lines and, consequently, the outcomes of the *in vitro* assays varies considerably (Backert & Hofreuter, 2013). As there is significant *C. jejuni* strain-to-strain variation, this is considered important in determining the outcomes in terms of virulence and survival of this pathogen. The range of *C. jejuni* strains present in our laboratory collection was, therefore, characterised in respect of their virulence and survival attributes. A common characteristic of *C. jejuni* cells is their ability to change the natural spiral cell shape to coccoid form when in stressed conditions (Ikeda & Karlyshev, 2012) and, so *C. jejuni* cells cultured for 24h

were used for the experimental work as after two days of growth it was possible to observe only a few coccoid cells in the bacterial population. *C. jejuni* strains 11168H, 81-176, G1 and X were present in our laboratory culture collection. In order to identify and distinguish between the different strains, primers for amplification of unique genes in these strains were designed. Amplification of *cj1435*, *dmhA*, *tagF* and *moaA* genes identified the 11168H, 81-176, G1 and X strains respectively. In addition, multiplex PCR using these primers helped in determining whether there was any contamination between these bacterial strains. In order to detect the virulence pVir and tetracycline resistance pTet plasmids, primers for amplification of the *virB11* and *tetO* were also designed. To determine which of these strains had a higher growth rate *in vitro*, growth curves in BHI broth were conducted for two days and it was possible to determine that *C. jejuni* 81-176 strain was able to grow better than the other wt strains.

As referred to previously, motility is a crucial factor for *C. jejuni* pathogenesis (Backert & Hofreuter, 2013) and, so, motility of these wt strains was determined in BHI soft agar plates. It was observed that *C. jejuni* strain 81-176 was the most motile, followed by 11168H, G1 and the least motile X strain. This may confer an advantage for strain 81-176 to survive better inside a host, and was, therefore, another reason why this strain was considered to be the best candidate for the co-culture experiments with amoebae. Biofilm formation is another common strategy for bacterial survival in the environment, especially when they are under harsh environmental conditions (Bronowski *et al*., 2014). Biofilm formation in distinct wt strains was determined by quantification of aggregates (flocs) in MH broth. Strains 11168H and 81-176 produced extensive floc formation in the broth medium, as opposed to both G1 and X strains. Overall, as *C. jejuni* strain 81-176 contains the virulence plasmid pVir, had the highest growth rate in BHI broth, was highly motile and formed extensive biofilm aggregates, it seemed one of the most promising candidate strains to study the interaction between *C. jejuni* and amoebae. Moreover this

invasive strain has been widely used for a number of studies in the *Campylobacter* research field (Hofreuter *et al*., 2006).

4.2. Generation of a chimeric *C. jejuni* strain

During the course of this study and as referred to previously, laboratory co-workers sequenced for the first time the G1 strain isolated from a Guillan-Barré patient (Lehri *et al.*, 2015). However, further to the initial sequencing, analysis of the genome of this strain using CLC genomics software revealed that the strain sequenced was not *C. jejuni* G1, but that of the 81-176 strain. It was discovered that the mixture between these two strains during the -80°C storage and subsequent plating on CBA medium with tetracycline concentration allowing growth of strain 81-176, but not G1, lead to the creation of a chimeric strain, which was named strain B7. This chimeric strain presented two large genome regions of the strain G1 integrated in the genome of 81-176. To distinguish between G1, 81-176 and chimeric B7 strains, primers for the *luxS* gene amplification (81-176 wt and G1-allele version) were designed and PCR together with the identification primers was conducted with these strains. This PCR also helped in determining the fraction of the chimeric strains in the culture mixture, which was approximately 25%. Although it was hypothesised that antibiotic selection pressure might have been the reason for the creation of this chimeric strain there was no difference in the tetracycline resistance levels between both the chimeric B7 and 81-176 strains. Examples of selective pressure that might possibly lead to the DNA exchange between these strains could be microbial competition for food and energy source. It would be interesting to unravel the mechanism by which this strain was naturally created as this might reveal an effective strategy utilised by bacteria for adaptation to selection from the surrounding environment. Nonetheless, the creation of this chimeric strain demonstrated how easily *Campylobacter* can exchange DNA. Since horizontal genetic exchange strongly influences the evolution

of many bacteria (Sheppard *et al*., 2011) it would be relevant to explore these results more deeply.

