The Response of *Campylobacter jejuni* to Pancreatic Enzymes

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration with the following exceptions: LC/MS/MS analysis was done by Dr Karen Homer, King's College London; chicken colonization experiments were performed by Dr Tristan Cogan (Bristol Veterinary School) and NMR spectroscopy, monomer analysis and linkage analysis of the polysaccharide was carried out by Professor Andy Laws (University of Huddersfield).

The contents have not been submitted for any other qualification.

This dissertation does not exceed the 60,000 word limit.

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PhD research often appears a solitary undertaking. However, it is impossible to maintain the degree of focus and dedication required for its completion without the help and support of many people.

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Abstract

Campylobacter jejuni is an important food-borne pathogen, and a major cause of bacterial gastroenteritis. Despite this, relatively little is known regarding the way in which *C. jejuni* colonises hosts, causes disease or survives in the environment during transmission between hosts. *C. jejuni* responds to a number of biological molecules found in the human intestinal environment such as bile salts leading to the induction of Campylobacter invasion antigens (Cia), and norepinephrine in response to which the bacterium shows increased virulence. In this study we investigated the response of *C. jejuni* to mammalian pancreatic α-amylase. The results of this study show that *C. jejuni* responds to pancreatic α-amylase with production of a mucoid colony phenotype that results from increased secretion of extracellular polysaccharide (EPS) identified as an α-dextran, further to this it was found that the amount of extracellular protein produced by *C. jejuni* was also increased, the proteins where identified using liquid chromatography mass spectrometry (LC-MS), a number of proteins associated with virulence were identified in the samples grown in the presence of α-amylase. In response to pancreatic α-amylase *C. jejuni* forms significantly increased biofilm and exhibits increased virulence in the *Galleria mellonella* and Caco-2 epithelial cell models. Furthermore *C. jejuni* pre exposed to amylase show significantly increased colonisation of chickens and increased resistance to stresses. It is also shown that *C. jejuni* is able to utilise α-amylase as a nutrient source, this was determined by growing the bacterium on a defined minimal media with the α-amylase as the only source of carbon present. The ability of *C. jejuni* to respond pancreatic amylase was shown to require proteolytic activity of Cj0511.

Contents

Chapter 1: Introduction

1.1 *Campylobacter* historical perspectives and background

In the early part of 1906, Sir John McFadyean and Sir Stewart Stockman, two British veterinary surgeons, isolated an unknown spiral-shaped organism from the aborted foetuses of sheep (McFadyean and Stockman, 1913; Zilbauer *et al*., 2008). The majority of cells observed were shaped like a comma, however some cells were described as being long and spiral. These cells were determined as actively motile; inoculation into liquid agar that was subsequently allowed to solidify exhibited a band of growth corresponding with growth at a region of reduced oxygen tension.
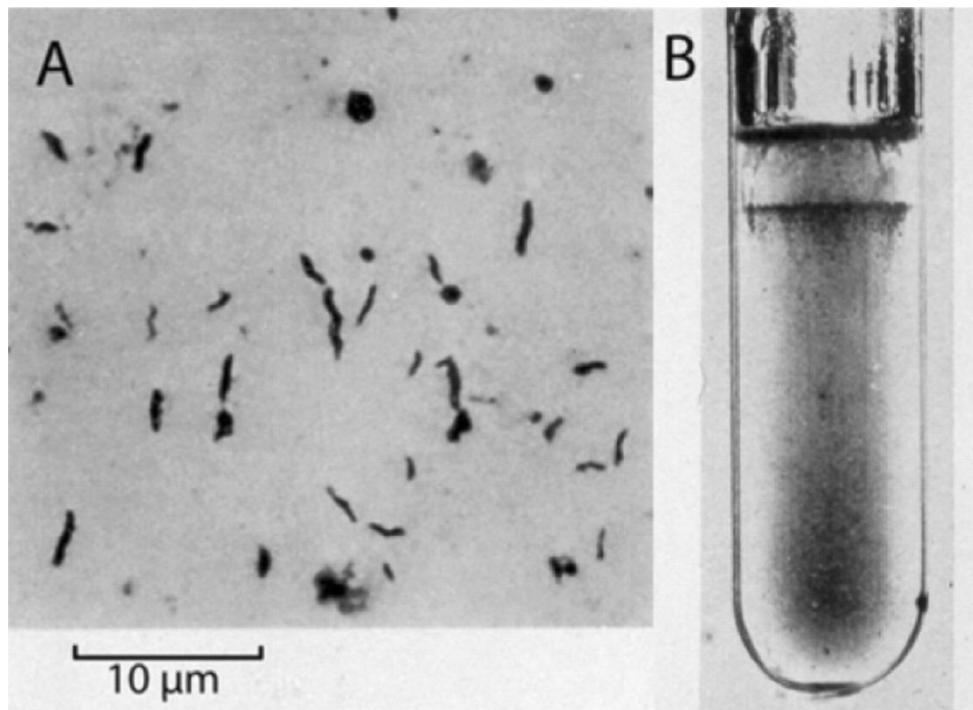
Figure 1.1. John McFadyean and Stewart Stockman's photographs of *Campylobacter* species (A) in smears of uterine exudate from an infected ewe. (B) and a culture of *Campylobacter* species in liquid agar which had then been allowed to solidify (Skirrow, 2006).

However, *Campylobacter* was likely to have been isolated approximately 20 years prior to the McFadyean and Stockman study. In 1886, Theodor Escherich had published a series of articles in the *Weekly Munich Medical Review* after making observations from examining the stool samples of 72 children suffering from intestinal diseases and found spiral-shaped bacteria in 35 of them. However, it was thought that this spiral-shaped bacterium played no role in causation of the disease (Butzler, 2004, Skirrow, 2006). The Escherich articles that were written and published in German remained unknown for nearly a century until the Third International *Campylobacter* Workshop held in Ottawa in 1985 and Manfred Kist presented Escherish's findings (Kist, 1985)

Further to the veterinary study of McFadyean and Stockman, in 1919 Theobald Smith investigated infectious abortions in cattle in the USA and isolated a bacterium that he described as spirillum. This organism was initially described as a *Vibrio fetus*, due to its spiral morphology, and was cultured on a medium constituted of agar, gelatin, and serum (Smith, 1919). And in 1931 Jones *et al*. credited winter dysentery in calves to an infection caused by what they called *Vibrio jejuni* (Jones *et al.,* 1931).

The first well-documented outbreak of human campylobacteriosis was in 1938 and took place in the US state of Illinois, where 355 inmates of 2 neighboring penitentiaries were affected (Levy, 1946). The outbreak was milk-borne and an organism resembling *Vibrio jejuni* was isolated from some of the infected. Then in 1947 Vinzent *et al.* isolated *V. fetus* from the blood of 3 pregnant women suffering from illness of unknown origin (Vinzent *et al.,* 1947). The illness persisted for approximately a month; consequently 2 of the women suffered an abortion. A decade later Elizabeth King observed similar findings to the Vinzent *et al*. (1947) study and called the organism a related *Vibrio*, although at that time there were only a handful of cases describing what we now know as campylobacteriosis King postulated that the true number was much higher and highlighted the importance of developing a suitable culturing method, to grow the bacterium (King, 1957).

This breakthrough came in 1968 when Dekeyser at the National Institute of Veterinary Research, Brussels, Belgium in collaboration with Buztler at the St Peter University Hospital, New Brunswick, Canada developed a method involving filtration of stool samples through 0.64 μm membrane filter and inoculation of the filtrates onto agar (Dekeyser *et al*., 1968). In 1977 a much simpler method of culturing both *C. jejuni* and *C. coli* was developed by Skirrow who described a method of directly culturing faeces onto blood agar containing trimethoprim, polymyxin and vancomycin (Skirrow, 1977). The growth condition were defined as 43°C in microaerobic conditions constituted of 5 % (v/v) oxygen 10 % (v/v) carbon dioxide and 85 % nitrogen (v/v)

1.2 *Campylobacter* taxonomy

The taxonomy of *Campylobacter* has changed considerably since the inception of the *Campylobacter* genus by Sebald and Veron in 1963, the name meaning 'twisting bacteria' in Greek. Under the original classification the genus contained only 2 taxa, the first being *C. fetus* and *C. bubulus* (Sebald and Veron, 1963).

The science of taxonomy comprises of 3 disciplines, identification, classification and nomenclature. Traditionally bacterial classification was based on cell morphology, growth requirements and biochemical tests. As mentioned before, *Campylobacter* was originally classified in the genus *Vibrio,* a genus which pre-1963 was very diverse and contained true *Vibrio spp*. such as *V. cholerae*. In 1963 Sebald and Veron discovered that *V. fetus* and *V. bubulus* were indeed different to other *Vibrio spp*. this discovery made when they applied the Hugh and Liefsen fermentation test as well as looking at the G+C ratio of genomic DNA (Sebald and Veron, 1963). Advancements in technology have allowed considerable progress in the methods used for bacterial classification, such as using 16S RNA sequencing. *C. jejuni* is today classified as belonging to the epsilon class of proteobacteria, order *Campylobacteriales*; this order also includes *Helicobacter* and *Wolinella*, these two combined with *Campylobacter* form a genus group known as the rRNA superfamily VI. The *Campylobacter*

genus contains 20 species, and 8 subspecies (Bergey's Manual of Systematic Bacteriology, 2005)

1.3 Epidemiology

Today, *C. jejuni* is one of the leading causes of bacterial gastroenteritis in the world, and a principal cause of diarrhoeal disease in England and Wales. According to the Health Protection Agency (HPA) there were 64, 608 reported cases of *Campylobacter spp.* infections in 2011 as in figure 1.2(HPA, 2012). In addition to this, the fact that the majority of *C. jejuni* infections do not require hospitalization means many cases go unreported and so the actual incidence is presumed to be considerably higher.
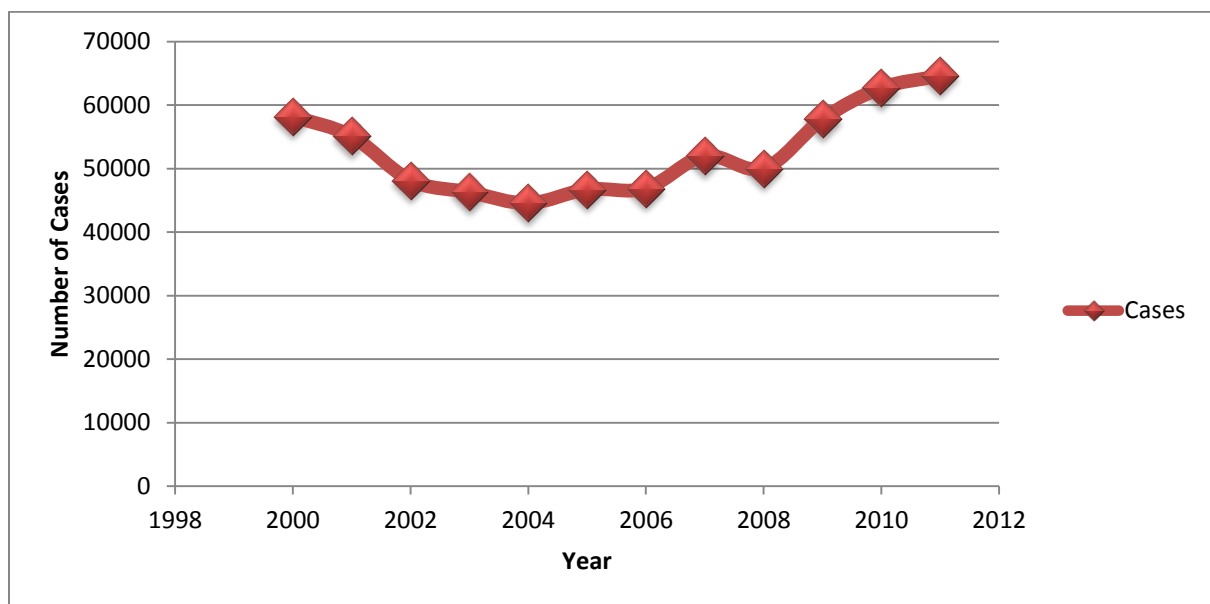
Figure 1.2. Incidence of Campylobacterioses per year in England and Wales from 2000 to 2011 (HPA. 2012).

The statistics show that by age group, ages 15-44 are the most affected with *Campylobacteriosis,* persons aged between 45-64 are the second most frequently infected. Furthermore the number of infections in this group has risen 25% in the last decade with 14, 355 cases in 2001 to 19,949 cases in 2011. (Fig 1.3. HPA, 2012).
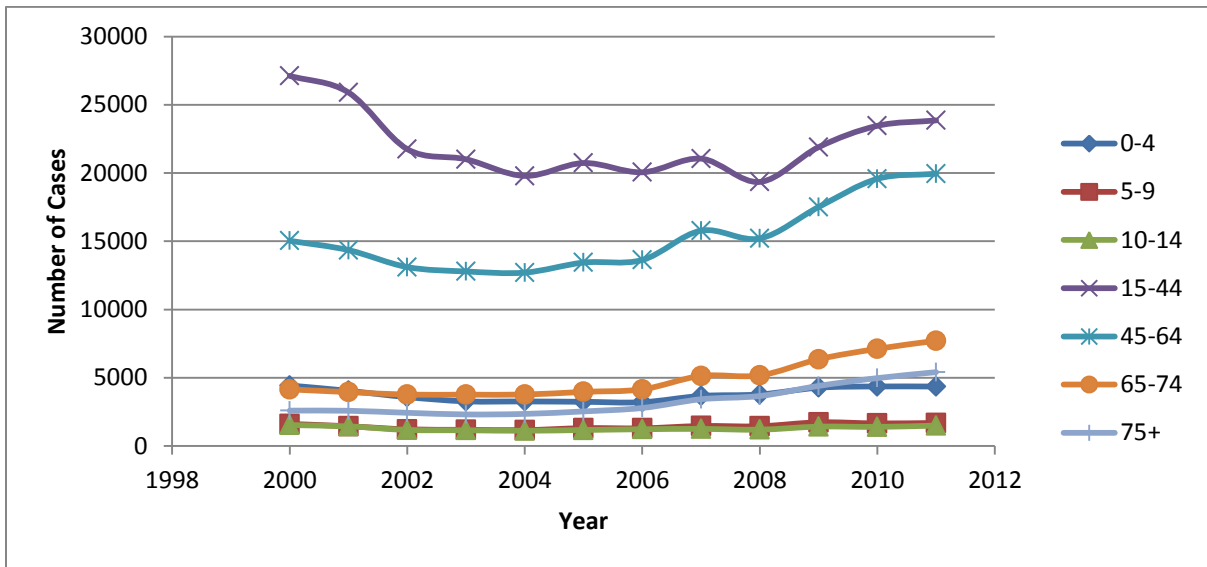
Figure 1. 3. Incidence of Campylobacterioses per age group in England and Wales from 2000 to 2011 (HPA, 2012).

Campylobacter spp. infections are present throughout the year but peak in the summer months (HPA. 2012) The Department for Environment Food & Rural Affairs (DEFRA) state that the economic impact of *Campylobacteriosis* is significant, due to affected individuals being unable to work. In 2009 the Food Standards Agency (FSA) conducted a survey of retail-bought chickens and reported that 66 % of all chickens sold in retail across the UK are infected with *Campylobacter spp.* (FSA. 2013).

The Centres for Disease Control and Prevention (CDC) in the United States of America report that approximately 13 cases are diagnosed per 100,000 people. However, they do estimate that over 2.4 million people are affected every year with the vast majority of cases are unreported. They also report that organism is isolated more regularly in males than females and predominantly in infants and young adults. It is thought that approximately 124 persons with *Campylobacter* infections die each year (CDC. 2012)

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Campylobacterioses is also a problem in developing countries and primarily affects children under the age of 5 years old, as an example the isolation rate is 17.7 % in Algeria 11% in Egypt and 13% in Bangladesh. *C. jejuni* infection in developing countries does not show seasonal variation as it does in developed countries.

1.4 The gastrointestinal tract

C. jejuni host environment is the human GI tract which consists of its own unique intestinal microflora, hometo a vast and diverse bacterial biomass of approximately 10^{14} bacterial almost 10 times the number of eukaryotic cells present in the human body, with an estimated 300-500 species (Simon and Gorbach, 1984).

In utero the intestine is sterile, immediately after birth the colonisation of the gastrointestinal tract begins; depending on the method of delivery different bacteria are acquired, from the maternal birth canal bifidobacteria and lactobacilli. If delivery occurs by caesarean section initial colonisation occurs by bacteria acquired from the ambient environment, this can affect the overall composition of the microflora (Borriello,1986).

Initial colonisation by pioneer bacteria influence the expression of genes in host epithelial cells and create an environment conducive to their own requirements, this in turn can result in the prevention of colonisation by bacteria the are introduced to the gastrointestinal tract later in life. Therefore this process of initial colonisation can have an impact on the permanent flora present throughout the lifetime of a person (Bengmark, 1998).

Babies who are fed formula milk have been shown to contain bacteriodes, clostridia and enteric bacteria in comparison to breast fed babies whose microflora is comprised of bifidobacteria and lactobacilli. Once the child begins on solid food the microbial intestinal composition begins to converge and the beginning of the complex permanent adult flora is formed, Gram negative

bacteria predominate and a wider range of genera are observed (Bengmark, 1998).

In fully grown adults the vast majority of bacteria are contained in the large intestine with up to 10^{12} cells/g of luminal content, this forms a highly dense and complex ecosystem. In comparison, the stomach and small intestine contains relatively fewer bacteria that adhere to the epithelial cell this is due to the conditions created in the upper section of the tract by bile and pancreatic secretion which do not allow ingested bacteria to persist. Furthermore, peristalsis that propels movement towards the ileum hinders colonisation by bacteria.

1.4 Pathogenesis

The infective dose of *C. jejuni* for humans is considered low, approximately 500 bacteria, compared to another Gram-negative enteric pathogen, *Vibrio cholerae* which requires between 10^3 to 10^8 cells for successful infection of the human host (Schmid-Hemple and Frank, 2007).

The onset of disease after ingestion of *C. jejuni* requires an incubation period of 24-72 hours, after which symptoms begin to present. Clinical symptoms include abdominal pain and diarrhoea that can be mild, non-inflammatory and non-bloody in some cases, and severe bloody diarrhoea in others. Disease is more severe in developed countries in comparison to developing countries. In developed countries disease is characterised by fever and abdominal pain accompanied with bloody diarrohea, in developing countries the disease is characterised by again fever and abdominal pain but the diarrhoea is non-bloody accompanied by vomiting and dehydration, in some cases the disease outcome can be more severe in developing countries because patients are often malnourished (Coker *et al.*, 2002). Furthermore in developing countries infants suffer from multiple infections which are followed by increasingly asymptomatic disease in older children and adults (Coker *et al.*, 2002).

For *C. jejuni* to cause an infection, the bacterium must first circumvent the barriers provided by the GI tract, be they mechanical or immunological. *C. jejuni* corkscrew morphology and high motility allow it to pass through the mucus layer of the GI tract which is the first line of defence; another feature which aids this process is the short *O*-side chain of its LOS which reduces non-specific binding to mucins (McSweegan *et al*., 1986).

Once this initial barrier has been penetrated the bacterium is able to it interact with epithelial cells using adherence mechanisms. A constituent of bile, sodium deoxycholate stimulates the production of *Campylobacter* invasion antigens or Cia proteins, the secretion of these proteins is not initiated until the bacterium is at the site of long-term colonisation (Malik-Kale *et al*., 2008). Some studies have suggested that *C. jejuni* is able to invade epithelial cells with intracellular bacteria being found in patient serum (Rivera-Amill *et al*., 2001)

Typically, in healthy individuals, infection is self-limiting and lasts up to 7 days. Antibiotics are only recommended to those with severe or prolonged symptoms and the antibiotic most commonly prescribed is erythromycin (Al-Abri *et al*., 2005, Zilbauer *et al*., 2008). Erythromycin has been the drug of choice for treatment of most *C. jejuni* gastrointestinal infections (Al-Abri *et al*., 2005). This agent is characterised by low toxicity, a narrow spectrum of activity and low cost (Allos and Blaser, 1995). The resistance of *C. jejuni* to erythromycin is less than 5% with little change in this resistance rate (Allos and Blaser, 1995)

In rare cases, patients can develop a serious complication as Guillain–Barré syndrome (GBS) and its variant variant Miller-Fisher syndrome (MFS). GBS is an autoimmune disorder resulting in paralysis of the peripheral nervous system. Approximately 66 % of all GBS patients have had *C. jejuni* intestinal infection one to three weeks prior to the onset of GBS, therefore *C. jejuni* is thought to be the fundamental predisposing factor associated with development and onset of GBS (Prendergast *et al*., 1998). With the decline of polio, GBS has now become the principal cause of acute neuromuscular paralysis, with an annual incidence of 1-2

cases per 100 000 in many parts of the world (Blaser *et al*., 1997; Buzby *et al*., 1997; Nachamkin, 1997; Wassenaar *et al.*, 2000).

Clinical features of GBS can include loss of tendon reflexes and symmetrical weakness that usually affects the lower limbs and progresses in an ascending fashion, and loss of proprioception. GBS can also affect the lower cranial and thoracic nerves that can result in respiratory difficulties. The majority of patients require hospital treatment and approximately one in three require treatment for respiratory failure (Schmidt-Ott *et al.*, 2006). Features such as fluctuations bowel function, blood pressure and heart rate can also be observed (Hahn, 1998, Hadden and Gregson, 2001).

A number of studies have stated that specific serotypes of *C. jejuni* are associated with the onset of GBS, (Kuroki *et al*., 1991). Studies by Kuroki *et al.* (1993) and Fujimoto *et al.* (1992) found *C. jejuni* O:19 to be predominant amongst Japanese patients suffering from GBS.

1.5 Virulence factors

1.5.1 Capsular Polysaccharides

It has been postulated that virtually every virulence factor associated with bacterial pathogenesis is secreted or positioned on the cell surface (Finlay and Falkow, 1997). Surface polysaccharides represent the predominant structures on all bacterial cell surfaces, and they are often important in the interactions between pathogens, their hosts and the environment

Capsular polysaccharide (CPS) plays an integral role in bacterial survival in the environment as well as contributing to virulence and pathogenesis. CPS is found on the surface of many bacteria, and can contribute to pathogenesis by providing structural variation, imitating host antigens and resisting phagocytosis and complement-mediated killing (Guerry and Szymanski, 2008). A significant

proportion of the *C. jejuni* genome is dedicated to polysaccharide synthesis as much as one-tenth of the genome might be devoted to carbohydrate synthesis (Mclennan et *al*., 2008).

C. jejuni has a high molecular weight capsular polysaccharide, whose synthesis is dependent on the *kps* gene cluster (Karlyshev *et al.*, 2000; Parkhill *et al.*, 2000). The CPS is a means of determining the Penner serotype, and has been shown to be phase variable (Karlyshev *et al.*, 2001; Bacon *et al.*, 2001). Bacon *et al.* (2001) showed that a non-capsulated *C. jejuni* 81-176 *kpsM* mutant showed a significant decrease in the ability to infect INT407 cells and reduced virulence in a diarrhoeal disease ferret model. Based on such evidence, CPS is considered one of the definite factors associated with virulence (Guerry and Szymanski, 2008).

1.5.2 Lipo-oligosaccharides

C. jejuni expresses a second surface carbohydrate, a lipo-oligosaccharide (LOS) which is anchored into the membrane by a segment of lipid A which is attached to an extracellular-positioned short oligosccahride unit via 2-keto-3-deoxy-octulosonic acid (KDO). The LOS is hyper-variable, consistent with a function in avoiding host immune action. The hyper variability arises from homopolymeric tract variation in genes, *cgtA* and *wlaN,* the former encodes an *N*-acetylgalactosaminyltransferase, and the latter encoding a β-1,3 galactosyltransferase (Linton *et al.*, 2000; Guerry *et al.*, 2002). Mutations in gene *cgtA* have been shown to increase resistance to normal human serum and intestinal epithelial cell invasion (Guerry *et al*, 2002; Guerry *et al*, 2000).

C. jejuni LOS are distinct from other bacterial LOS as they are able to imitate the structure of human gangliosides. This ability to imitate causes antibodies targeted against the *C. jejuni* LOS ganglioside to react with host gangliosides resulting in the development of autoimmune neuropathies such as GBS (Guerry and Szymanski, 2008).

1.5.3 Flagella

C. jejuni possesses either uni-polar or bi-polar unsheathed flagella providing the bacterium with a high degree of motility. Motility is required to overcome the peristalsis present the gut and is criterion for entry into the mucous layer and subsequent colonization of host tissue (Wassenaar *et al.*, 1993). Flagella also play a role in chemotactic motility: *C. jejuni* exhibits chemotaxis towards amino acids that are present in abundance in the gastrointestinal tract of the host (Young *et al.*, 2007).

Mutation in the response regulator that controls flagellar rotation *CheY*, exhibit decreased virulence in the ferret mode (Yao *et al.*,1997) The function of the flagellar apparatus is not restrained only to motility; constituents of the flagellar apparatus are used to export certain proteins. The flagellar export apparatus is used not only for the export of flagellin subunits but also for the export of other proteins, the so-called *Campylobacter* invasion antigens (CiaA-H) that are required for adhesion to and invasion of epithelial cells (Konkel *et al.*, 2004).

These antigens are produced during coculture with INT407 epithelial cells, insertional mutagenesis of the *ciaB* gene resulted in a significant reduction in *C. jejuni* invasion (Konkel *et al*, 1999). However, Novik *et al* (2010) found that a 81-176 *ciaB* mutant did not exhibit any measurable deficiency in its ability to invade T84 epithelial cells (Novik *et al.*, 2010)

Genes *flgB*, *flgC* and *flgE2* are involved flagellar filament assembly and mutations in these genes resulted in the inability to secrete protein CiaB, demonstrating a function in flagellar assembly and export. To successfully secrete CiaB either FlaA or FlaB are required, additionally *in vitro* cell invasion of INT407 cells is dependent on secretion of these Cia antigens and motility. (Konkel *et al.*, 2004).

1.5.4 Cytolethal distending toxin (CDT)

Other bacterial species, such as *Escherichia coli* and *Helicobacter hepaticus,* produce a CDT that is related to that of *C. jejuni.* CDT functions by causing eukaryotic cells to stop in the G1/S or G2/M transition cell cycle preventing them from entering the mitotic cell cycle (Whitehouse, *et al.*, 1998).

CDT is composed of 3 subunits known as CdtA, CdtB and CdtC, which are membrane-associated proteins and are required for the induction of IL-8 (Lara-Tejero and Galan., 2000). Transfection with CdtB into host cell leads to the same outcome as seen with complete CdtABC complex suggesting that CdtB is the toxic component of the of the 3 subunits, furthermore CdtB has been shown to share homology with mammalian DNase I (Lara-Tejero and Galan., 2000). CdtB causes damage to DNA by phosphorylating protein H2AX, which contributes to the structure of DNA by nucleosome formation.

It has been suggested that CdtA and CdtC play a pivotal role in delivering CdtB to the host cell nucleus to allow the protein to operate as a DNase (Lara-Tejero and Galan, 2001). However the complete role of CDT in the pathogenesis of *C. jejuni* disease remains undetermined.

1.5.5 Glycosylation Pathways

C. jejuni posses both *O*-linked and *N*-linked glycosylation pathways. The *N*-linked system is responsible for post-translational modification of proteins by modifying asparagine residues. The system is encoded by a single gene cluster termed the protein glycosylation locus (*pgl*). *N*-linked glycosylation is an important virulence factor (Hendrixson and DiRita, 2004; Young *et al.*, 2002). Karlyshev *et al.* (2004) reported that a *pglH* mutant was unable to glycosylate numerous proteins, showed a reduced ability to adhere to and invade human epithelial cells, and was unable to colonize chicks.

O-linked glycosylation functions by modifying serine or threonine residues on flagellin, the flagellin proteins are *O*-glycosylated and this is essential for successful construction of flagella. The importance of flagella for motility and the flagellar export apparatus for the secretion of the Cia proteins means that *O*-linked glycosylation has a direct impact on motility and ultimately affects adhesion, invasion and virulence (Guerry *et al*., 2006).
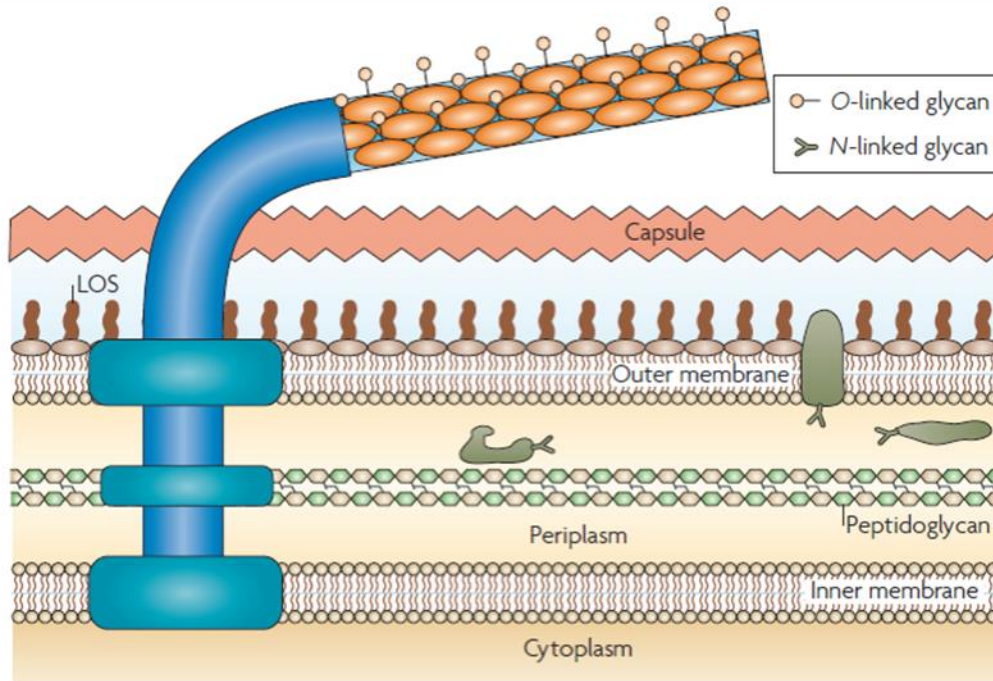
Figure 1.4. The *Campylobacter jejuni* glycome and surface structures. Showing flagellum, capsule and LOS (Young *et al*., 2007)

1.5.6 Adherence mechanisms

In order to successfully colonise the host microbial pathogens must first adhere to host surfaces. In many Gram-negative and Gram-positive bacteria this adherence is supported by appendage like structures known as pili. Essentially there are 2 types of bacterial adherence mechanisms the first being fimbriae or pilus, and the second being afimbrial adhesins. *C. jejuni* has no genetic

homologues for genes encoding pilus-like structures (Finlay and Falkow., 1997; Parkhill *et al*., 2000)

Although *C. jejuni* does not possess any specific adhesion organelles it does produce a number of adhesins, one these is JlpA a surface exposed lipoprotein which binds Hsp90 resulting pro-inflammatory responses by activation of NF-κB. Mutants of *jlpA* showed markedly reduced adherence to Hep-2 cells when compared to the wild-type strains (Jin *et al*, 2001). CapA is another lipoprotein thought to be an adhesin, mutants lacking CapA have shown reduced colonisation in a chick model and reduced adherence to *in vitro* Caco-2 cells, indicating its role in adhesion (Ashgar *et al*., 2007).

C. jejuni synthesises a 37 kDa outer membrane protein termed CadF which binds to a constituent of the extracellular matrix of human colonic tissue known as fibronectin. Mutants unable to synthesize CadF exhibited significantly reduced colonization of a chicken model, and reduced adherence and invasion *in vitro* (Monteville *et al*., 2003; Konkel, 2005). A study by Flanagan *et al*. (2009) showed that fibronectin-like protein A (FlpA) promotes binding of *C. jejuni* to host epithelia and plays a role in the colonistaion of chickens (Flanagan *et al*., 2009).

The *peb1A* a periplasimic gene product acts as an adhesin and is required for adherence to HeLa cells and also colonisation of mice. Peb1 has been shown to be an L-glutamate and L-asparagine binding protein component of an ABC-transporter, it is required to metabolise these amino acids and strains mutations in *peb1A* are unable to grow if these amino acids are the major constituents of growth media (Leon-Kempis *et al*., 2006).

1.6 Biofilms

Bacteria can exist either as planktonic cells or as a biofilm. Bacteria favour growing after attachment to a surface, then form an enclosed microbial community within a matrix known as a biofilm (Jenkinson and Lamont, 2005).

Microbial biofilms where first described in 1936 by Zobell and Anderson who studied the multiplication of bacteria in sea water.

There are a number of hypotheses as to why bacteria form biofilms. The primary reason is defence. The microorganisms inside the biofilm matrix are able to avoid antimicrobial agents; in some cases a 100-fold increase in antibiotic concentration has been shown to be required to kill the biofilm organisms compared with that required to kill the same microbes in planktonic form (Jefferson, 2004). Existing as a biofilm also allows organisms to withstand host immune responses such as phagocytosis and as a community they can endure changes in pH and starvation of nutrients (Jenkinson and Lamont, 2005).

The best characterised bacterial biofilms are oral biofilms. If we look at the mechanism of biofilm formation in the oral cavity we see that the process begins when a pioneer organism forms a primary colony on salivary pellicle after attaching to specific surface receptors. In the oral cavity this pioneer organism may one of a number of *Streptococci,* and the surface they preferentially adhere to is tooth enamel (Petersen *et al.*, 2006).

After this initial step, the primary colonising bacteria form a link for other species of bacteria such as *Actinomyces* and *Fusobacterium* to attach, subsequent growth and expansion allows other species that are present as planktonic bacteria to attach and form a diverse species biofilm (Jenkinson and Lamont, 2005).

Another reason for biofilm formation specifically in the mouth, is that it is a favourable site for bacteria to grow as it has a constant supply of nutrients and exhibits surfaces which are ideal for growth and survival as well as inevitable multiplication. Therefore growing as biofilm is preferential to remaining an attached community (Jefferson, 2004)

It has been hypothesised that in natural habitats bacteria are predominantly found in mixed species biofilms rather than planktonic cells, and are therefore multi-cellular organisms. Bacteria in biofilms do exhibit behaviour similar to a

multi-cellular organism. For example, bacteria in biofilms develop architecture, they are built with various species, providing numerous micro-environments in which they can interact with their surroundings. This enables them to adjust their metabolic processes to maximize the use of available substrates and to protect themselves from detrimental conditions (Jenkinson and Lamont, 2005).

The gastrointestinal tract is covered with a layer of mucus which is produced by goblet cells that are present within the intestinal epithelium, they also produce and secrete mucins which form the thick gel-like properties of the mucus layer and vary in thickness from 48 to 273 µm. In healthy individuals, this mucus layer provides protection so the host cells are never exposed to luminal content. (Pullan *et al*., 1994).

C. jejuni 81-176 has been shown to form microcolonies and biofilms on human intestinal tissue, and it has been suggested that biofilm formation on the intestine is prerequisite for causing diarrhoea in humans (Haddock *et al*., 2010).

Joshua *et al.* (2006) showed that *C. jejuni* is able to form both attached and unattached biofilm. These unattached aggregates of biofilm known as 'flocs' are more resistant to environmental stresses and are thought to be important in survival and pathogenesis of the organism. Further to this, *C. jejuni* has the ability to form attached biofilm on the surfaces of domestic and farming watering supplies and these attached bacteria could potentially act as an active reservoir of infection for humans and domestic animals (Reeser *et al* ., 2007).

Biofilm-forming ability of *C. jejuni* is decreased in the presence of nutrient rich media, concurring with the idea that it is a stress response. Reeser *et al.* (2007) reported that biofilm formation is significantly decreased when *C. jejuni* is grown in nutrient-rich media. They also found that motility is an important factor for optimal biofilm formation; *flaAB* mutants, which are deficient in a motile flagellum, and *luxS* mutants, which are deficient in a quorum sensing signal molecule called autoinducer-2 (AI-2), showed significantly decreased ability to form biofilm (Reeser *et al.,* 2007).

One of the major withstanding questions with regards to *C. jejuni* biofilms is the composition of the extracellular polymeric matrix or EPM. The EPM is what holds the biofilm together, providing structure and strength; for most bacterial biofilms the EPM is constituted of polysaccharide. However in the case of *C. jejuni* biofilms, the best characterised polysaccharide, the CPS, is not involved in biofilm formation as shown by Joshua *et al.* (2006) who reported no reduction in the biofilm forming capacity of a *C. jejuni* NCTC 11168 *kpsM* mutant (CPS deficient).

1.7 Metabolism of *C. jejuni*

The metabolism of *C. jejuni* is not yet fully understood. It is an asaccharolytic organism unable to metabolise exogenous sugars as a carbon source due to a deficiency of phosphofructokinase, a glycolytic enzyme which acts upon fructose 6-phosphate (Hofreuter *et al.*, 2008).

Instead, *C. jejuni* metabolises amino acids as a carbon source, with a preference for apartate, serine, glutamate and proline (Leach *et al.*, 1997; Guccione *et al.*, 2008). The organism only has a limited number of catabolic enzymes which utilise the above named amino acids as well as asparagine and glutamine (Velayudhan *et al.*, 2004).

1.7.1 Serine

The L-isomer of serine is preferentially utilised by *C. jejuni* as it is able to convert L-serine into pyruvate, this conversion occurs when serine is transported into the cell via SdaC a serine transporter and after which protein SdaA an active serine deaminase which converts L-serine to pyruvate. (Mendz *et al.*, 1997). Pyruvate is a major metabolic intermediate that allows the utilisation of amino acids for energy generation through biosynthetic pathways (Mendz *et al.*, 1997). *C. jejuni* metabolises pyruvate through the citric acid cycle and pyruvate:flavodoxin oxidoreductase (Guccione *et al.*, 2008).

Inhibition of SdaA and SdaC synthesis resulted in complete impairment of chock colonistaion, showing that metabolistaion of L-serine is essential for colonisation (Velayudhan *et al*., 2004).

1.7.2 Proline

Proline which is a constituent of mucins and is abundant in the gastrointestinal tract has been found by Wright *et al*. (2009) to be a less favoured in comparison aspartate, glutamine and serine metobilisation. Proline is utilised through two proteins PutA a bifunctional proline dehydrogenase/delta-1-pyrroline-5-carboxylatedehydrogenase and PutP is a sodium/proline symporter. PutAP proteins are homologues to ones seen in both *E. coli* and *Helicobacter* spp and PutA has been to be a key a factor for its motility and subsequent stomach colonisation (Krishnan *et al*., 2008; Nakajima *et al*., 2008).

1.7.3 Asparagine

It has been suggested that asparagine metabolisation plays a role in tissue specific colonisation; this conclusion is based on mutation in gene *ansB*, a asparaginease, resulting in decreased colonisation of the liver in mice however this reduction in colonisation was not observed in the murine intestine (Hofreuter *et al*., 2008).

Although *C. jejuni* possess an asparaginase, many strains do not possess a functioning asparagine transporter, and are unable to ultilise asparagine. Strains such as 81-176 have an extended *ansB* gene by approximately 40 bp which encodes a Sec dependent signal peptide and this peptide allows the AnsB protein to transported from cytoplasm where it is generally found to the periplasm, where it can function by deaminating asparagine to aspartate (Hofreuter *et al*., 2008).

1.7.4 Glutamine

Some *C. jejuni* strains such as 81-176 synthesis γ-glutamyltranspeptidase (GGT) which enables them to directly utilise glutamine as a principal nutrient source (Hofreuter *et al*., 2006). However, Hofreuter *et al*. (2008) showed that strain *C. jejuni* NCTC11168 does not utilise glutamine as a source of carbon despite having a complete functional glutamine uptake system.

This uptake system contains components of amino acid ABC transporter PaqP and PaqQ, glutamatehydrogenase encoded by *gltBD* which converts glutamine and 2-oxoglutarate into glutamate and a glutamine synthase encoded by *glnA* which catalyses the process of converting glutamate back into glutamine.

1.7.5 Short chain fatty acids

C. jejuni is also able to metabolise short chain fatty acids acetate and lactate which are abundant in the gastrointestinal tract of humans. When other primary metabolites are exhausted *C. jejuni* switches to acetate and lactate metabolism, acetate that is a by-product of the metabolism of L-serine. No acetate transporter has been documented in *C.jejuni* except for an annotated acetate permease encoded by *actP* in *C. jejuni* subsp. *doylei* (Parker *et al*., 2007). Wright *et al*. (2009) showed that acetate can be used as a primary source of carbon *in vitro*.