4.3. Contribution of CmeABC pump and sequence variation of CmeB protein to tetracycline resistance

The emergence and spread of bacterial resistance to antibiotics is an increasing problem that is becoming a major public health concern (O´Neill, 2014). Understanding antibiotic resistance mechanisms in *Campylobacter* may open a new direction in comprehending how bacteria adapt to antibiotic treatment and thus, in this study the tetracycline resistance mechanism in *C. jejuni* G1 strain was explored. Further to genome sequencing of strain G1, it was observed that this strain presented a large difference in the sequence of the *cmeB* gene when compared with the *cmeB*s from the reference strains 11168 and 81-176 (Lehri *et al*., 2015). Protein blast analysis revealed that the CmeB from strain G1 shared only 81% identity (100% query cover) with both 11168H and 81-176 strains, whilst the CmeBs from the latter strains shared 99% identity. In addition, despite the fact that both G1 and 11168H strains do not contain the tetracycline resistance plasmid pTet, the former strain was more resistance to tetracycline. So, it was hypothesised that the difference in the *cmeB* sequence might be the cause for the different levels in Tet resistance between these *C. jejuni* strains.

To test this hypothesis, G1, 81-176 and 11168H *cmeB* mutants were constructed. In addition, to test the effect of *cmeB* and pTet in AR, this plasmid was transferred from *C. jejuni* 81-176 to G1 strain. Tetracycline susceptibility assays revealed that the G1 strain was not only more resistant to tetracycline, but also to several other antibiotics than strains 11168H and 81-176 and that its *cmeB* may be the principal factor responsible for the differences in AR. Also, when the pTet plasmid was present in G1 strain, a huge

increase in Tet resistance of G1 was observed. This suggests a role of *cmeB* and pTet in antibiotic resistance (Iovine, 2013).

As demonstrated in this study, genetic exchange of elements involved in antibiotic resistance can result in a dramatic increase in *C. jejuni* antibiotic resistance levels. This is in accordance with other studies that found CmeB sequence variants presented a greater and more powerful efflux of antibiotics (Cagliero *et al*., 2006; Yao *et al.,* 2016). Consequently these variants also displayed enhanced antibiotic resistance (Cagliero *et al*., 2006; Yao *et al.,* 2016). The CmeB from *C. jejuni* G1 strain shared a high similarity with two "super" efflux pumps variants previously discovered, the 154KU CmeB (Cagliero *et al*., 2006) and the RE-CmeB variants (Yao *et al.,* 2016), indicating that CmeB from the G1 strain could also be a super efflux variant. In particular, in this study it was possible to generate bacteria with tetracycline resistance levels, which significantly exceeded those of all parental strains. Such exchanges are likely to occur in the environment, not only via conjugation e.g. involving a transfer of the pTet plasmid, but also via transformation, as many strains of *C. jejuni,* as previously mentioned, are naturally competent and can easily acquire DNA released due to lysis of the cells carrying antibiotic resistance genes (Young *et al*., 2007). In addition, the studies showing that regulation of the expression of the *cmeABC* efflux pump potentiates the differences in *Campylobacter* antibiotic resistance (Lin *et al.,* 2005; Cagliero *et al.,* 2007; Perez-Boto *et al.,* 2015), we and others (Cagliero *et al.*, 2006; Yao *et al.,* 2016) suggest that *cmeB* sequence variation may also be responsible for these differences. However, other contributors (e.g. CmeR; CmeA/C; second efflux pump, etc.) cannot be excluded and further evidence is required to claim that antibiotic resistance is mainly due to the CmeB transporter.

This data confirms that originally sensitive *C. jejuni* strains might easily become more resistant to antibiotics, and that this is an effective strategy, which may be utilised by bacteria for adaptation to selective pressure, which poses a serious threat to public health.

4.4. *C. jejuni* is able to survive and multiply within the *Acanthamoebae* host

The association of bacteria with free-living amoebae may have significant implications for human health (Goni *et al*., 2014). Also, there are conflicting accounts in the literature regarding the interaction between *C. jejuni* and *A. polyphaga* which may be explained by different methodologies used or different strains of *C. jejuni* and *Acanthamoebae* species (Vieira *et al*., 2015). In this present study therefore, the interaction between these microorganisms was investigated for a clear knowledge regarding the *Campylobacter-Acanthamoebae* interaction. Luckily, *A. polyphaga* is very easy to handle experimentally, shares similarities with macrophages and has been widely used to unravel bacterial virulence factors (Sandstrom *et al*., 2011; Tosetti *et al*., 2014; Guimaraes *et al*., 2016).