Lactate, a product of carbohydrate fermentation by gut microflora is produced in profuse quantities but is taken up by many gastrointestinal bacteria keeping the overall concentration of lactate in the colon reasonably low. Lactate is transported into the cell via a L-lactate permease encoded by *lctP*, however lactate transport is not completely dependent on lctP a mutation in in this gene resulted in decreased lactate metabolisation leading to the proposition that another yet unidentified transporter and/or mechanism is involved (Thomas *et al*., 2011).

Once lactate has been transported into the cell it converted in pyruvate by a number of methods. The first involves three genes, *Cj0073c*, *Cj0074c*, and *Cj0075c* (*lldEFG/lutABC*) encode a non-flavin iron–sulfur containing oxidoreductase, and convert s L-lactate to pyruvate through deamination. The second a flavin and iron–sulfur containing enzyme Cj1585c (Dld) also shows L-acetate dehydrogenase activity. *C. jejuni* has also been shown to utilise the D-isomer of lactate (Thomas *et al*., 2011).
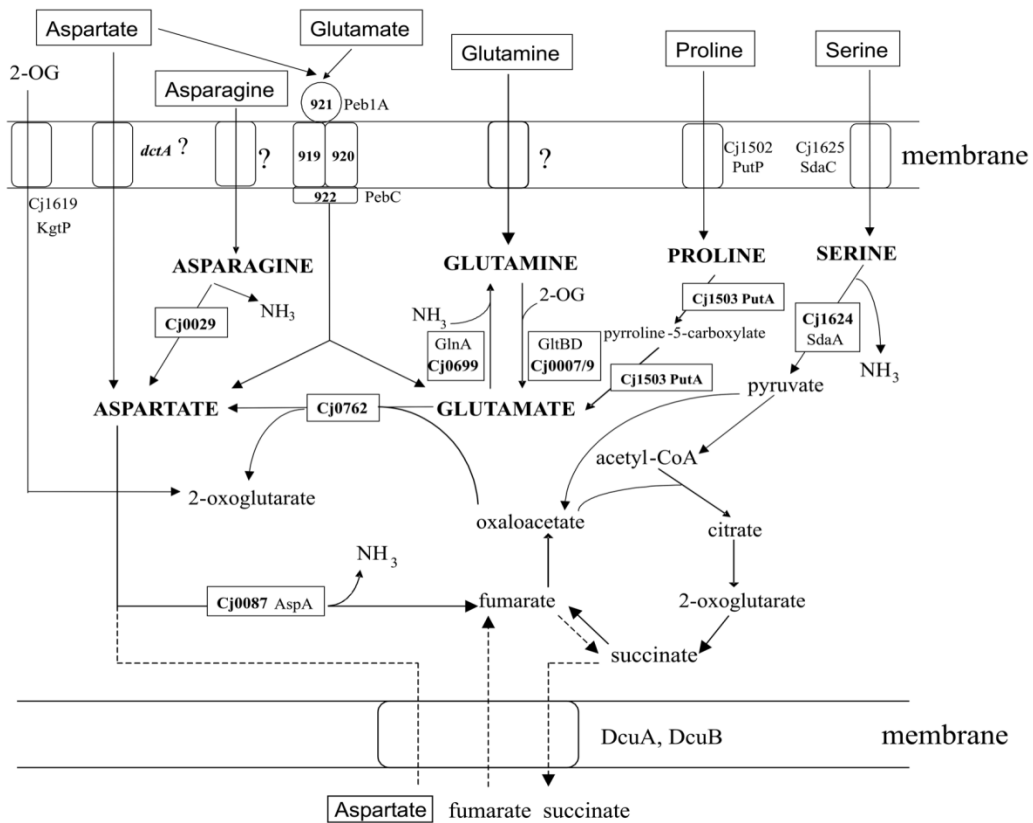
Figure 1.5: The predicted amino acid transport and catabolism pathways in *C. jejuni* NCTC11168. Dashed arrows represent pathways of importance under oxygen-limited conditions. Solid arrows represent predicted functions of pathways under microaerobic conditions (Guccione *et al*., 2008).

1.8 Models for the study of *C. jejuni* virulence and colonisation

Despite the advances in the study of *C. jejuni* virulence, the mechanisms by which *C. jejuni* colonises the host and causes disease are still relatively poorly understood. A major reason for this the lack of a practical animal model to study Campylobacterioses.

A variety of animal models have been proposed for study of *C. jejuni*-mediated disease these include; the ferret model which was used by Bacon *et al.* (2001) to elucidate that the phase-variable Capsular polysaccharide (CPS) was a significant virulence factor. Other models such as the rabbit ileal loop method have been used, this particular model was first used by Everest *et al.* (1993) to examine pathological changes in rabbit ileal tissue after *C. jejuni* infection. Primates such as infant rhesus macaques (*Macaca mulatta*) and the New World monkey *Aotus nancymae* have also been used (Russell *et al.*, 1993; Jones *et al.*, 2006). However ethical concerns and inadequate representation of human campylobacterioses has prevented the widespread application of these models.

1.8.1 Chicken

Campylobcater jejuni is able to colonise the avian gastrointestinal tract, and the contamination of broiler chicken meat is a principle source of Campylobacterioses. However, this colonisation is asymptomatic and is considered to be a commensal association with the host (Newell, 2001). With the problems and limitations of the models described above, the chicken colonisation model has become a frequently used colonisation model.

Campylobcater jejuni predominantly colonises the caecum of chicken, generally between 10^6 to 10^8 cfu/g (Beery *et al.*, 1988). This ability to successfully colonise the chicken gastrointestinal tract is a multifactorial process; the model has been

used to elucidate genes and mechanisms that are vital for colonisation of, and survival within the host.

Chicken colonisation models for *C. jejuni* are categorised into 2 groups, the first uses chicks that are up to 2 days old and the second group use chickens between 14 and 16 days. Although the model does not provide a true representation of human disease, the model has been used to determine colonisation factors such as motility, chemotaxis, two-component regulatory systems and Oxidative and nitrosative stress response among many others (Lin *et al*., 2003; Raphael *et al*., 2005: Karlyshev *et al*., 2002; Bras *et al*., 1999; Atack and Kelly, 2009).

1.8.2 *Galleria mellonella*

In 2010 Champion *et al*, reported that the larvae of *Galleria mellonella* or Greater Wax Moth can be successfully infected by *C. jejuni* and this model can be utilised to screen for genes involved in virulence.

Insect models of infection have been used as alternatives to mammalian models due to their cost effectiveness and non-requirement of ethical consent. The most commonly used invertebrate for infection is the nematode species *Caenorhabditis elegans*, it has been used to study marine bacteria such as *Shewanella frigidimarina* and *Aeromonas hydrophila*. However the limitations of the *C. elegans* model are its inability survive at 37°C they also lack functional homologues of the mammalian immune system such as phagocytic cells. Using insect larvae overcomes these disadvantages because they can survive at 37°C and have structures known as hemocytes which function in a similar fashion as components of the human innate immune system (Champion *et al*., 2010).

Senior *et al*. (2011) used the model to show that *C. jejuni* can survive within *G. mellonella* causing significant damage to the gut of these larvae.

1.8.3 Murine model

The mouse model is a useful tool for the study of bacterial pathogenicity. However murine models for the study of *C. jejuni* infection, have disadvantages including sporadic colonization and absence of clinical manifestations of disease; this is partially due to mice which have a normal commensal microflora exhibiting colonisation resistance against *C. jejuni* (Friedman *et al*., 2000). Therefore germfree mice, which have a restricted gut microflora, have been an attractive model and have been used with success (Hodgson *et al*., 1998). Chang and Miller (2006) used the limited flora murine colonisation model to characterise virulence determinants required for colonisation and described the model as a tractable, reproducible tool which they used to define host responses to colonisation (Chang and Miller, 2006). Furthermore, a number of studies have induced clinical manifestations of disease such as bloody diarrhea, and humoral immune responses in isolator-raised germfree mice (Yrios and Balish, 1986a; Yrios and Balish, 1986b; Jesudason *et al*., 1989; Youssef *et al*., 1987).

However, germfree mice have abnormal gut lymphoid tissue development and therefore lack a complete innate immune system (Shroff and Cebra, 1995). Mice lacking MyD88 an innate immunity adaptor protein, which plays an essential role in Toll-like receptor (TLR) signalling, were colonised by *C. jejuni* but did not develop intestinal inflammation, a key clinical symptom (Watson *et al*., 2007). Furthermore, the outcomes of *C. jejuni* infection vary between genetically engineered mice; this highlights the lack of a robust, reproducible mouse model (Mills *et al*., 2012)

1.8.4 Intestinal cell invasion model

As an alternative to using live animals to model Campylobacterioses *in vitro* epithelial cells are utilised. Cell lines used to study *C. jejuni* infection include the INT407 cell line, a human embryonic intestinal cell line, and the Caco-2 human colon adenocarcinoma line (Friis *et al*., 2009).

These cells are used to assess the ability of *C. jejuni* to adhere to the extracellular cell surface of the epithelial cells and the ability to invade the epithelial cells. Of the two cell lines the Caco-2 cell line is considered favourable to study including columnar shape, become polarised and form tight junctions and express microvilli (Bak *et al*., 1999).

It has been shown that data obtained from *in vitro* cell lines accurately mimics that obtained using animal models of infection, for example mutations of genes *cheY* and *kpsM* in strain 81-176 exhibited reduced virulence in a ferret disease model these results corroborated with the findings of cell culture results showing reduced adherence and invasion (Yao *et al.*, 1997; Bacon *et al.*, 2001).

1.9 Aims of study

C. jejuni has evolved to respond to a number of biological molecules found in the host intestinal environment. The bacterium responds to human bile salts (sodium deoxycholate) leading to the induction of *Campylobacter* invasion antigens (Cia), which are required for host cell invasion (Malik-Kale *et al*., 2008). *C. jejuni* also responds to norepinephrine, a neurotransmitter which has been found to spill over into the intestine. When grown in iron-limited medium in the presence of norepinephrine, *C. jejuni* shows an increased growth rate, motility and ability to invade epithelial cells (Cogan *et al*., 2009).

Therefore it is possible that *C. jejuni* has evolved to respond to other biological molecules specific to the host intestinal environment. The pancreas is the predominant digestive gland in the human body; it is a dual functioning gland possessing both endocrine and exocrine features. This exocrine function plays a major role in digestion of carbohydrates, proteins and lipids (Farges *et al*., 1996). Pancreatic α-amylase functions by cleaving the α1-4 glycosidic bonds of polysaccharides to generate dextrin, maltose or maltotriose. Also, hyperamylasia has been reported in clinical cases of severe campylobacterioses (Tositti *et al*., 2001; Peterson and Besendorfer, 2012).

The aims of this study are to determine whether C. jejuni is able to recognise and respond to mammalian pancreatic α-amylase. Furthermore, to characterise how the responses of C. jejuni to pancreatic α-amylase affects the virulence of the organism by assessing changes in colony morphology through secretion of exo-polymers, subsequent biofilm formation and resistance to stresses. Furthermore, the effects of α-amylase on growth rate, virulence and colonisation as well as elucidating the molecular basis of the response.

Chapter 2: Materials and methods

2.1 Growth media and reagents

All the media and supplementary components used for bacterial growth are summarized below.

2.1.1 Media

Table 2.1. Bacterial growth medium.

Medium name	Components and Source
Luria-Bertani Broth (LB)	As supplied by Oxoid
Mueller Hinton Broth (MHB)	Oxoid
Charcoal Mueller Hinton Agar (CMHA)	MHB 1.5 % bacteriological agar (Oxoid) 0.4 % Charcoal (BDH)
Minimum Essential Media-α (MEM-α) agar	MEM-α (Sigma) 20 mM sodium pyruvate 1.5 % bacteriological agar (Oxoid)
Brucella Broth (BB)	Beckton Dickinson
Columbia Blood Agar (CBA)	Oxoid Typical formula: 2.3% w/v Special peptone 0.1% w/v Starch 0.5% w/v Sodium chloride 1% w/v Agar 7% sterile defibrinated horse blood (E & O Laboratories)
Nutrient Agar	Oxoid
Dulbecco's modified essential media (DMEM)	Gibco
Super Optimal broth with Catabolite repression (SOC)	Promega
Standard American Petroleum Institute	KCl 3.35 mM

(SAPI) medium	KH$_2$PO$_4$ 1.84 mM
	MgSO$_4$ 1.01 mM
	NH$_4$NO$_3$ 6.25 mM
	Tris 50 mM
	FeSO$_4$ 10 µM
	pH adjusted to 7.5 using HCl.
	1.5 % bacteriological agar
Starch Agar	Nutrient agar (Oxoid
	0.4 % (v/v) starch (Sigma-S9765)

2.1.2 Supplementary components

Table 2.2. Supplementary components.

Supplementary Component	Source
α-amylase from hog pancreas (powder)	Sigma-Aldrich (cat no. 10080)
α-amylase from *Aspergillus oryzae* (aqueous solution)	Sigma-Aldrich (cat no. A8220)
Trypsin from porcine pancreas (powder)	Sigma-Aldrich (cat no. T7409)
Mucin from porcine stomach (powder)	Sigma-Aldrich (cat no. M2378)
Albumin from bovine serum (BSA) (powder)	Sigma-Aldrich (cat no. A2153)
Bile from porcine extract (powder)	Sigma-Aldrich (cat no. B8631)
Protease inhibitor cocktail	Sigma-Aldrich (cat no. P8465)
Azocasein solution	Sigma-Aldrich (cat no. A2765)
Lugol iodine solution	Sigma-Aldrich (cat no. L6146)
Starch	Sigma-Aldrich (cat no. S9765).
Live/Dead BacLight stain	Invitrogen (L-7012)
Foetal bovine serum	Sigma-Aldrich (cat no. F6178)
Non-essential amino acids	Sigma-Aldrich (cat no. M7145)
Penicillin streptomycin solution	Sigma-Aldrich (cat no. P4333)

2.2 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2.3.

Table 2.3. Strains and plasmids used in this study.

Strains and plasmids	Characteristics	Reference
C. jejuni 11168H	Hypermotile variant of *C. jejuni* NCTC 11168	Karlyshev *et al.* (2002)
C. jejuni 81-176	Clinical isolate from human diarrhoeal sample	Korlath *et al.* (1985)
C. jejuni 11168H *kpsM*::*aphA3*	Mutation of gene encoding ABC transporter involved in capsule assembly, by insertion of Kan[r] gene *aphA-3* resulting in inability to express CPS	Karlyshev *et al.* (2001)
C. jejuni 11168H *waaF*::*aphA3*	Mutation of gene encoding heptosyltransferase II by insertion of Kan[r] gene *aphA-3* resulting in a truncated core oligosaccharide	Campylobacter Resource Facility
C. jejuni 11168H *waaC*::*aphA3*	Mutation of gene encoding heptosyltransferase I by insertion of Kan[r] gene *aphA-3* resulting in the expression of a severely truncated LOS	Campylobacter Resource Facility
C. jejuni 11168H *spoT*::*aphA3*	Mutation of gene that governs stringent response by insertion of Kan[r] gene *aphA-3* resulting. Resulting mutant is unable to express and increased level of CFW-binding polysaccharide	This study

C. jejuni 11168H *Cj0511*::*aphA3*	Mutation of gene encoding for secreted carboxyl-terminal protease.	Campylobacter Resource Facility
11168H *Cj0511*::*aphA3* pWJ4	Complemented strain containing *Cj0511* gene and Cam[r] inserted into pseudogene *Cj0046*	This study
E. coli JM109	Cloning host (F¢ (traD36*, pro*AB+ *lac*Iq, D(*lac*Z)M15*)* *end*A1 *rec*A1 *hsd*R17(rk-, mk+) *mcr*A *sup*E44 l- *gyr*A96 *rel*A1 D*(lacproAB) thi-*1)	Promega
Plasmids		
pJMK30	pUC19 derivative carrying *C. coli* Kan[r] gene *aphA-3*	van Vliet *et al.* (1998)
pGEM-T-Easy	Cloning vector enables TA cloning	Promega
pWJ1	pGEM-T-Easy derivative with *C. jejuni* 11168H *spoT* gene	This study
pWJ2	pWJ1derivative with *aphA3* inserted into *spoT* gene at the *Bgl*II site	This study
pET151/D-TOPO	Protein expression vector containing T7 promoter, lac operator,ribosome binding site, Initiation ATG, and polyhistidine region	Invitrogen
pWJ3	pET151D-TOPO derivative with *C. jejuni* *Cj0511*cloned downstream of ribosome binding site.	This study
pC46fdxA	pC46 derivative with *fdxA* promoter, Cam[r] selection marker.	Institute of Food Research

| pWJ4 | Derivative of pC46fdxA containing *Cj0511* | This study |

All *C. jejuni* strains were stored at -70 °C in Brucella broth supplemented with 15 % (v/v) glycerol. Bacteria were revived by plating on CBA and grown at 37 °C in an atmosphere of 5 % (v/v) CO_2 for 48 hours.

2.2.1 Antibiotic selections

The antibiotics used in this study are listed in Table 2.4. Stock solutions were prepared at 1000x concentration.

Table 2.4. Antibiotics used for culture of *C. jejuni* and *E. coli*.

Antibiotic	Diluent	Final concentration (µg/ml)
Ampicillin (Amp)	Filtered dH_2O	100
Chloramphenicol (Cam)	Ethanol	20
Kanamycin (Kan)	Filtered dH_2O	40

2.3 α-Amylase Agar

α-Amylase powder from hog pancreas, were all made into a suspension using distilled water and added into the respective media at a final concentration of 5 µg/ml (100 nM). To improve visualization of *C. jejuni* colonies, 0.4 % (w/v) charcoal was used.

2.4 DNA Techniques

2.4.1 Plasmid purification

Plasmid preparation from *E. coli* and *C. jejuni* was performed by using the Spin column plasmid miniprep kit (NBS Biologicals) according to the manufacturer's instructions.

2.4.2 Genomic DNA extraction

C. jejuni genomic DNA was extracted using the Promega Wizard Genomic DNA Purification Kit according to the manufacturer's instructions.

2.4.3 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis in 0.8 % (w/v) agarose (Sigma-Aldrich) in 1× Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) containing 0.1g/ml ethidium bromide. Samples were run in 1×loading buffer (0.05 % (w/v) bromophenol blue, 5 % (v/v) glycerol in dH_2O at 80-100 V for 1 h. The 1 kb plus DNA ladder (New England Biolabs) was included as a size marker. The DNA bands were detected under ultraviolet light at 302 nm using an Alpha Innotech Alpha Imager.

2.4.4 DNA purification from gel or solution

PCR amplified DNA fragments were purified by using Qiagen PCR purification columns. Gel purified DNA fragments were excised from agarose gels and purified using a Qiagen Gel Purification Kit.

2.4.5 PCR protocol

Where amplicons were to be used for TA cloning, amplification was carried out using a 1:4 mix of Vent$_R$® DNA polymerase and *Taq* DNA polymerase (New England Biolabs); otherwise PCR used Vent$_R$® DNA polymerase or *Taq* DNA polymerase. The primers used for PCR are listed in Table 2.5

Table 2.5. Primers for PCR amplification.

Primer name	Sequence (5'>>>3')
WJ1	TATTGCTTGAACAATTAATCG
WJ2	TCTTTAGCATATTCATAAAGC
WJ3	TTGGCGTATAACATAGTATCG
WJ10F	GTCGTCTCACATGTTGAAAACAAAACG
WJ10R	GTCGTCTCACATGTTATTGTCCTTGTTTG
WJ11	GGAACACAGCAGAGCACTTG
WJ12	CCTGGAGAAGTATTAGATAGTAGCG
CJ1AF	CACCAAAGTTGATCAAAAAGAAGAGC
CJ1AR	CTATTGTCCTTGTTTGATATTTAA
PfdxAF	GTAAATTTTTGATTATCAAAATTTACATTATTTAAG

Reaction mix

Qiagen PCR buffer	1×
dNTPs	0.2 mM
Forward primer	0.5 µM
Reverse primer	0.5 µM
Template DNA	~0.2 ng /µl
$Vent_R^{®}$/*Taq* DNA polymerase	0.05 unit /µl

Reaction conditions:

Pre-amplification denaturation	96 °C	2 min
Denaturation	94 °C	30 s
Annealing	$T_m - 5$ °C	30 s, 35 cycles
Extension	72 °C	1min/kb
Final extension	72 °C	7 min
Pause	4 °C	∞

2.4.6 Restriction Digestion

Restriction endonucleases were purchased from New England Biolabs, and digests performed according to the manufacturer's guidelines for each enzyme.

2.4.8 Ligation

Prior to ligation, the concentrations of vector and DNA fragments were determined using a NanoDrop 1000 spectrophotometer (NanoDrop), and the molar concentration ratio of vector / inserts was calculated. A 1:3 molar ratio of vector / insert was used in ligation reactions with T_4 DNA ligase (New

England   Biolabs),   and   performed   according   to   manufacturer's recommendations.

2.5 Electrocompetent Cells Preparation

C. jejuni 11168H was inoculated onto CBA plates and incubated at 37 °C with 5 % CO_2 for 24 hours. The growth was harvested into 1 ml cold electroporation buffer (EPB: 272 mM sucrose, 15% (v/v) glycerol, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4, pH 7.4). After centrifuging at 13,200 rpm for 8 min at 4 °C in a microcentrifuge (Eppendorf), the supernatant was discarded and the pellet was resuspended in 1 ml cold EPB. This wash step was repeated 3 times, after which cells were suspended in 1 ml EPB and used immediately.

2.6 Electroporation

Approximately 3 µg of plasmid DNA was mixed with 150 µl of electrocompetent cells, followed by incubation on ice for 20 min. This mixture was then transferred into a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad). Electroporation of cells was performed with a Bio-Rad Gene Pulser system at 2.5 kV, 200 Ω, and 25 mF. Immediately after electroporation, 100 µl of SOC medium was added to the cuvette. The contents of the cuvette were then plated on CBA and grown for 24 hours at 37 °C in microaerobic conditions.

For selection of kanamycin resistant (kan^r) colonies, growth was removed from the CBA plates and suspended in 1 ml MHB, this was then plated onto CBA supplemented with 40 µg/ml kanamycin, and grown for 48 hours in microaerobic conditions at 37 °C.

2.7 Growth curve

C.jejuni 11168H was revived from frozen stock on to CBA and grown for 48 hours. A single colony was inoculated into 10 ml Brucella broth (Oxoid) and grown overnight at 37 °C in an atmosphere of 5 % CO_2 with shaking at 50 rpm. The overnight culture (OD_{600} = 0.5-0.7) was diluted into 40 ml Brucella broth with or without 100 nM of amylase to OD_{600} = 0.1 and incubated at 37 °C in an atmosphere of 5 % CO_2 with shaking at 50 rpm. The optical density at 600 nM ($OD_{600)}$ was recorded hourly.

2.8 EPM Purification

The growth was removed from 4 MH agar plates with or without 100 nM hog pancreatic α-amylase and suspended in 5 ml phosphate buffered saline (PBS; Sigma-Aldrich). For proteomics analysis, the preparation was done in the presence and absence of 1 µl protease inhibitor cocktail (Sigma) prepared according to the supplier's instructions and then diluted 1 in 10. The suspension was washed at 200 rpm on a rotary platform for 1.5 hours at 30 ºC then vortexed for 15 min and washed at 200 rpm for a further 1.5 hours at room temperature. The bacteria were removed by centrifugation at 1,800 x g for 15 min and the material in the supernatant was precipitated by addition of 4 volumes of ice cold acetone which was left at 4 °C overnight. The precipitate was recovered by centrifugation at 450 x g for 5 min, washed in water and dried in a Speedvac SPD1010 (Thermo Scientific). Finally, the pellet was dissolved in 1 ml of distilled water and, if not used immediately, was stored at -70°C.

2.9 Carbohydrate Assay

Total carbohydrate was measured using the phenol-sulphuric acid assay (Dubois *et al.*, 1956) with glucose as the standard.

2.10 Protein Assay

The acetone-precipitated material was assayed for protein using a Micro BCA Protein Assay Kit (Pierce, Thermo Scientific) according to the manufacturer's instructions.

2.11 Carbohydrate characterisation

Crude EPM samples were dissolved in doubly-distilled water (20 mL) and small sugars were removed by dialysis (14K MWCO) for 72 hours at 4 ºC, against three changes of distilled water per day. The contents of the dialysis bag were freeze dried and the resulting polysaccharide was characterised using a combination of NMR spectroscopy (Laws *et al.*, 2008), monomer

analysis (Gerwig *et al.*, 1978) and linkage analysis (Stellner *et al.*, 1973). For comparison, the same experiments were performed with a commercial α-dextran standard (Sigma-Aldrich). Details of the experimental procedures used to characterise the EPM samples have been reported elsewhere (Laws *et al.*, 2008).

2.12 Proteomics

An equal volume of 2 x sample buffer [0.5M Tris-HCl pH 6.8, 1.0 M dithiothreitol 5% (w/v) SDS (sodium dodecyl sulfate), 0.02% bromophenol blue, 20 % glycerol] was added to each sample and heated for 5 min at 90 ˚C. The samples were electrophoresed in a 10% SDS-PAGE gel at 100 V in running buffer [25 mM Trizma, 192 mM glycine, 0.1 % (w/v) SDS]. After electrophoresis, the gels were fixed in 2% (v/v) acetic acid, 40% (v/v) methanol at room temperature for 1 hour and stained in 16% Colloidal Coomassie Brilliant Blue (Sigma), 20 % (v/v) methanol for 2 hours at room temperature on a rotary platform shaker at 30 rpm. The gel was rinsed in 5% (v/v) acetic acid, 25 % (v/v) methanol for 2 minutes at room temperature, destained in 25 % (v/v) methanol on a rotary platform shaker 30 rpm and stored in water in sealed acetate at 4˚C. For LC/MS/MS, the bands were excised from the gel, digested with trypsin and analysed by LC-MS/MS according to standard methods. MS data were used to interrogate the Cj database using Bioworks version 3.2 hits are listed according to the 11168H database.

2.13 Autoagglutination assay

The autoagglutination assay is based on the phenomenon that bacterial cells which strongly agglutinate do not remain in the aqueous phase resulting in the reduction of OD_{600}. The method is based upon that described by Misawa and Blaser (2000). Briefly, bacteria were harvested from MH agar which was supplemented with and without 100 nM (5 µg/ml) hog pancreatic α-amylase and suspended in Brucella broth to an OD_{600} of 1.0; 1 ml of this suspension was left to stand in a cuvette at room temperature. The OD_{600} was measured at 6, 12, 24 and 48 hours.

2.14 Azocaesin assay

Total proteolytic activity was determined using the azocaesin hydrolysis method (Tomarelli., *et al* 1949). Briefly, 2.5 ml 2.50% (w/v) azocasein solution (Sigma-A2765) was mixed with 1.5 ml 60 µM sodium bicarbonate buffer (NaHCO$_3$), pH 8.3 at 37°C. To this 1 ml of the protease (trypsin or recombinant Cj0511) was added and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 4 ml 5.0 % (v/v) trichloroacetic acid and filtered through a 0.45 µm filter, before being added to 3 ml 500 mM sodium hydroxide solution. The OD$_{440}$ was recorded. Blanks (test tubes without samples) were run in all cases and trypsin was used as a control. The following formula was used to determine A$_{440}$ nm: ΔA440nm = A440nm Test - A440nm Blank. The absorbance was plotted against increasing concentrations of protein.

2.15 Starch-iodine assay of amylase degradation by recombinant Cj0511

Starch agar plates were made by incorporating nutrient agar (Oxoid) with 0.4 % (v/v) starch (Sigma-Aldrich). Once the agar had set, five equal sized wells were cut into the agar using the rim of a sterile 200 µl pipette tip. Next 1, 2, 3 and 4 mg of recombinant Cj0511 were incubated with 5 µg hog pancreatic α-amylase in volume of 1 ml at 37 °C in a water bath for 6 hours, after which 100 µl of each sample was loaded into the plate and incubated aerobically overnight at 37 °C. As a control 5 µg/ml hog pancreatic α-amylase alone was loaded into a well. The plate was flooded with Lugol iodine solution (Sigma) and resulting halos which indicate α-amylase activity were viewed over a white light.

2.16 *Galleria mellonella* killing assay

Galleria mellonella larvae (Cornish Crispa Co., England) were stored in a breathable container on wood chips at 11°C. Killing assays were done as described by Champion *et al* (2010). *G. mellonella* larvae weighing between 0.2 and 0.3 g were selected. *C. jejuni* strains grown in the presence and absence of 100 nM (5 µg/ml) hog pancreatic α-amylase on MH agar were harvested in PBS and the OD_{600} was adjusted to 0.6 (~1×10^6 CFU/ml). Infectious doses were confirmed by viable counts on CBA supplemented with 7% horse blood. The larvae were placed in a petri dish and put on ice for 5 min, then 10 µl of the inoculum was injected into the right foreleg of the larvae using PBS as the negative control. Survival after 24 hours was assessed by stimulating the underside of the larvae; an absence of a response was recorded as dead.

2.17 Interaction of GFP- *C. jejuni* with *G. mellonella*

Green fluorescent protein (GFP)-tagged *C. jejuni* 11168H was cultured under microaerobic conditions at 37 °C on MH agar with and without 100 nM amylase. Growth was harvested in PBS and the OD_{600} was adjusted to 0.6 (~1×10^6 CFU/ml). Infectious doses were confirmed by viable counting CBA supplemented with 7% horse blood.10 µl of inoculum was injected into the right foreleg of *G. mellonella* larvae weighing between 0.2 and 0.3 g. The larvae were incubated at 37 °C and removed at 3 hours and 6 hours before being chilled on ice for 5 min. Next the larvae were swabbed with 70% ethanol prior to the aseptic removal of the bottom 2 mm of the body and the haemocoel was drained into an eppendorf. To pellet the haemocytes, the haemocoel was centrifuged at 200 rpm for 5 min and 10 µl of the supernatant was dropped onto a slide. Slides were examined using a Bio-Rad confocal microscope attached to Olympus BX51 upright microscope.

2.18 Biofilm assay

Biofilm formation was determined on borosilicate glass test tubes using crystal violet as described previously by Mclennan *et al.* (2008) and Reuter *et al.* (2010). Briefly, a single colony of each strain was inoculated into Brucella broth and grown for 16 hours at 37°C in an atmosphere of 5% CO_2 with shaking at 50 rpm. The overnight cultures were diluted to an OD_{600} = 0.1 and 2 ml of this culture was added to each test tube. To test biofilm growth in the presence of amylase, 100 nM (5 μg/ml) hog pancreatic α – amylase (Sigma-Aldrich) was supplemented to one set of test tubes and incubated for 48 hours at 37°C in 5% CO_2 with agitation. For crystal violet (CV) staining, tubes were washed with distilled water and dried for 30 min at 45°C. 0.1% (w/v) CV (Sigma, UK) was added for 5 min. Each tube was washed with dH_2O and dried at 45°C. Bound CV was solubilised with 80% ethanol-20% acetone, and OD_{600} measured. .

2.19 Biofilm formation for confocal microscopy

A single colony was inoculated into 10 ml Brucella broth (Beckton Dickinson) and grown with shaking at 50 rpm for 16 hours at 37°C in 5% CO_2. The culture was diluted to OD600 = 0.1 and 4 ml added to each well of a 6-well tissue culture plate (Sarstedt). To test the effect of α-amylase on biofilm formation 100 nM was supplemented into one set of wells. Two glass coverslips were immersed in the broth in each well and placed upright against each other and incubated for 48 hours at 37 °C with 5% CO_2 with shaking at 5 rpm. For confocal microscopy, the coverslips were removed from the culture, washed gently in PBS, stained with Live/Dead BacLight stain (Invitrogen) according to the manufacturer's instructions and visualised with Bio-Rad confocal microscope attached to Olympus BX51 upright microscope.

2.20 Kinetics of biofilm formation

A single colony of (GFP)-tagged *C. jejuni* 11168H was inoculated into 10 ml Brucella broth (Beckton Dickinson) and grown with shaking at 50 rpm for 16 hours at 37°C in 5% CO_2. The culture was diluted to OD_{600} = 0.1 and 4 ml

added to each well of a 6-well tissue culture plate (Sarstedt). To test the effect of α-amylase on biofilm formation 100 nM was supplemented into one set of wells. Two glass coverslips were immersed in the broth in each well and placed upright against each other and incubated for 48 hours at 37 °C with 5% CO_2 with shaking at 5 rpm. To observe the changes during formation of the biofilm, at time points 3, 6, 12, 24 and 48 hours, the slides were removed, washed three times with sterile PBS and visualised using a confocal microscope.

2.21 Scanning electron microscopy (SEM) of 5-day old biofilms

To validate the CV staining data, SEM was used to visualize biofilms of reference strain 11168H in the presence and absence of 100 µM α-amylase at the ultra-structural level. Bacterial biofilms were grown microaerobically at 37°C on 0.2 µm polycarbonate filter disks (Millipore) placed on MHA plates the disks were removed and placed on fresh plates every 24 hours for a total of 5 days. The sample was processed as shown in Table 2.6.

Table 2.6. SEM sample preparation

	Chemical	Temperature	Time	Repetitions
Primary fixation	2.5% glutaraldehyde in distilled water	room or 0-4°C	16 hours	1
Wash	distilled water	room or 0-4°C	5-10 minutes	3-5
Secondary fixation	1-4% osmium tetroxide in distilled water	room or 0-4°C	20 minutes	1
Wash	distilled water	room or 0-4°C	5-10 minutes	3-5
Dehydration	25% ethanol 50% ethanol 70-75% ethanol 90-95% ethanol 100% ethanol	room or 0-4°C	5 minutes 5 minutes 5 minutes 5 minutes 5 minutes 5-10 minutes	1 1 1 1 2
Place porous container in small amount of 100% ethanol in petri dish				
Critical point dry				
Coated with gold				

Images were obtained using JEOL JSM 5410 LVSEM at 5.0 kV

2.22 Preparation of extract from chicken pancreas

A crude extract from twenty chicken pancreases that had been stored at -70ºC for four months was prepared using methods adapted from Madhusudhan *et al.*, (1987). The pancreases were partially thawed at room temperature, 1 ml of buffer added (0.05 M Tris, 0.9% (w/v) NaCl, 0.05 M $CaCl_2$, pH 8.0) and homogenised using a Ultra-Turrax T8 (IKA Labortechnik). The homogenate was centrifuged at 1,000 x g for 20 min, the supernatant recovered and the pH adjusted to 8.0 with NaOH. The amount of amylase present in the extract was determined using a starch iodine agar assay (see above) using the purified hog pancreatic α-amylase preparation from Sigma as a standard. An

appropriate volume of pancreatic extract was used in the biofilm assay to give a final concentration of 1 µM of α-amylase.

2.23 Resistance to Environmental stress

For determining resistance to environmental stress, cultures were grown microaerobically on MHA with and without 100 nM pancreatic α-amylase for 48 hours. The growth was removed and suspended in PBS to OD_{600} of 2.0. To determine survival at low temperature, 5.0 ml of this suspension was left at 4 °C and aliquots removed every 2 days for viable counting on CBA. To determine survival under ambient conditions, bacteria were harvested and OD_{600} adjusted to 2.0 in 5 ml Mueller-Hinton broth this was left in a screw cap tube on the bench and aliquots removed every 2 days for viable counting on Columbia blood agar plates. To determine survival at high temperature, 1.0 ml of the suspension was incubated at 65 °C and aliquots removed every 3 min for viable counting. To determine survival at low pH, the bacteria were collected OD_{600} adjusted to 2.0 in 1 ml PBS. The bacteria were then pelleted using a bench-top centrifuge (13,200 rpm), and the supernatant removed. Next the pellets were resuspended in 1 ml PBS (pH 2 or 4 adjusted using HCL). The suspensions were then incubated at 37 °C and aliquots removed every 15 min for viable counting.

2.24 Interaction and invasion of Caco-2 cells

Human colon cancer cells (Caco-2) were stored in liquid nitrogen and cultured in 5% CO_2 at 37°C in Dulbecco's modified essential media (DMEM) supplemented with 10 % (v/v) foetal bovine serum (FBS), 1 % (v/v) non-essential amino acids, 100 µg/ml streptomycin and 100 U/ml penicillin. To a confluent monolayer of approximately 10^6 epithelial cells, 10^8 bacteria were added (multiplicity of infection (MOI) of 100:1). Infected monolayers were incubated at 37°C in 5% CO_2 for 3, 6 and 24 hours. For determination of the total numbers of interacting bacteria, the monolayers were washed three times with PBS and the cells were lysed by adding 0.2 % (v/v) Triton X-100. Dilutions were performed and plated on CBA for determination of viable

counts. To determine the numbers of bacteria having invaded the Caco-2 cells, the monolayers were incubated with DMEM containing gentamicin (150 µg/ml) for 2 hours to kill extracellular bacteria. The monolayers were washed three times with PBS, lysed by the addition of 0.2 % (v/v) Triton X-100, and the bacteria were enumerated by viable counting on Columbia blood agar.

2.25 Interaction with T84 epithelial cells

T84 epithelial cells were grown in complete DMEM/F12 HAM media in rat collagen pre-coated transwell dishes until the transepithelial electrical resistance (TEER) measurement was > 800 Ω to ensure formation of tight-junctions in the monolayer (usually >14 days). Co-culture studies were performed at an MOI of 10 in DMEM/F12 HAM + 10% FBS for either 6 hours or 24 hours. CFU counts were subsequently performed by serial dilution and plating on BA plates. IL-8 levels were determined from the supernatant of the apical surface at 24 hours post-infection by ELISA (eBioscience). TEER measurements were conducted at 24 hours post-infection.

2.26 Infection of chickens

Day old broiler chickens were obtained from a commercial supplier and housed in biosecure housing. The study was performed under UK Home Office licence and approval by the local ethical review committee. Birds were fed a commercial diet, had constant access to food and water and had a gradual increase and decrease in light at the beginning and end of each day, as part of a 12 hour light and 12 hour darkness cycle. When the birds were 21 days old, groups of 30 were infected by oral gavage with a 10^5 cfu dose of *C. jejuni* strain 11168H or the *Cj0511* mutant grown on agar with or without 100 µM amylase and resuspended in MH broth. Fifteen birds were euthanased at 4 and 7 days post infection (d.p.i) and ileum and liver samples removed for the culture of *Campylobacter*. Ileum samples were serially diluted and spread plated on to modified charcoal cefoperazone deoxycholate agar (mCCDA;Oxoid Ltd) and these plates were incubated at 37^oC for 48 hours under microaerobic conditions of 2% H_2, 10% CO_2, 5% O_2 in N_2. Liver samples were homogenised and enriched in modified Exeter broth incubated

with minimal headspace at 37°C for 48 h followed by plating on to mCCDA. Differences in the number of birds colonised were assessed using a Chi-square test. Differences in the number of bacteria found at each site were analysed using a Kruskal-Wallis test with Dunn's multiple comparison test.

2.27 Statistical Analysis

Statistical analysis was conducted on data using Minitab 15 statistical software (Minitab). The statistical test used was the 2 paired students T-test, equal variance was assumed. For environmental stress analysis, one way ANOVA was used in SPSS 16.0 and again equal variance was assumed. In both cases, p values <0.05 were considered significant and p values <0.001 were considered highly significant.

Chapter 3: Exposure to pancreatic α-amylase induces exo-polymers

3.1 Introduction

Campylobacter jejuni is one of the most common causes of gastroenteritis in the developed world, with a predicted 500,000 cases per year in the United Kingdom, and 2.5 million cases per year in the United States of America (Blaser, 1997). The consensus is that the vast majority of cases are transmitted through either handling or consumption of contaminated undercooked poultry (Friedman *et al.*, 2004). The infective dose of *C. jejuni* for humans is considered low, approximately 500 bacteria, compared to another Gram-negative enteric pathogen, *Salmonella typhimirium* which requires $>10^5$ cells for successful infection of the human host (Kothary and Babu, 2007).

The onset of disease after ingestion of *C. jejuni* requires an incubation period of 24-72 hours, after which symptoms develop. Clinical symptoms include abdominal pain and diarrhoea which can be mild, non-inflammatory and non-bloody, in some cases and severe, inflammatory and bloody in others. Disease is more severe in developed countries in comparison to developing countries (Smith *et al.*, 2002). In developed countries, disease is characterised by fever, abdominal pain and sometimes accompanied by bloody diarrhoea; in developing countries the disease is characterised again by fever and abdominal pain but the diarrhoea is often non-bloody accompanied by vomiting and dehydration. In some cases the disease outcome can be more severe in developing countries because patients are often malnourished (Smith *et al.*, 2002).