The efficiency by which *C. jejuni* strains interact with cultured cells depends on the specific properties of the strain (Young *et al*., 2007; Backert & Hofreuter, 2013). Selection of the *C. jejuni* strain to be used in this study was, therefore, very important, because it could determine the outcome of the infection. *C. jejuni* strain 81-176 was selected for this study after careful consideration because it was a highly virulent strain (Bacon *et al*., 2000; Hofreuter *et al*., 2006) and it was shown to have a higher potential to invade amoebic cells than the other strains tested (G1, 11168H and X). Nonetheless, it would be interesting to test the capability of survival within amoebae of other *C. jejuni* strains to check if the positive interaction between strain 81-176 and *A. polyphaga* may be also extended to other strains.

In order to survive, *Campylobacter* must be able to sense, adapt, and respond to temperature fluctuations (Stintzi, 2003). To study survival of *C. jejuni* inside amoebae, the temperatures of 25°C and 37°C were selected. At 37°C during longer incubation periods with *A. polyphaga*, *C. jejuni* may escape to the environment and begin to multiply. Further, re-infection of other amoebae cells by a high number of bacterial cells

may occur, resulting in toxicity to the amoebae and lysis and thus, this may be confused with intracellular multiplication of *C. jejuni* within *A. polyphaga*. If gentamicin is not applied before the lysis of amoebae with Triton during these times (standard gentamicin protection assay) (Mavri & Mozina, 2012), the extracellular bacterial numbers may also be included and intracellular numbers might be a result of re-infection. So, to avoid this it was decided to add an extra gentamicin step from the 24h time-point onwards (modified version). Compared with the standard gentamicin assay, a much more significant reduction in the intracellular bacterial numbers was observed at both 25ºC and 37ºC. This modified method allowed for quantification of intracellular bacteria only leading to more reliable results. In future, it would be noteworthy to investigate the interaction between *C. jejuni* and *A. polyphaga* at 42°C as this is the chicken body temperature and it was previously observed that at, this temperature, amoebic cells changed to the cyst shape. This could be considered relevant to human health as it is known that amoebic cysts may play a role in the contamination and persistence of pathogenic bacteria in food-related environments, because they allow for internalised foodborne pathogens to survive the physical and chemical disinfection methods used in the food industry (Lambrecht *et al*., 2015).

At 25°C a decline in the intracellular bacteria was detected 5h after gentamicin treatment. However, it should be noted that even if strain 81-176 was able to reside within an amoeba for a short time period, this might be sufficient time to increase the risk of infection by this pathogen. The data presented here also demonstrated prolonged survival of *C. jejuni* 81-176 in the presence of amoebae, which is in accordance with previous results (Bui *et al.*, 2012b). These results confirm that the presence of amoebae enhances the survival of *C. jejuni* in the environment. According to Bui *et al.*, this is likely due to the depletion of dissolved oxygen by amoeba, thus creating the microaerophilic environment optimal for *C. jejuni* growth (Bui *et al*., 2012b). Additionally, it was

observed viable *C. jejuni* after 4 days incubation in PYG medium aerobically without amoebae. Because coccoid cells are viable but not culturable and coccoid cell formation in *C. jejuni* is stimulated by stress conditions, such as, starvation and oxidative stress, it would be expected no viable counts. Although in some bacteria viable but non culturable forms are able to resuscitate and convert to culturable and fully infective forms, in *Campylobacter* whether they can resuscitate or not is a controversial issue (Ikeda & Karlyshev, 2012). The confliting results may be due to variation in the stress conditions used, leading to different types of coccoid cell formation. There are reports of reversion of coccoid forms into culturable forms after acid treatment (Chaveerach *et al*., 2003) and also, according to some studies using animal models of infection, the coccoid forms of *C. jejuni* are be able to convert to fully infectious forms (Jones *et al*., 1991; Saha *et al*., 1991). However, it was noticed that some of these results were also controversial due to irreproducibility of the data and that this process deserves further investigation (Medema *et al*., 1992; Van de Giessen *et al*., 1996).