Typically, in healthy individuals, infection is self-limiting and lasts up to 7 days (Zilbauer *et al.*, 2008). In some cases, patients can develop serious complication as Guillain–Barré syndrome (GBS) and its variant Miller-Fisher syndrome (MFS). These syndromes are is autoimmune neuropathies of the peripheral nervous system, which are characterized by acute flaccid paralysis.

Despite the prevalence of *C. jejuni* infections in humans, the understanding of the pathogenesis of disease at the molecular level is still insufficient in comparison to other enteric pathogens such as *Escherichia coli, Salmonella spp., Shigella spp.* and *Yersinia spp.* (Young *et al*., 2007). This lack of understanding of pathogenesis and thus lack of any specific control measures can be attributed in part to the absence of a suitable, reproducible small-animal model system to study *C. jejuni* host interactions (Dorrell and Wren, 2007).

A number of animal models have been proposed but all have major disadvantages. Models using mammalian hosts such as ferrets and rhesus monkeys have been shown to accurately imitate human disease (Bell and Manning, 1990; Russell *et al*., 1993). Nevertheless, drawbacks include the cost of facilities required to house and handle such animals, the lack of host genetic manipulation techniques and the ethical issues surrounding the use of primates as disease hosts. Furthermore the long generation time for the animals renders them impractical for use in the majority of laboratories.

The natural host of *C. jejuni* are chickens and a number of studies have used chicks and chickens to study *C. jejuni* colonisation, however they are unable to truly replicate human campylobacteriosis (Hendrixson and Di Rita 2004).

The model most commonly used is the mouse which was first used 30 years ago (Blaser *et al*., 1983). The results obtained from mouse models have differed significantly from severe diarrhoea to non-colonisation which has been explained by differences in the mouse strains used and the routes of inoculation (Baqar *et al*., 1996; Blaser *et al*., 1989). Knockout mouse models, deficient in expressing pro-inflammatory cytokines, and mice with limited enteric flora have all been studied, but the results of *C. jejuni* infections in these models vary between genetically engineered mice. This clearly shows that there is no robust, accurate and reproducible model for the *in vivo* study of *C. jejuni* (Mansfield *et al*., 2007)*.

The lack of a model has led to the use of tissue culture assays as an

alternative. Studies have shown that *C. jejuni* is able to both adhere to, and invade, a number of intestinal epithelial cell lines such as INT 407 (Konkel *et al*., 1992), Caco-2 (Everest *et al*., 1992) and T-84 cells (Monteville and Konkel 2002). However, the reported interactions of *C. jejuni* with intestinal epithelial cells *in vitro* are frequently low, with invasion being reported at less than one per cent. This is not considered accurate when compared to clinical data (Friis *et al*., 2005).

A rational explanation for this is that these experiments are conducted in conditions which are not truly representative of the gastrointestinal (GI) tract of humans. The human GI tract extends from the mouth to the anus, and includes the stomach, large and small intestines. It is home to a plethora of microorganisms making up the human intestinal microflora, a vast and diverse bacterial biomass of approximately 10^{14} bacterial cells, almost 10 times the number of eukaryotic cells present in the human body, with an estimated 500-1000 species (Xu and Gordon, 2003).

The pH and oxygen concentration of the GI tract vary greatly. Therefore, bacteria travelling through the GI tract will encounter aerobic, microaerophilic and anaerobic conditions. The pH of the stomach is between 1.5 to 3.0 and changes quickly to approximately pH 6.0 in the duodenum, the pH then progressively increases to approximately pH 7.4 in the ileum and then decreases to 5.7 in the caecum, and again gradually increases to pH 6.7 in the rectum (Fallingborg, 1999).

Altering the oxygen concentrations when conducting *in vitro* assays results in changes in virulence. A microaerophilic environment increases the ability of *E. coli* to invade Caco-2 cells when compared to experiments conducted under aerobic conditions (Pietzak *et al*., 1998). A study by Cottet *et al*. (2002) involved co-culture of epithelial cells with *Helicobacter pylori* under aerobic conditions at the basolateral surface and microaeropilic conditions at the apical surface using a device called the vertical diffusion chamber (VDC). The results showed an increase in expression of virulence factor CagA and a significant increase in bacterial adherence under microaerophilic conditions (Cottet *et al*., 2002).

A similar model was utilised for the first time for *C. jejuni*, by Mills *et al*. (2012). The results showed significantly increased interaction with epithelial cells complemented by greater basolateral secretion of proinflammatory interleukin-8 (IL-8) highlighting the necessity to perform experiments in conditions that replicate *in vivo* conditions as closely as possible to elucidate the pathogenic mechanisms of *C. jejuni* (Mills *et al*., 2012).

Bacteria travelling through the GI tract encounter numerous secretions and compounds from organs in the gut. Secretions such as saliva which contains mucus and salivary amylase, gastrin in the stomach, bile and pancreatic juices composed of numerous enzymes and hormones in the small intestine.

Enteric pathogens have been shown to react to specific intestinal compounds such as gastrin, a peptide hormone secreted by G cells in the antrum of the stomach, small intestine and pancreas. Gastrin at physiological concentrations promotes growth *of H. pylori in vitro* in a dose-dependent manner and the effects were specific to gastrin and not stimulated by cholecystokinin, somatostatin and pentagastrin (Chowers *et al*., 1999).

C. jejuni also responds to a constituent of gastrointestinal secretions, such as bile. Bile performs a pivotal role in the dispersion and absorption of lipids; it is composed of ~ 50% bile acids produced by the liver from cholesterol. Bile acids include cholates and deoxycholates (DOC) (Elliot, 1985). Studies have shown that bile stimulates expression of virulence factors by *Vibrio parahaemolyticus* (Pace *et al*., 1997) and *Shigella spp*. (Pope *et al*., 1995). Furthermore, Yang *et al*. (2013) showed that bile stimulated virulence cascade in *V. cholerae* through periplasmic cysteine oxidation. Malik-Kale *et al*. (2008) showed that culturing *C. jejuni* with DOC stimulates an increase in epithelial cell invasion; the study concluded that interaction with DOC prepares the bacterium to invade cells though activating the production of Cia proteins. Furthermore, microarray analysis showed an increase in the expression of virulence genes *ciaB, cmeABC* (multidrug efflux pump), *dccR* (two-component signal transduction system), and *tlyA* (hemolysin A) when *C. jejuni* is grown in

the presence of DOC (Malik-Kale *et al*., 2008).

The human GI tract also has a nervous system component consisting of approximately 100 million neurones and norepinephrine (NE) is the main neurotransmitter in this system (Hansen, 2003). It has been shown that NE levels in the gut of rats are increased in response to sepsis by increasing expression of tyrosine hydroxylase, a catecholamine biosynthetic enzyme (Zhou *et al*., 2004). A number of Gram-negative pathogens such as *Pseudomonas aeruginosa* and *Yersinia enterolitica* have been shown to respond to catecholamines by increasing growth rate (Lyte and Ernst, 1992). Furthermore *E. coli* O157 also responds to NE by increasing virulence factor expression as well as adherence to epithelial cells *in vivo* in a bovine intestinal loop model (Walters and Sperandio, 2006; Vlisidou *et al*., 2004).

When *C. jejuni* was cultured with NE in iron limited media, growth rate, motility and invasion of Caco-2 cells were increased in comparison with cultures grown in the absence of NE. Additionally, *C. jejuni* exposed to NE produced extensive breakdown of tight junctions in cell monolayers, indicating that this may contribute to the epithelial destruction that is characteristic of severe campylobacteriosis (Cogan *et al*., 2007).

The mucus layer which is present in the GI tract provides protection to mucosal epithelial cells against mechanical, chemical, microbial and enzymatic damage. The main components of the mucus are glycoproteins known as mucins (Neutra and Forstner, 1987; Strugala *et al*., 2003). *C. jejuni* has shown significant chemotaxis towards mucin and it has been demonstrated that the bacteria is able to bind to mucin (Hugdahl *et al*., 1988; Szymanski *et al*., 1995)

Of all mucins, MUC2 is the most common and is secreted by goblet cells in the small intestine and colon (Allen *et al*., 1998). Tu *et al*. (2008) examined, using RT-PCR, the expression of 20 virulence genes in response to MUC2 and showed that genes encoding cytolethal distending toxin protein (*cdtABC*), vacuolating cytotoxin (*vacB*), *C. jejuni* lipoprotein (*jlpA*), Campylobacter

invasion antigen B (*ciaB*) and the multidrug efflux system (*cmeAB*) were all increased. It was concluded that *C. jejuni* utilizes MUC2 as an environmental signal to control expression of genes with functions that include colonization and pathogenicity (Tu *et al*., 2008).

Further research on the response of *C. jejuni* to mucins has shown *C. jejuni* to possess a genomic island (*Cj0480c–Cj0490*) that is up-regulated in the presence of both L-fucose and mucin and allows L-fucose to be used as a substrate for growth. When a fucose permease (*Cj0486*) was mutated, this resulted in loss of the ability to utilise L-fucose and consequently the mutant showed decreased colonisation of the piglet model of human campylobacterioses (Stahl *et al*., 2011).

3.1.2 Mucoid bacteria

Bacteria such as *Burkholderia cepacia* (Bartholdson *et al*., 2008)*, Streptococcus pneumoniae* (Allegrucci and Sauer, 2007) *and Pseudomonas aeruginosa* (Friedl *et al*., 1992) all have the ability to express mucoidy. Some bacteria such as *B. cepacia* and *P. aeruginosa* express mucoidy through secretion of an exopolymeric material or EPM (Bartholdson *et al*., 2008; Tielen *et al*., 2010). These EPM can consist of either polysaccharide, extracellular proteins, DNA and lipids, in some cases the EPM can be a combination of all these substrates (Costerton *et al*., 1995).

In nature, bacteria persist in organised microbial communities which are encapsulated in an EPM known as a biofilm. These communities exhibit a number of advantages over unicellular growth, including increased survival in harsh conditions (Davey *et al*., 2003). Biofilms often exhibit increased antimicrobial resistance and protection against predation, dehydration and phagocytosis (Davey *et al*., 2003).

Biofilm formation commences when planktonic bacteria irreversibly attach to biotic or abiotic surfaces. Once in the biofilm mode of growth, bacteria assume a biofilm-specific phenotype, drastically different from that expressed in planktonic cells (Mah and O'Toole, 2001). Bacteria within biofilms assume

specific changes in protein expression particularly proteins associated with resistance to oxidative damage, exopolysaccharide production, phospholipids synthesis and membrane transport. Furthermore, biofilm-specific phenotype can trigger mechanisms responsible for antimicrobial resistance, enhanced virulence and persistence (Costerton *et al*., 1995).

Joshua *et al.* (2005) showed that *C. jejuni* is able to form both attached and unattached biofilms. These unattached aggregates of biofilm known as 'flocs' are more resistant to environmental stresses and are thought to be important in survival and pathogenesis of the organism. Further to this, *C. jejuni* has the ability to form attached biofilm on the surfaces of domestic and farming watering supplies and these attached bacteria could potentially act as an active reservoir of infection for humans and domestic animals (Reeser *et al* ., 2006).

3.1.3 *C. jejuni* EPM

One of the major outstanding questions with regards to *C. jejuni* biofilms is the composition of the EPM. The EPM is what holds the biofilm together, providing structure and strength. In the case of *C. jejuni* biofilms, the best characterised polysaccharide, the CPS, is not involved in biofilm formation as shown by Joshua *et al.,* (2005) who reported no reduction in the biofilm forming capacity of a *C. jejuni* NCTC 11168 *kpsM* mutant. KpsM consists of six putative trans-membrane regions and is situated in the inner membrane, and functions as a component of an ABC transporter involved in capsule transport through the inner membrane (Karlyshev *et al*., 2000).

Another polysaccharide produced by *C. jejuni* is a calcoflour white (CFW)-reactive polysaccharide. CFW is a fluorescent stain which binds β1-3 and β1-4 sugar linkages (McLennan *et al*., 2008). These authors showed that a *C. jejuni* biofilm produces a CFW-reactive polysaccharide. They also found biofilm formation is up-regulated in the absence of the stringent response, a stress response generally triggered by nutrient deprivation, which is controlled by SpoT. When *spoT* was mutated, an increase in the level of CFW-binding

polysaccharide and in the amount of biofilm was observed. Furthermore, there is evidence that LOS and N- and O-linked glycoproteins are not involved in biofilm formation. McLennan *et al.* (2008) tested a *gne* mutant and found no defect in biofilm formation. The *gne* gene encodes a bifunctional UDP-GlcNAc/Glc 4 epimerase involved in the synthesis of CPS, LOS, and *N*-linked carbohydrates (Bernatchez *et al*., 2005).

3.1.4 Hypothesis

As highlighted above *C. jejuni* has evolved to respond to a number of biological molecules found in the host intestinal environment including bile salts and norepinephrine. Initial data showed that supplementation of charcoal MHA (CMHA) or MEM-α with pancreatin (a commercial preparation of porcine pancreatic enzymes used for the treatment of pancreatic deficiency), results in *C. jejuni* exhibiting a mucoid morphology. The hypothesis examined in this chapter is that *C. jejuni* can also detect and respond to specifically mammalian pancreatic α-amylase, and this enzyme is responsible for the mucoid morphology seen upon pancretin supplementation.

3.2 Results

3.2.1 Pancreatic α-amylase induces a colony morphology change in *C. jejuni*

When hog pancreatic α-amylase was incorporated into charcoal MHA (CMHA) or MEMα, a change in colony morphology was observed. Figure 1 shows the typical colony morphology observed on charcoal MHA with and without 100 µM hog pancreatic α-amylase. The colonies on pancreatic α-amylase supplemented CMHA plates (panel A) are larger and more mucoid than the colonies on CMHA without amylase (panel B). This response is specific to hog pancreatic amylase and was not evident in response to amylase from *Aspergillus oryzae* or human salivary amylase. Other *C. jejuni* strains were tested (81-176, 81116, G1, X), all showing a similar response however the oral bacterium *Campylobacter rectus* did not show this response (Table 3.1). Further to this, porcine mucin, porcine bile, porcine pancreatic trypsin and bovine serum albumin (BSA) were tested at a range of concentrations and no change in phenotype was observed (Table 3.1). No difference was observed between strains in the degree of mucoidy in response to enzyme supplementation. Furthermore to identify the lowest concentration of amylase at which mucoidy was induced, varying concentrations of amylase were incorporated into CMHA. The results showed that the lowest concentration at which mucoidy was visible was at 100 µM (Figure 3.1). The hog α-amylase preparation was shown to contain only pancreatic α-amylase and no other proteins, see Appendix 1.
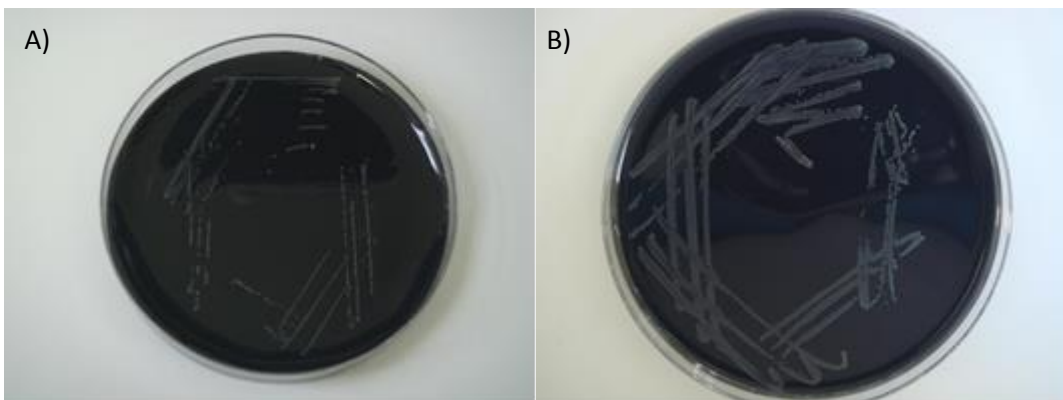
Figure 3.1. *C. jejuni* 11168H streaked on CMHA (panel A) and CMHA with 100 µM hog pancreatic α-amylase (panel B).

Table 3.1: Appearance of mucoidy in response to medium supplementation with pancreatic enzymes of all strains tested and the respective response to the individual components tested. All results were tested on CMHA and MEM-α with identical results. Mucoidy was marked on a scale +++ being very mucoid and – being non-mucoid

Strain	pancreatic α-amylase	α-amylase from *A. oryzae*	human salivary α-amylase	BSA	Trypsin	Mucin	Bile
11168H	+++	-	-	-	-	-	-
81-176	+++	-	-	-	-	-	-
Strain X	+++	-	-	-	-	-	-
G1	+++	-	-	-	-	-	-
81116	+++	-	-	-	-	-	-
C. rectus	-	-	-	-	-	-	-

3.2.2 Determining the carbohydrate content of *C. jejuni* exposed to α-amylase

To determine if the mucoid phenotype observed in the presence of α-amylase is a result of increased carbohydrate secretion, the EPM was prepared from bacteria (for methods see Chapter 2 section 2.9) grown on increasing concentrations of α-amylase and the carbohydrate content determined (Figure 2). The results showed that carbohydrate secretion significantly increased at 100 nM of α-amylase, the estimated physiological concentration. The increase in carbohydrate secretion was not dose dependent and no further increase was observed when concentrations above 100 nM were tested.
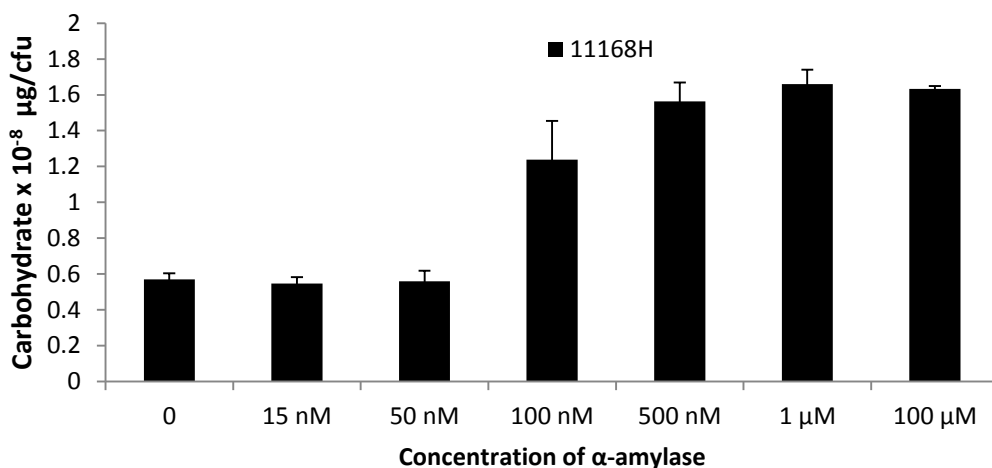
Figure 3.2. Graph showing the amount of extracellular carbohydrate produced by 11168H grown on MHA supplemented with increasing concentrations of hog pancreatic α-amylase. Data shown is the mean and standard deviation from three independent experiments.

3.2.3 Previously defined glycans are not associated with increased carbohydrate secretion

To determine if the increase in carbohydrate secretion in response to α-amylase involved the known glycan structures, the following mutants were tested alongside the wild-type strains, 11168H and 81-176. The *kpsM* mutant was included in these experiments to see whether any increase in secreted polysaccharide involves CPS expression. Both *waaC* and *waaF* mutants were also included in these experiment to test whether any increase in carbohydrate secretion is due to LOS. Finally a *spoT* mutant was constructed (for construction of *spoT* mutant see Appendix 1) in order to see if the previously described CFW-binding polysaccharide was involved. There was a significant increase in extracellular carbohydrate when all strains were grown with 100 nM pancreatic α-amylase compared to bacteria grown without α-amylase (Figure 3.3; 11168H, *spoT*, *waaC* and *waaF p*= 0.0001; 81-176 and *kpsM p*=0.001). Further, the magnitude of the increase was similar for all strains.
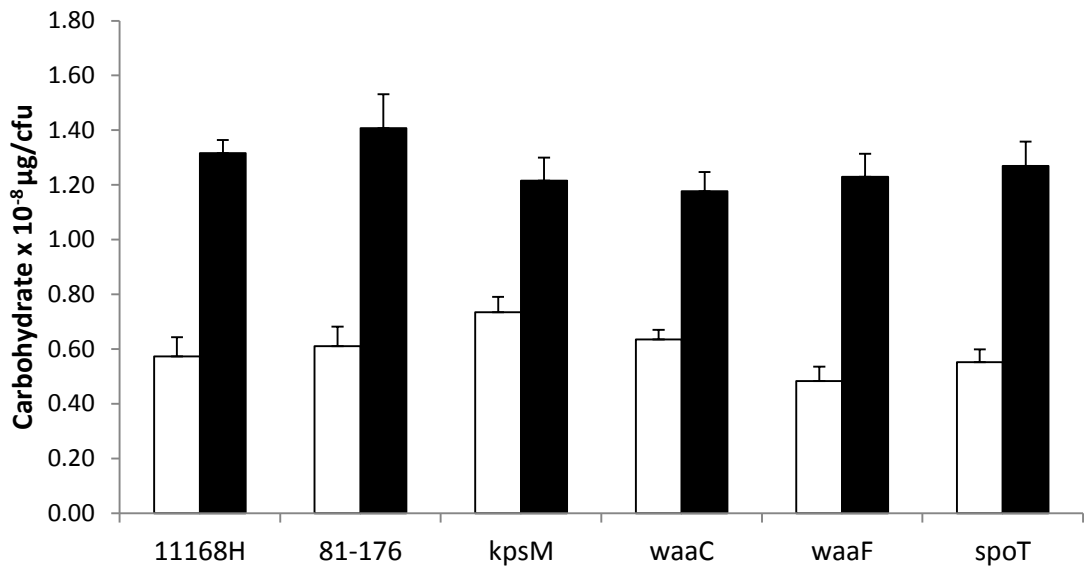
Figure 3.3. Graph showing extracellular carbohydrate in *C. jejuni* strains grown in the presence (black bars) or absence (white bars) of 100 nM pancreatic α-amylase. Data shown is the mean and standard deviation from three independent experiments.

3.2.4 Protein secretion is increased in response α-amylase

To determine whether protein secretion was affected in response α-amylase, total protein assays were performed on each strain grown with and without the presence of 100 nM pancreatic α-amylase. An increase in the total secreted protein was observed in all the four strains grown on charcoal MHA supplemented with pancreatic α-amylase compared to growth without α-amylase (Figure 3.4; 11168H, *spoT, waaC* and *waaF p*= 0.001; 81-176, *kpsM p*=0.0001).
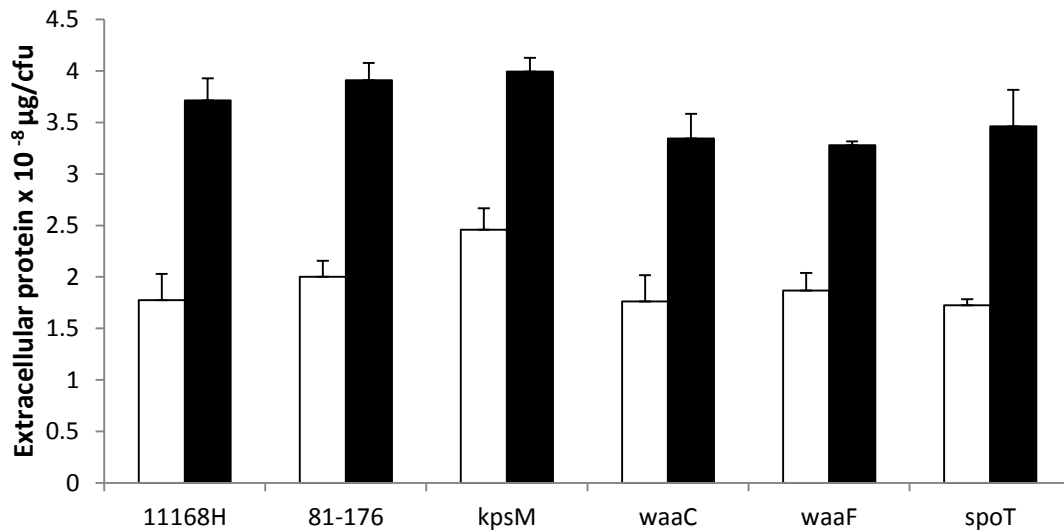
Figure 3.4. Graph showing extracellular protein in *C. jejuni* strains grown in the presence (black bars) or absence (white bars) of 100 nM pancreatic α-amylase. Data shown is the mean and standard deviation from three independent experiments.

3.2.5 Growth promotion by pancreatic α-amylase

Since the colonies produced on amylase-containing plates were larger as well as being more mucoid, the growth of *C. jejuni* 11168H in the presence and absence of 100 nM hog pancreatic α-amylase was compared in Brucella broth and MEMα (Figure 3.5). In both cases, α-amylase supplementation resulted in an increased rate of *C. jejuni* growth. Furthermore there was a higher final culture density in both cultures, this increase was more pronounced in MEMα (Figure 3.5B). The generation time in Brucella broth was 256 minutes in comparison to Brucella broth supplemented with amylase which was reduced to 178 minutes. For bacteria grown in MEMα, the generation time was 248 minutes, reduced to 172 minutes in MEMα supplemented with amylase. To assess if this response was specific to pancreatic α-amylase, α-amylase from *A. oryzae* was tested at a range of concentrations (100 nM, 1 µM and 100 µM) in both Brucella broth and MEM-α; this showed no effect on growth dynamics of *C. jejuni* 11168H.
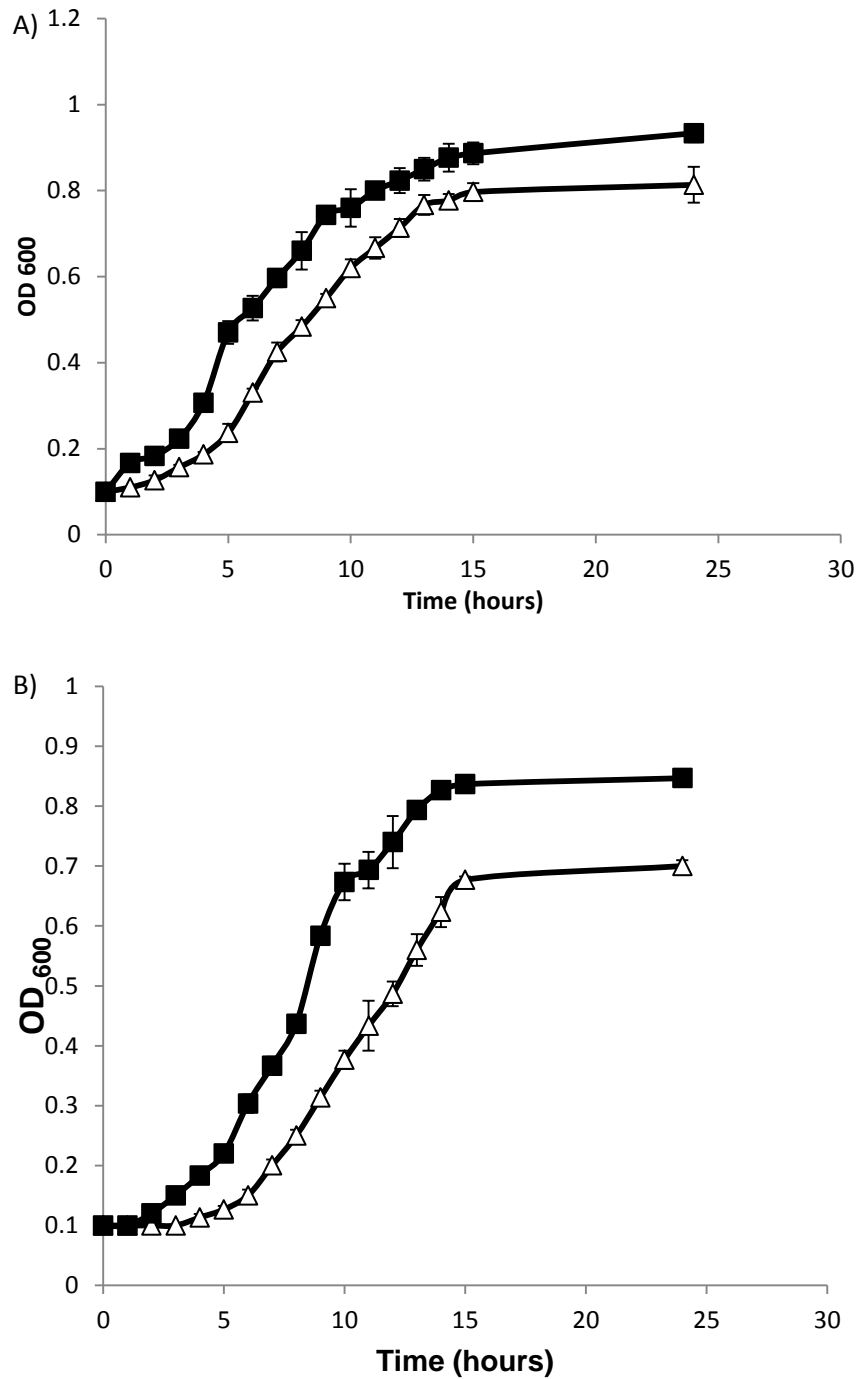
Figure 3.5. Growth of *C. jejuni* 11168H at 37°C in (A) Brucella broth with (filled squares) and without (open triangles) 100 nM pancreatic α-amylase and (B) MEM α with (filled squares) and without (open triangles) 100 nM pancreatic α-amylase. The optical density (OD_{600}) was measured at hourly intervals. Data shown is the mean and standard deviation from three independent experiments.

3.2.6 Reintroduction of mucoidy to clinical isolate

The colony morphology of *C. jejuni* laboratory strains differs from clinical isolates, with these isolates being wet mucoid and translucent. Initially a clinical isolate *C. jejuni* JW1 was passaged to see if this would result in loss of mucoidy. The first culture shows wet, mucoid translucent colonies typical of clinical *C. jejuni* isolates. After repeated sub-culture, the colonies changed, and after the fourth passage the colonies were non-mucoid and smaller (Figure. 3.6D). Next, to see if mucoidy could be re-introduced, the isolate was recovered from the fourth sub-culture and grown on CHMA containing 100 µM α-amylase and, as shown in Figure 3.6E, the mucoidy of the colonies was restored. The same result was obtained with a second clinical strain.
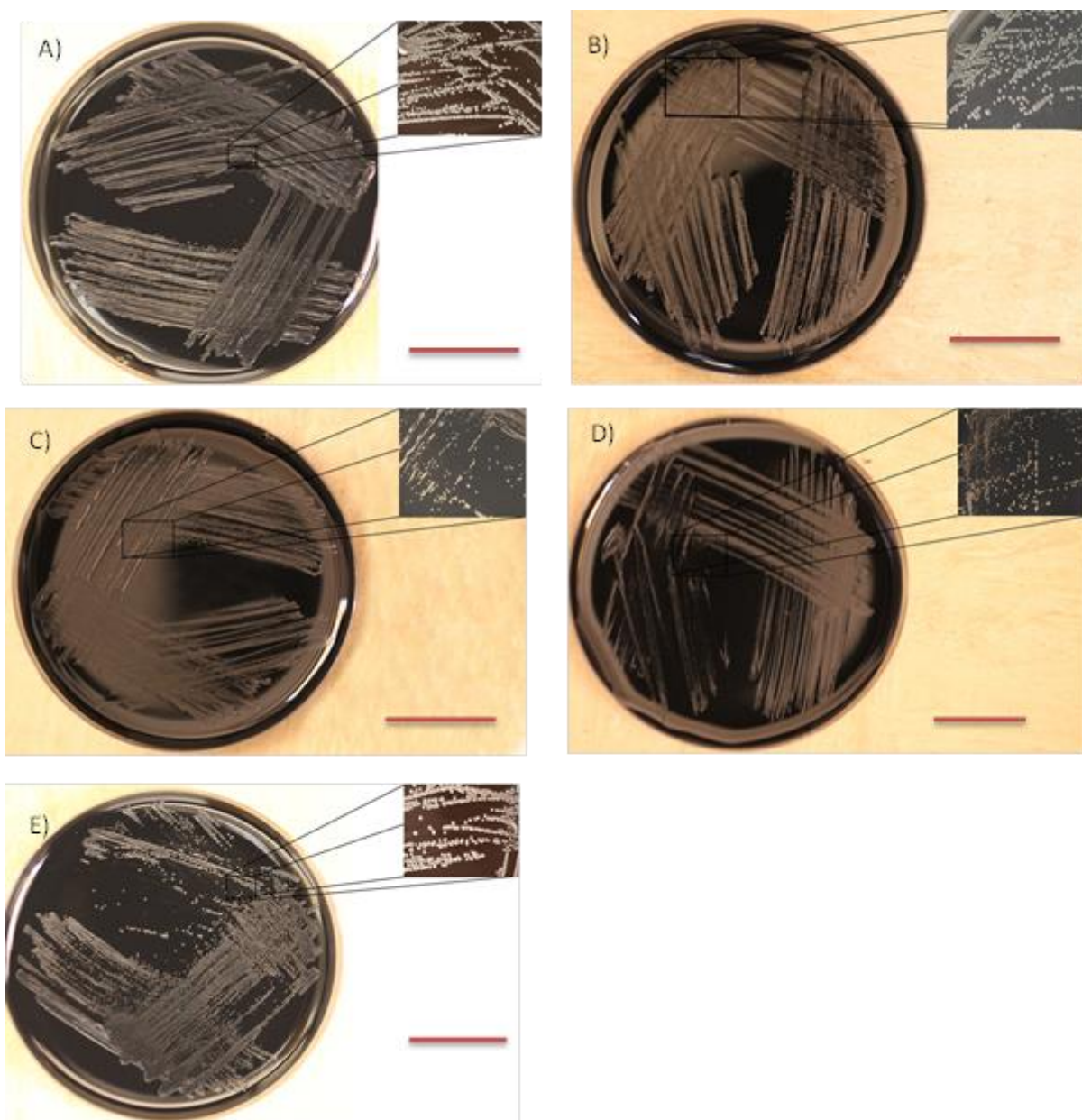
Figure 3.6. Clinical *C. jejuni* JW1 isolate A-D sub-culture 1-4 on CMHA, E sub-culture 5 on CMHA supplemented with 100 μM α-amylase. Scale bar represents 2.25 cm.

3.2.7 Identity of the polysaccharide component of the EPM

Preparations of EPM from *C. jejuni* 11168H and the *kpsM* mutant grown in the presence of 100 nM α-amylase were analysed using ^{1}H and ^{13}C-NMR and provided identical results to an α-dextran standard (Figure 3.7).

Furthermore, a number of 2D-NMR (COSY, HSQC & HMBC) were used to verify the identity of the EPM was an α-dextran. Monomer analysis

substantiated that glucose was the only monosaccharide present additionally analysis of the linkage showed the glucan to be composed entirely of 1,6-glycosidic linkage. All the sugar analysis was conducted by Professor Andrew Laws, University of Huddersfield.
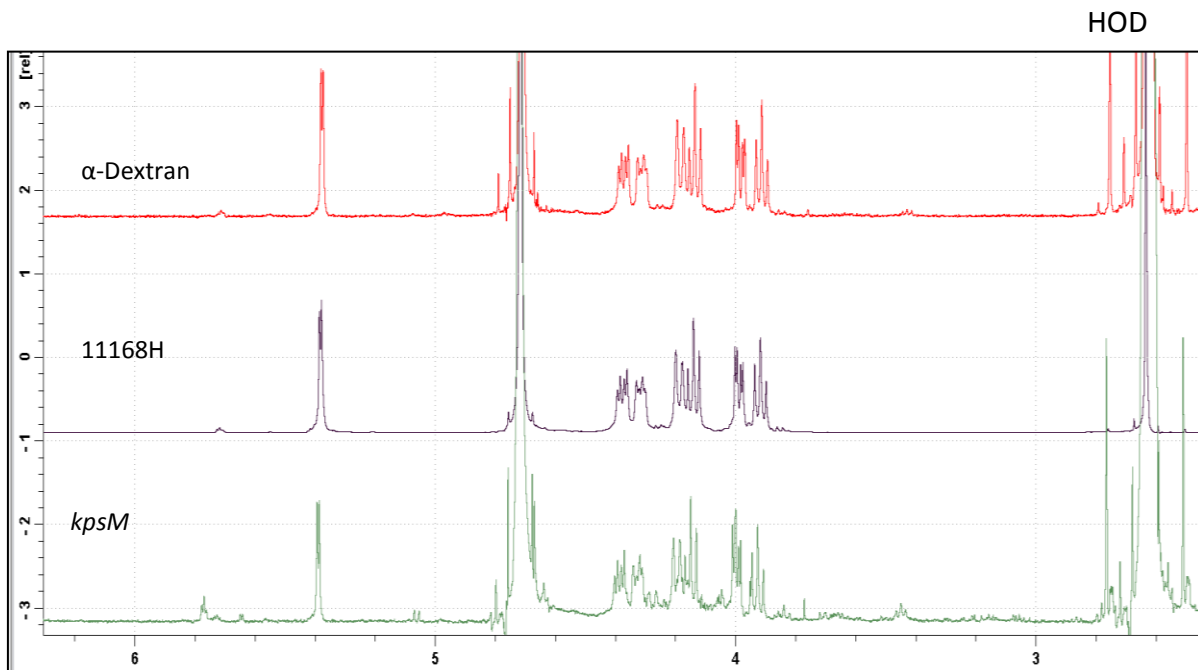
Figure 3.7. ^{1}H and ^{13}C-NMR spectra of EPS isolated from *C. jejuni* 11168H and kpsM mutant grown with 100 nM α-amylase supplementation. Both EPS samples showed identical spectra to a commercially available α-dextran standard.

3.2.8 Protein identification

Preparations of EPM from *C. jejuni* 11168H grown in the presence and absence of 100 nM α-amylase were run on SDS-PAGE gel; equal concentrations of each sample were loaded into the well, the gel profiles shown are indicative of those seen in three independent experiments. Samples grown in the presence of amylase showed a substantially different profile in comparison with sample grown in the absence of amylase (Figure 3.8). The bands were cut and analysed using LC-MS/MS and the hits are listed according to the 11168H database (Table 3.2).
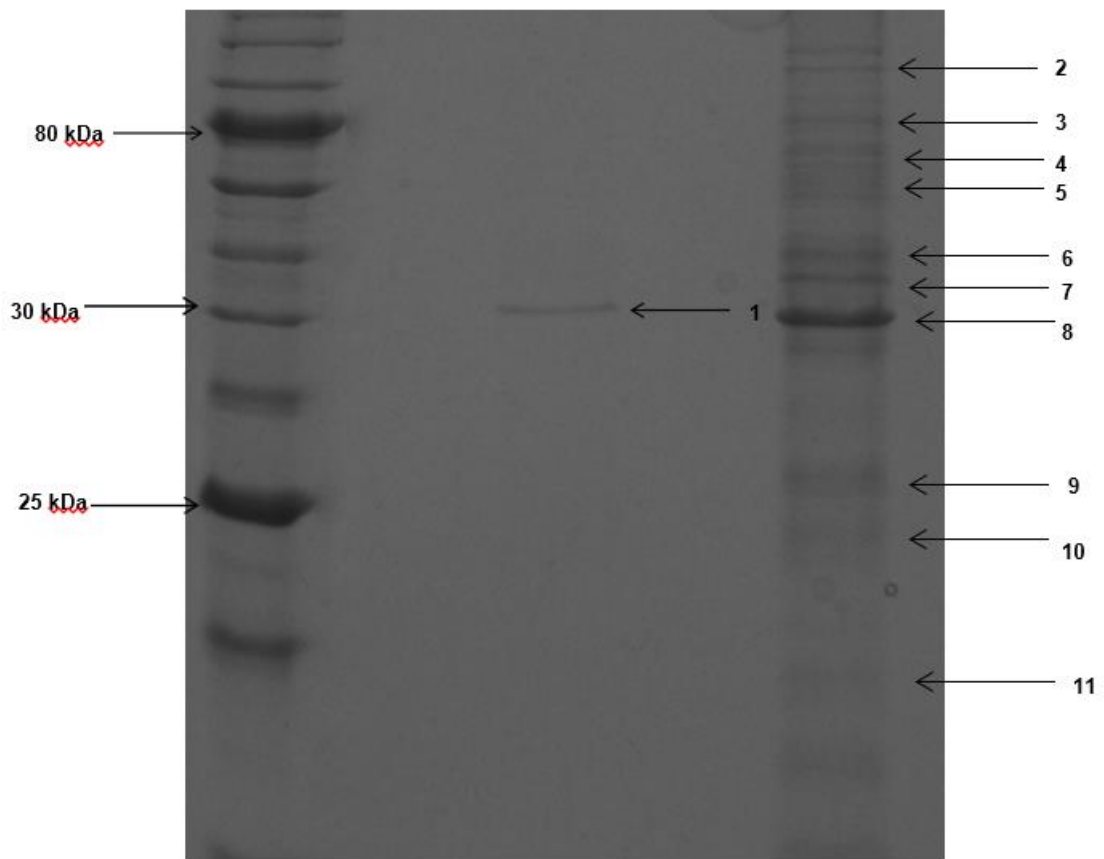
Figure 3.8. Lane 1: 10-250 kDa protein ladder (NEB). Lane 2: Precipitate *C. jejuni* 11168H grown in the absence of 100 nM α-amylase. Lane 3: Precipitate *C. jejuni* 11168H grown in the presence of 100 nM α-amylase. Bands were excised from the SDS-PAGE gel shown, digested in-gel with trypsin and analysed by LC-MS/MS.