At 37°C, the initial decrease in the number of viable bacteria was followed by a remarkable increase in the bacterial numbers after 48h of incubation. A similar trend was already reported for *S. enterica* and *L. monocytogenes* (Anacarso *et al.*, 2012). The authors named this first phase of viability decrease as the eclipse phase, which occurs probably due to an initial use of the bacteria as food source, or just due to a prolonged lag or adaptation phase, followed by an active intracellular growth (Anacarso *et al.*, 2012). Intracellular growth was confirmed by phase-contrast microscopy where *C. jejuni* cells were seen bursting out of the amoeba cells to the extracellular medium, a result of intracellular bacterial multiplication.

The results obtained in this study are in agreement with the studies supporting *C. jejuni* intracellular survival or multiplication inside *A. polyphaga* (Axelsson-Olsson *et al*., 2005, 2007, 2010a,b; Snealling *et al* 2005, 2008; Baré *et al*., 2010; Olofsson *et al*., 2013;

Grieskspoor *et al*., 2013), thus contradicting with the studies from Bui *et al*., that support an extracellular mode of survival only (Bui *et al*., 2012a,b).

Based on the information available in the literature and in the data presented here, we suggest a hypothetical model describing the mechanism of interaction between *C. jejuni* and its amoeba host (Fig. 4.1). According to this model, the intracellular bacteria acquired from the environment (at 25°C in our experiments) multiply at 37°C (conditions simulating host temperature). Upon ingestion of a product (e.g. water or milk) contaminated with amoebae, the latter are lysed releasing large amounts of bacteria causing the disease. A global search for other bacterial factors involved in the interaction between *C. jejuni* and amoebae could be based on differential expression studies (transcriptomics and proteomics).

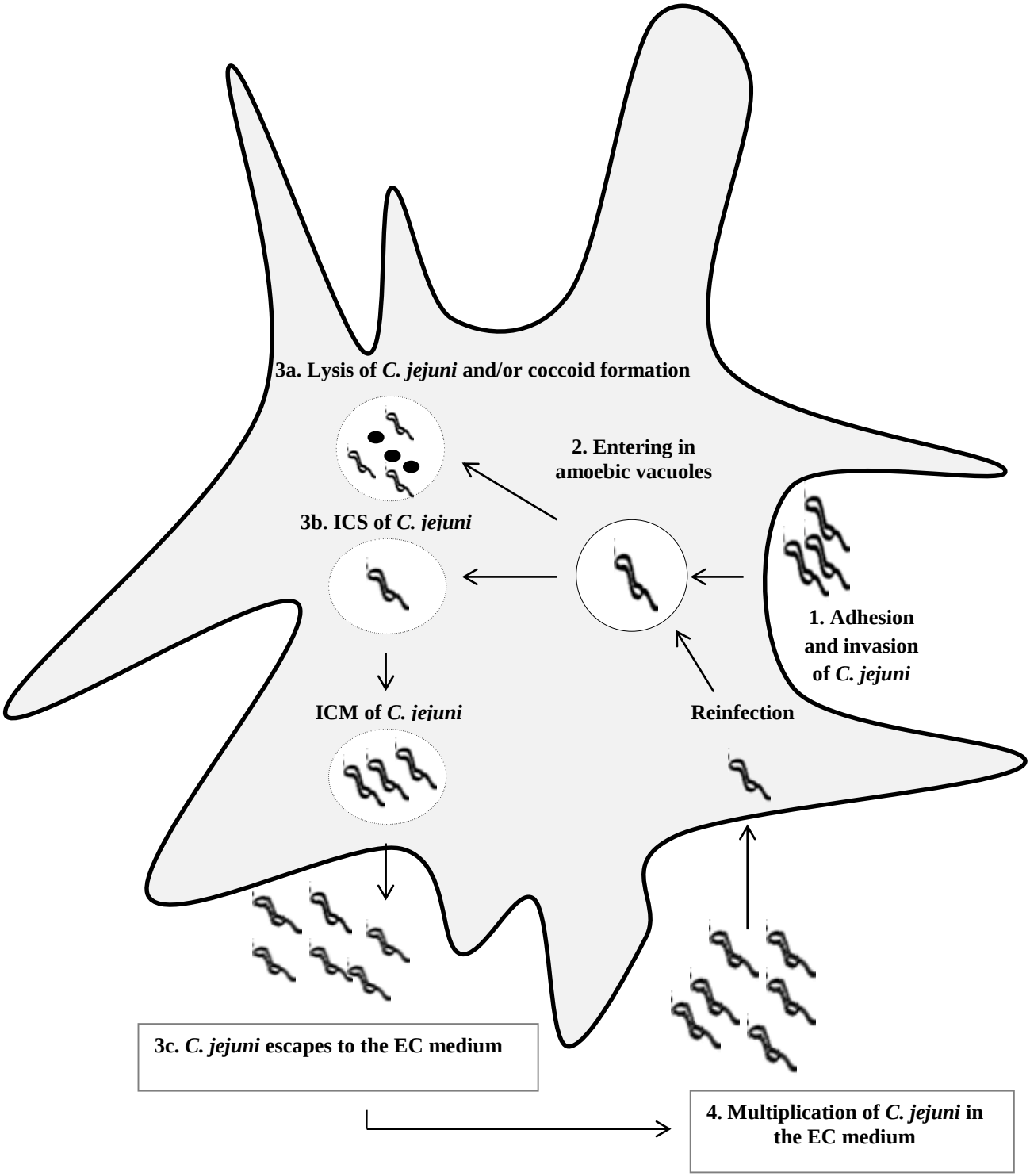


Figure 4.1. Hypothetical model of the interaction between *C. jejuni* 81-176 and *A. polyphaga*. The following possible stages of bacterial entry are depicted: 1, adhesion to and invasion of amoebic cells via phagocytosis; 2, gathering within amoebic vacuoles (Baré *et al*., 2010; Olofsson *et al*., 2013); 3a, bacteria degradation and/or coccoid formation; 3b), intracellular survival (ICS) followed by intracellular multiplication (ICM) and 3c, release of *C. jejuni* into the EC medium (Grieskspoor *et al*., 2013); 4), *C. jejuni* is able to multiply and re-infect other amoebic cells. Stages 1, 3a-b and 4 are based on the observations reported in this study.

4.5. Role of CmeB transporter and capsule production in the interaction between *C. jejuni* and *A. polyphaga*

As several CmeB homologues in other bacterial species were shown to have a role in virulence (Hirakata *et al.,* 2002; Jerse *et al.,* 2003; Chan & Chua, 2005; Buckley *et al.,* 2006; Bina *et al.,* 2008a; 2008b; Padilla *et al.,* 2010), the concept that efflux pumps may indeed act as virulence determinants has increased and could be expanded to *C. jejuni*. Although the CmeABC of *C. jejuni* was previously reported to be required for colonisation of the intestinal tract of the chicken (Lin *et al.,* 2003) its role in host cell interaction had not previously been determined. In this study, the role of CmeB in *C. jejuni* pathogenesis was explored using *Acanthamoeba polyphaga* as an *in vitro* host model.

The results presented here show that CmeB is required for survival and replication of *C. jejuni* 81-176 within amoebae and that this phenomenon was not an artefact of the detergent used in the co-culture experiments. These observations support the theory that efflux pumps may indeed act as virulence determinants. CmeB contribution to survival within amoebae might be related to the efflux pumps capability to pump out and confer resistance to host-derived antimicrobial agents (such as toxins or antimicrobial peptides) or because these pumps are able to export virulence determinants (Piddock, 2006).

As previously mentioned, different *C. jejuni* strains display wide differences in the sequence of their *cmeB* genes affecting the function of this efflux transporter (Cagliero *et al.,* 2006). For this reason the role of strain-dependency of *cmeB* in survival and multiplication of *C. jejuni* in *A. polyphaga* cannot be excluded. In the future it would be interesting to include other *C. jejuni* strains in a similar study to check whether CmeB contributes to survival of other strains in amoebae.

Capsule was found to be implicated in the interaction between *E. coli* and *Streptococcus suis* with amoebae (Jung *et al.,* 2007; Bonifait *et al.,* 2011). So, the role of capsule in the

interaction between *C. jejuni* and this eukaryotic organism was explored in this study. Interestingly, it was found that capsule production is also required for survival of *C. jejuni* 81-176 and G1 strains inside amoeba, but whether it is also implicated in multiplication still needs further confirmation. Because different *C. jejuni* strains have different capsular polysaccharide structures (CPS) and this might also have an impact on the outcome of infection, it would be interesting to identify the role of various types of CPS in survival of *C. jejuni* within amoebae.