Table 3.2. Identities of extracellular proteins in *C. jejuni* grown in the presence (bands 2-11) and absence (band 1) of hog pancreatic α-amylase.

Band No.[1]	Accession number[2]	Locus tag	Protein identity[3]	Peptides matched[4]	Coverage[5]	MW (kDa)[6]
1	YP_002344650	Cj1259	Major outer membrane protein*†	6	30.7	45.6
2	YP_002344856	Cj1476c	Pyruvate ferredoxin/flavodoxin oxidoreductase	3	3.1	131.2
3	YP_002344242	Cj0835c	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	3	5.9	92.7
	YP_002343725	Cj0284c	Chemotaxis protein CheA*#	2	4.0	85.2
	YP_002344890	Cj1511c	Formate dehydrogenase, alpha subunit, selenocysteine-containing	2	3.8	103.8
4	YP_002343927	Cj0493	Elongation factor G*	11	23.2	76.6
	YP_002343962	Cj0531	Isocitrate dehydrogenase, NADP-dependent	9	13.2	82.2
	YP_002343704	Cj0262c	Methyl-accepting chemotaxis protein*	8	18.3	72.7
	YP_002343604	Cj0144	Methyl-accepting chemotaxis protein*	8	31.8	72.2
	YP_002344885	Cj1506c	Methyl-accepting chemotaxis protein*	4	6.6	77.3
	YP_002343846	Cj0409	Fumarate reductase flavoprotein subunit	3	5.6	73.7
	YP_002343589	Cj0129c	OMP85 family outer membrane protein†	2	3.4	83.1
	YP_002344625	Cj1234	Glycyl-tRNA synthetase subunit beta	2	3.9	76.4
5	YP_002344612	Cj1221	Chaperonin GroEL*#	8	22.2	57.8
	YP_002344292	Cj0893c	30S ribosomal protein S1	6	16.5	62.7
	YP_002344069	Cj0640c	Aspartyl-tRNA synthetase	4	8.2	66.0
	YP_002343950	Cj0518	Heat shock protein 90	4	8.9	69.5
	YP_002344166	Cj0759	Molecular chaperone DnaK*	3	7.1	67.3
	YP_002344331	Cj0933c	Oxaloacetate decarboxylase, alpha subunit, putative	3	6.8	65.7
	YP_002344509	Cj1116c	Cell division protein FtsH	3	6.2	70.9
	YP_002343510	Cj0039c	GTP-binding protein TypA (BipA)	2	4.2	66.4
	YP_002345014	Cj1645	Transketolase	2	3.6	69.5

6	YP_002343565	Cj0105	F_0F_1 ATP synthase subunit alpha*	8	18.4	54.7
	YP_002343547	Cj0087	Aspartate ammonia-lyase*	9	22.4	51.6
	YP_002343829	Cj0392c	Pyruvate kinase	5	12.7	53.7
	YP_002344561	Cj1170c	50 kDa outer membrane protein†	5	12.4	53.2
	YP_002344588	Cj1197c	Aspartyl/glutamyl-tRNA amidotransferase subunit B	4	11.2	52.8
	YP_002344453	Cj1058c	Inosine 5'-monophosphate dehydrogenase	3	8.5	52.0
	YP_002343802	Cj0365c	outer membrane channel protein CmeC*	3	7.7	55.3
	YP_002344779	Cj1394	Adenylosuccinate lyase	2	5.9	51.2
	YP_002343651	Cj0193c	Trigger factor (peptidyl-prolyl cis/trans isomerase)	2	4.7	50.8
	YP_002344106	Cj0688	Phosphate acetyltransferase$^{\pm}$	2	4.5	56.1
	YP_002344408	Cj1013c	cytochrome c biogenesis protein	2	2.2	123.3
7	YP_002343904	Cj0470	Elongation factor Tu*	9	31.1	43.5
	YP_002343567	Cj0107	F_0F_1 ATP synthase subunit beta*	6	19.1	50.7
	YP_002344581	Cj1190c	Methyl-accepting chemotaxis protein	4	12.6	50.9
	YP_002343552	Cj0092	Probable periplasmic protein*	3	9.2	49.1
	YP_002343489	Cj0017c	DsbB family disulfide bond formation protein*	3	7.1	56.6
	YP_002344680	Cj1290c	Biotin carboxylase	2	6.1	49.0
	YP_002344228	Cj0821	UDP-N-acetylglucosamine pyrophosphorylase	2	8.6	47.8
8	YP_002344650	Cj1259	Major outer membrane protein*†	13	52.6	45.6
	YP_002343964	Cj0533	Succinyl-CoA synthase, beta subunit	2	6.2	41.6
9	YP_002344802	Cj1419c	Putative methyltransferase	5	21.2	29.6
	YP_002343909	Cj0475	50S ribosomal protein L1	4	24.0	24.9
	YP_002344152	Cj0734c	CjaC, predicted periplasmic solute binding protein†	2	10.4	27.7
10	YP_002344869	Cj1489c	Cytochrome c oxidase, cbb3-type, subunit II*	2	10.9	24.8
11	YP_002343573	Cj0113	Peptidoglycan-associated lipoprotein Omp18*	5	33.3	17.7

[1] Band number as shown on the SDS PAGE gel in Figure 3.8.

[2,3] Accession number and protein identity as given in the *C. jejuni* NCTC 11168 re-annotated genome sequence database (Gundogdu *et al.*, 2007).

[4] Number of peptides observed in mass spectra matching to the protein in the database.

[5] Percentage amino acid coverage (peptides observed/theoretical from sequence data).

[6] Theoretical molecular weight (kDa) of proteins as given in the *C. jejuni* NCTC 11168 re-annotated genome sequence database.

* Protein identified in outer membrane vesicles (Elmi *et al.*, 2012).

[†] Protein contains predicted N-terminal secretion signal.

[#] Protein up-regulated in biofilms (Kalmokoff *et al.*, 2006)

± Demonstrated functional role in biofilms (Joshua *et al.*, 2006).

3.3 Discussion

The expression of a mucoid phenotype is not an uncommon phenomenon, a number of Gram-negative bacteria are able to express a mucoid phenotype, including *B. cepacia, P. aeruginosa, V. cholera* and *V. parahaemolyticus,* which is the result of EPM secretion (Bartholdson *et al*., 2008; Enos-Berlage and McCarter, 2000; Qiu *et al.,* 2007). In the case of *B. cepacia,* the increased EPM production is due to interaction with onion skin which the organism encounters within its life cycle (Bartholdson *et al*.,2008).

It has previously been shown that *C. jejuni* is able to recognise and respond to a number of biological compounds that it encounters in the human GI tract including constituent of bile DOC (Malik-Kale *et al*., 2008), neurotransmitter NE (Cogan *et al*., 2006) and mucin constituent MUC2 (Tu *et al*., 2008). The results of this chapter show that *C. jejuni* is also able to recognise mammalian pancreatic α-amylase specifically and responds with the production of exo-polymers.

The EPM expressed was shown to contain carbohydrate which was independent of previously characterised glycans in *C. jejuni.* The *kpsM* mutant which is CPS deficient, *waaC* and *waaF* mutants which are LOS deficient all showed increases in carbohydrate secretion in response to α-amylase at wild-type levels indicating that none of these glycans are involved. The fact that the *spoT* mutant (which is reported to over-express the CFW-reactive polysaccharide) only displays mucoidy in the presence of α-amylase, and shows similar levels of EPM as the wild-type strain, 11168H, in both the presence and absence of pancreatic amylase, clearly indicates that the CFW-reactive polysaccharide is not involved in the response, highlighting that the EPM is composed of a novel polysaccharide.

NMR analysis showed the polysaccharide component of the EPM consisted of 1,6 linked glucose monomers, producing an α-dextran. The polysaccharide component of many bacterial EPM's retain backbone structures which are composed 1,3- or 1,4-β-linked hexose residues (Sutherland, 2001). These sequences exhibit a very rigid structure which are resistant to deformation and

are generally very poorly soluble. However polysaccharides of EPMs which are composed of 1,2-α- or 1,6-α-linkages observed in dextrans provide a more flexible structure (Sutherland, 2001). Fast flow around a biofilm results in stresses around the biofilm and can lead to eventual detachment. Having visco-elastic flexible biofilm structures enables the bacteria to deform under the stresses placed upon them and thus remain attached (Young, 2006). This is presumably an advantage for an enteric bacterium such as *C. jejuni* which has to withstand significant peristalsis in the small intestine.

C. jejuni is asaccharolytic and is unable to utilise glucose as a primary source of carbon, nevertheless genome sequencing and experimental data have identified homologues for the Embden-Meyerhof pathway, which provides both glycolysis and gluconeogenesis pathways. However, in *C. jejuni* these enzymes are considered to be predominately gluconeogenic (Mendz *et al*., 1997). Furthermore it has been determined that *C. jejuni* requires gluconeogenesis to produce glucose derived polysaccharides, such as the one observed in this study (Gaynor *et al*., 2004).

The data presented in this chapter shows that the increase in extracellular proteins and the dextran secreted in response to pancreatic α-amylase, clearly demonstrates the EPM to be constituted of a mixture of protein and polysaccharide, however it would be advantageous to further extend this analysis to look for the presence of lipids and nucleic acids.

The colony morphology of clinical *C. jejuni* isolates are wet and mucoid and differ considerably in comparison to laboratory strains such as *C. jejuni* 11168H. When fresh clinical isolates JW1 and JW2 were passaged mucoid morphology was lost and after the fourth passage resembled the non-mucoid small colonies of *C. jejuni* 11168H. However, upon supplementation of α-amylase into the growth medium, the original mucoidy observed was restored; this highlights the mucoid phenotype seen in response to α-amylase is clinically relevant and mucoidy observed in fresh clinical isolates is due to recent exposure to α-amylase. This emphasises that *C. jejuni* laboratory strains commonly used in research have, in the sequence of consecutive *in*

vitro passage, become significantly differentiated from non-passaged clinical isolates; leading to current research performed with laboratory strains possibly missing important pathophysiological mechanisms and not being truly clinically relevant.

As an example *P. aeruginosa* PAO1 which was first isolated by B.W. Holloway (1955) in Australia, has been passaged for decades and have become effectively non-pathogenic; studies using this strain has shown phenotypically diverse biofilm from different laboratory under the same conditions (Fux *et al*., 2005).

Another finding of this study is the ability of pancreatic α-amylase to promote growth of *C. jejuni*. The results presented here have shown that supplementation of α-amylase results in increased growth kinetics in liquid culture. Growth promotion by intestinal secretions has been documented previously; norepinephrine has been shown to significantly increase the rate of growth of *C. jejuni* (Cogan *et al.,* 2009). Furthermore *Helicobacter pylori* exhibits growth promotion in response to gastrin a gastrointestinal hormone produced by g cells in the duodenum and in the pyloric antrum of the stomach (Chowers *et al*.,1999)

It has been predicted that the concentration of pancreatic α-amylase in the human GI tract is between 5-15 nM, however this is considered an underestimate (Slaughter *et al*., 2000) and the actual value could be higher. Therefore the minimum concentration of α-amylase required for expression of the phenotype observed in this study was 100 nM is physiologically relevant. Interestingly hyperamylasia has been reported in clinical cases of severe campylobacterioses (Tositti *et al*., 2001; Peterson and Besendorfer, 2012).
C. jejuni encounters both bile and pancreatic α-amylase at the same point in the small intestine, the pancreatic duct connects to the common bile duct before the hepatopancreatic ampulla, after which both ducts pierce the medial region of the duodenum. Malik-Kale *et al*. (2008) proposed that encountering DOC would prime *C. jejuni* to invade epithelial cells within the human GI tract, this idea of the bacterium using biological compounds as environmental

signals is supported by Tu *et al*. (2008) who suggested *C. jejuni* uses MUC2 as an environmental cue to regulate expression of genes associated with pathogenicity. The results presented in this chapter indicate a similar role for pancreatic α-amylase. LC-MS/MS analysis showed the expression of a number of surface associated proteins specifically in bacteria grown in the presence of α-amylase. Interestingly a number of the identified proteins in the α-amylase grown bacteria, are secreted in outer membrane vesicles as shown by Elmi *et al.* (2012). The data set obtained shows a number of proteins involved chemotaxis such as transmembrane sensor proteins methyl-accepting chemotaxis protein, which enables bacteria to detect concentrations of molecules extracellularly (Gestwicki *et al*., 2000). Other proteins are involved in metabolism cycle of *C. jejuni* such as pyruvate ferredoxin/oxidoredctase and pyruvate kinase which is involved in glycolysis. Also, proteins such as CjaC which are components of ABC type transport systems and are involved in pathogenicity were identified (Pawalec *et al*., 2000).

This chapter has shown that *C. jejuni* is able to respond to specifically mammalian pancreatic α-amylase in response to which it produces a clinically relevant mucoid phenotype through secretion of exo-polymers composed of proteins and a dextran not previously described in *C. jejuni*.

Chapter 4: The role of Cj0511 in the response to α-amylase

4.1 Introduction

Pathogenic bacteria have a selection of factors which cause virulence either directly or indirectly. The ability of *C. jejuni* to persist in diverse environments that are experienced by the bacterium through its life cycle is due to its ability to adapt to these conditions.

4.1.1 Bacterial Proteases

Bacterial proteases are a group of enzymes which perform proteolysis, initiating protein catabolism through hydrolysis of peptide bonds which string together amino acids to make peptides. All living organisms produce proteases; they can be endopeptidases which cleave internal peptide bonds, or exo-peptidases which act on amino or carboxyl termini of proteins. Microbial proteases are can be divided into 6 groups based on the essential catalytic residue at their respective active site metalloproteases, serine, threonine, cysteine, aspartate and glutamic proteases.

The majority of metalloproteases are zinc containing, zinc is an essential constituent of many proteins which a play a pivotal role in all aspects of metabolism across different phyla. They are ubiquitous and found in both Gram-negative and Gram-positive bacteria.

4.1.2 Lon proteases

A model organism in which peptidases have been studied is *E. coli*. Lon proteases are cytoplasmic serine proteases consisting of hexameric rings of single peptide chains carrying an ATPases domain and a chaperone activity domain. Lon recognises sequences abundant with aromatic residues which are exposed in unfolded proteins but concealed in folded proteins (Gur and Sauer, 2008). In *E. coli* these particular proteases degrade non-native proteins (Tsilibaris *et al*., 2006).

Lon proteases also play an important role in *Salmonella typhimurium*. Mutants that are deficient in the ability to express Lon displayed a phenotype that showed increased invasion of epithelial cells, increased expression of pathogenic proteins as well as stimulating apoptosis in macrophages. However, this did not lead to increased virulence in an animal model, with *S. typhimurium* mutants deficient in Lon requiring a 50% increase in lethal dose, and also showed decreased survival in response to oxidative stress (Takaya *et al*., 2002).

4.1.3 Clp protease

Another set of protease are Clp proteases, these consist of 2 heptameric rings of ClpP subunits flanked by hexameric rings of one Clp AtPases. Clp proteases are tubular shaped and the associated Clp ATPases are phylogentically determined, with Gram-negative bacteria generally having ClpA and ClpX, whereas the Gram-positive bacteria contain ClpX, ClpC and ClpE (Butler *et al*., 2006; Frees *et al*., 2004). In *Listeria monocytogenes* a foodborne pathogen, it has been suggested that ClpP play a fundamental role in degrading inhibitors of listeriolysin which is an essential virulence factor (Gaillot *et al*., 2000). Furthermore, mutation of *clpC* and *clpP* genes reduce transcription of *actA*-encoding proteins which initiate host actin polymerisation (Gaillot *et al*., 2000). Clp proteins play an active role in virulence of *S. pneumonia*, the causative agent of pneumonia, mutation of *clpP* results in significantly reduced survival in an intratracheal inoculation mice model (Kwon *et al*., 2004).

4.1.4 HtrA proteases

DegP also known more commonly as HtrA are highly conserved set of proteins which exhibit both *in vivo* proteolytic and chaperone activity. HtrA is localised in the periplasm of Gram-negative bacteria in comparison to ClpP and Lon that are ATP-dependent cytoplasmic peptidases (Meltzer *et al*., 2008). In Gram-positive bacteria that do not have extramembranous compartments, HtrA is located at a single domain of the cellular membrane.

The predominant role of chaperone activity is to direct outer membrane proteins through the periplasm and proteolytic activity ensures degradation of unfolded periplasmic proteins (Rosch and Caparon, 2005).

Investigations in *in vivo* virulence models have shown that mutation of *htrA* of *S. typhimurium* (Humphreys *et al*., 1999), *Y. enterocolitica* (Li *et al*., 1996) and *L. monocytogenes* (Yamamoto *et al*., 1996) show attenuated virulence. HtrA is involved in degradation of damaged proteins which build up during unfavourable conditions in the macrophages. Bacteria such as *Y. enterocolitica* are thus able to survive phagocytosis and other pathogens such as *L. monocytogenes* which are intracellular facultative survive within macrophages during infection by replicating. Within these environments the bacteria encounter a number of antimicrobial compounds such as reactive oxygen radicals, which in turn leads to unfolded damaged proteins amassing.

4.1.5 FtsH Proteases

FtsH proteins are located in the cytoplasmic membrane, they incorporate a core cystolic area and a transmembrane fragment situated at the N-terminus. These proteins are ubiquitous in eubacteria, and are essential for viability in some organisms. FtsH functions by degrading a number of mis-folded and transient membranous proteins. *Staphylococcus aureus* mutants that are FtsH deficient, when injected into mice exhibited lower recovery from lesions (Lithgow *et al*., 2004).

4.1.6 Proteases in *C. jejuni*

C. jejuni is an Epsilonproteobacteria and is distantly related to member of Gammaproteobacteria such *E. coli* which has been a model organism to study proteases. Scrutiny of the *C. jejuni* genome shows homologues for ClpA, ClpX and Lon that are found in *E. coli* (Hoskins *et al*., 1998).

For *C. jejuni,* growth at high temperatures results in the accumulation of misfolded proteins and it has been shown both Lon and ClpP are both

required for growth under these conditions (Cohn *et al.*, 2007). Characterisation of both *clpP* and *lon* mutants showed changes in phenotypes, such as lower levels of autoagglutination, decreased motility and decreased invasion of INT407 epithelial cells and it was concluded these proteases contribute to virulence of *C. jejuni* (Cohn *et al.,* 2007).

C. jejuni also maintains homologues of HtrA protease which is required for both heat and oxygen tolerance, and for interaction with human epithelial cells (Bronsted *et al.*, 2005). Bronsted *et al.* (2005) showed that a *C. jejuni htrA* mutant exhibited reduced growth at 44°C and in the presence of puromycin, a peptide inhibitor, and further showed a reduced ability to adhere and invade INT407 epithelial cells.

4.1.7 Cj0511

Gene *Cj0511* encodes a predicted product with similarity to tail-specific proteases. The amino acid sequence of Cj0511 shows the highest similarity to *Bartonella bacilliformis* peptidase CtpA, similar protease is found in *Brucella suis* and *Burkholderia mallei* and are required for survival within macrophages and for complete virulence in mice (Bandara *et al.,* 2008; Bandara *et al*., 2005) In *E. coli* CtpA is a periplasmic protease and has been shown to be responsible for C-terminal processing of penicillin-binding protein 3 (PBP-3), furthermore it has been shown *in vitro* to degrade proteins with non-polar C termini (Hara *et al.,* 1991).

Mutation of the *Cj0511* gene in *C. jejuni* 11168H resulted in reduced colonisation of chickens, this decrease seen in the chicken model was shown to be independent of any changes is flagella expression which appear in *C. jejuni* because of phase variation, with the mutant strain showing motility at wild-type levels (Personal communication Dr A. Karlyshev). Champion *et al.* (2010) showed that a *Cj0511* mutant exhibited a reduced kill in *G. mellonella* model.

4.1.8 Outer membrane vesicles

The mechanisms by which *C. jejuni* is able to deliver virulence factors into host cell remain unclear as the bacterium lacks the classical virulence-associated secretion systems that are possessed by other enteric pathogens such as *Salmonella spp*., and uses a flagellar export system which Konkel *et al*. (2004) showed is necessary for the invasion of INT 407 epithelial cells. A number of Gram-negative bacteria, instead of secreting proteins into the surrounding environment, where they are liable to get broken down by host proteases, use outer membrane vesicles (OMVs) as a way of delivering active proteins and other biological products into the host cell (Ellis and Kuehn, 2010).

Utilising OMVs allows bacteria to secrete a number of virulence factors together rather than secrete them individually. OMVs have been shown to be involved in moderating host responses and delivering virulence factors for a number of Gram-negative bacteria (Lara-Tejero and Galan, 2001). OMVs are spherical nanostructures consisting of outer membrane proteins, LOS, phospholipids, and several periplasmic proteins and generally have a diameter between 10 to 500 nm (Kuehn and Kesty, 2005; Mashburn-Warren *et al.,* 2008) However, *C. jejuni* OMVs differ in size and were found to be between 10-250 nm (Elmi *et al*., 2012)

OMVs in *C. jejuni* were first described in 1982 by Logan and Trust and evidence of the biological importance of *C. jejuni* to OMVs has recently been determined with Lindmark *et al.* (2009) showing that active CDT is released inside OMVs by strain 81-176. Elmi *et al*. (2012) conducted proteome analysis on OMVs produced by *C. jejuni* 11168H and identified 151 including the protease HtrA and the predicted protease Cj0511.

4.1.9 Hypothesis

Based on the findings of Elmi *et al*. (2012) who showed that the OMVs of *C. jejuni* contained Cj0511, it was postulated that this protease was involved in processing α-amylase as a signal for the response observed in Chapter 3. Therefore the hypothesis being addressed in this chapter is that *Cj0511* activity is necessary for the response seen when *C. jejuni* is grown in the presence of pancreatic α-amylase. To test this, a *Cj0511* mutant was obtained (Campylobacter Resource Facility, LSHTM) and a complemented strain was constructed by insertion of a functional *Cj0511* gene into *Cj0046* under control of the *pfdxA* promoter by using plasmid pC46*fdxA*. Additionally recombinant Cj0511 was prepared by cloning and expression of a histidine-tagged protein in *E. coli*

4.2 Results

4.2.1 Functional Cj0511 is required to increase carbohydrate secretion in response to α-amylase

The mucoid phenotype, documented in Chapter 3, was not apparent when *C. jejuni* 11168H *Cj0511* mutant was grown with α-amylase supplementation, as shown in figure 4.1 which shows11168H wild-type exhibiting a mucoid phenotype, and Cj0511 mutant exhibiting a non-mucoid phenotype; when both strains were grown on CMHA supplemented with 100 µM α-amylase.
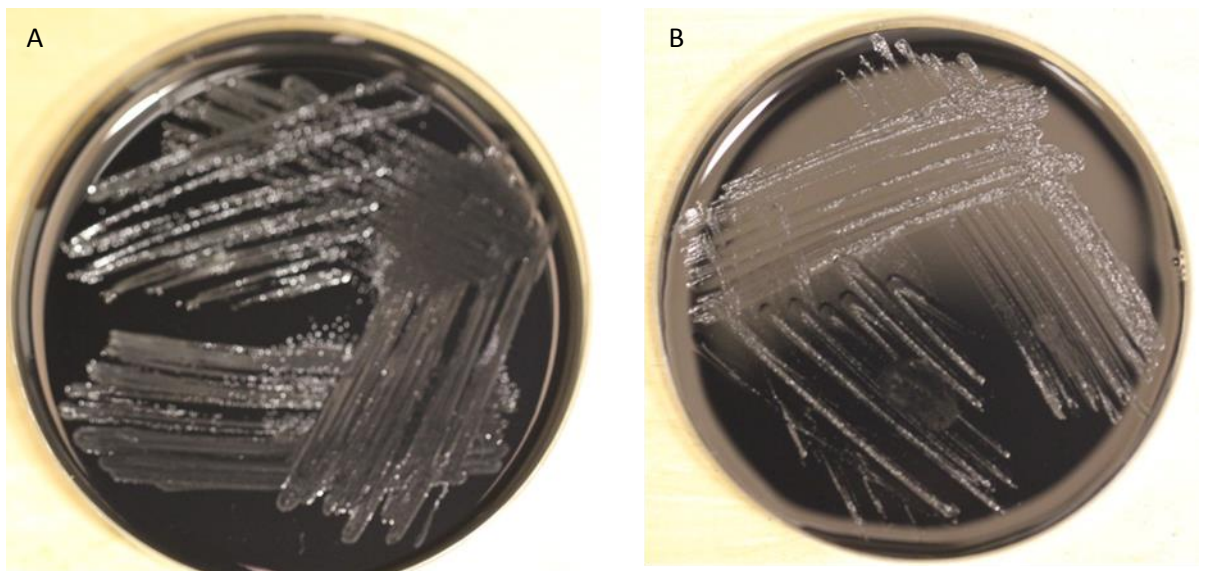
Figure 4.1 *C. jejuni* 11168H exhibiting a mucoid phenotype (Panel A) and *Cj0511* mutant exhibiting a non-mucoid phenotype (Panel B) streaked on CMHA supplemented with 100 µM α-amylase.

However, the lowest concentration that carbohydrate increase is exhibited is 100 nM and this is not visible by eye, therefore a carbohydrate analysis was conducted to test if the *Cj0511* mutant was able to express increased carbohydrate in response to rising concentrations of pancreatic α-amylase as exhibited by the wild-type strain. Furthermore the complemented *Cj0511* strain (for complementation of *Cj0511* see Appendix 2) was tested. The results

clearly show that *Cj0511* was unable to express increased carbohydrate in response to increasing concentrations of amylase as exhibited by strain 11168H, further to this the complemented strain exhibited an increase in carbohydrate secretion which was comparable to the wild-type strain (Figure 4.2).
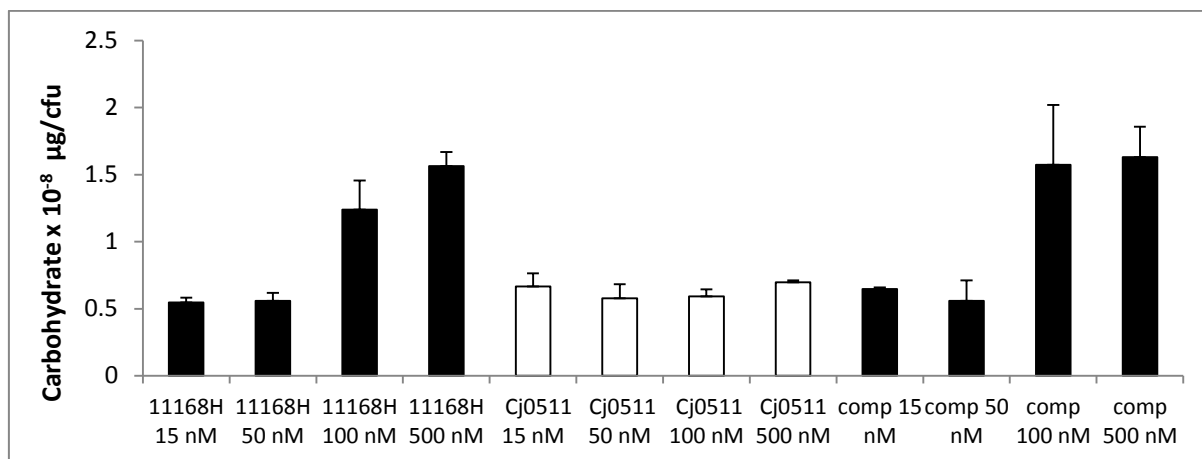
Figure 4.2. Graph showing the amount of extracellular carbohydrate produced by *C. jejuni* 11168H, the *Cj0511* mutant and the complemented strain in the presence of pancreatic α-amylase. Data shown is the mean and standard deviation from three independent experiments.

4.2.3 Functional Cj0511 is required to grow on agar with α-amylase as the sole source of carbon

As pancreatic amylase appears to increase the growth rate of *C. jejuni* as shown in Chapter 3, the question of whether the bacterium was able to use pancreatic enzymes as a carbon source was investigated. Growth on a defined minimal medium (SAPI) was observed on plates supplemented with 100 µM pancreatic α-amylase, as a positive control 20 mM sodium pyruvate was used, the results are summarised in Table 4.1. The wild-type strain and the *Cj0511* complemented strain showed growth on SAPI when supplemented with pancreatic α-amylase; however no growth was observed by the *Cj0511* mutant in the presence of pancreatic α-amylase. All of the tested strain exhibited growth on sodium pyruvate. To test if the response was specific to pancreatic α-amylase growth SAPI was supplemented with α- amylase from *A. oryzae*, pancreatic trypsin, porcine gastric mucin, bile, BSA and lactose at a range of concentrations; no growth by any of the strains was observed.

Further to this, no growth was observed on plates which were supplemented with microbial α-amylase. These results show that *C. jejuni* is able to grow on minimal defined medium containing pancreatic α-amylase as the sole source of carbon. The data also indicates that *C. jejuni* can use only pancreatic α-amylase as a carbon source and not α-amylase from a microbial source, furthermore this ability is dependent on functional Cj0511.

Table 4.1. Growth of *C. jejuni* 11168H, the *Cj0511* mutant and the complemented strain on SAPI medium containing different sole sources of carbon.

Strain	Hog α-amylase	α-amylase from *A. oryzae*	BSA	Trypsin	Mucin	Bile	Lactose	Pyruvate
11168H	Yes	No	No	No	No	No	No	Yes
11168H *Cj0511*	No	No	No	No	No	No	No	Yes
11168H Comp	Yes	No	No	No	No	No	No	Yes

4.2.4 Recombinant Cj0511 is proteolytically active

To test if recombinant Cj0511 (For expression of recombinant protein see Appendix 3) has proteolytic activity, azocasein was used as a substrate and trypsin as a control. Azocasein is casein conjugated with an azo dye. When an active protease is incubated with azocasein, hydrolysis of the casein liberates the free dye into the supernatant which can be quantified by measuring

absorption at 440 nm. Figure 4.3 shows trypsin exhibited higher activity when compared to an equal concentration of Cj0511. The results clearly show that Cj0511 exhibits proteolytic activity.
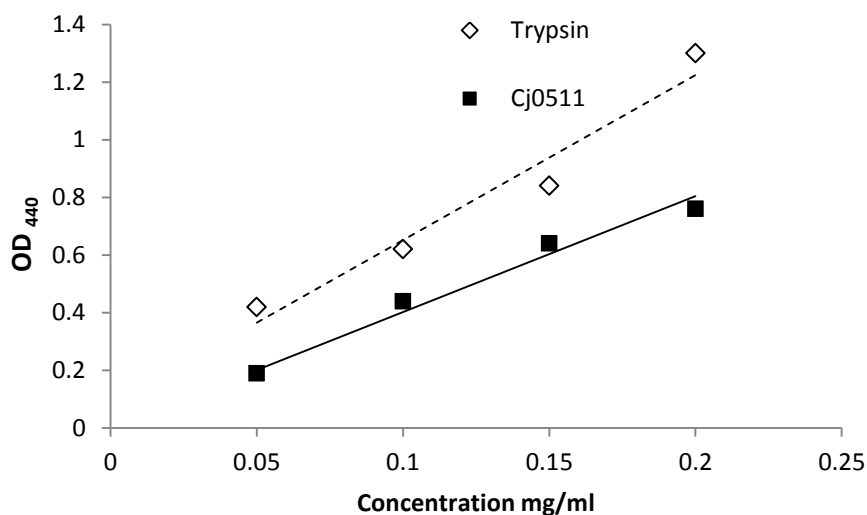
Figure 4.3. Analysis of proteolytic activity of recombinant Cj0511 using azocaesin as substrate with equal concentrations of trypsin.

4.2.5 Recombinant Cj0511 degrades α-amylase

Final concentrations of 0,1,2,3 and 4 mg/ml recombinant Cj0511 were pre-incubated at 37˚C with 5 µg/ml hog pancreatic α-amylase and then loaded into an agar plate containing starch. The resulting halos were visualised over white light as shown in Figure 4.4. The results show a dose-dependent decrease in the ability of α-amylase to hydrolyse starch.
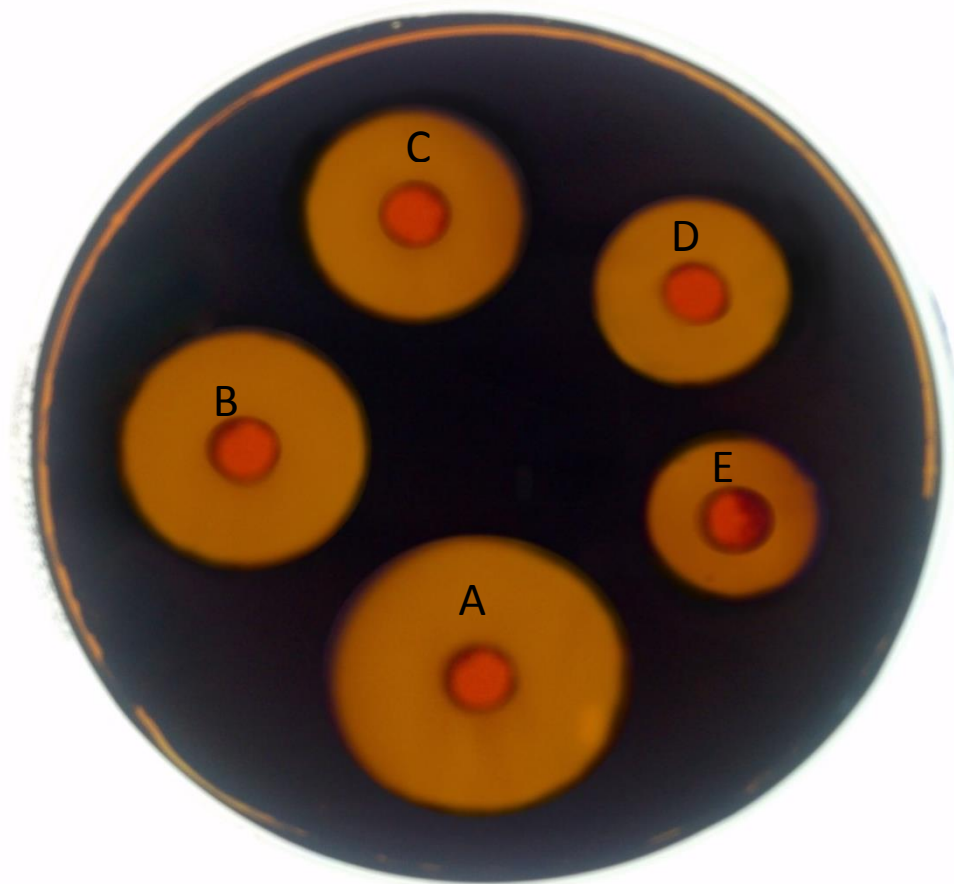
Figure 4.4. Nutrient agar plates containing 0.4 % (v/v) starch inoculated with A) Control 5 µg/ml hog pancreatic α-amylase. B) 1 mg/ml Cj0511, C) 2 mg/ml Cj0511, D) 3 mg/ml Cj0511, E) 4 mg/ml Cj0511 pre-incubated with 5 µg/ml hog pancreatic α-amylase at 37˚C.

4.3 Discussion

The study of proteases secreted by bacteria and their subsequent function in host environments is of significant importance. In general, proteases affect pathogenesis in two ways. They are involved in protein quality control which is necessary for the turnover of unfolded proteins produced in hostile environments within the host (Ingmer and Brondsted, 2009). There is evidence that suggests a conserved role in specific and controlled proteolysis of regulatory proteins in response to temporal, spatial or environmental stimuli

(Ingmer and Brondsted, 2009). These extra-cytoplasmic peptidases are attractive targets for the development of antimicrobial drugs. It has been highlighted that secreted peptidases are important virulence factors, *C. jejuni* produces a number of peptidases, of these Cj0511 has been found to be cell surface-associated (Prokhorova *et al*., 2006, Elmi *et al*., 2012). However the exact function of this protease has yet to be elucidated. Champion *et al* (2010) showed that a *Cj0511* mutant was less toxic in *G. mellonella* model and concluded that the predicted protease-encoding gene warrants further investigation.

This study has shown that induction of EPM in *C. jejuni* in response to α-amylase (Chapter 3) requires a functioning *Cj0511* gene. In contrast to the wild-type strain, *C. jejuni* 11168H, the *Cj0511* mutant was unable to express increased levels of carbohydrate in response to α-amylase, however, when the mutant was complemented with a wild-type copy of *Cj0511,* wild-type response was restored.

In chapter 3 it was shown that exposure to physiological concentrations of mammalian pancreatic α-amylase during growth in liquid culture promoted growth of *C. jejuni* 11168H. However, when mammalian pancreatic α-amylase was incorporated into SAPI agar as the sole source of exogenous carbon the *Cj0511* mutant was unable to grow, whereas the wild-type strain did. Furthermore upon complementation of *Cj0511* this ability to grow on α-amylase-supplemented SAPI agar was restored. This data suggests that proteolytic activity of Cj0511 on α-amylase is required for *C. jejuni* to utilise α-amylase   as a nutrient source.

Subsequently recombinant Cj0511 was tested first for activity on azocaesin using trypsin as control, and determined the protein as proteolytically active. Secondly, pre-incubation of α-amylase with Cj0511 resulted in a reduction in the activity of α-amylase. The combination of caesinolytic activity and reduced amylase activity suggests that the reduction in amylase activity is due to proteolytic degradation of amylase.

The results show that *C. jejuni* is able to grow on agar with pancreatic α-amylase as the sole source of carbon, the *Cj0511* mutant was unable to do this, when the complemented strain was tested wildtype response was restored. This together with the proteolytic degradation of amylase suggests the ability to metabolise as an energy source is depended of Cj0511 activity. Tripeptide and dipeptide metabolism in *E. coli* K12 have been shown to begin by proteolytic breakdown and peptide uptake. The primary mode of uptake of these peptides in bacteria is via ATP binding cassette (ABC) transporters. The substrate-binding protein (SBP) of these transporters captures extracellular peptides and delivers them to membrane-bound components for transport. Bacterial peptide-binding SBPs that function in ABC transporters have evolved predominantly to function as generalists, recognising peptides of particular lengths but with low or no sequence specificity. However, within the peptide-specific SBPs are examples that appear to have evolved subsequently to recognise specific peptides. *C. jejuni* contains homologues of a number of ABC transoprters and SBP; furthermore it also has homologues for tri/dipeptide transporters, making the above response feasible.

This study shows that Cj0511 degrades pancreatic α-amylase. According to the MEROPS peptidase database (Sanger, UK) Cj0511 is predicted to be a C-terminal processing peptidase and belongs to clan SK and family S41. Chlamydial protease-like activity factor (CPAF) from *Chlamidya spp.* is also in this family and it has been shown to be associated in the proteolysis of at least 16 host proteins (Chen *et al*., 2012). Similar C-terminal proteases in organisms such as *E. coli* have been shown to be involved in the universal degradation of proteins tagged by a C-terminal sequence. Therefore based on the sequence homology shared between Cj0511 and the aforementioned proteases it can be assumed that Cj0511 functions in a similar fashion; processing pancreatic α-amylase to produce a C-terminal peptide that can be can be taken up by ABC transporters and recognised by *C. jejuni.* The finding that stable and proteolytically active Cj0511 can be expressed by *E. coli* is highly relevant as this will facilitate in future work with development of specific inhibitors. The study of bacterial proteases as future drug targets is promising. Slomiany *et al*. (1992) found successful treatment of *H. pylori* infection using

anti-ulcer drug ebrotidine was in part due to its strong inhibitory action of *Helicobacter* proteases involved in degrading gastric mucin, which is a growth promoter in *H. pylori* (Chowers *et al*., 1999).