Since motility was shown to be a crucial factor in *C. jejuni* host cell invasion and biofilm formation and contributes to pathogenesis (Bolton, 2015), the involvement of CmeB in these processes was tested. No statistically significant difference in the motility halo formed by the mutant strain compared with the wt was detected. No statistically significant differences were also observed for biofilm formation between these strains. As opposed to other studies that showed the involvement of an efflux pump in both flagellar motility and biofilm (Kvist *et al*., 2008; Webber *et al*., 2009) these data demonstrated that neither of these processes were responsible for the decrease of the *cmeB* mutant strain survival within the *A. polyphaga* host. The *C. jejuni* CmeG, a putative efflux transporter, was shown to be involved in oxidative stress response (Jeon *et al*., 2011), and so, it was hypothesised that CmeB could also contribute towards the oxidative defence in *Campylobacter*. This is of particular relevance since reactive oxygen species (ROS) are produced by the immune system of hosts (such as phagocytes), and are toxic, or deadly for bacterial cells (Paiva & Bozza, 2014). Unexpectedly, mutation of *cmeB* did not affect resistance to hydrogen peroxide of both *C. jejuni* strains 81-176 (Fig. 3. 47) and G1 (data not shown). The reason why CmeB was not involved in oxidative defence is not known, but it may be that the CmeDEF and CmeG are the major contributors for the detoxification process of ROS.

In summary, this study is the first to report that a *C. jejuni* 81-176 efflux pump was advantageous for its survival in amoebae and, by using an accurate gentamicin protection method, it was demonstrated that the interaction with *A. polyphaga* was beneficial for this foodborne pathogen.

4.6. Conclusion

This study provides up to date experimental data regarding the interaction between *Campylobacter* and *Acanthamoebae*. The results presented here help to clarify the conflicting accounts in the literature as to whether *C. jejuni* was able to survive and/or multiply inside the *A. polyphaga* host. This is considered to be a clinically relevant interaction since both these microorganisms can co-exist in poultry farms and are resistant to the existing biosecurity measures, thus increasing the risk of infection with *C. jejuni*. So, deciphering the type of interaction between *C. jejuni* and *A. polyphaga* and the molecular mechanisms involved it is considered that this has improved our knowledge regarding the *Campylobacter* lifestyle and thus will help in the development of drugs against this foodborne pathogen. Because CmeABC is required for *C. jejuni* antibiotic resistance and virulence, this efflux pump is a promising target for interventions to combat *C. jejuni* infections.

Additionally, it was demonstrated how easily *C. jejuni* can acquire DNA from other strains, and thus display an extensive genetic variation. This proves how relevant this organism is to human health (e.g. increased AR) and that rigorous care is needed when working with this pathogen so that it does not accumulate unwanted mutations.

4.7. Future work

C. jejuni fluorescent strain 81-176/pCPE11/28/GFP strain is available in the laboratory collection. This strain was able to invade *A. polyphaga* as efficiently as the 81-176 wt strain and so, it would be interesting to conduct confocal microscopy on *A. polyphaga* cells infected with this GFP strain. This would allow visualisation of the location of this pathogen within amoebae as *C. jejuni* bacteria will appear green due to GFP and amoebae will appear in both blue (due the 4', 6-diamidino-2-phenylindole that stains the nuclei) and red (due to phalloidin that will stain the actin cytoskeleton).

Although it is suggested that CmeB and capsule production are involved in the interaction between *Campylobacter* and *Acanthamoeba,* many bacterial factors that might participate in this interaction remain unknown. A promising approach in this direction would be a gene expression study (transcriptomics analysis) aimed at the identification of the bacterial genes differentially regulated during invasion. Despite the low intracellular concentration obtained for *C. jejuni* inside the amoebae cells, there may be improved techniques to be able to obtain a higher intracellular bacterial concentration and thus, be able to extract sufficient RNA for RNA-seq analysis. In addition, since capsule was found to be involved in survival of *C. jejuni* inside *A. polyphaga*, it would be interesting to know whether it is also implicated in multiplication of this pathogen inside this host and whether it is involved in the different outcomes of amoebae infection by the different *C. jejuni* strains.