Chapter 5: Biofilm Formation and Stress Survival

5.1 Introduction

5.1.1 History of biofilm formation

In their natural environment bacteria are most commonly found in close contact with surfaces and form multicellular aggregates known as biofilms. A biofilm can be defined as a structured community of bacterial cells enclosed in a self-produced extracellular polymeric matrix (EPM) and adherent to an inert or living surface (Costerton *et al.*,1999). This tendency to exist as a multicellular community is comparable to other living cells which are capable of unicellular growth but reside within multicellular communities (Davey and O'Toole, 2000). The perception of unicellularity in bacteria is based on the pure culture paradigm, since bacteria can be diluted to a single cell and studied in liquid culture, which was advocated in the Koch's postulates by Dr Robert Koch (Koch, 1876). The study of planktonic bacteria has become the standard method to study a variety of bacterial activities.

The sessile mode of bacterial growth was not described until 1933, when Arthur Henrici, an American microbiologist studying fresh water bacteria stated , "It is quite evident that for the most part the water bacteria are not free floating organisms, but grow on submerged surfaces; they are of the benthos rather than the plancton"(Henrici 1933). Then in 1940 H. Heukelekian and A. Heller published their work in the *Journal of Bacteriology*, stating "Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as bacterial slime or colonial growth attached to surfaces" (Heukelekian and Heller. 1940)

Ten years later it was reported that bacteria introduced into a bottle disappear from the liquid phase; concurrently there was a rapid increase in the number of bacteria attached to the surface of the bottles. This bacterial adhesion was accompanied by an increase in the amount of suspended organic material which was termed 'mucilaginous' in other words gelatinous or mucoid. Zobell

concluded that the bacteria attached themselves to the glass surface where they encountered a concentration of organic nutrients much higher than would have been found in the bulk fluid (Zobell, 1943).

5.1.2 Growing biofilms *in vitro*

Today there are a number of ways to grow biofilms and study them under laboratory conditions. The first method is culturing bacteria on solid agar. In the strictest sense a colony grown on an agar plate is a biofilm; they are colonies of bacteria growing attached to a solid surface.

Another method, considered a gold standard for biofilm study, is continuous culture using a flow cell, in which a submerged biofilm is formed on the internal surface of the cell (Christensen *et al.,* 1999). This method allows the biofilm to be tested against antimicrobials and other environmental stresses, and also allows analysis by confocal scanning laser microscopy (CSLM). The use of microscopy has allowed the determination of biofilm architecture and composition using a number of fluorescent staining methods (O'Toole and Kolter, 1998). Once the biofilm is formed it is stained with dyes such as crystal violet or safranin, which are subsequently solubilised, and the absorbance is taken as a measure of biofilm formation (O'Toole *et al.*, 1999).

The environment in which bacteria find themselves determines the architecture of the biofilm and this can vary greatly in response to miniscule changes in the ambient environment, for example it has been shown that biofilm formation by *Bacillus subtilis* strain 3610 varies on medium solidified with different concentrations of agar, the morphology of the biofilm decreases in size and structure as the concentration of agar increases (Branda *et al.*, 2006).

Difference in biofilm architecture can arise from differences in constituents which make up the extracellular matrix or EPM. The EPM of biofilms has been shown to be composed of polysaccharide, protein and/or extracellular DNA as well as phospholipids (Davey and O'Toole, 2000; Chandra *et al.*, 2001).

5.1.3 Biofilm Formation

Biofilm formation can be divided into four stages: initial attachment, irreversible attachment, maturation and detachment (Watnick and Kolter, 2000).

Once a bacterium approaches the surface it is to attach to, van der Waals forces of attraction bring the bacterium close to the surface and are active at a distance of a few nanometres. Bacterial cell surfaces and the surfaces of many environmental surfaces possess a net negative electrostatic charge resulting in a repelling force as the bacterium move towards the surface; this is effective at a distance of 10 nm. Once the bacterium has closed the distance between itself and the surface to approximately 5 nm through secondary Gibbs free energy, receptors on the bacterial cell surface such as pili and adhesins begin to interact with the surface (Hermansson, 1999).

When the bacterium is in such close proximity to the surface, the flow velocity is reduced resulting in a reduction in friction and turbulence which encourages attachment. Once this physical contact is achieved, the initial attachment which maybe a transient reversible attachment or a stable irreversible attachment occurs (Kolari, 2003). Once attachment has taken place, the pioneer bacteria that are the initial colonisers begin to recruit other bacteria through quorum sensing leading to the formation of microcolonies; these pioneer bacteria facilitate the arrival of other bacteria by providing more diverse adhesion sites. Studies in *Pseudomonas aeruginosa* have shown that initial attachment causes the quorum sensing induction of genes responsible for the production of compounds found in the EPM for developing biofilms such as the genes of the alginate pathway. Alginate is a major component of EPM in this organism. It has been shown that attachment causes a major change in phenotypic expression in biofilm forming *P. aeruginosa* and may involve a change in expression of at least 30% of the genome (Davies *et al.*, 1998).

Once microcolonies are formed, the bacteria begin producing the EPM, which consist primarily of polysaccharides but can be composed of a mixture of extracellular protein and DNA.

5.1.4 Role of the extracellular polymeric matrix (EPM)

The structure and architecture of biofilm are provided by the EPM which, in many cases is highly hydrated and tightly bound to the underlying substratum. The structure of the matrix is heterogeneous within a bioflm and the formation of channels allows the transport of oxygen and essential nutrients to the cells present within the matrix (Lewandonski, 2000).

It has been found that bacteria such as *Klebsiella pneumonia* present within a biofilm grow slower than bacteria in planktonic culture; this is due to changes in oxygen concentration and limited nutrients (Wentland *et al.,* 1996). Cells detach from the biofilm as a result of either cell growth and division or the removal of biofilm aggregates that contain masses of cells. The microorganisms inside the biofilm matrix are able to avoid antimicrobial agents and in some cases a 100-fold increase in antibiotic concentration has been shown to be required to kill the biofilm organisms compared with that required to kill the same bacteria in planktonic form (Jefferson, 2004).

There are three possible explanations for this phenomenon. The first is that the EPM impedes diffusion of, or chemically reacts with, the antimicrobial. Studies have shown the EPM of *P. aeruginosa* 579 composed mainly of acetylated β-D-mannuronic acid and α-L-guluronic acid is capable of binding tobramycin (Hoyle *et al.*, 1992)

Secondly, as bacteria present within a biofilm exhibit reduced growth rates, this minimises the rate at which antimicrobial agents are taken up into the cell. A study in *Staphylococcus epidermidis* found that ciprofloxacin activity was influenced by the cell cycle with daughter cells being more susceptible than other populations in the biofilm (DuGuid *et al.*, 1992). The actions of antibiotics such as penicillin, which target cell wall synthesis, affect only bacteria which are growing. The depletion of substrates and accumulation of waste products which have an inhibitory effect results in bacteria entering a stationary phase

in which they are protected from antimicrobial killing, as demonstrated in *E. coli* with increasing generation times resulting in the bacteria showing increasing phenotypic tolerance to β-lactam antibiotics (Tuomanen *et al.*, 1986). These findings are further supported by direct visualisation of metabolically inactive regions within *P. aeruginosa* biofilms grown in continuous culture (Goto *et al.*, 1999).

Finally, the availability of oxygen may affect susceptibility. Tresse *et al.* (1995) demonstrated that agar enclosed *E. coli* demonstrate a decreased susceptibility to aminoglycoside antibiotics as a result of decreased uptake of the antibiotic by the oxygen-deprived cells (Tresse *et al.,* 1995).

5.1.5 *C. jejuni* biofilm EPM

One of the major withstanding questions with regards to *C. jejuni* biofilms is the composition of the EPM. The EPM is what holds the biofilm together, providing structure and strength. In the case of *C. jejuni* biofilms, the best characterised polysaccharide, the CPS, is not involved in biofilm formation as shown by Joshua *et al.,* (2005) who reported no reduction in the biofilm forming capacity of a *C. jejuni* NCTC 11168 *kpsM* mutant. KpsM consist of six putative trans-membrane regions and is situated in the inner membrane, and is a component of an ABC transporter involved in capsule transport through the inner membrane (Karlyshev *et al.*, 2000).

Another polysaccharide which has been described, is a calcoflour white (CFW) - reactive polysaccharide. CFW is a fluorescent stain which binds β1-3 and β1-4 sugar linkages. McLennan *et al.* (2008) showed that *C. jejuni* biofilm produces a CFW-reactive polysaccharide; they found biofilm formation is up-regulated in the absence of a stringent response, a stress response generally triggered by nutrient deprivation, which is governed by SpoT. When *spoT* was mutated, an increase in the level of CFW-binding polysaccharide was observed (McLennan *et al.,* 2008).

Furthermore, LOS and *N-* and *O*-linked glycoproteins have been shown to not be involved in biofilm formation. These are all glycans which have been ruled

out. McLennan *et al* (2008) tested a *gne* mutant and found no defect in biofilm formation. Gene *gne* encodes a bifunctional UDP-GlcNAc/Glc 4 epimerase involved in the synthesis of CPS, LOS, and *N*-linked carbohydrates (Bernatchez *et al.*, 2005).

5.1.6 Environmental stresses

C. jejuni remains a paradox: in the laboratory it requires fastidious conditions to grow, however in its natural environment it is able to survive surprisingly well. The ability of *C. jejuni* to transmit between hosts and cause diseases may be explained partly by the low infectious dose in comparison to other gastrointestinal pathogens such as *Salmonella typhimurium* and *Vibrio cholerae* (Schmid-Hemple and Frank, 2007).

This may account for the bacterium's ability to cause infection but does not explain how the bacterium is able to survive environmental stress. The stresses endured by *C. jejuni* from its natural reservoir in the chicken caeca to the GI tract of humans, is varied involving changes in temperature, pH and oxygen concentrations.

Examples of proteins which have an integral role in the ability of *C. jejuni* to adapt to environmental changes include HtrA that prevents periplasmic proteins from aggregating by degrading proteins which are misfolded during stress (Li *et al.*, 1996) Studies have shown transcription of *dnaJ* is up-regulated when *C. jejuni* encounters temperature stress. DnaJ plays a significant role in the thermotolerance of *C. jejuni* by facilitating protein folding (Stintzi, 2003).

Alternatively, mucoid growth of bacteria which can be defined as 'biofilms' have been shown to exhibit increased survival to environmental stresses. Mucoid strains of *P. aeruginosa* which are characterised by excessive amounts of the exo-polysaccharide alginate showed a significant increase in viability within chlorinated water in comparison to isogenic non-mucoid cells. When alginate was removed, survival was reduced (Grobe *et al.*, 2001).

5.1.7 Hypothesis

The hypotheses being addressed in this chapter are:

1. Induction of the EPM by exposure to pancreatic α-amylase promotes biofilm formation in *C. jejuni*.

2. Induction of the EPM increases resistance to environmental stresses.

5.2 Results

5.2.1 *C. jejuni* exhibits increased autoagglutination in response α-amylase

Autoagglutination (AAG) activity is thought to be a precursor for micro-colony formation and can be measured by assessing the reduction in absorbance at OD_{600} over time, because the suspended bacteria precipitate at the base of the cuvette after they agglutinate. Figure 5.1 shows that both *C. jejuni* 11168H and 81-176 wild-type strains grown in the presence (Chapter 2: 2.13) of 100 nM α-amylase have higher rates of AAG than bacteria grown in the absence of α-amylase.
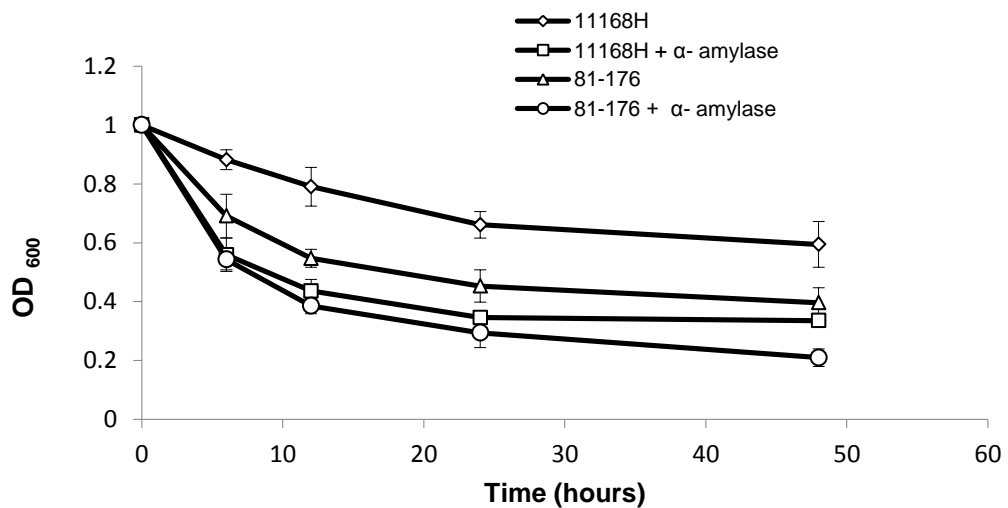
Figure 5.1. Autoagglutination activity of *C.jejuni* 11168H and 81-176 grown in the presence and absence of 100 nM α-amylase. The optical density at 600 nm ($OD_{600)}$ measured at 6, 12, 24 and 48 hours. Results shown are means from three independent experiments. Errors bars represent standard deviation.

5.2.2 Quantification of biofilm formation using crystal violet (CV) staining

The ability of *C. jejuni* strains to form biofilm on borosilicate glass test tubes at the liquid/gas interface was assessed in the presence and absence of 100 nM α-amylase. All the strains which were tested exhibited highly significant (p <0.001) increase in biofilm formation in the presence of α-amylase, with the exception of the *Cj0511* mutant (Figure 5.2). The magnitude of the increase seen in biofilm formation in response to α-amylase by carbohydrate structure mutants (*kpsM, spoT, waaC* and *waaF)* was similar to reference strain 11168H and strain 81-176, indicating that none of the known carbohydrate structures, capsule (*kpsM*), CFW binding polysaccharide (*spoT*) nor LOS (*waaC* and *waaF*) are involved in the response to α-amylase. Furthermore, these experiments definitively indicate that the ability of *C. jejuni* to form increased biofilm in response to amylase is dependent on Cj0511 as shown by the lack of response to amylase in the *Cj0511* mutant and its restoration in the complemented strain; which showed a similar magnitude of increase as reference strain 11168H.
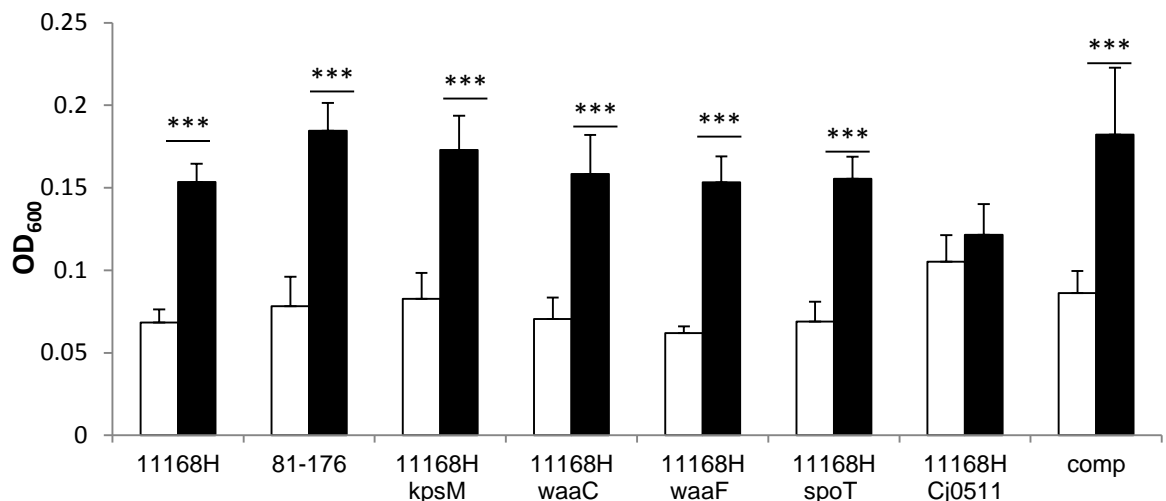
Figure 5. 2. *C. jejuni* biofilms grown in the presence (black bars) and absence (white bars) of pancreatic α-amylase. Results are presented as the mean OD_{600} of solubilised CV in three independent experiments. Errors bars represent standard deviation. Statistical significance was analysed using students T-test (*** = p <0.001).

5.1.3 Biofilm analysis by CLSM reveals a three-dimensional structure

Confocal laser scanning microscopy (CLSM) in conjunction with Live/Dead staining was used to visualise bacteria within an intact biofilm. Forty eight hour old *C. jejuni* 11168H biofilms grown on glass coverslips in the presence or absence of 100 nM α-amylase were compared. Confocal image and model of biofilms in the absence of α-amylase show a scarce number of bacteria adhering to the coverslip surface and no significant depth of biofilm structure in the 3-D model (Figure 5.3A-C). In contrast biofilm grown in the presence of α-amylase show a large concentration of bacteria adhering in the form of microcolonies separated by dark regions which are probably water filled channels (Figure 5.3B). Construction of a 3-D model and subsequent analysis of the X-Z plane, clearly show the development of characteristic matrix structure which has a constant depth of 160 microns (Figure 5.3D). Images shown are representative of three independent experiments.
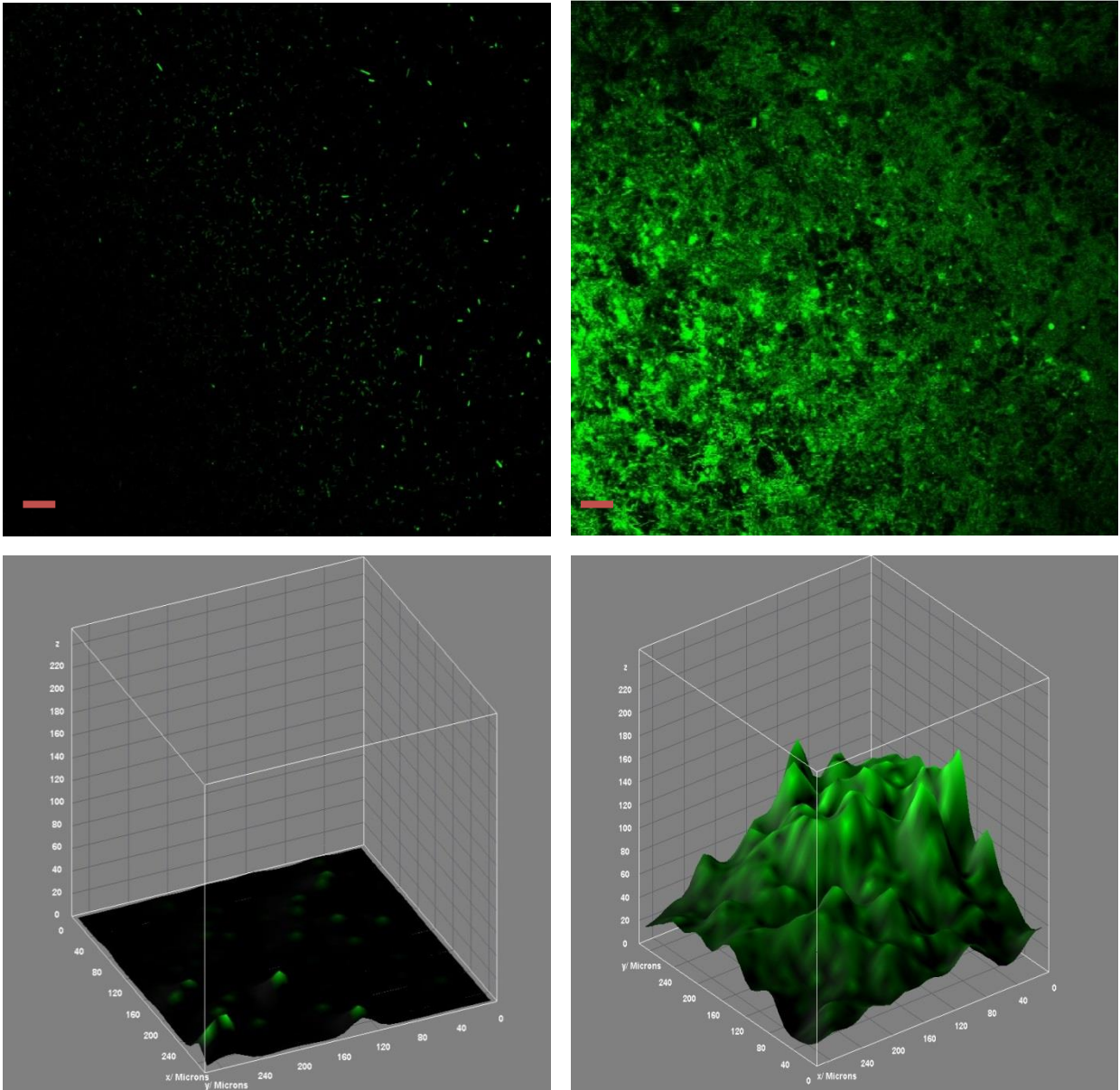
Figure 5.3. Confocal microscope images of Live/Dead stained *C. jejuni* 11168H biofilm attached to a glass coverslip at 48 hours images taken at x40 magnification A) 11168H without hog pancreatic α-amylase, B) 11168H with 100 nM hog pancreatic α-amylase. Graphical depiction of vertical density of biofilm C) 11168H without α-amylase, D) 11168H with 100 nM α-amylase. The digitally manipulated 3D images and graphical representation of the biofilm were produced using ImageJ software (NIH, USA). Scale bar represent 10μm

5.1.4 Biofilm kinetics using a strain expressing green fluorescent protein (GFP)

The increased biofilm observed in response to α-amylase suggests that the rate of growth of biofilm in the presence and absence of amylase may differ. Therefore biofilm formation in the presence and absence of 100 nM α-amylase was monitored by CLSM in a time course over 48 hours using a GFP-tagged strain of *C. jejuni* 11168H. At 3 hours (Figure. 3 A and F), no difference in the attachment of bacteria was seen with sparse attached cells being seen in both sample. At 6 hours, the formation of microcolonies was observed on both sets of coverslips, with no evident differences observed. At 12 hours, there is a visible increase in the number of bacterial cells attached to the coverslip in the presence of α-amylase. At 24 hours the difference observed between the sets was clearly visible; the α-amylase supplemented culture had grown into a biofilm with a large biomass of cells attached to the surface, in comparison to sparse isolated clumps of attached cells observed in the biofilm grown without α-amylase. At 48 hours, there was further biomass accumulation in the presence of amylase. The image is indicative of a mature biofilm with large amounts of cellular biomass covering the surface of the coverslip. At this same time point the bacteria growing in the absence of amylase were beginning to show signs of biomass localisation and initial biofilm formation, however this is substantially lower and slower than the bacteria grown in the presence of α-amylase. Images shown are representative of three independent experiments.
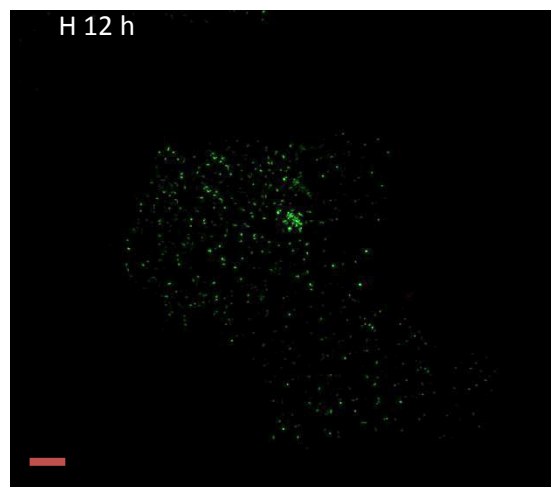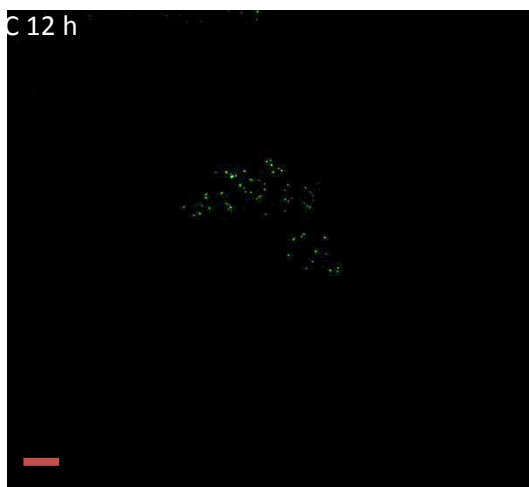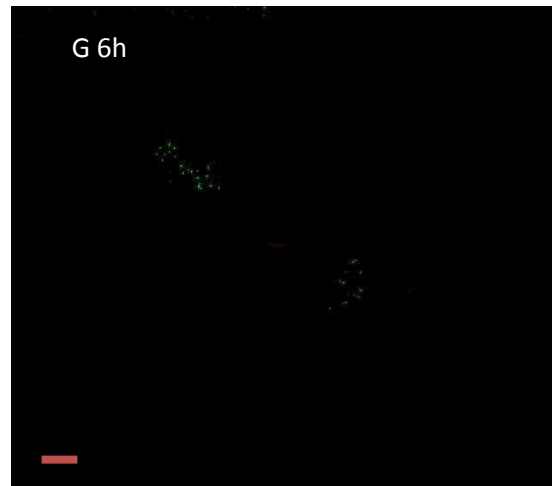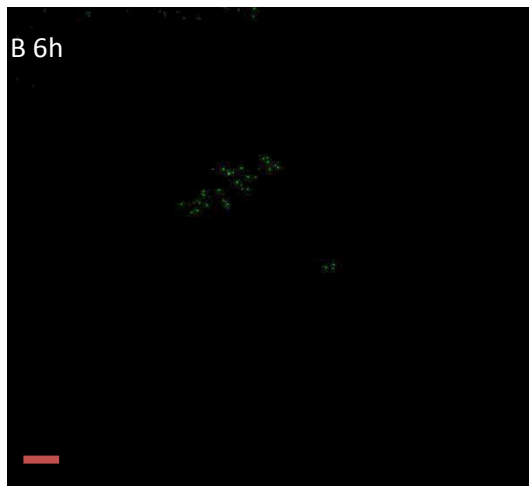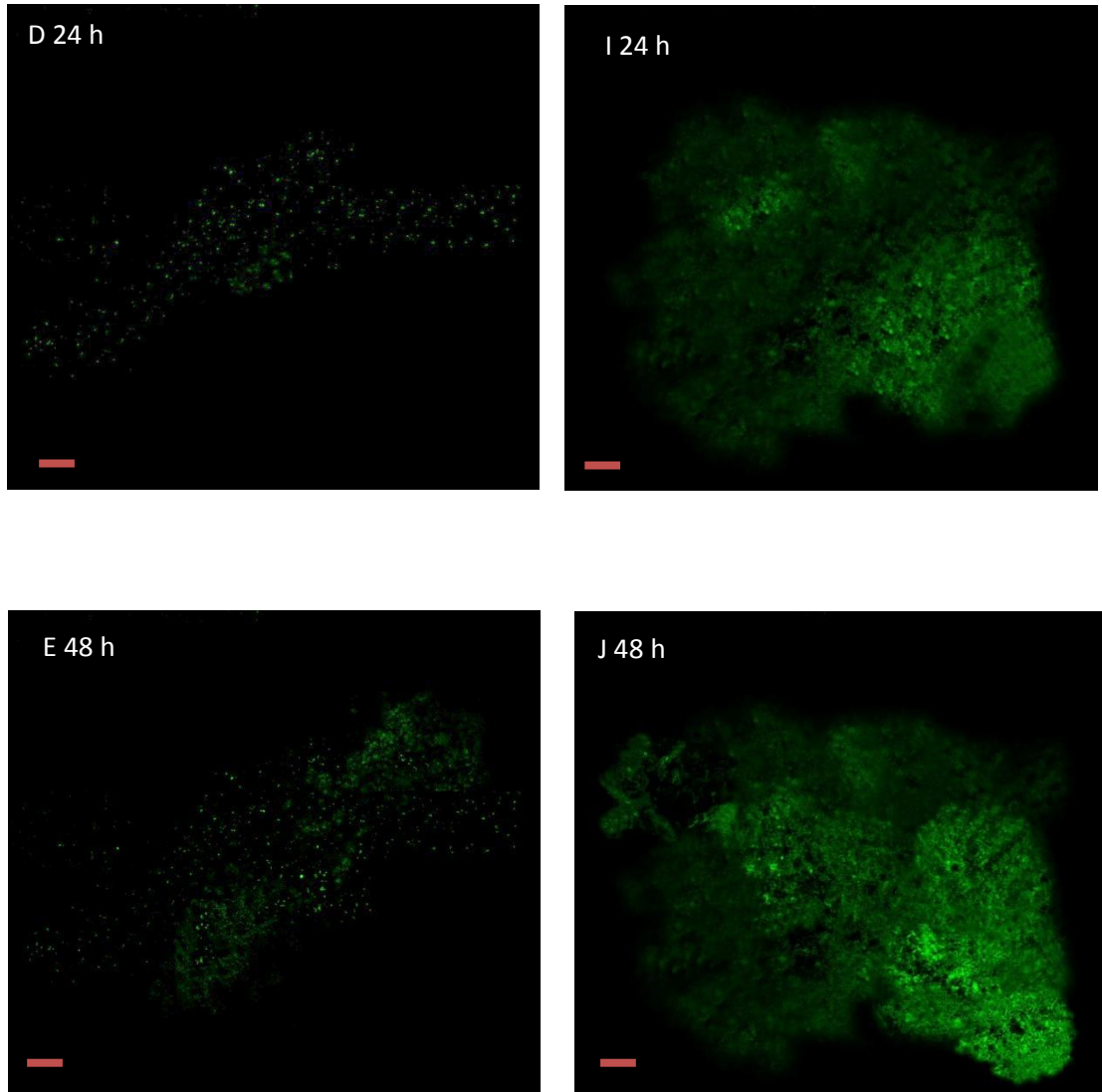
Figure 5. 4. Kinetics of biofilm formation over 48 hours by GFP-tagged *C. jejuni* 11168H. Images were taken using confocal microscopy x20 lens at time points 3, 6, 12, 24 and 48 hours. Images A-E are grown in Brucella broth only and images F-J are supplemented with 100 nM hog pancreatic α-amylase. Scale bars represent 50 μm. Images shown are representative of three independent experiments.

5.1.5 Electron microscopy show distinct differences in biofilm structure

To validate the CV staining data, SEM was used to visualize biofilms of 11168H in the presence and absence of α-amylase at the ultra-structural level. Biofilms were grown on polycarbonate filter disks placed on MHA plates, the disks were removed and placed on fresh plates every 24 hours for a total of 5 days, and processed for SEM. The concentration of α-amylase was increased to 100 nM see if the structural differences in biofilm would increase and the time of 5 days was chosen to ensure that biofilm maturation was achieved. Figure 5.5 shows that biofilms grown in the presence of α-amylase show high density of cells attached to the polycarbonate surface in multiple layers with evidence of mucoid secretions between the cells (highlighted by red arrows). In contrast biofilm grown in absence of α-amylase show only sparse bacteria adhered to the polycarbonate surface, at identical magnifications. Images shown are representative of three independent experiments.
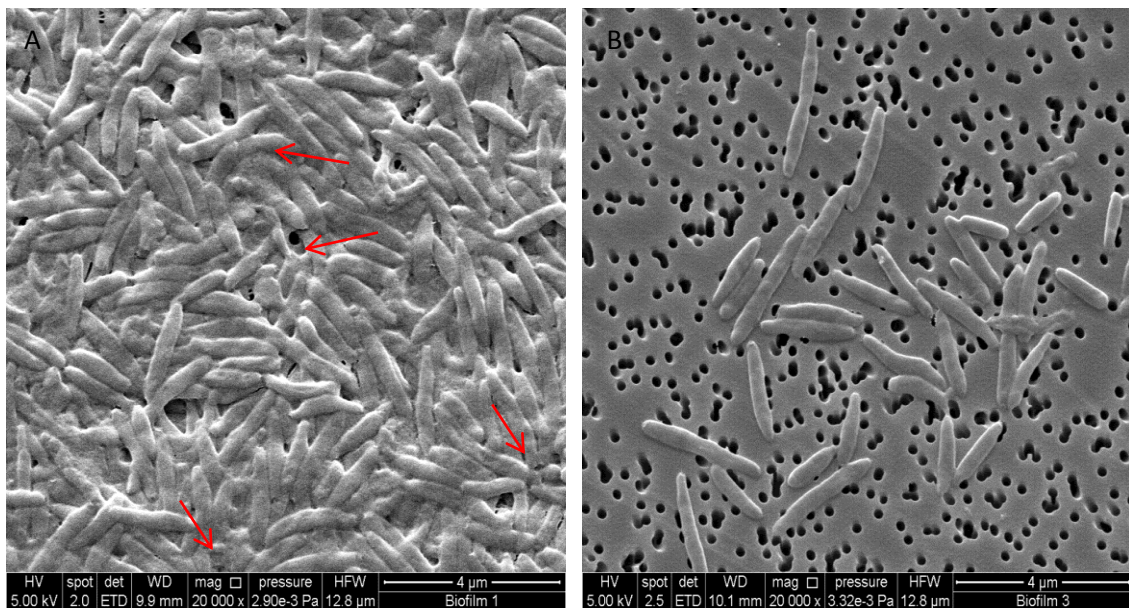
Figure 5.5. SEM of 5-day biofilms formed on 0.2 μm polycarbonate filter disks placed on MHA with (A) and without (B) 100 nM pancreatic α-amylase. Images shown are representative of three independent experiments.

5.1.5 *C. jejuni* **exhibits increased autoagglutination and biofilm formation in response to chicken pancreatic extract**

To test whether *C. jejuni* 11168H was able to respond to chicken pancreatic α-amylase, autoagglutination and biofilm formation were examined. A crude chicken pancreas extract was prepared and tested. An increase in autoagglutination was observed with the crude pancreatic extract that was of similar magnitude to the increase apparent with pancreatic α-amylase (Figure 5.6A). An increase in biofilm formation of similar magnitude (2.2-fold, $p<0.0001$) as the increase apparent with pancreatic α-amylase (Figure 5.6B) was also observed. As a control in both experiments, 0.05 M Tris pH 8.0 was supplemented into the growth medium as this was the buffer used to prepare the pancreatic extract.

A

B
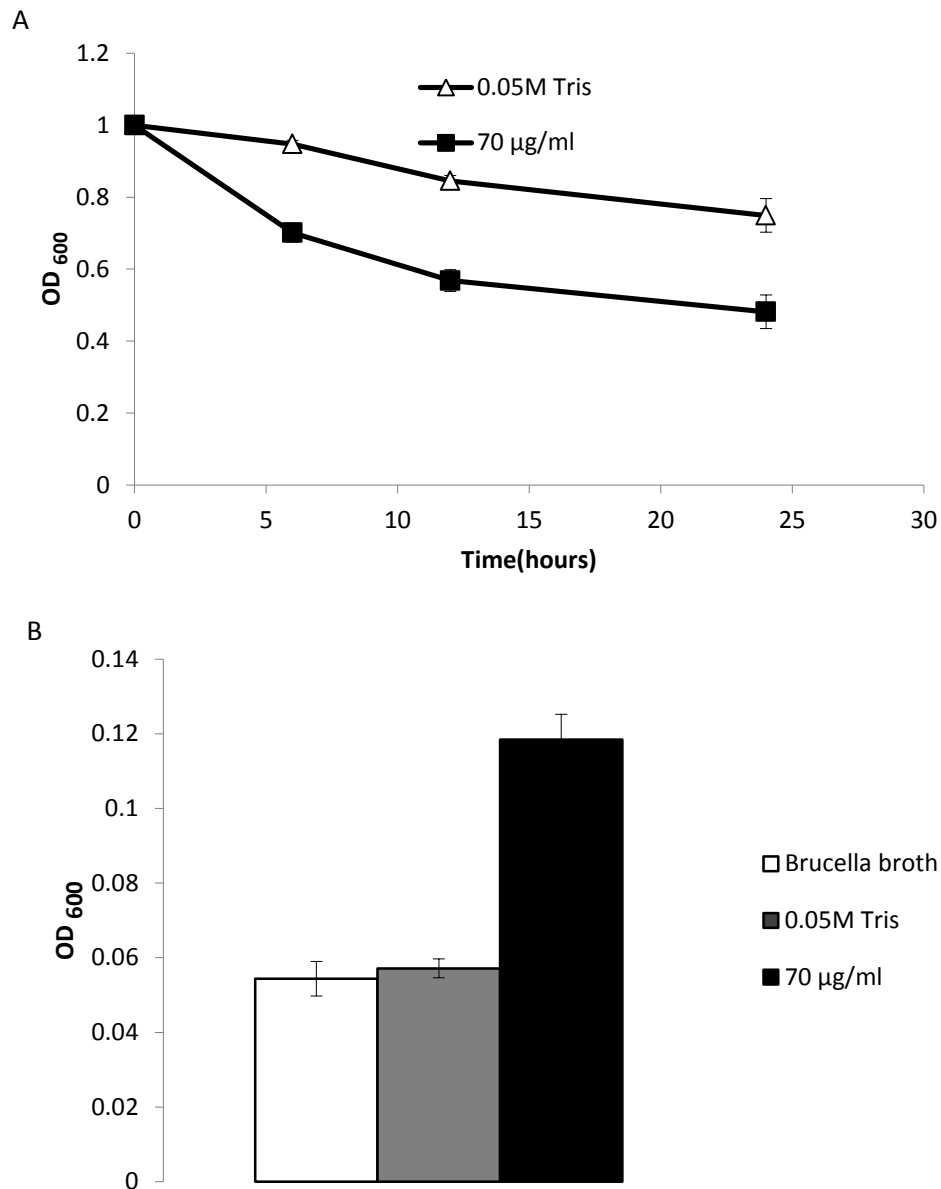
Figure 5.6. A) Autoagglutination activity of *C. jejuni* 11168H in the presence of 70 µg/ml α-amylase and 0.05 M Tris. The optical density at 600 nm (OD_{600}) was measured at 6, 12, and 24 hours. Results shown are means from three independent experiments. Error bars represent standard deviation. B) *C. jejuni* biofilms grown in the presence and 70 µg/ml α-amylase, 0.05 M Tris and in Brucella broth. Results are presented as the mean OD_{600} of solubilised CV in three independent experiments. Error bars represent standard deviation. Statistical significance was calculated using students T-test (*** $p < 0.001$).

5.3 Survival of environmental stresses

5.3.1 EPM promotes increased survival under ambient conditions

To determine if pre-exposure of *C. jejuni* to α-amylase promotes survival in ambient conditions, *C. jejuni* pre-exposed and non-exposed to 100 nM hog pancreatic α-amylase were suspended in broth and viable counts were performed. *C. jejuni* pre-exposed to α-amylase were able to survive 10 days longer under ambient conditions in comparison to non-mucoid cells ($p<0.05$; Figure 5.7).
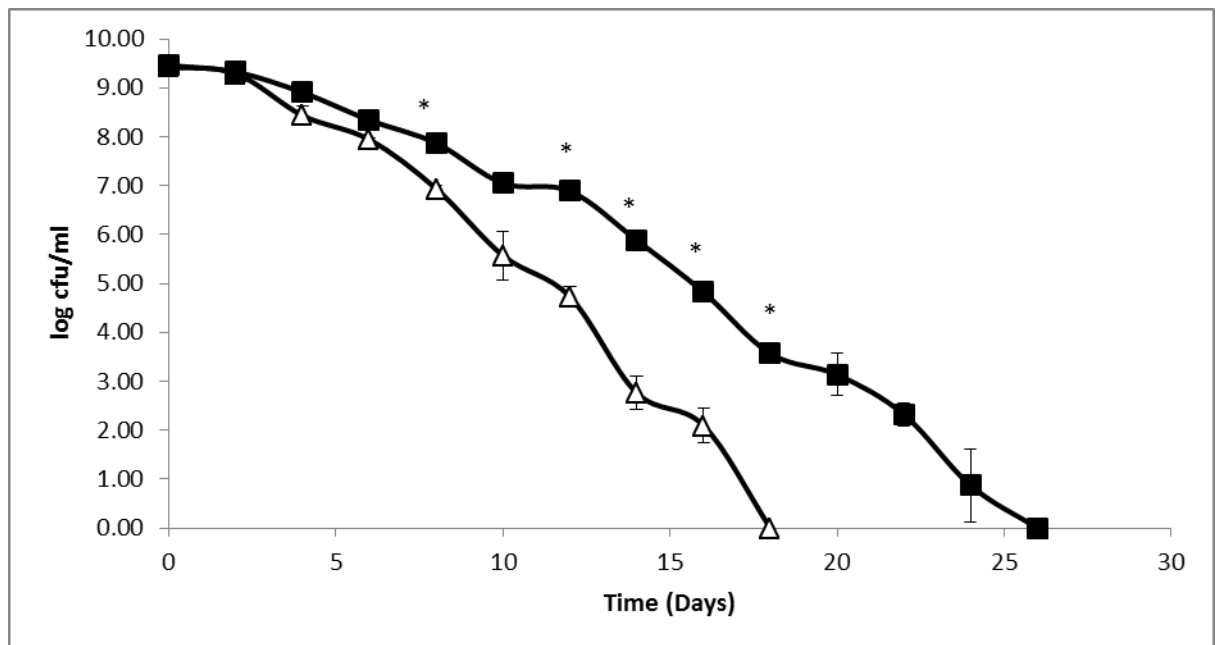
Figure 5.7. Survival of *C. jejuni* 11168H pre-exposed (filled square) or not pre-exposed (open triangle) to α-amylase in Mueller-Hinton broth under aerobic conditions at room temperature. Results are mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * ($p<0.05$) as determined by one way ANOVA.

5.3.2 EPM promotes increased survival at refrigeration temperature

To determine if pre-exposure to α-amylase affects survival under refrigeration, bacteria exposed and not pre-exposed bacteria were incubated aerobically at 4°C. *C. jejuni* pre-exposed to α-amylase were able to survive 8 days longer than bacteria that were not pre-exposed, exhibiting significantly ($p<0.05$) lower susceptibility to refrigeration (Figure 5.8).
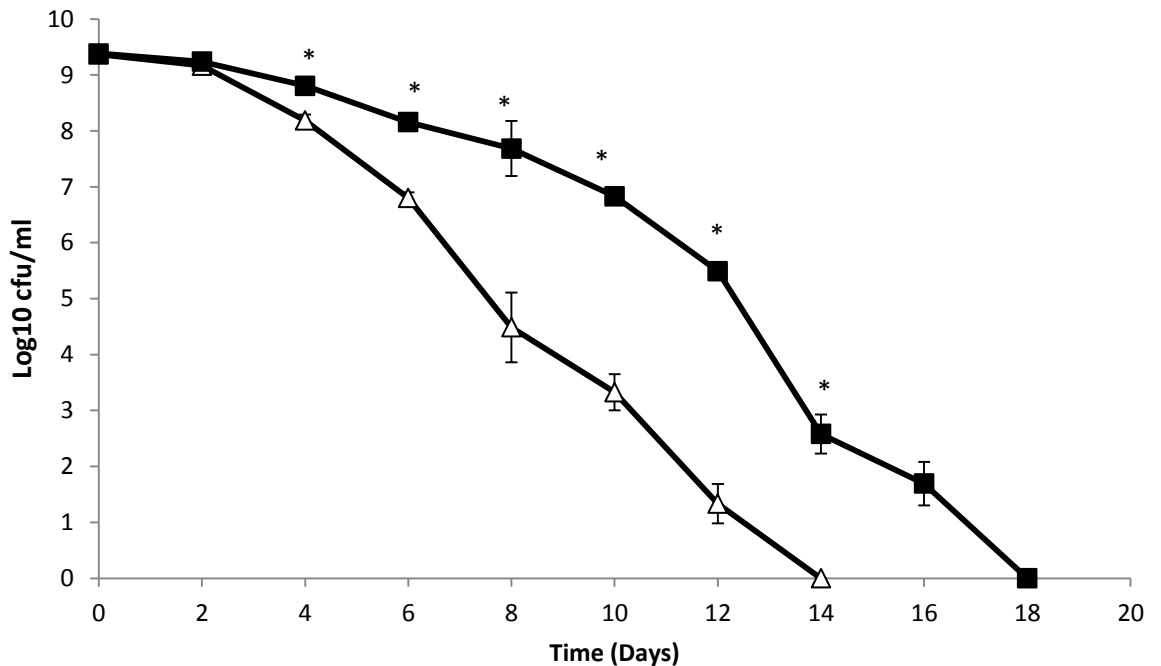
Figure 5.8. Survival of *C. jejuni* 11168H pre-exposed (filled square) or not pre-exposed (open triangles) to 100 nM hog pancreatic α-amylase in PBS under aerobic conditions at 4°C. Results are mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * ($p<0.05$) as determined by one way ANOVA.

5.3.3 EPM promotes resistance to high temperature

To assess the effect of high temperature on bacteria pre-exposed or not to α-amylase, *C. jejuni* grown in the presence and absence of 100 nM hog pancreatic α-amylase was suspended in PBS and incubated at 55°C, 60°C and 65°C. Bacteria pre-exposed to α-amylase showed significantly lower susceptibility to high temperature compared to bacteria that were not pre-exposed (Figure 5.9).

Non-exposed cells exhibit greater total log reduction 7.8, when compared to pre-exposed bacteria which showed a log reduction of just 4.6 over 21 minutes incubation at 55 °C (Figure 5.9 A).

When incubated at 60°C, the rate of reduction for non-exposed cells was 1.0 log per minute in comparison to 0.5 log per minute for pre-exposed cells. The difference between the two sets of cells was not significant at 3 minutes, however at later time points (6, 9, and 12 minutes), the difference in survival was significant ($p<0.05$; Figure 5.9 B).

At 65°C, the rate of reduction was increased to 1.5 log per minute for non-exposed cells and 0.7 log per minute for pre-exposed bacteria. Again at 3 minutes the difference between the two was considered non-significant, however at 6 minutes the difference was significant ($p<0.05$; Figure 5.9 C).
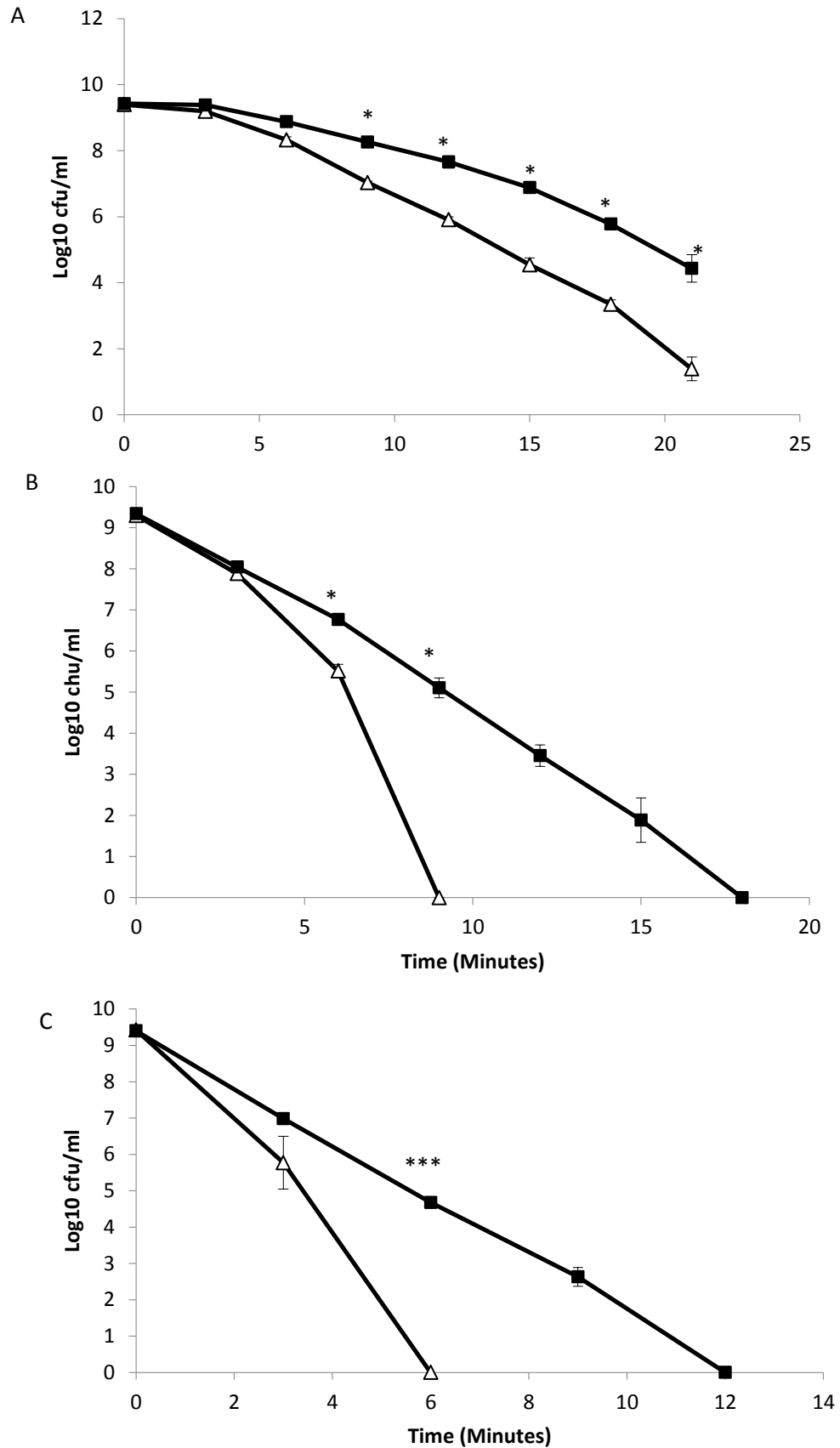
Figure 5.9. Survival of *C. jejuni* 11168H pre-exposed to α-amylase (filled squares) and with no pre-exposure (open triangles) in PBS under aerobic conditions at A) 55°C, B) 60°C and C) 65°C. Results shown are the mean counts of three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * (*p*<0.05) as determined by one way ANOVA.

2.3.4 EPM promotes survival in acidic conditions

To assess if pre-exposure to α-amylase affects survival under acidic conditions, bacteria were grown on agar in the presence or absence of 100 nM hog pancreatic amylase, and suspended in PBS which was buffered to pH 4.0 or pH 2.0. Bacteria pre-exposed to α-amylase showed significantly ($p<0.05$) higher pH tolerance in comparison to bacteria that were unexposed. The rate of reduction at pH 4.0 for pre-exposed cells was 0.04 log per minute and 0.03 log per minute for unexposed cells (Figure 5.10 A). At pH 2.0 unexposed cells showed a log reduction 0.077 per minute and for pre-exposed cells a log reduction 0.044 per minute was observed (Figure 5.10 B).

A
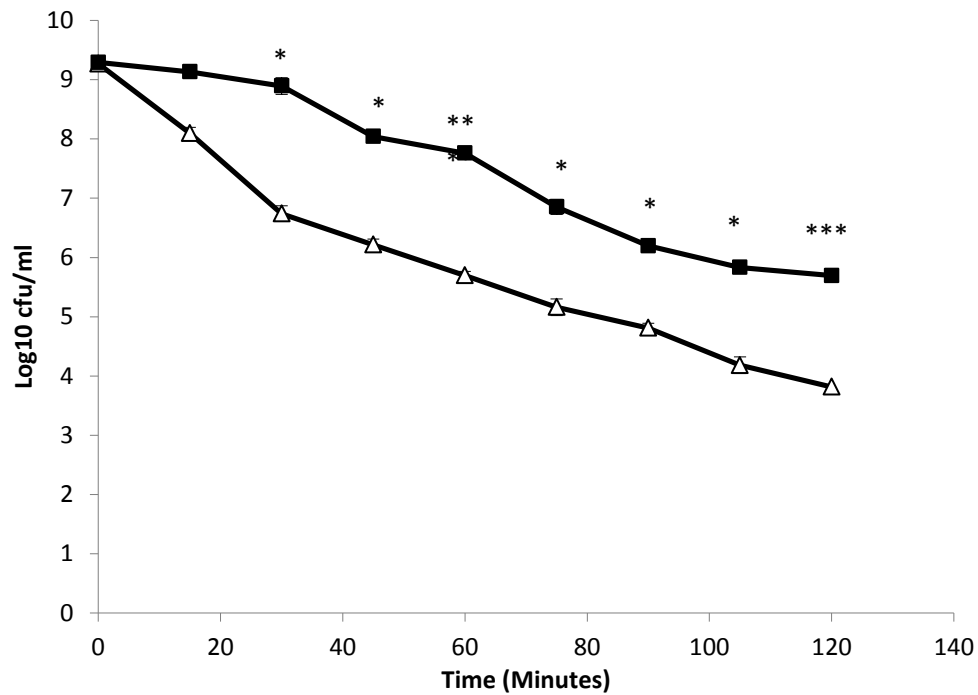
B
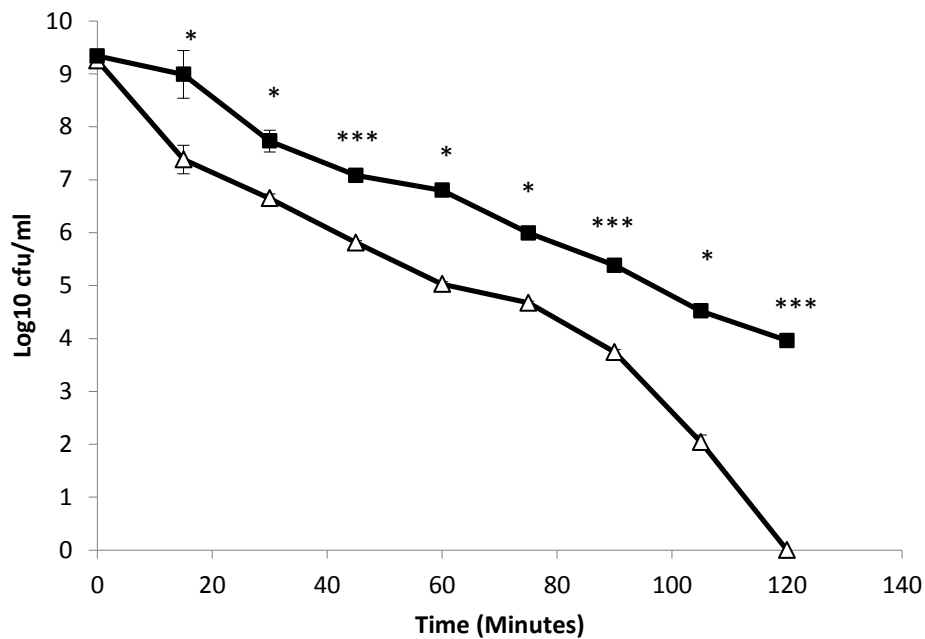
Figure 5.10: Survival of *C. jejuni* 11168H pre-exposed (filled squares) or not pre-exposed (open triangles) to 100 nM hog pancreatic in PBS under aerobic conditions at A) pH 4.0 B) pH 2.0. Results shown are the means viable counts in three independent experiments. Error bars represent standard deviation. Statistical significance is denoted by * (*p*<0.05) as determined by one way ANOVA.

5.3 Discussion

One of the challenges in understanding the diseases caused by *C. jejuni* is how the bacterium is able to survive in the environment and be transmitted from host to host. One of the explanations put forward is that existing in a biofilm allows *C. jejuni* to survive stresses encountered in the environment (Reuter *et al.*, 2010). However, there is little known about the factors which initiate *C. jejuni* biofilm formation, and determining these factors is important for developing adequate control measures.

The data presented in this chapter show that *C. jejuni* produces significantly more biofilm in response to pancreatic α-amylase using the crystal violet quantification method and this response is independent of the previously documented carbohydrate structures (CPS, LOS and CFW-binding polysaccharide). The data presented in Chapter 3 shows that exposure to pancreatic α-amylase induces the secretion of an EPM comprised of a novel α–dextran, whilst the results presented in this chapter show that this promotes formation of a biofilm. Furthermore the response to α-amylase requires Cj0511 activity, since the *Cj0511* mutant showed no increase in biofilm formation in response to α-amylase, this in contrast with the complemented strain which showed a wild-type response. The *Cj0511* mutant exhibited a slight increase in biofilm formation when grown without α-amylase in comparison to the wild-type strain. Although this increase was not significant, an explanation could be that production of the predicted extracellular protease may inhibit biofilm formation. It has been shown that extracellular proteases Aur and SspA of *S. aureus* inhibit biofilm formation, furthermore it has been suggested that protein-mediated biofilm formation is controlled by expression of extracellular proteases in *S. aureus* (Marti *et al.*, 2010).

It has been proposed that the ability of *C. jejuni* to survive harsh conditions in a biofilm is related to the area covered, thickness and architecture of biofilms (Trachoo and Frank 2002). When the architecture of the *C. jejuni* 11168H biofilm was assessed using CLSM; the biofilm formed in the presence α-

amylase was approximately 8 times thicker, and showed a clear increase in biofilm across both X-Y and X-Z planes.

Biofilm formation kinetics was monitored using a GFP-labelled *C. jejuni* strain. Once in the human small intestine *C. jejuni* encounters mechanical pressure through the intestine by peristalsis caused by smooth muscle and mesentery. This movement is not particularly fast, a typical peristaltic wave lasts only a few seconds and travelling a few centimetres per second (Young, 2006). The results presented here suggest that biofilm is formed significantly faster in response to physiological concentrations of α-amylase which would provide a considerable advantage in colonising the host. Furthermore studies have shown that strains isolated from humans and chicken, which are pre-exposed to α-amylase, form micro-colonies on smooth surfaces quickly during incubation and then the colonies progressively grow and form interconnections forming a mature biofilm similar to the results displayed in this study (Moe *et al.*, 2009).

The primary sequence between mammalian and avian pancreatic amylases are 80% identical, *C. jejuni* 11168H was able respond to chicken pancreatic extract which contained approximately 70 μg/ml α-amylase by significantly increasing both autoagglutination and biofilm formation. This result is of considerable importance in two ways. Firstly, it is well known that *C. jejuni* is able to colonise the cecum of chickens in considerable numbers, forming *in vivo* biofilms would assist this process of colonisation and it has been suggested that biofilm formation is necessary for contamination of broiler meat with *C. jejuni* (Kudirkiene *et al.*, 2012). Secondly *C. jejuni* excreted into the slaughterhouse environment by chickens are pre-exposed to α-amylase and thus, based on the data from this study, form increased biofilm. This is supported by findings showing that colonised chickens shed up to 10^7 *C. jeuni* CFU/g of faeces and these isolates along with exhibiting mucoidy have been shown to form biofilm faster and thicker (Mead *et al.*, 1995; Moe *et al.*, 2009). The ability of food borne pathogens to form biofilms on environmental surfaces is an important matter, *C. jejuni* has been shown to form biofilm on stainless steel, glass and polypropylene, and is subsequently more resistant to antimicrobials than planktonic cells (Neutres and Chen. 2009). Further to

this, *C. jejuni* has the ability to form attached biofilm on the surfaces of domestic and farming watering supplies and these attached bacteria could potentially act as an active reservoir of infection for humans and domestic animals (Reeser *et al*., 2006). Biofilm formation in *C. jejuni* was first described by Joshua *et al.* (2005). Existing as a biofilm allows organisms to withstand host immune responses such as phagocytosis and as a community they can endure changes in pH, temperature and starvation of nutrients (Jenkinson and Lamont, 2005). Joshua *et al.* (2005) showed that *C. jejuni* is able to form both attached and unattached biofilm. The unattached aggregates of biofilm known as 'flocs' are more resistant to ambient air and may be important in survival and pathogenesis of the organism.

Campylobacter spp. lack the genes encoding the RpoS global stress response mechanism, the oxidative stress response factor SoxRS (positive regulators for the response to superoxide stress) as well as other stress response factors such as Lrp (global regulator of metabolism), ProU (high affinity osmoregulatory uptake of compatible solutes), CspA (major cold shock protein) and RpoH (alternative sigma factor regulating the heat shock response) (Park, 2002; Nachamkin and Blaser, 2001).

Studies have shown that acidic treatment at pH 4.5 has a significant bactericidal effect on *C. jejuni*, it has been recommended that the addition of organic acids in water supplies in poultry farms could significantly reduce the transmission of *C. jejuni* (Murphy *et al.*, 2003). The data obtained in this study documents that *C. jejuni* secreting EPM are able to withstand acidic conditions at pH 4 and pH 2 significantly longer highlighting that current control measures using acidic treatment in domestic water supplies are inadequate. Further to this, decreased acid sensitivity which is promoted by EPM secretion is necessary to colonise the human GI tract, C. *jejuni* must pass the inhospitable acidic conditions of the stomach where pH values as low as 2.0 are present, and the emptying time for the stomach is approximately 2 hours (Smith, 2003). Therefore *C. jejuni* must be able to withstand an acidic pH of approximately 2 for this amount of time if it is to reach its site of colonisation in the small intestine (Smith, 2003). EPM production in other bacteria such as by *E. coli* O157:H7 confer resistance to acidic stresses and it has been proposed that

this resistance allows the bacterium to persist in the GI tract (Mao *et al*., 2006). A study by Murphy *et al.* (2003a) showed a novel stress response to acid stress by a natural *C. jejuni* isolate, in which extracellular proteins are accumulated on the surface of the bacterium protecting it from acid stress. In chapter 3 it was described that a significantly increased quantity of extracellular protein and carbohydrate secreted in response to α-amylase, from this we can deduce that these secretions play an active role in stress tolerance (Murphy *et al*., 2003a).

Mesophilic bacteria such as *C. jejuni* must possess the capacity to resist cold temperatures (Panoff *et al*., 1999). These mechanisms are thought to be highly conserved; one method of tolerating chilled conditions is to alter the fatty acid composition in the outer membrane. *Listeria spp.* respond to cold conditions by increasing levels of branched fatty acids and *Salmonella spp.* increase levels of unsaturated fatty acids in cellular membranes (Russel, 2002). Although a similar response has been observed in *C. coli,* this was not the case for *C. jejuni*; a study by Hazeleger *et al.* (1998) found no significant changes in fatty acid composition.

Chilled storage is an important control measure in the food chain, studies have shown growth of *C. jejuni* at 31°C and ATP generation has been observed at temperatures as low as 4°C (Hazeleger *et al*.,1998). The FSA (2013) reported that 66% retail bought chicken are contaminated with *C. jejuni,* poultry is generally stored under refrigeration conditions and the ability to persist at such a low temperature would provide the bacterium with an advantage when transmitting from chicken to humans. *C. jejuni* 11168H grown in the presence of pancreatic α-amylase was shown to survive significantly longer in nutrient-deprived conditions at 4°C. This is supported by the view that existing within an EPM allows *C. jejuni* cells to survive starvation conditions and unfavourable temperatures (Reeser *et al*., 2007).

It has been reported that high temperature treatment of *C. jejuni* at 50°C and 60°C significantly reduces the bacterial load on the carcasses of chickens (Li *et al*., 2002). Specifically, treatment of broilers with hot water in slaughter houses is considered a means to eradicate *C. jejuni* in poultry, and this

method is reasonably successful when using it against *C. jejuni* not secreting EPM. However the results obtained in this chapter show that *C. jejuni* expressing the EPM exhibit a significantly lower susceptibility to heat treatment at 55°C, 60°C and 65°C. This ability to survive high temperature can explain why *C. jejuni* are able to survive and persist on broilers after heat treatment and inadequate cooking, playing an integral role in transmission of *C. jejuni.*

The phenomenon of EPM expression and its relevance to stress survival is probably best described in *P. aeruginosa,* the bacteria is able to secrete extracellular alginate, which confers protection against many stresses such as desiccation changes in pH and chlorination. Bacteria expressing alginate reveal enhanced survival compared with isogenic non-alginate producing cells in chlorinated water. Removal of alginate from bacteria abolishes chlorine resistance; conversely the addition of alginate to washed cells enhances cell survival (Grobe *et al.*, 2001).

Another feature of cells secreting EPM is their ability to autoagglutinate. Agglutination of bacteria was discovered by Chartin Roger in 1889. Autoagglutination has been noted in a number of Gram-negative pathogens, including *Vibrio cholerae, Neisseria gonorrhoeae*, and *Yersinia* (Chiang *et al.*, 1995; Laird *et al.*,1980). Furthermore there is practical evidence of a correlation between autoagglutination and biofilm formation (Reuter *et al.*, 2010; Howard *et al.*, 2009). The data shows that bacteria grown in the presence of α-amylase, expressing the EPM, show an increased autoagglutination activity, which may play a role in increased virulence, a hypothesis which is addressed in the next chapter.

In conclusion the findings of this chapter clearly show supplementation of physiological relevant concentrations of mammalian pancreatic α-amylase result in significant increase in the biofilm forming ability of *C. jejuni*, this increase in biofilm-forming ability is independent of all the major defined polysaccharide structures, additionally biofilm forming capacity was directly related to activity of Cj0511. The biofilm formed in response to α-amylase

formed quicker and was significantly thicker (8 times) in the X-Y and X-Z planes when assessed with CLSM. EPM producing *C. jejuni,* formed in response to α-amylase, were able to withstand environmental stresses such as changes in aerobic conditions, temperature and pH significantly longer than non-EPM producing cells.

This chapter is the first description of *C. jejuni* responding to a gastrointestinal constituent, α-amylase by increasing formation of biofilm. The findings presented are pertinent when considering development of future control strategies, as the majority of current research would not account for the fact that *C. jejuni* expelled from chicken faeces into the environment in a broiler house will be expressing EPM and will be more resistant to stress than *in vitro* grown bacteria.

Chapter 6: Role of the EPM in interaction with eukaryotic cells

6.1 Introduction

The efforts to reduce *Campylobacter* infections in humans is unequivocally connected to a further understanding of the biology of the pathogen and particularly the virulence mechanisms contributing to colonisation and pathogenesis (Dasti *et al*., 2010).

Despite the advances in the study of *C. jejuni* virulence through genome sequencing, the mechanisms by which *C. jejuni* colonises the host and causes disease are still relatively poorly understood in comparison to other enteric pathogens. A major reason for this is the lack of a practical animal model to study campylobacteriosis.

6.1.1 Pathophysiology of *C. jejuni* infection

Human infection begins after oral consumption of *C. jejuni*, with a low infective dose of 500 to 800 CFU (Berberian *et al*., 1994). After consumption, the ingested bacteria pass through the acidic environment of the stomach where a large percentage of the bacteria are eradicated (Smith. 2003). However this can be affected by the buffering capacity of food. The bacteria which survive and pass into the small intestine have the ability to attach to the mucus which covers intestinal epithelial cells. At this point the bacteria are able to replicate within the intestinal environment.

The outcome of this in humans can be two-fold: first, the bacteria can cause diarrheal disease, or the bacteria can remain in the intestine asymptomatically (Cawthraw *et al*., 2000).

6.1.2 Sources of *C. jejuni* infection

Epidemiological studies have shown that the majority of *C. jejuni* infections arise from exposure to contaminated food especially undercooked or raw chicken (Friedman *et al.,* 2003). Handling and ingesting raw undercooked poultry are predominately linked to *C. jejuni* infection; however infections can arise from drinking raw milk and consuming red meat, cattle and swine have been determined to maintain *Campylobacter spp*. Furthermore, untreated water has been determined as a potentially significant source of *C. jejuni* contamination, generally outbreaks are irregular but a number of epidemiological studies have traced the source of contamination back to water sources (Smith *et al*., 2005). An interesting phenomenon, particularly in water supply pipes on farms, is the formation of *C. jejuni* biofilms which act as a reservoir for *C. jejuni* to enter the human food chain. Although the general consensus is that eating undercooked poultry meat is the most important cause of Campylobacteriosis (EFSA, 2010).

6.1.3 Immune response to C. *jejuni*

Intracellular *C. jejuni* have been observed in individuals suffering from campylobacteriosis, supporting the notion that *C. jejuni* invades epithelial cells *in vivo* as occurs in *in vitro* experiments with cell lines (van Spreeuwel *et al*., 1985).

However, the mechanism of invasion has not been elucidated, a difficulty exasperated by the variation among *C. jejuni* strains. Many invasive bacteria use microfilament-dependent mechanisms of entry, however *C. jejuni* utilises microtubule and/or microfilment polymerisation which are required for maximum invasion (Monteville *et al*., 2003)

When internalised, *C. jejuni*-containing vacuoles (CCV) have been shown to progress along microtubules to the perinuclear region of the cell by interactions with dynein (Hu and Kopecko, 1999). CCV allows *C. jejuni* to survive within epithelial cells allowing by averting delivery to lysosomes (Watson and Galan, 2008).

The responses of intestinal epithelial cells to *C. jejuni* is characterised by cytokine induction, particularly IL-8, a pro-inflammatory cytokine (Hickey *et al*., 2000). The induction of IL‑8 results in the recruitment of dendritic cells, macrophages and neutrophils, the interaction between these cells and *C. jejuni* results in a considerable pro-inflammatory response (Hickey *et al*., 2000).

Infection of *in vitro* epithelial cell lines such as Caco‑2 and T84 results in the activation of ERK and p38 which are members of MAP kinase family proteins for T84 cells. ERK activation is essential for the stimulation of IL‑8. Similar observations have been observed with human intestinal tissue explants (MacCallum *et al*., 2005). Systemic diseases are rare in immunocompetent individuals (0.15%-0.8%), with diseases being restricted to the intestine in most cases (Kapperud *et al*., 1992; Skirrow *et al*., 1993).

6.1.4 Colonisation and disease models

According to Newell (2001), animal models for *C. jejuni* are required to:

a) Confirm the identification and enable the characterization of putative bacterial virulence factors.
b) Validate *in vitro* models of pathogenic mechanisms.
c) Investigate the role of host mechanisms in the induction of clinical symptoms.
d) Determine host immune responses and investigate surrogates of protective immunity.
e) Measure and test the efficacy of therapeutic or prophylactic treatments such as vaccines or antibiotics.

A variety of animal models have been proposed for study of *C. jejuni*-mediated diseases. These include the ferret model which was used by Bacon *et al.* (2001) to show that the phase variable CPS is a virulence factor. Other models include the rabbit ileal loop model which was first used by Everest *et al.* (1993) to examine pathological changes in ileal tissue after *C. jejuni*

infection, and primates such as infant rhesus macaques (*Macaca mulatta*) and the New World monkey *Aotus nancymae* (Russell *et al.*, 1993; Jones *et al.*, 2006). However ethical concerns and inadequate representation of human campylobacteriosis have prevented the widespread application of these models.

6.1.5 Chicken colonisation model

C. jejuni is able to colonise the avian gastrointestinal tract, and the contamination of broiler chicken meat is a principal source of infection (Friedman *et al.*, 2004). However, this colonisation is asymptomatic and is considered to be a commensal association with the host (Newell, 2001). *C. jejuni* predominantly colonises the caecum of chicken, generally between 10^6 to 10^8 cfu/g (Beery *et al.*, 1988). This ability to successfully colonise the chicken gastrointestinal tract is a multifactorial process and the model has been used to elucidate colonisation and survival factors.

Chicken colonisation models for *C. jejuni* are categorised into 2 groups: the first uses chicks that are up to 2 days old and the second uses chickens between 14 and 16 days old. The model has been used to determine colonisation factors, including motility, chemotaxis, two-component regulatory systems and oxidative and nitrosative stress responses (Lin et al., 2003; Raphael *et al.*, 2005; Karlyshev *et al.*, 2002; Bras *et al.*,1999; Atack and Kelly, 2009).

However this high level of colonisation does not result in a pathological inflammatory response and does not result in similar symptoms observed in humans. *In vitro* data has shown that *C. jejuni* stimulates intracellular nitric oxide synthase, IL-1 and IL-6 by chicken macrophages and epithelial cells, furthermore heterophils are also stimulated in response to *C. jejuni* but epithelial invasion is not common (Bar-Shira and Friedman, 2006). Although the above immune response has been observed, chickens have either developed tolerance or the response is diminished by an unknown mechanism.

6.1.6 *Galleria mellonella* infection model

In 2010 Champion *et al.* reported that the larvae of *G. mellonella*, the Greater Wax Moth can be successfully infected by *C. jejuni* and this model can be utilised to screen for genes involved in virulence.

Insect models of infection have been used as alternatives to mammalian models due to their cost effectiveness and non-requirement of ethical consent. The most commonly used invertebrate for infection is the nematode species *Caenorhabditis elegans*, which has been used to study *Yersinia* pathogenicity (Joshua *et al*., 2003). However, there are limitations when using the *C. elegans* model, as it is unable to survive at 37°C and it lacks functional homologues of the mammalian immune system such as phagocytic cells (Mylonakis *et al*., 2007). Using insect larvae overcomes these disadvantages because they can survive at 37°C and have structures known as haemocytes that function in a similar fashion to human phagocytic cells, are able to ingest bacterial pathogens and generate bactericidal compounds such as superoxide (Bergin *et al*., 2005). Senior *et al.* (2011) showed that *C. jejuni* can survive within *G. mellonella* causing significant damage to the gut of these larvae.

6.1.4 Intestinal cell invasion model

As an alternative to using whole animals to model campylobacteriosis, epithelial cell lines are utilised. Two cell lines are predominantly used to study *C. jejuni* infection: INT407 which is a human embryonic intestinal cell line, and the Caco-2 human colon adenocarcinoma line (Friis *et al.*, 2005).

These cells are used to assess the ability of *C. jejuni* to adhere to and invade the epithelial cells. Of the two cell lines, Caco-2 cells are considered favourable to study columnar shape, polarity, formation of tight junctions and expression of microvilli (Friis *et al*, 2005).

It has been shown that data obtained from *in vitro* cell lines accurately mimics that obtained using animal models of infection. For example, mutation of

genes *cheY* and *kpsM* in strain 81-176 exhibited reduced virulence in a ferret disease model and these results corroborated with the findings of cell culture results showing reduced adherence and invasion (Yao *et al*, 1997; Bacon *et al*, 2001).

Tissue culture models are also used to study bacterial translocation which is the first step in the passage of viable bacteria from the gastrointestinal (GI) tract to extraintestinal sites. *C. jejuni* has been shown to exhibit translocation across polarised epithelial cells and it has been determined that this process is important factor in pathogenesis (Louwen *et al*., 2012).

6.1.5 Hypothesis

The hypothesis being addressed in this chapter is that exposure of *C. jejuni* to physiologically-relevant concentration of mammalian pancreatic α-amylase results in an increase in virulence in the *G. mellonella* model and increased adhesion and interaction with Caco-2 cells, as well as increased colonisation in 21 day old chickens.

6.2 Results

6.2.1 Exposure of *C. jejuni* to pancreatic α-amylase results in increased kill in *G. mellonella*

To establish if bacteria grown in the presence of pancreatic amylase show increased virulence in the *G. mellonella* model, the percentage of larval kill was assessed after 24 h of infection. Ten larvae were infected with approximately 10^6 (CFUs) of *C. jejuni* strains 11168H, 81-176, the *kpsM* and *Cj0511* mutants and the *Cj0511* complemented strain, all of which had been cultured on MH agar with and without amylase. With the exception of the *Cj0511* mutant, strains grown in the presence of amylase resulted in increased lethality (Figure 6.1). *C. jejuni* 11168H showed a mean kill of one out of 10 larvae when grown in the absence of amylase, compared to a mean of 8.7 when pre-exposed to amylase. *C. jejuni* 81-176 showed mean kills of 9.3 and 2.3 out of ten larvae, respectively, when grown with or without amylase. Similar results were obtained with the *kpsM* mutant with a mean kill of 3.3 out of 10 larvae in the absence of amylase compared to 7.6 out of 10 in the presence of amylase as shown in figure 6.1. The *Cj0511* mutant showed a mean kill of 3 when the inoculum was prepared without amylase compared to a mean of 4.6 when the bacteria were grown with amylase. The complemented strain displayed killing capacity similar to the wild-type strain 11168H with a mean kill of 1.3 out of 10 larvae in the absence of amylase compared to 9.3 out of 10 in the presence of amylase. The control group which were injected with PBS showed no kill.
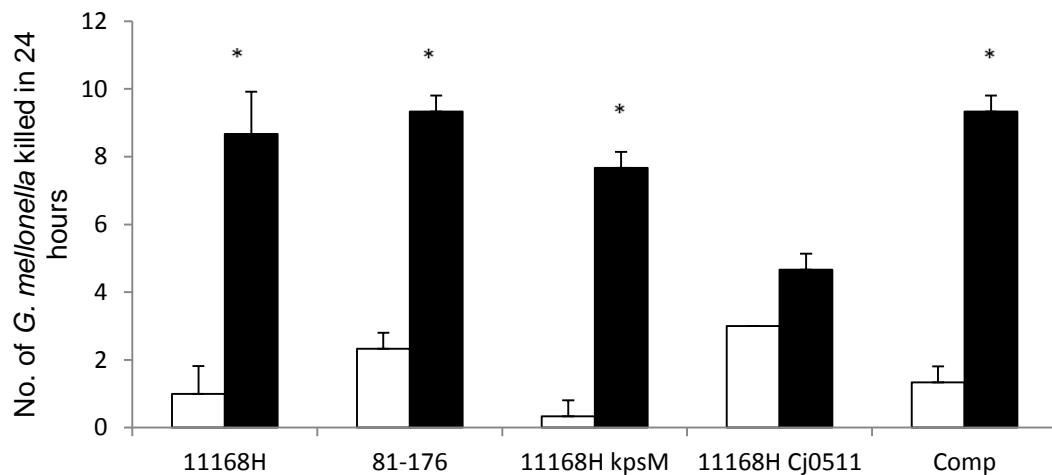
Figure 6.1. Killing capacity of *Campylobacter jejuni* grown on MH agar with (black bars) or without (white bars) 100 nM hog pancreatic α-amylase. 11168H, 81-176, *kpsM* mutant. Sets of ten *G. mellonella* larvae were used. Means of three experiments are shown and the error bars show the standard deviations. Statistical significance was analysed using students T-test (* $p < 0.05$).

6.2.2 Exposure to pancreatic amylase delays formation of coccoid forms in *G. mellonella* larvae

In this study, GFP-labelled *C. jejuni* 11168H was injected into the right foreleg of the *G. mellonella* larvae. The haemocoel of infected larvae were collected, the autoflourescent hemocytes were removed by low speed centrifugation and examined by fluorescent microscopy.

Figure 6.2 shows that *C. jejuni* 11168H-GFP grown on agar in the absence of α-amylase became coccoid at 3 hours (Figure 6.2B), whereas bacteria pre-exposed to α-amylase remained the characteristic rod shape at 3 hours (Figure 6.2C) and exhibited coccoid shape only at 6 hours (Figure 6.2D). As a positive control 11168H-GFP was grown in Brucella broth and the fluorescent image of characteristic rod-shaped cells is shown Figure 6.2A. All hemolymph controls which are autoflourscent showed no fluorescence. Images shown are representative of three independent experiments.
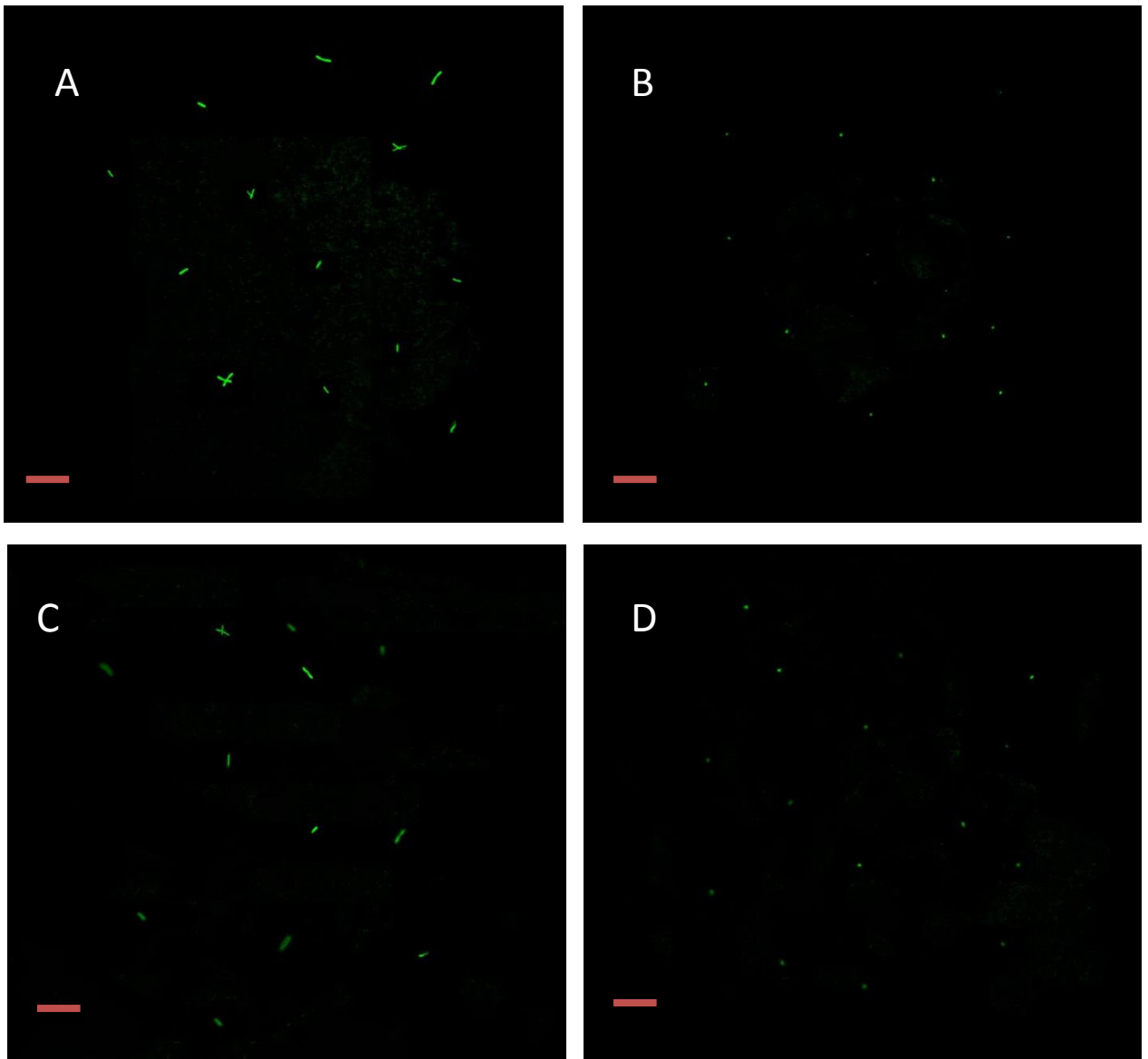
Figure 6.2. *C. jejuni* 11168H-GFP interaction with *G. mellonella* larvae. A) *C. jejuni* 11168H-GFP grown in Brucella broth for 16 h at 37℃ microaerobically. B) *C. jejuni* 11168H-GFP grown without amylase, 3 h post infection. C) *C. jejuni* 11168H-GFP grown with 100 nM hog pancreatic α-amylase, 3 hour post infection, D) *C. jejuni* 11168H-GFP grown with 100 nM pancreatic amylase, 6 hour post infection. Scale bar represents 10μm. Images taken at x40 magnification and are representative of three independent experiments.