As previously mentioned, amoebae and macrophages share similarities in their cellular structure, motility, physiology and in their ability to capture their prey (Ruqaiyyah & Naveed, 2011). It would be interesting to know whether the KpsM and CmeB are involved in survival of *C. jejuni* in macrophages, because it would appear likely given the results obtained in this study with *A. polyphaga.* This would be clinically relevant since

only few bacterial factors have been found to be involved in survival of this pathogen in macrophages (Backert & Hofreuter, 2013).

As previousely discussed, it is important to explore whether *Campylobacter* cells are culturable under the growth conditions used in the experiment investigating extracellular survival in the presence of amoebae. As there is controversial data in the literature this should help clarifying if *C. jejuni* coccoid cell formation can be a reversible process or not.

It was noted that there was a large difference in the sequence of *cmeB* from strain G1 when it was compared with the *cmeB* from strains 11168H and 81-176. In order to prove that amino acid sequence contributes to the enhanced antibiotic efflux function it was attempted to transform the *cmeB* gene from strains G1 and 11168H in the G1/*cmeB*::*kan*^{r} mutant so that the former could be under the regulation of the same promoter in G1 strain. This would allow for further analysis of the tetracycline resistance of these strains. Although a similar strategy confirming this hypothesis was recently demonstrated by Yao *et al.*, (2016), it would still be relevant to try to conclude this part of the work especially since it used a different strategy than the one published.

The chimeric B7 strain was naturally constructed via horizontal gene transfer during the *in vitro* growth of two different *C. jejuni* strains. It would be interesting to explore the reason behind this genetic material exchange and if this mechanism is reproducible both *in vitro* and *in vivo*. For the *in vivo* growth, as amoebae was shown to support *C. jejuni* growth at 37°C, the former microorganism would be infected by both 81-176 and G1 strains for a determined period of time after which, amoebae will be lysed and the bacterial genomic DNA would be extracted and further sequenced to check for mutations.

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Appendix

Published articles

- Vieira, Ana; Ramesh, Amritha; Seddon, Alan & Karlyshev, Andrey: "CmeABC multidrug efflux pump contributes to antibiotic resistance and promotes *Campylobacter jejuni* survival and multiplication in *Acanthamoeba polyphaga*", AEM, 2017 (doi: 10.1128/AEM.01600-17). This article was selected for inclusion in 'Spotlight' section of the Applied and Environmental Microbiology journal.

- Vieira, Ana; Seddon, Alan & Karlyshev, Andrey. "Review: *Campylobacter-Acanthamoeba* interactions", Microbiology, 2015 (doi: 10.1099/mic.0.000075)

- Lehri, Burhan; Kukreja, Kushneet; Vieira, Ana; Zaremba, Milena; Bonney, Kwasi & Karlyshev, Andrey: "Specific features of *Campylobacter jejuni* strain G1 uncovered by genome sequencing", FEMS Microbiology Letters, 2014 (doi: 10.1093/femsle/fnu064)

Presentations & Posters

- IhSHA Conference 2016 at Kingston University – poster presentation. Won the 3rd prize for the poster presentation: "The *Campylobacter jejuni* multidrug efflux pump transporter CmeB is involved in interaction with *Acanthamoeba polyphaga*" (June, 2016)

- The Microbiology Society Annual Conference 2016 – Liverpool, UK – 20th to 24th March, 2016 – Poster presentation: "Molecular mechanisms of

Campylobacter jejuni pathogenesis and survival in the environment" (https://www.microbiologysociety.org/event/annual-conference/annual-conference-2016.html)

- Faculty Research and Enterprise Development Day Conference at Kingston University – oral presentation: "Molecular mechanisms of *Campylobacter jejuni* resistance to antibiotics and survival in the environment" (June, 2015)

- IhSHA Conference 2015 at Kingston University – oral presentation – Won the 1st prize for the oral presentation: "Antibiotic resistance in *Campylobacter jejuni* and the search for novel antibacterials" (June, 2015) (http://sec.kingston.ac.uk/news/2015/the-former-school-of-life-sciences-students-won-the-1st-prize-at-the-3rd-ihsha-conference-at-kingston-university/)