6.2.3 Exposure of *C. jejuni* to pancreatic α-amylase results in increased interaction and invasion of Caco-2 cells

Interaction (adhesion and invasion) with human Caco-2 cells was assessed using *C. jejuni* 11168H, the *Cj0511* mutant and the complemented strain grown on MH agar with and without 100 nM pancreatic α-amylase. Approximately 10^8 were added to a confluent monolayer of ~10^6 cells at an MOI 1:100.

Both the wild-type and the complemented strains pre-exposed to amylase displayed a significantly (*p<0.05*) increased ability to interact with Caco-2 cells, whereas the *Cj0511* mutant showed no increase in interaction when pre-exposed to amylase compared to unexposed bacteria (Figure 6.3A). The increase was the most pronounced in the early stage of the infection (3-fold increase at 3 hours). At 6 hours and 24 hours, the increase in the number of interacting bacteria caused by pre-exposure to amylase was 2-fold and 1.6-fold, respectively.

When invasion of Caco-2 cells was tested exclusively (Figure 6.3B), a significant (*p<0.05*) increase in the numbers of invading bacteria as a result of pre-exposure to amylase was observed at all-time points in the case of strains 11168H and the complemented strain, whereas the *Cj0511* mutant did not show an increase in response to amylase pre-exposure.
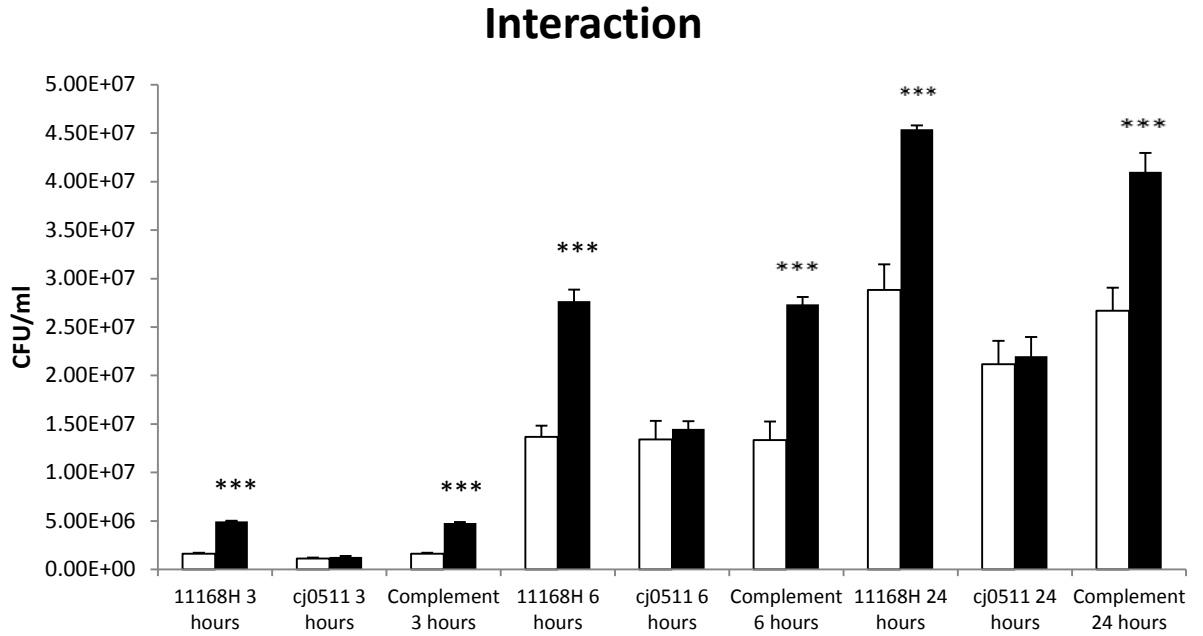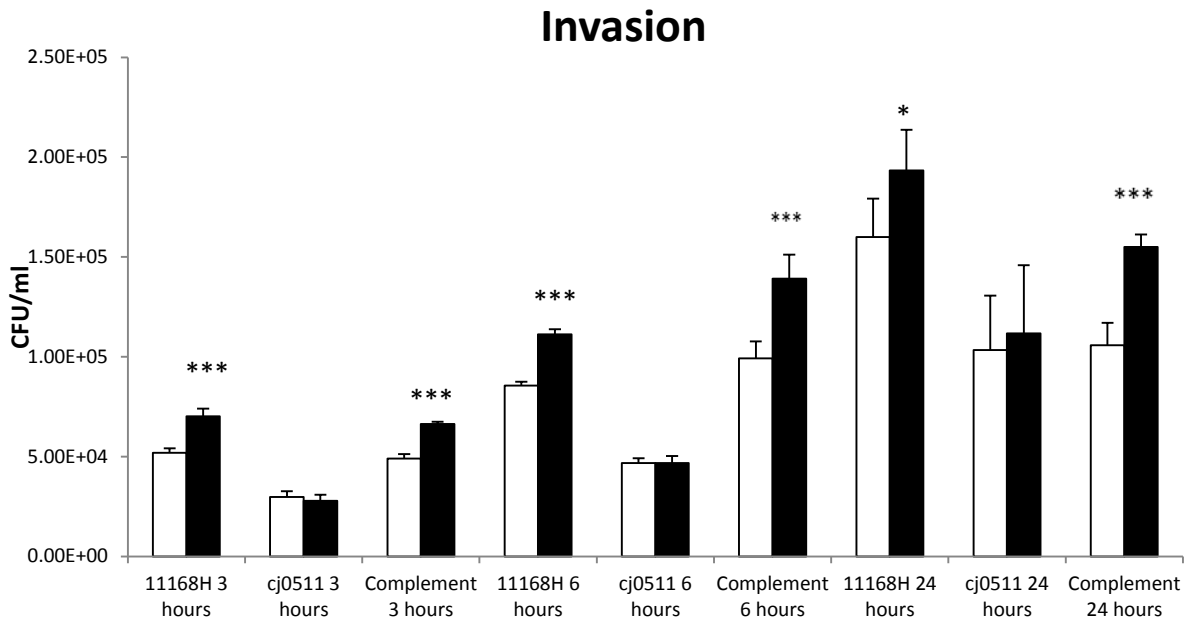
Figure 6.3. A) Interaction B) invasion of Caco-2 cells by *C.jejuni* 11168H, the *Cj0511* mutant and the complemented strain grown on MH agar with (black bars) and without (white bars) 100 nM hog pancreatic α-amylase at 3 hours, 6 hours and 24 hours. Statistical significance was analysed using students T-test (*** *p* <0.001).

6.2.5 *C. jejuni* pre-exposed to pancreatic α-amylase exhibits increased translocation in T84 cells

Translocation across polarised T84 cells by *C. jejuni* 11168H, the *Cj0511* mutant and the complemented strain was assessed after 8 hours of infection (Figure 6.4). The results showed that in both the wild-type and the complemented strain pre-exposure to α-amylase resulted in significantly ($p<0.05$) increased translocation of T84 cells compared to bacteria not pre-exposed to α-amylase. However, the *Cj0511* mutant did not show a significant increase in translocation in response to amylase pre-exposure.
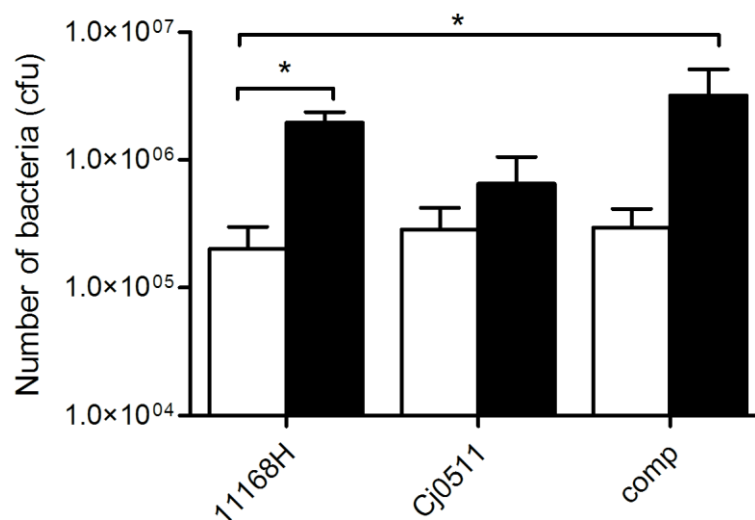
Figure 6.4.Translocation of T84 cells by at 8 hours. *C. jejuni* 11168H, the *Cj0511* mutant and its complemented strain were grown in the presence and absence of 100 nM hog pancreatic amylase (*$p<0.05$).

6.2.6 *C. jejuni* pre-exposed to pancreatic α-amylase exhibit increased colonisation of chickens

To determine if pre-exposure to α-amylase would alter the ability of *C. jejuni* to colonise chickens, 21 day old broilers chickens were infected with *C. jejuni* 11168H or the *Cj0511* mutant which were grown in the presence and absence of α-amylase (Figure 6.5).

The results showed no significant difference at day 4, however at day 7 strain 11168H which was pre-exposed to α-amylase before infection showed a significant increase (p=0.005) with 11/15 birds colonised, in comparison to the same strain not pre-exposed to α-amylase which colonised 5/15 birds. The *Cj0511* mutant did not show a difference in colonisation when pre-exposed and not pre-exposed bacteria were compared (Figure 6.5A).

Upon further examination, chickens infected with *C. jejuni* 11168H pre-exposed to amylase showed a significant increase (p=0.03) in the numbers of colonising bacteria in the ileum at days 4 and 7, compared to the *Cj0511* also pre-exposed to α-amylase (Figures 6.5B, 6.5C).
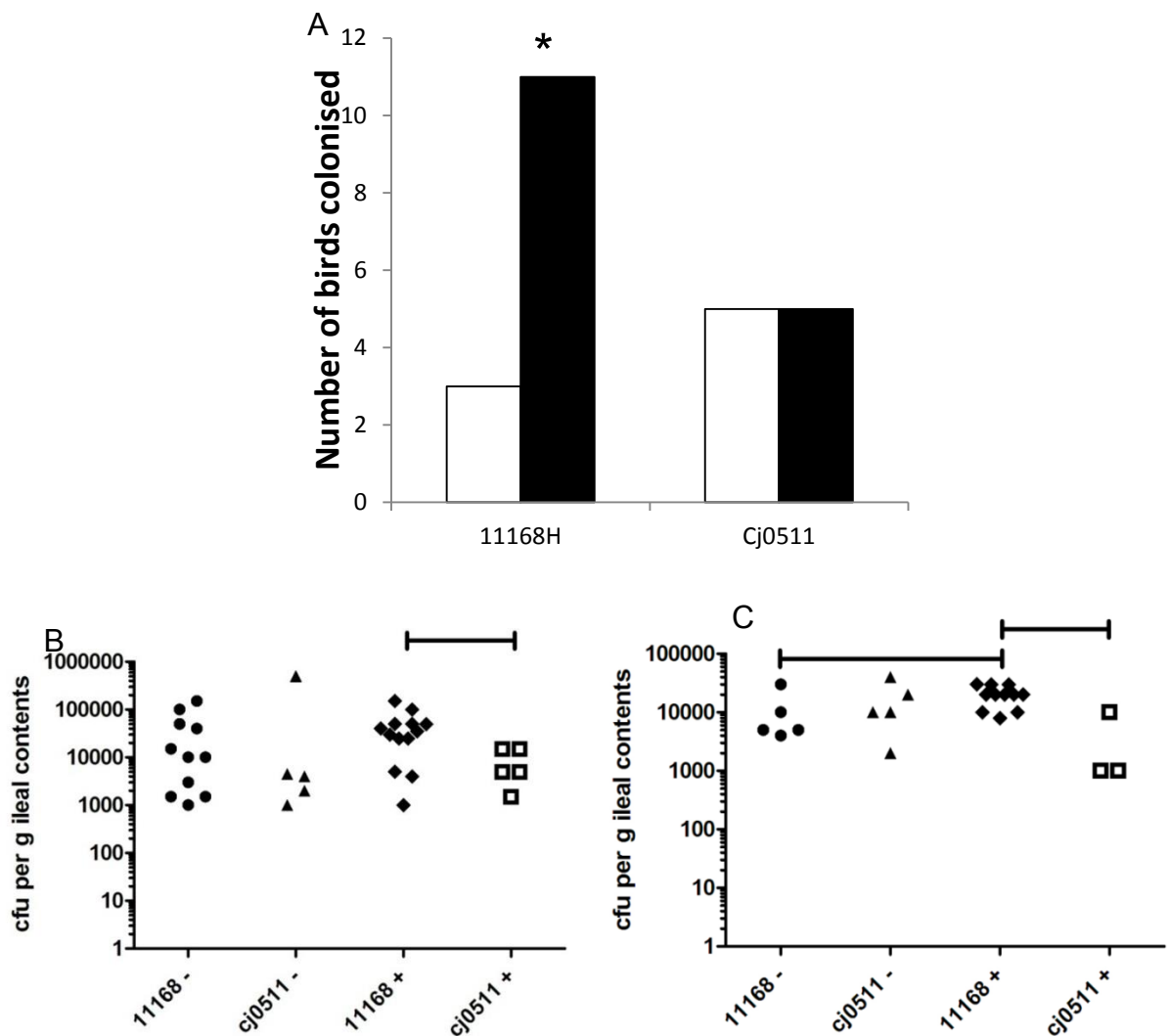
Figure 6.5. A. Number of birds from which *Campylobacter* was isolated from the ileum at 7 days post infection. *C. jejuni* was grown with (black bars) or without hog pancreatic α- amylase (white bars). Bars indicate significant difference between groups. n=15 birds per group.

B. CFU of *Campylobacter* per gram of ileal contents in birds 4 days post infection. Bars indicate significant difference by Dunn's multiple comparison test. n=15 birds per group.

C. CFU of *Campylobacter* per gram of ileal contents in birds 7 days post infection. Bars indicate significant difference by Dunn's multiple comparison test. n=15 birds per group.

6.3 Discussion

The results of this study have shown that pre-exposure of *C. jejuni* to physiologically relevant concentrations of mammalian pancreatic α-amylase results in increased virulence in the *G. mellonella* model. Both wild-type strains and the *kpsM* mutant showed increased virulence in response to amylase exposure indicating that the EPM is a virulence factor in this model. The *Cj0511* mutant did not show an increase in virulence in response to amylase in contrast to the complemented strain which showed an increase similar to that of the wild-type. This indicates that Cj0511 activity is necessary for the response to amylase and is a virulence factor.

Champion *et al.* (2010) reported that a *Cj0511* mutant was significantly attenuated in killing *G. mellonella* in comparison to the wild-type *C. jejuni* strain 11168H, however the data reported here did not show a similar decrease for the *Cj0511* mutant. This difference in results could be explained by the fact that Champion *et al.* (2010) used broth-grown bacteria whereas here the bacteria were grown on agar, a factor shown by Champion *et al.* (2010) to influence virulence in the *G. mellonella* model.

One of the main advantages of using the *G. mellonella* model is the resemblance between the vertebrate and invertebrate innate immune systems, which can be exploited. Kemp and Massey (2007) showed that *G. mellonella* have a cuticle which functions in a similar way to mammalian skin, operating as a physical barrier which, when breached, results in the induction of humoral immune response with the release of factors such as antimicrobial peptides. Furthermore, *G. mellonella* induces a cellular response to invading microorganisms, and hemocytes phagocytose bacteria in similar fashion to human neutrophils (Kemp and Massey, 2007). Therefore, using such a model allows us to predict how *C. jejuni* would behave in the human GI tract. A study by Senior *et al.* (2011) showed that upon entry into the larvae, *C.jejuni* begins to undergo a change to a coccoid shape after 3 hours. The reason for the change of *C. jejuni* from being rod shaped to coccoid has been debated however the consensus is that it is a response to oxidative stress and nutrient

deprivation and coccoid forms are therefore a degenerative state and are likely candidates for viable but non-culturable cells (Harvey and Leach 1998; Hudock *et al*., 2005). Interestingly a study by Beumer *et al.* (1992) concluded that oral administration of coccoid *C. jejuni* to mice and human volunteers resulted in none of the symptoms associated with *C. jejuni* infection. The findings described in this chapter show that *C.jejuni* 11168H-GFP which was grown in the absence of amylase was coccoid at 3 hours, however the *C.jejuni* 11168H-GFP grown in the presence of amylase were still rod shaped at 3 hours and became coccoid by 6 hours. This indicates that EPM-secreting cells are able to withstand oxidative stresses, nutrient deprivation and action of hematocyes and maintain its characteristic shape for a longer period of time longer *in vivo* allowing leading to increased virulence.

The Caco-2 intestinal cell model was used to determine interaction and invasion of epithelial cells. The findings show that pre-exposure of *C. jejuni* to physiologically relevant concentrations of α-amylase significantly increased both interaction and invasion. It was also demonstrated that the protease, Cj0511, is necessary for the response. The *Cj0511* mutant when grown without pancreatic α-amylase, showed no major difference in interaction with Caco-2 cells when compared to the wild-type strain; however when invasion of epithelial cells, a distinct reduction in the density of bacteria invading Caco-2 cells was observed. In many cases it has been suggested that secreted extracellular proteases assist in tissue invasion through breaking down extracellular matrix proteins and polysaccharides, allowing bacteria to invade deeper.

It was previously shown that induction of EPM results in significantly increased autoagglutination and biofilm formation (chapter 5) are both markers for virulence. Micro-colony formation is a perquisite for biofilm formation, *in vitro* data obtained by Haddock *et al.* (2010) showed microcolony formation on human intestinal tissue, and concluded this to be a principal step in the ability of *C. jejuni* to cause diarrhoea in humans (Haddock *et al*., 2010). The results obtained in this chapter clearly highlight that induction of EPM through exposure to α-amylase results in significantly higher rates of adherent cells,

emphasising that biofilm formation by *C. jejuni in vivo* is an important step in the bacterium to cause disease, furthermore it underscores the fact that exposure to α-amylase is a significant and necessary step in the ability to colonise eukaryotic cells.

The invasiveness of *C. jejuni* is an important virulence determinant, supported by a number of studies using animal models such as the chicken embryo, hamster and piglet (Field *et al*., 1986; Humphrey *et al.* 1986; Babakhani *et al*., 1993).

The data obtained here shows an increased invasion by EPM-producing bacteria, this increase was significant however the magnitude of change observed was not as high as the magnitude of change observed with interaction. This brings to light the EPM secretion predominantly assists adhesion and not invasion directly of eukaryotic cells, and this increased attachment subsequently results in increased invasion.

A study by Everest *et al.* (1992) found isolates recovered from patients suffering from inflammatory colitis showed significantly higher levels of invasiveness than isolates from patients suffering from non-inflammatory campylobacteriosis. Furthermore, it has been determined that clinical isolates show increased invasion when compared to environmental isolates (Newell *et al*., 1985). An explanation for this can be found in the fact that clinical isolates have been exposed to pancreatic α-amylase prior to excretion in faeces, it is a well-known phenomenon that clinical isolates exhibit mucoidy, a response highlighted previously in Chapter 3. Chapter 3 described that mucoidy was associated with exposure to pancreatic α-amylase and is therefore the phenotype that would be expressed *in vivo*, mucoidy has been definitively associated with increased virulence in *C. jejuni* (Babakhani, 1993). Substantiating that EPM induction is vital for the pathogenesis seen clinically. Whereas environmental strains are unlikely to encounter pancreatic amylase and thus do not express EPM in their life cycle and thus exhibit reduced virulence.

Translocation is a mechanism of virulence used by a number of enteropathogens such as *Salmonella* sp., *Yersinia* sp., *Shigella* sp. and *Listeria* sp. to propagate within the host. The exact mechanism by which *C. jejuni* undergoes translocation is not known, however there is evidence that it requires a functional flagellum and requires *de novo* protein synthesis (Bras and Ketley, 1999; Finlay and Falkow, 1997). The wild-type strain 11168H pre-exposed to pancreatic amylase showed significantly more translocation across T84 cells than the unexposed bacteria. When the *Cj0511* mutant pre-exposed to amylase was tested it did not show a significant increase in translocation in comparison to the mutant grown in the absence of amylase, however the complementation of this gene resulted in restoration of the response seen in the wild-type. The first step to translocation is bacterial adhesion to the colonic cells (Bras and Ketley, 1999); our data has shown that bacteria grown with amylase supplementation show greater interaction and thus adhesion to Caco-2 epithelial cells. Based on this we can assume that greater number of bacteria adhering to the cells would result in an increased number of translocating bacteria.

The data presented in this chapter has also shown that *C. jejuni* 11168H grown with 100 µM amylase, showed a significantly increased ability to colonise 21-day old chickens. This again underlines the fact that EPM induction via exposure to amylase is essential to forming an attachment to eukaryotic cells, as described in chapter 5 it was demonstrated that *C. jejuni* is also able to respond to chicken pancreatic extract containing amylase, by increasing autoagglutination and biofilm formation by secretion of EPM; A number of studies have shown increased colonisation after *in vivo* passaging through chicks (Sang *et al.,* 1989; Cawthraw *et al*., 1996) and in fact enhanced colonisation has been shown by even inadequately colonising isolates after several *in vivo* passages (Stern *et al*., 1988). These studies suggest that passaging through a chicken and thus exposing the bacteria to pancreatic α-amylase would induce EPM secretion; validating that EPM induction is paramount to successful colonisation in chickens. As in the environment *C. jejuni* encounters a number of environmental stresses in the chicken intestinal tract, which inhibits optimal growth (Hermans *et al*., 2011).

However the consistent nature of colonisation of the chicken GI tract by *C. jejuni* point towards the bacterium having regulatory systems that confer protection towards a hostile environment within and outside the host. The exact mechanism by which *C. jejuni* is able to maintain itself within this hostile environment has not been previously elucidated, however this study is the first to demonstrate the role of EPM in conferring protection of *C. jejuni* to stresses as shown in Chapter 5. This protective mechanism is expected to be the same within the chicken caeca, allowing the bacteria to resist stresses and colonise, indeed analysis of five *C. jejuni* isolate obtained from chickens have been shown to be covered by dextrans (Personal communication Dr E. Allan).

In summary the data shown in this chapter illustrates bacteria grown in the presence of physiological-relevant concentrations of pancreatic α-amylase show significantly increased virulence in the *G. mellonella* and cell culture models; as well as significantly increased colonisation of 21 day old chickens. Based on the results of this chapter it can be concluded that current research conducted using laboratory strains are not truly representative of the phenotypes found *in vivo* and therefore the virulence data obtained is not accurately demonstrating pathogenicity; however supplementing growth media with pancreatic amylase and thus inducing EPM would allow researchers to more accurately study *C. jejuni* virulence. Another finding of this chapter is the validation of the *G. mellonella* model with *in vitro* Caco-2 epithelial cell assays in both cases bacteria grown with α-amylase supplementation showed similar increase in virulence, in the case of *G. mellonella* an increased kill was observed over 24 hours in comparison to increased interaction and invasion of Caco-2 cells.

Chapter 7: Final Discussion

7.1 Significance of the study

This study has generated results that have a considerable impact on the understanding of *C. jejuni* biology. The data presented in this thesis shows, for the first time, that *C. jejuni* is able to detect the presence of physiological concentrations of pancreatic α-amylase and responds by secretion of EPM. This response resulted in a change in colony morphology to a mucoid phenotype through the secretion of EPM which includes the secretion of extracellular proteins and a novel polysaccharide that consists of 1,6-linked glucose residues, producing an α-dextran. Bacteria such as *B. cepacia* (Bartholdson *et al.*, 2008)*, E. faecalis* (Battone *et al.*, 1998) *, S. pneumoniae* (Allegrucci and Sauer, 2007) and *P. aeruginosa* (Friedl *et al.*, 1992) all have the ability to express mucoidy. Some bacteria such as *B. cepacia* and *P. aeruginosa* express mucoidy through secretion of an EPM (Cérantola *et al.*, 2006; Bartholdson *et al.*, 2008; Tielen *et al.*, 2010). These EPM can consist of either polysaccharide, extracellular proteins, DNA and lipids, in some cases the EPM can be a combination of all these substrates (Costerton *et al.*, 1995).

Secretion of EPM is a prerequisite to biofilm formation and has been observed in a number of enteric bacteria, such as *V. cholerae* (Yildiz and Schoolnik, 1999*), H. pylori* (Stark *et al.*, 1999) and *S. typhimurium* (Ledeboer and Jones. 2005). Existing encased within EPM provides a number of advantages to bacteria including the ability to evade immune responses. For example, in *P. aeruginosa* which when expressing mucoidy through secretion of alginate shows significantly reduced killing by macrophages (Leid *et al.*, 2005).

The results of this study further show *C. jejuni* pre-exposed to α-amylase and thus expressing EPM exhibit increased virulence in a *G. mellonella* model and increased colonisation of chickens. These results could be explained by the enhanced ability of *C. jejuni* to form a biofilm *in vivo*, a conclusion supported by data showing increased biofilm formation in response to chicken pancreatic α-amylase in this study. Haddock *et al.* (2010) described the formation of *C. jejuni* micro-colonies and biofilms on human intestinal tissue obtained from

biopsies; they concluded that this was an essential step in causing diarrhoeal disease in humans. A study by Purdy and Watnick (2011) discovered biofilm formation was necessary for *V. cholerae* to colonise the intestine of arthropods, which are the environmental host for the organism.

One of the major challenges regarding control of *C. jejuni* transmission is the ability of the bacterium to persist in broiler houses and colonise chicken caeca in such high numbers. These bacteria are able to withstand hot water, acid and chemical treatment, data presented here shows that *C. jejuni* is able to respond to chicken pancreatic amylase by increasing autoagglutination and biofilm formation. Therefore *C. jejuni* excreted in the faeces of chickens would have been pre-exposed to amylase and producing EPM. The results of this study query many of the suggested control strategies. Studies had previously suggested using organic acids to treat water supplies in poultry farms to reduce transmission of *C. jejuni*, with treatment at pH 4.5 showing a significant bactericidal effect (Murphy *et al.*, 2003). The data reported here shows that EPM-expressing *C. jejuni* exhibit significantly increased resistance to acid stress at pH 4.0 and pH 2.0. EPM production in other bacteria such as by *E. coli* O157:H7 confer resistance to acid stress and it has been proposed that this resistance allows the bacterium to persist in the GI tract (Mao *et al.*, 2006). Studies of EPM production by Bifidobacteria have shown that bacteria expressing EPM exhibit increased resistance to bile salts and low pH (Alp and Aslim, 2010). Furthermore it has been suggested high temperature treatment at 50°C and 60°C significantly reduces the *C. jejuni* load on the carcasses of chickens (Li *et al.*, 2002). However the data obtained here showed that *C. jejuni* expressing EPM exhibit a significantly lower susceptibility to heat treatment at 55°C, 60°C and 65°C.

Cold storage is another stress endured by *C. jejuni* and the FSA (2013) have recently reported that 66% retail bought chicken are contaminated with *C. jejuni*. The United States Department of Agriculture (USDA) as well as the FSA recommend all poultry to be refrigerated at 4°C. This study has shown

that *C. jejuni* expressing EPM is able to survive significantly longer in nutrient deprived conditions at 4°C. Expression of EPM also increases survival at refrigeration temperatures in *E. coli* O157:H7, another food-borne pathogen, compared to bacteria which do not express the EPM (Lee and Chen. 2004).

The phenomenon of EPM expression and its relevance to stress survival is probably best described in *P. aeruginosa,* the bacteria is able to secrete extracellular alginate, which confers protection against many stresses such as desiccation, changes in pH and chlorination. Bacteria expressing alginate reveal enhanced survival compared with isogenic non-alginate producing cells in chlorinated water. Removal of alginate from bacteria abolishes chlorine resistance; and the addition of alginate to washed cells enhances cell survival (Grobe *et al.*, 2001)

Data obtained here has shown fresh clinical isolates of *C. jejuni* exhibit a mucoid phenotype as a result of exposure to pancreatic α-amylase. Mucoidy has been conclusively associated with increased virulence in *C. jejuni* (Babakhani, 1993). Furthermore, clinical strains, which would have encountered amylase in the human GI tract, show increased invasion when compared to environmental isolates (Newell *et al.*, 1985).

Standardised *in vitro* bacterial growth conditions contrast significantly from the complex and fluctuating conditions seen *in vivo*, regardless of this the majority of research is conducted using reference strains which become adapted to laboratory conditions through decades of passaging. Reference strains such as *C. jejuni* NCTC 11168 which was originally isolated from the faeces of a diarrheic patient in 1977 by Martin Skirrow have been passaged for well over three decades. Through this successive *in vitro* passaging, the bacterium has significantly changed from the original clinical equivalent and lost many of the pathophysiological mechanisms that are present in clinical strains. This is highlighted by the reduced interactions of laboratory *C. jejuni* strains with intestinal epithelial cells, with invasion being reported at less than one per cent, which is not accurate when compared to clinical data (Friis *et al.*, 2005).

To understand the biology of *C. jejuni* it is imperative that researchers attempt to simulate the natural environment of the host as accurately as possible, as shown by this study.

Research has been conducted using secretions found in the GI tract and the results have been fascinating. When *C. jejuni* was cultured with NE, growth rate, motility and invasion of Caco-2 cells were increased in comparison with cultures grown in the absence of NE. Additionally, these bacteria produced extensive breakdown and disruption of cell monolayers, indicating that this may contribute to the epithelial destruction that is characteristic of severe campylobacteriosis (Cogan *et al.*, 2007). Another study by Quoc *et al.* (2008) concluded that *C. jejuni* utilizes MUC2 a constituent of mucin as an environmental signal to control expression of genes associated with pathogenicity. Furthermore, Malik-Kale *et al.* (2008) showed that culturing *C. jejuni* with DOC activates pathogenic behaviour by exhibiting an increase in epithelial cell invasion; the study concluded that interaction with DOC prepares the bacterium to invade cells though activating the production of Cia proteins. In another gastrointestinal bacterium, *H. pylori,* gastrin at physiological concentrations has been shown to promote growth *in vitro* (Chowers *et al.*, 1999).

7.2 Further work

Further work is necessary to extend and build upon the data presented in this study. Although it was determined that Cj0511 activity is vital for the bacterium to respond to α-amylase, the molecular mechanisms behind recognition of α-amylase and elucidating the signal transduction pathway need to be investigated. This could be done through screening a *C. jejuni* transposon mutagenesis library on CHMA agar supplemented with and without pancreatic α-amylase to check for conformity of phenotype on both sets of plates, with colonies not showing mucoidy in response to amylase being further characterised. A mutagenesis library could be constructed using a mariner transposon based system which has a recognition sequence of TA and would provide a good degree of genome coverage; it has previously been

demonstrated to produce random *in vivo* transposon mutagenesis of *C. jejuni* (Golden *et al.*, 2000).

With the discovery that the *C. jejuni* EPM consists partly of a dextran it would be advantageous to assess whether commercially available dextranases have the ability to degrade the EPM, this in turn would lead to the possibility of incorporating such enzymes into treatment of *C. jeuni* in commercial broiler houses to make the bacteria more susceptible to the already used control treatments such as using scalding water to wash chicken carcasses after slaughter. Furthermore the optimum pH range for dextranase activity is pH 5.0-6.0, with the lower end of the range more preferable, this would be ideal to incorporate into acidic treatment of water to treat chicken faeces, which would inevitably contain *C. jejuni* pre-exposed to α-amylase and thus expressing EPM.

The results of this study have demonstrated that activity of secreted protease Cj0511 is essential for *C. jejuni* to respond to pancreatic α-amylase, a valuable therapeutic measure would be to inhibit the activity of Cj0511. The finding that stable and proteolytic active Cj0511-His protein can be expressed by *E. coli* is highly relevant as this will facilitate in future work with development of specific inhibitors. The study of bacterial proteases as future drug targets is promising. Slomiany *et al.* (1992) found successful treatment of *H. pylori* infection using anti-ulcer drug ebrotidine was in part due to a strong inhibitory action on *Helicobacter* proteases involved in degrading gastric mucin, which is a growth promoter in *H. pylori* (Chowers *et al.*, 1999). Protease inhibitors have been used to treat viral infections such as hepatitis and HIV, Saquinavir an antiretroviral drug that was the first approved protease inhibitor for the treatment of HIV inhibits the function of both HIV-1 and HIV-2 proteases.

Protein–protein interactions play an integral role in the majority of biological processes, developing small molecule inhibitors to proteases are appealing opportunities for therapeutic intervention. Recently, it was shown in mice that inhibition of lethal factor protease activity from *Bacillus anthracis* can protect against anthrax infection (Moayeri *et al.*, 2013). Developing similar inhibitors to

Cj0511 would be valuable tools in the development of future control measures for *C. jejuni.*

In summary, the data generated in this thesis has improved our understanding of *C. jejuni* biology in particular it has highlighted that pancreatic α-amylase acts as a signal for initiating pathogenic behaviour and changing to a phenotype which promotes survival. Furthermore, this study has elucidated the relevance of Cj0511 activity in the ability of *C. jejuni* to persist in the food chain and cause disease, this in combination with the identification of a α-dextran as the main component of *C. jejuni* EPM are useful for the development of effective control measures against *C. jejuni.* The data from this PhD has also emphasised the importance of simulating the host intestinal environment as closely as possible when studying *C. jejuni* to allow novel insights into pathogenic mechanisms.

References

Al-Abri, S. S., N. J. Beeching, et al. (2005). "Traveller's diarrhoea." <u>Lancet Infect Dis</u> **5**(6): 349-360.

Allegrucci, M. and K. Sauer (2007). "Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms." <u>J Bacteriol</u> **189**(5): 2030-2038.

Allen, A., D. A. Hutton, et al. (1998). "The MUC2 gene product: a human intestinal mucin." <u>Int J Biochem Cell Biol</u> **30**(7): 797-801.

Allos, B. M. and M. J. Blaser (1995). "Campylobacter jejuni and the expanding spectrum of related infections." <u>Clin Infect Dis</u> **20**(5): 1092-1099; quiz 1100-1091.

Alp, G., B. Aslim, et al. (2010). "The role of hemagglutination and effect of exopolysaccharide production on bifidobacteria adhesion to Caco-2 cells in vitro." <u>Microbiol Immunol</u> **54**(11): 658-665.

Ashgar, S. S., N. J. Oldfield, et al. (2007). "CapA, an autotransporter protein of Campylobacter jejuni, mediates association with human epithelial cells and colonization of the chicken gut." <u>J Bacteriol</u> **189**(5): 1856-1865.

Atack, J. M. and D. J. Kelly (2009). "Oxidative stress in Campylobacter jejuni: responses, resistance and regulation." <u>Future Microbiol</u> **4**(6): 677-690.

Atack, J. M. and D. J. Kelly (2009). "Oxidative stress in Campylobacter jejuni: responses, resistance and regulation." <u>Future Microbiol</u> **4**(6): 677-690.

Babakhani, F. K. and L. A. Joens (1993). "Primary swine intestinal cells as a model for studying Campylobacter jejuni invasiveness." <u>Infect Immun</u> **61**(6): 2723-2726.

Bacon, D. J., C. M. Szymanski, et al. (2001). "A phase-variable capsule is involved in virulence of Campylobacter jejuni 81-176." <u>Mol Microbiol</u> **40**(3): 769-777.

Bak, A., O. S. Gudmundsson, et al. (1999). "Acyloxyalkoxy-based cyclic prodrugs of opioid peptides: evaluation of the chemical and enzymatic stability

as well as their transport properties across Caco-2 cell monolayers." <u>Pharm Res</u> **16**(1): 24-29.

Bandara, A. B., D. DeShazer, et al. (2008). "A disruption of ctpA encoding carboxy-terminal protease attenuates Burkholderia mallei and induces partial protection in CD1 mice." <u>Microb Pathog</u> **45**(3): 207-216.

Bandara, A. B., N. Sriranganathan, et al. (2005). "Carboxyl-terminal protease regulates Brucella suis morphology in culture and persistence in macrophages and mice." <u>J Bacteriol</u> **187**(16): 5767-5775.

Baqar, S., A. L. Bourgeois, et al. (1996). "Murine intranasal challenge model for the study of Campylobacter pathogenesis and immunity." <u>Infect Immun</u> **64**(12): 4933-4939.

Bartholdson, S. J., A. R. Brown, et al. (2008). "Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the Burkholderia cepacia complex." <u>Microbiology</u> **154**(Pt 8): 2513-2521.

Beery, J. T., M. B. Hugdahl, et al. (1988). "Colonization of gastrointestinal tracts of chicks by Campylobacter jejuni." <u>Appl Environ Microbiol</u> **54**(10): 2365-2370.

Bell, J. A. and D. D. Manning (1990). "Reproductive failure in mink and ferrets after intravenous or oral inoculation of Campylobacter jejuni." <u>Can J Vet Res</u> **54**(4): 432-437.

Bengmark, S. (1998). "Ecological control of the gastrointestinal tract. The role of probiotic flora." <u>Gut</u> **42**(1): 2-7.

Berberian, L. S., Y. Valles-Ayoub, et al. (1994). "Expression of a novel autoantibody defined by the VH3-15 gene in inflammatory bowel disease and Campylobacter jejuni enterocolitis." <u>J Immunol</u> **153**(8): 3756-3763.

Bergin, D., E. P. Reeves, et al. (2005). "Superoxide production in Galleria mellonella hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils." <u>Infect Immun</u> **73**(7): 4161-4170.

Bernatchez, S., C. M. Szymanski, et al. (2005). "A single bifunctional UDP-GlcNAc/Glc 4-epimerase supports the synthesis of three cell surface glycoconjugates in Campylobacter jejuni." <u>J Biol Chem</u> **280**(6): 4792-4802.

Beumer, R. R., J. de Vries, et al. (1992). "Campylobacter jejuni non-culturable coccoid cells." Int J Food Microbiol **15**(1-2): 153-163.

Blaser, M. J. (1997). "Epidemiologic and clinical features of Campylobacter jejuni infections." J Infect Dis **176 Suppl 2**: S103-105.

Blaser, M. J. (1997). "Epidemiologic and clinical features of Campylobacter jejuni infections." J Infect Dis **176 Suppl 2**: S103-105.
    Gram-negative bacteria of the genus Campylobacter and of related genera

Blaser, M. J. and W. R. Brown (1989). "Campylobacters and gastroduodenal inflammation." Adv Intern Med **34**: 21-42.

Blaser, M. J., D. J. Duncan, et al. (1983). "Experimental Campylobacter jejuni infection of adult mice." Infect Immun **39**(2): 908-916.
    HA-ICR adult mice were studied to develop an animal model for Campylobacter

Borriello, S. P. and F. E. Barclay (1986). "An in-vitro model of colonisation resistance to Clostridium difficile infection." J Med Microbiol **21**(4): 299-309.

Branda, S. S., F. Chu, et al. (2006). "A major protein component of the Bacillus subtilis biofilm matrix." Mol Microbiol **59**(4): 1229-1238.

Bras, A. M., S. Chatterjee, et al. (1999). "A novel Campylobacter jejuni two-component regulatory system important for temperature-dependent growth and colonization." J Bacteriol **181**(10): 3298-3302.

Bras, A. M. and J. M. Ketley (1999). "Transcellular translocation of Campylobacter jejuni across human polarised epithelial monolayers." FEMS Microbiol Lett **179**(2): 209-215.

Butler, S. M., R. A. Festa, et al. (2006). "Self-compartmentalized bacterial proteases and pathogenesis." Mol Microbiol **60**(3): 553-562.

Butzler, J. P. (2004). "Campylobacter, from obscurity to celebrity." Clin Microbiol Infect **10**(10): 868-876.

Buzby, J. C., B. M. Allos, et al. (1997). "The economic burden of Campylobacter-associated Guillain-Barre syndrome." J Infect Dis **176 Suppl 2**: S192-197.

Cawthraw, S. A., L. Lind, et al. (2000). "Antibodies, directed towards

Campylobacter jejuni antigens, in sera from poultry abattoir workers." Clin Exp Immunol **122**(1): 55-60.

Cawthraw, S. A., T. M. Wassenaar, et al. (1996). "Increased colonization potential of Campylobacter jejuni strain 81116 after passage through chickens and its implication on the rate of transmission within flocks." Epidemiol Infect **117**(1): 213-215.

Champion, O. L., A. V. Karlyshev, et al. (2010). "Insect infection model for Campylobacter jejuni reveals that O-methyl phosphoramidate has insecticidal activity." J Infect Dis **201**(5): 776-782.

Chandra, J., D. M. Kuhn, et al. (2001). "Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance." J Bacteriol **183**(18): 5385-5394.

Chiang, S. L., R. K. Taylor, et al. (1995). "Single amino acid substitutions in the N-terminus of Vibrio cholerae TcpA affect colonization, autoagglutination, and serum resistance." Mol Microbiol **17**(6): 1133-1142.

Chowers, M. Y., N. Keller, et al. (1999). "Human gastrin: a Helicobacter pylori--specific growth factor." Gastroenterology **117**(5): 1113-1118.

Christensen, H., K. Jorgensen, et al. (1999). "Differentiation of Campylobacter coli and C. jejuni by length and DNA sequence of the 16S-23S rRNA internal spacer region." Microbiology **145 (Pt 1)**: 99-105.

Cogan, T. A., A. O. Thomas, et al. (2007). "Norepinephrine increases the pathogenic potential of Campylobacter jejuni." Gut **56**(8): 1060-1065.

Cohn, M. T., H. Ingmer, et al. (2007). "Contribution of conserved ATP-dependent proteases of Campylobacter jejuni to stress tolerance and virulence." Appl Environ Microbiol **73**(24): 7803-7813.

Costerton, J. W. (1995). "Overview of microbial biofilms." J Ind Microbiol **15**(3): 137-140.

Costerton, J. W. (1999). "Introduction to biofilm." Int J Antimicrob Agents **11**(3-4): 217-221; discussion 237-219.

Costerton, J. W., P. S. Stewart, et al. (1999). "Bacterial biofilms: a common cause of persistent infections." Science **284**(5418): 1318-1322.

Cottet, S., I. Corthesy-Theulaz, et al. (2002). "Microaerophilic conditions permit to mimic in vitro events occurring during in vivo Helicobacter pylori infection and to identify Rho/Ras-associated proteins in cellular signaling." J Biol Chem **277**(37): 33978-33986.

Dasti, J. I., A. M. Tareen, et al. (2010). "Campylobacter jejuni: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms." Int J Med Microbiol **300**(4): 205-211.

Davey, M. E., N. C. Caiazza, et al. (2003). "Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosa PAO1." J Bacteriol **185**(3): 1027-1036.

Davey, M. E. and J. W. Costerton (2006). "Molecular genetics analyses of biofilm formation in oral isolates." Periodontol 2000 **42**: 13-26.

Davies, D. G., M. R. Parsek, et al. (1998). "The involvement of cell-to-cell signals in the development of a bacterial biofilm." Science **280**(5361): 295-298.

Dorrell, N. and B. W. Wren (2007). "The second century of Campylobacter research: recent advances, new opportunities and old problems." Curr Opin Infect Dis **20**(5): 514-518.

Duguid, I. G., E. Evans, et al. (1992). "Effect of biofilm culture upon the susceptibility of Staphylococcus epidermidis to tobramycin." J Antimicrob Chemother **30**(6): 803-810.
        Biofilms of Staphylococcus epidermidis were cultured at various specific growth

Elliott, W. H. and R. Shaw (1985). "High-performance liquid chromatography of bile acids." Methods Enzymol **111**: 51-63.

Elmi, A., E. Watson, et al. (2012). "Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells." Infect Immun **80**(12): 4089-4098.

Enos-Berlage, J. L. and L. L. McCarter (2000). "Relation of capsular polysaccharide production and colonial cell organization to colony morphology in Vibrio parahaemolyticus." J Bacteriol **182**(19): 5513-5520.

Everest, P. H., H. Goossens, et al. (1992). "Differentiated Caco-2 cells as a model for enteric invasion by Campylobacter jejuni and C. coli." J Med

Microbiol **37**(5): 319-325.

Everest, P. H., H. Goossens, et al. (1993). "Pathological changes in the rabbit ileal loop model caused by Campylobacter jejuni from human colitis." J Med Microbiol **38**(5): 316-321.

Everest, P. H., H. Goossens, et al. (1993). "Pathological changes in the rabbit ileal loop model caused by Campylobacter jejuni from human colitis." J Med Microbiol **38**(5): 316-321.

Fallingborg, J. (1999). "Intraluminal pH of the human gastrointestinal tract." Dan Med Bull **46**(3): 183-196.

Farges, M. C., M. P. Vasson, et al. (1996). "Supplementation of oral nutrition with pancreatic enzymes improves the nutritional status of aged endotoxemic rats." Nutrition **12**(3): 189-194.

Field, L. H., V. L. Headley, et al. (1986). "The chicken embryo as a model for campylobacter invasion: comparative virulence of human isolates of Campylobacter jejuni and Campylobacter coli." Infect Immun **54**(1): 118-125.

Finlay, B. B. and S. Falkow (1997). "Common themes in microbial pathogenicity revisited." Microbiol Mol Biol Rev **61**(2): 136-169.

Frees, D., A. Chastanet, et al. (2004). "Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in Staphylococcus aureus." Mol Microbiol **54**(5): 1445-1462.

Friedl, P., B. Konig, et al. (1992). "Effects of mucoid and non-mucoid Pseudomonas aeruginosa isolates from cystic fibrosis patients on inflammatory mediator release from human polymorphonuclear granulocytes and rat mast cells." Immunology **76**(1): 86-94.

Friedman, C. R., R. M. Hoekstra, et al. (2004). "Risk factors for sporadic Campylobacter infection in the United States: A case-control study in FoodNet sites." Clin Infect Dis **38 Suppl 3**: S285-296.

Friedman, M., P. R. Henika, et al. (2003). "Antibacterial activities of phenolic benzaldehydes and benzoic acids against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica." J Food Prot **66**(10): 1811-1821.

Friis, L. M., M. Keelan, et al. (2009). "Campylobacter jejuni drives MyD88-independent interleukin-6 secretion via Toll-like receptor 2." Infect Immun **77**(4): 1553-1560.

Fujimoto, S., N. Yuki, et al. (1992). "Specific serotype of Campylobacter jejuni associated with Guillain-Barre syndrome." J Infect Dis **165**(1): 183.

Fux, C. A., M. Shirtliff, et al. (2005). "Can laboratory reference strains mirror "real-world" pathogenesis?" Trends Microbiol **13**(2): 58-63.

Gaillot, O., E. Pellegrini, et al. (2000). "The ClpP serine protease is essential for the intracellular parasitism and virulence of Listeria monocytogenes." Mol Microbiol **35**(6): 1286-1294.

Gaynor, E. C., S. Cawthraw, et al. (2004). "The genome-sequenced variant of Campylobacter jejuni NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes." J Bacteriol **186**(2): 503-517.

Gestwicki, J. E., A. C. Lamanna, et al. (2000). "Evolutionary conservation of methyl-accepting chemotaxis protein location in Bacteria and Archaea." J Bacteriol **182**(22): 6499-6502.

Golden, N. J., A. Camilli, et al. (2000). "Random transposon mutagenesis of Campylobacter jejuni." Infect Immun **68**(9): 5450-5453.

Goto, T., Y. Nakame, et al. (1999). "In vitro bactericidal activities of beta-lactamases, amikacin, and fluoroquinolones against Pseudomonas aeruginosa biofilm in artificial urine." Urology **53**(5): 1058-1062.

Guccione, E., R. Leon-Kempis Mdel, et al. (2008). "Amino acid-dependent growth of Campylobacter jejuni: key roles for aspartase (AspA) under microaerobic and oxygen-limited conditions and identification of AspB (Cj0762), essential for growth on glutamate." Mol Microbiol **69**(1): 77-93.

Guerry, P., C. P. Ewing, et al. (2000). "Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of Campylobacter jejuni." Infect Immun **68**(12): 6656-6662.

Guerry, P., C. P. Ewing, et al. (2006). "Changes in flagellin glycosylation affect

Campylobacter autoagglutination and virulence." Mol Microbiol **60**(2): 299-311.

Guerry, P. and C. M. Szymanski (2008). "Campylobacter sugars sticking out." Trends Microbiol **16**(9): 428-435.

Guerry, P., C. M. Szymanski, et al. (2002). "Phase variation of Campylobacter jejuni 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro." Infect Immun **70**(2): 787-793.

Gur, E. and R. T. Sauer (2008). "Recognition of misfolded proteins by Lon, a AAA(+) protease." Genes Dev **22**(16): 2267-2277.

Hadden, R. D. and N. A. Gregson (2001). "Guillain--Barre syndrome and Campylobacter jejuni infection." Symp Ser Soc Appl Microbiol(30): 145S-154S.

Haddock, G., M. Mullin, et al. (2010). "Campylobacter jejuni 81-176 forms distinct microcolonies on in vitro-infected human small intestinal tissue prior to biofilm formation." Microbiology **156**(Pt 10): 3079-3084.

Hahn, A. F. (1998). "Guillain-Barre syndrome." Lancet **352**(9128): 635-641.

Hara, H., Y. Yamamoto, et al. (1991). "Cloning, mapping, and characterization of the Escherichia coli prc gene, which is involved in C-terminal processing of penicillin-binding protein 3." J Bacteriol **173**(15): 4799-4813.

Harvey, P. and S. Leach (1998). "Analysis of coccal cell formation by Campylobacter jejuni using continuous culture techniques, and the importance of oxidative stress." J Appl Microbiol **85**(2): 398-404.

Hazeleger, W., C. Arkesteijn, et al. (1994). "Detection of the coccoid form of Campylobacter jejuni in chicken products with the use of the polymerase chain reaction." Int J Food Microbiol **24**(1-2): 273-281.
.

Hazeleger, W. C., J. D. Janse, et al. (1995). "Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of Campylobacter jejuni." Appl Environ Microbiol **61**(7): 2713-2719.

Hazeleger, W. C., J. A. Wouters, et al. (1998). "Physiological activity of Campylobacter jejuni far below the minimal growth temperature." Appl Environ Microbiol **64**(10): 3917-3922.

Hendrixson, D. R. and V. J. DiRita (2004). "Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract." Mol Microbiol **52**(2): 471-484.

Hermans, D., K. Van Deun, et al. (2011). "Colonization factors of Campylobacter jejuni in the chicken gut." Vet Res **42**(1): 82.

Hickey, T. E., A. L. McVeigh, et al. (2000). "Campylobacter jejuni cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells." Infect Immun **68**(12): 6535-6541.

Hofreuter, D., V. Novik, et al. (2008). "Metabolic diversity in Campylobacter jejuni enhances specific tissue colonization." Cell Host Microbe **4**(5): 425-433.

Hoskins, J. R., M. Pak, et al. (1998). "The role of the ClpA chaperone in proteolysis by ClpAP." Proc Natl Acad Sci U S A **95**(21): 12135-12140.
.

Hoyle, B. D., J. Alcantara, et al. (1992). "Pseudomonas aeruginosa biofilm as a diffusion barrier to piperacillin." Antimicrob Agents Chemother **36**(9): 2054-2056.

Hu, L. and D. J. Kopecko (1999). "Campylobacter jejuni 81-176 associates with microtubules and dynein during invasion of human intestinal cells." Infect Immun **67**(8): 4171-4182.
.

Hudock, J. F., A. C. Borger, et al. (2005). "Temperature-dependent genome degradation in the coccoid form of Campylobacter jejuni." Curr Microbiol **50**(2): 110-113.

Hugdahl, M. B., J. T. Beery, et al. (1988). "Chemotactic behavior of Campylobacter jejuni." Infect Immun **56**(6): 1560-1566.

Humphrey, C. D., D. M. Montag, et al. (1986). "Morphologic observations of experimental Campylobacter jejuni infection in the hamster intestinal tract." Am J Pathol **122**(1): 152-159.

Ingmer, H. and L. Brondsted (2009). "Proteases in bacterial pathogenesis." Res Microbiol **160**(9): 704-710.

Jefferson, K. K. (2004). "What drives bacteria to produce a biofilm?" FEMS Microbiol Lett **236**(2): 163-173.

Jenkinson, H. F. and R. J. Lamont (2005). "Oral microbial communities in sickness and in health." Trends Microbiol **13**(12): 589-595.

Jin, S., A. Joe, et al. (2001). "JlpA, a novel surface-exposed lipoprotein specific to Campylobacter jejuni, mediates adherence to host epithelial cells." Mol Microbiol **39**(5): 1225-1236.

Jones, F. R., S. Baqar, et al. (2006). "New World monkey Aotus nancymae as a model for Campylobacter jejuni infection and immunity." Infect Immun **74**(1): 790-793.

Jones, F. R., S. Baqar, et al. (2006). "New World monkey Aotus nancymae as a model for Campylobacter jejuni infection and immunity." Infect Immun **74**(1): 790-793.

Joshua, G. W., C. Guthrie-Irons, et al. (2006). "Biofilm formation in Campylobacter jejuni." Microbiology **152**(Pt 2): 387-396.
        32799, 33084 and 31485 did not form flocs, and floc formation was reduced in

Kakuda, T. and V. J. DiRita (2006). "Cj1496c encodes a Campylobacter jejuni glycoprotein that influences invasion of human epithelial cells and colonization of the chick gastrointestinal tract." Infect Immun **74**(8): 4715-4723.

Kapperud, G., E. Skjerve, et al. (1992). "Risk factors for sporadic Campylobacter infections: results of a case-control study in southeastern Norway." J Clin Microbiol **30**(12): 3117-3121.

Karlyshev, A. V., P. Everest, et al. (2004). "The Campylobacter jejuni general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks." Microbiology **150**(Pt 6): 1957-1964.

Karlyshev, A. V., D. Linton, et al. (2000). "Genetic and biochemical evidence of a Campylobacter jejuni capsular polysaccharide that accounts for Penner serotype specificity." Mol Microbiol **35**(3): 529-541.

Karlyshev, A. V., D. Linton, et al. (2002). "A novel paralogous gene family involved in phase-variable flagella-mediated motility in Campylobacter jejuni."

Microbiology **148**(Pt 2): 473-480.

Karlyshev, A. V., M. V. McCrossan, et al. (2001). "Demonstration of polysaccharide capsule in Campylobacter jejuni using electron microscopy." Infect Immun **69**(9): 5921-5924.

Kolari, M., J. Nuutinen, et al. (2003). "Colored moderately thermophilic bacteria in paper-machine biofilms." J Ind Microbiol Biotechnol **30**(4): 225-238.
.

Konkel, M. E., B. J. Kim, et al. (1999). "Bacterial secreted proteins are required for the internaliztion of Campylobacter jejuni into cultured mammalian cells." Mol Microbiol **32**(4): 691-701.

Konkel, M. E., J. D. Klena, et al. (2004). "Secretion of virulence proteins from Campylobacter jejuni is dependent on a functional flagellar export apparatus." J Bacteriol **186**(11): 3296-3303.

Konkel, M. E., J. D. Klena, et al. (2004). "Secretion of virulence proteins from Campylobacter jejuni is dependent on a functional flagellar export apparatus." J Bacteriol **186**(11): 3296-3303.

Krishnan, K. and A. M. Flower (2008). "Suppression of DeltabipA phenotypes in Escherichia coli by abolishment of pseudouridylation at specific sites on the 23S rRNA." J Bacteriol **190**(23): 7675-7683.

Kudirkiene, E., M. T. Cohn, et al. (2012). "Phenotypic and genotypic characterizations of Campylobacter jejuni isolated from the broiler meat production process." Curr Microbiol **65**(4): 398-406.

Kuehn, M. J. and N. C. Kesty (2005). "Bacterial outer membrane vesicles and the host-pathogen interaction." Genes Dev **19**(22): 2645-2655.

Kuroki, S., T. Haruta, et al. (1991). "Guillain-Barre syndrome associated with Campylobacter infection." Pediatr Infect Dis J **10**(2): 149-151.

Kuroki, S., T. Saida, et al. (1993). "Campylobacter jejuni strains from patients with Guillain-Barre syndrome belong mostly to Penner serogroup 19 and contain beta-N-acetylglucosamine residues." Ann Neurol **33**(3): 243-247.

Laird, W. J. and D. C. Cavanaugh (1980). "Correlation of autoagglutination and virulence of yersiniae." J Clin Microbiol **11**(4): 430-432.

Lara-Tejero, M. and J. E. Galan (2000). "A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein." <u>Science</u> **290**(5490): 354-357.

Laws, A., Chadha, M., Chacon-Romero, M., Marshall, V. and Maqsood, M. (2008) 'Determination of the structure and molecular weights of the exopolysaccharide produced by Lactobacillus acidophilus 5e2 when grown on different carbon feeds' <u>Carbohydrate Research</u>, **343** (2):301-307

Lara-Tejero, M. and J. E. Galan (2001). "CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity." <u>Infect Immun</u> **69**(7): 4358-4365.

Leach, S., P. Harvey, et al. (1997). "Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of Campylobacter jejuni." <u>J Appl Microbiol</u> **82**(5): 631-640.

Ledeboer, N. A. and B. D. Jones (2005). "Exopolysaccharide sugars contribute to biofilm formation by Salmonella enterica serovar typhimurium on HEp-2 cells and chicken intestinal epithelium." <u>J Bacteriol</u> **187**(9): 3214-3226.

Lee, S. M. and J. Chen (2004). "Survival of Escherichia coli O157:H7 in set yogurt as influenced by the production of an exopolysaccharide, colanic acid." <u>J Food Prot</u> **67**(2): 252-255.

Leid, J. G., C. J. Willson, et al. (2005). "The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing." <u>J Immunol</u> **175**(11): 7512-7518.

Li, Y., H. Yang, et al. (2002). "Effect of high-temperature inside-outside spray on survival of campylobacter jejuni attached to prechill chicken carcasses." <u>Poult Sci</u> **81**(9): 1371-1377.

Lin, J., O. Sahin, et al. (2003). "Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of Campylobacter jejuni." <u>Infect Immun</u> **71**(8): 4250-4259.

Lin, J., O. Sahin, et al. (2003). "Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of Campylobacter jejuni." <u>Infect Immun</u> **71**(8): 4250-4259.

Lindmark, B., P. K. Rompikuntal, et al. (2009). "Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from Campylobacter

jejuni." <u>BMC Microbiol</u> **9**: 220.

Linton, D., M. Gilbert, et al. (2000). "Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of Campylobacter jejuni." <u>Mol Microbiol</u> **37**(3): 501-514.

Lithgow, J. K., E. Ingham, et al. (2004). "Role of the hprT-ftsH locus in Staphylococcus aureus." <u>Microbiology</u> **150**(Pt 2): 373-381.

Louwen, R., E. E. Nieuwenhuis, et al. (2012). "Campylobacter jejuni translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures." <u>Infect Immun</u> **80**(9): 3307-3318.

Lyte, M. and S. Ernst (1992). "Catecholamine induced growth of gram negative bacteria." <u>Life Sci</u> **50**(3): 203-212.

MacCallum, A., G. Haddock, et al. (2005). "Campylobacter jejuni activates mitogen-activated protein kinases in Caco-2 cell monolayers and in vitro infected primary human colonic tissue." <u>Microbiology</u> **151**(Pt 8): 2765-2772.

MacCallum, A., S. P. Hardy, et al. (2005). "Campylobacter jejuni inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions." <u>Microbiology</u> **151**(Pt 7): 2451-2458.

Mah, T. F. and G. A. O'Toole (2001). "Mechanisms of biofilm resistance to antimicrobial agents." <u>Trends Microbiol</u> **9**(1): 34-39.

Malik-Kale, P., B. H. Raphael, et al. (2007). "Characterization of genetically matched isolates of Campylobacter jejuni reveals that mutations in genes involved in flagellar biosynthesis alter the organism's virulence potential." <u>Appl Environ Microbiol</u> **73**(10): 3123-3136.

Mansfield, L. S., J. A. Bell, et al. (2007). "C57BL/6 and congenic interleukin-10-deficient mice can serve as models of Campylobacter jejuni colonization and enteritis." <u>Infect Immun</u> **75**(3): 1099-1115.

Marti, M., M. P. Trotonda, et al. (2010). "Extracellular proteases inhibit protein-dependent biofilm formation in Staphylococcus aureus." <u>Microbes Infect</u> **12**(1): 55-64.

Mashburn-Warren, L., R. J. McLean, et al. (2008). "Gram-negative outer membrane vesicles: beyond the cell surface." Geobiology **6**(3): 214-219.

McLennan, M. K., D. D. Ringoir, et al. (2008). "Campylobacter jejuni biofilms up-regulated in the absence of the stringent response utilize a calcofluor white-reactive polysaccharide." J Bacteriol **190**(3): 1097-1107.

McSweegan, E. and R. I. Walker (1986). "Identification and characterization of two Campylobacter jejuni adhesins for cellular and mucous substrates." Infect Immun **53**(1): 141-148.

Meltzer, M., S. Hasenbein, et al. (2008). "Allosteric activation of HtrA protease DegP by stress signals during bacterial protein quality control." Angew Chem Int Ed Engl **47**(7): 1332-1334.

Mendz, G. L., G. E. Ball, et al. (1997). "Pyruvate metabolism in Campylobacter spp." Biochim Biophys Acta **1334**(2-3): 291-302.

Mills, D. C., O. Gundogdu, et al. (2012). "Increase in Campylobacter jejuni invasion of intestinal epithelial cells under low-oxygen coculture conditions that reflect the in vivo environment." Infect Immun **80**(5): 1690-1698.

Misawa, N. and M. J. Blaser (2000). "Detection and characterization of autoagglutination activity by Campylobacter jejuni." Infect Immun **68**(11): 6168-6175.

Moayeri, M., D. Crown, et al. (2013). "Small molecule inhibitors of lethal factor protease activity protect against anthrax infection." Antimicrob Agents Chemother.

Moe, K. K., J. Mimura, et al. (2010). "The mode of biofilm formation on smooth surfaces by Campylobacter jejuni." J Vet Med Sci **72**(4): 411-416.

Monteville, M. R. and M. E. Konkel (2002). "Fibronectin-facilitated invasion of T84 eukaryotic cells by Campylobacter jejuni occurs preferentially at the basolateral cell surface." Infect Immun **70**(12): 6665-6671.

Monteville, M. R., J. E. Yoon, et al. (2003). "Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization." Microbiology **149**(Pt 1): 153-165.

Monteville, M. R., J. E. Yoon, et al. (2003). "Maximal adherence and invasion of INT 407 cells by Campylobacter jejuni requires the CadF outer-membrane protein and microfilament reorganization." Microbiology **149**(Pt 1): 153-165.

Murphy, C., C. Carroll, et al. (2003). "Identification of a novel stress resistance mechanism in Campylobacter jejuni." J Appl Microbiol **95**(4): 704-708.

Murphy, C., C. Carroll, et al. (2003). "Induction of an adaptive tolerance response in the foodborne pathogen, Campylobacter jejuni." FEMS Microbiol Lett **223**(1): 89-93.

Mylonakis, E. (2008). "Galleria mellonella and the study of fungal pathogenesis: making the case for another genetically tractable model host." Mycopathologia **165**(1): 1-3.

Nachamkin, I. (1997). "Microbiologic approaches for studying Campylobacter species in patients with Guillain-Barre syndrome." J Infect Dis **176 Suppl 2**: S106-114.

Nachamkin, I., J. Liu, et al. (2002). "Campylobacter jejuni from patients with Guillain-Barre syndrome preferentially expresses a GD(1a)-like epitope." Infect Immun **70**(9): 5299-5303.

Nachamkin, I., H. Ung, et al. (2002). "Increasing fluoroquinolone resistance in Campylobacter jejuni, Pennsylvania, USA,1982-2001." Emerg Infect Dis **8**(12): 1501-1503.

Nakajima, K., S. Inatsu, et al. (2008). "Possible involvement of put A gene in Helicobacter pylori colonization in the stomach and motility." Biomed Res **29**(1): 9-18.

Neutra, M. R., T. L. Phillips, et al. (1987). "Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch." Cell Tissue Res **247**(3): 537-546.

Newell, D. G. (2001). "Animal models of Campylobacter jejuni colonization and disease and the lessons to be learned from similar Helicobacter pylori models." Symp Ser Soc Appl Microbiol(30): 57S-67S.

O'Toole, G. A. and R. Kolter (1998). "Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis." Mol Microbiol **28**(3): 449-461.

O'Toole, G. A., L. A. Pratt, et al. (1999). "Genetic approaches to study of biofilms." Methods Enzymol **310**: 91-109.

Pace, J. L., T. J. Chai, et al. (1997). "Effect of bile on Vibrio parahaemolyticus." Appl Environ Microbiol **63**(6): 2372-2377.

Parker, C. T., W. G. Miller, et al. (2007). "Common genomic features of Campylobacter jejuni subsp. doylei strains distinguish them from C. jejuni subsp. jejuni." BMC Microbiol **7**: 50.

Parkhill, J., B. W. Wren, et al. (2000). "The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences." Nature **403**(6770): 665-668.

Pawalec, G. (2000). "Meeting report: first conference of the EU-supported Thematic Network on Immunology and Ageing in Europe (ImAginE), Schloss Hohentubingen, April, 2000." Exp Gerontol **35**(8): 1095-1103.

Petersen, F. C., N. A. Ahmed, et al. (2006). "LuxS-mediated signalling in Streptococcus anginosus and its role in biofilm formation." Antonie Van Leeuwenhoek **90**(2): 109-121.

Pope, L. M., K. E. Reed, et al. (1995). "Increased protein secretion and adherence to HeLa cells by Shigella spp. following growth in the presence of bile salts." Infect Immun **63**(9): 3642-3648.

Prokhorova, T. A., P. N. Nielsen, et al. (2006). "Novel surface polypeptides of Campylobacter jejuni as traveller's diarrhoea vaccine candidates discovered by proteomics." Vaccine **24**(40-41): 6446-6455.

Pullan, R. D., G. A. Thomas, et al. (1994). "Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis." Gut **35**(3): 353-359.

Purdy, A. E. and P. I. Watnick (2011). "Spatially selective colonization of the arthropod intestine through activation of Vibrio cholerae biofilm formation." Proc Natl Acad Sci U S A **108**(49): 19737-19742.

Quoc, V. T., McGuckin, M. A, et al. (2008). "Campylobacter jejuni response to human mucin MUC2: modulation of colonization and pathogenicity determinants" J Med Microbiol 57 (7): 795-802

Raphael, B. H., S. Pereira, et al. (2005). "The Campylobacter jejuni response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization." J Bacteriol **187**(11): 3662-3670.

Raphael, B. H., S. Pereira, et al. (2005). "The Campylobacter jejuni response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization." J Bacteriol **187**(11): 3662-3670.

Reeser, R. J., R. T. Medler, et al. (2007). "Characterization of Campylobacter jejuni biofilms under defined growth conditions." Appl Environ Microbiol **73**(6): 1908-1913.

Reuter, M., A. Mallett, et al. (2010). "Biofilm formation by Campylobacter jejuni is increased under aerobic conditions." Appl Environ Microbiol **76**(7): 2122-2128.

Rivera-Amill, V., B. J. Kim, et al. (2001). "Secretion of the virulence-associated Campylobacter invasion antigens from Campylobacter jejuni requires a stimulatory signal." J Infect Dis **183**(11): 1607-1616.

Rosch, J. W. and M. G. Caparon (2005). "The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in Streptococcus pyogenes." Mol Microbiol **58**(4): 959-968.

Russell, R. G., M. O'Donnoghue, et al. (1993). "Early colonic damage and invasion of Campylobacter jejuni in experimentally challenged infant Macaca mulatta." J Infect Dis **168**(1): 210-215.

Russell, R. G., M. O'Donnoghue, et al. (1993). "Early colonic damage and invasion of Campylobacter jejuni in experimentally challenged infant Macaca mulatta." J Infect Dis **168**(1): 210-215.

Sang, F. C., S. M. Shane, et al. (1989). "Enhancement of Campylobacter jejuni virulence by serial passage in chicks." Avian Dis **33**(3): 425-430.

Schmidt-Ott, R., H. Schmidt, et al. (2006). "Improved serological diagnosis stresses the major role of Campylobacter jejuni in triggering Guillain-Barre syndrome." Clin Vaccine Immunol **13**(7): 779-783.

Senior, N. J., M. C. Bagnall, et al. (2011). "Galleria mellonella as an infection model for Campylobacter jejuni virulence." J Med Microbiol **60**(Pt 5): 661-669.

Simon, G. L. and S. L. Gorbach (1984). "Intestinal flora in health and disease." Gastroenterology **86**(1): 174-193.

Skirrow, M. B. (2006). "John McFadyean and the centenary of the first isolation of Campylobacter species." Clin Infect Dis **43**(9): 1213-1217.

Skirrow, M. B., D. M. Jones, et al. (1993). "Campylobacter bacteraemia in England and Wales, 1981-91." Epidemiol Infect **110**(3): 567-573.

Slomiany, B. L., J. Piotrowski, et al. (1992). "Effect of sucralfate on the degradation of human gastric mucus by Helicobacter pylori protease and lipases." Am J Gastroenterol **87**(5): 595-599.

Smith, C. K., P. Kaiser, et al. (2005). "Campylobacter jejuni-induced cytokine responses in avian cells." Infect Immun **73**(4): 2094-2100.

Smith, J. L. (2003). "The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions." J Food Prot **66**(7): 1292-1303.

Smith, S. I., M. N. Otuonye, et al. (2002). "Prevalence of Campylobacter species among HIV/AIDS patients in Nigeria." Br J Biomed Sci **59**(3): 162-163.

Smith, S. I., M. N. Otuonye, et al. (2002). "Prevalence of Campylobacter species among HIV/AIDS patients in Nigeria." Br J Biomed Sci **59**(3): 162-163.

Stahl, M., L. M. Friis, et al. (2011). "L-fucose utilization provides Campylobacter jejuni with a competitive advantage." Proc Natl Acad Sci U S A **108**(17): 7194-7199.

Stahl, M., L. M. Friis, et al. (2011). "L-fucose utilization provides Campylobacter jejuni with a competitive advantage." Proc Natl Acad Sci U S A **108**(17): 7194-7199.

Stark, R. M., G. J. Gerwig, et al. (1999). "Biofilm formation by Helicobacter pylori." Lett Appl Microbiol **28**(2): 121-126.

Stern, N. J., J. S. Bailey, et al. (1988). "Colonization characteristics of Campylobacter jejuni in chick ceca." Avian Dis **32**(2): 330-334.

Stintzi, A. (2003). "Gene expression profile of Campylobacter jejuni in response to growth temperature variation." J Bacteriol **185**(6): 2009-2016.

Strugala, V., A. Allen, et al. (2003). "Colonic mucin: methods of measuring mucus thickness." Proc Nutr Soc **62**(1): 237-243.

Sutherland, I. (2001). "Biofilm exopolysaccharides: a strong and sticky framework." Microbiology **147**(Pt 1): 3-9.

Szymanski, C. M., M. King, et al. (1995). "Campylobacter jejuni motility and invasion of Caco-2 cells." Infect Immun **63**(11): 4295-4300.

Takaya, A., T. Tomoyasu, et al. (2002). "The ATP-dependent lon protease of Salmonella enterica serovar Typhimurium regulates invasion and expression of genes carried on Salmonella pathogenicity island 1." J Bacteriol **184**(1): 224-232.

Thomas, M. T., M. Shepherd, et al. (2011). "Two respiratory enzyme systems in Campylobacter jejuni NCTC 11168 contribute to growth on L-lactate." Environ Microbiol **13**(1): 48-61.

Tielen, P., M. Narten, et al. (2011). "Genotypic and phenotypic characterization of Pseudomonas aeruginosa isolates from urinary tract infections." Int J Med Microbiol **301**(4): 282-292.

Tomarelli, R. M., J. Charney, et al. (1949). "The use of azoalbumin as a substrate in the colorimetric determination or peptic and tryptic activity." J Lab Clin Med **34**(3): 428-433.

Tositti, G., P. Fabris, et al. (2001). "Pancreatic hyperamylasemia during acute gastroenteritis: incidence and clinical relevance." BMC Infect Dis **1**: 18.

Trachoo, N., J. F. Frank, et al. (2002). "Survival of Campylobacter jejuni in biofilms isolated from chicken houses." J Food Prot **65**(7): 1110-1116.

Tresse, O., T. Jouenne, et al. (1995). "The role of oxygen limitation in the resistance of agar-entrapped, sessile-like Escherichia coli to aminoglycoside and beta-lactam antibiotics." J Antimicrob Chemother **36**(3): 521-526.

Tsilibaris, V., G. Maenhaut-Michel, et al. (2006). "Biological roles of the Lon ATP-dependent protease." Res Microbiol **157**(8): 701-713.

Tu, Q. V., M. A. McGuckin, et al. (2008). "Campylobacter jejuni response to human mucin MUC2: modulation of colonization and pathogenicity

determinants." J Med Microbiol **57**(Pt 7): 795-802.

Tuomanen, E., R. Cozens, et al. (1986). "The rate of killing of Escherichia coli by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth." J Gen Microbiol **132**(5): 1297-1304.

Velayudhan, J., M. A. Jones, et al. (2004). "L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by Campylobacter jejuni." Infect Immun **72**(1): 260-268.

Vlisidou, I., M. Lyte, et al. (2004). "The neuroendocrine stress hormone norepinephrine augments Escherichia coli O157:H7-induced enteritis and adherence in a bovine ligated ileal loop model of infection." Infect Immun **72**(9): 5446-5451.

Walters, M. and V. Sperandio (2006). "Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli." Infect Immun **74**(10): 5445-5455.

Wassenaar, T. M., B. N. Fry, et al. (2000). "Genetic characterization of Campylobacter jejuni O:41 isolates in relation with Guillain-Barre syndrome." J Clin Microbiol **38**(2): 874-876.

Wassenaar, T. M., B. A. van der Zeijst, et al. (1993). "Colonization of chicks by motility mutants of Campylobacter jejuni demonstrates the importance of flagellin A expression." J Gen Microbiol **139 Pt 6**: 1171-1175.

Watnick, P. and R. Kolter (2000). "Biofilm, city of microbes." J Bacteriol **182**(10): 2675-2679.

Watson, R. O. and J. E. Galan (2005). "Signal transduction in Campylobacter jejuni-induced cytokine production." Cell Microbiol **7**(5): 655-665.

Wentland, E. J., P. S. Stewart, et al. (1996). "Spatial variations in growth rate within Klebsiella pneumoniae colonies and biofilm." Biotechnol Prog **12**(3): 316-321.

Wright, J. A., A. J. Grant, et al. (2009). "Metabolite and transcriptome analysis of Campylobacter jejuni in vitro growth reveals a stationary-phase physiological switch." Microbiology **155**(Pt 1): 80-94.

Xu, Z. R., C. H. Hu, et al. (2003). "Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male

broilers." <u>Poult Sci</u> **82**(6): 1030-1036.

Yao, R., D. H. Burr, et al. (1997). "CheY-mediated modulation of Campylobacter jejuni virulence." <u>Mol Microbiol</u> **23**(5): 1021-1031.

Yildiz, F. H. and G. K. Schoolnik (1999). "Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation." <u>Proc Natl Acad Sci U S A</u> **96**(7): 4028-4033.

Young, K. T., L. M. Davis, et al. (2007). "Campylobacter jejuni: molecular biology and pathogenesis." <u>Nat Rev Microbiol</u> **5**(9): 665-679.

Zhou, M., H. Hank Simms, et al. (2004). "Increased gut-derived norepinephrine release in sepsis: up-regulation of intestinal tyrosine hydroxylase." <u>Biochim Biophys Acta</u> **1689**(3): 212-218.

Zilbauer, M., N. Dorrell, et al. (2008). "Campylobacter jejuni-mediated disease pathogenesis: an update." <u>Trans R Soc Trop Med Hyg</u> **102**(2): 123-129.

Appendix 1

A1.1. Purity of hog pancreatic α-amylase preparation

Preparations of hog pancreatic α-amylase (Sigma cat no. 10080) dissolved in dH$_2$0 at concentration of 1 mg/ml were analysed by Dr Karen Homer (Kings College). Samples were run on SDS-PAGE gel (Figure A1.1)., the gel profile shown is indicative of those seen in three independent experiments The bands were cut and analysed using LC-MS/MS and the hits are listed according to the UniprotKB/SwissProt database from EBI. The data showed only pancreatic α-amylase as the sole protein present in the sample (Table A1.1).
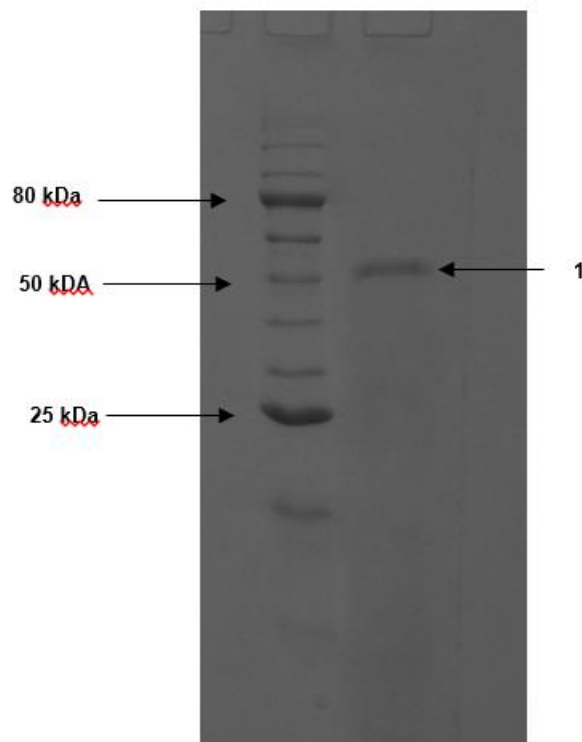
Figure A1.1. Lane 1: 10-250 kDa protein ladder (NEB). Lane 2: hog pancreatic α-amylase. Bands were excised from the SDS-PAGE gel shown, digested in-gel with trypsin and analysed by LC-MS/MS.

Table A1.1. Identity of protein present hog pancreatic α-amylase preparation (Sigma-Aldrich)

Band No.[1]	Accession number[2]	Protein identity[3]	Peptides matched[4]	Coverage[5]	MW (kDa)[6]
1	P00690	Pancreatic alpha-amylase	6	40	57.1

[1] Band number as shown on the SDS PAGE gel in Figure 3.8.

[2, 3] Accession number and protein identity as given in the the UniprotKB/SwissProt database from EBI.

[4] Number of peptides observed in mass spectra matching to the protein in the database.

[5] Percentage amino acid coverage (peptides observed/theoretical from sequence data).

[6] Theoretical molecular weight (kDa)

Appendix 2

A2.1 Construction of *spoT* mutant

A *spoT* mutant of *C. jejuni* 11168H was generated. This mutant was constructed by insertion of a ~1.4 kb fragment carrying the kanamycin-resistance gene, (*aphA-3*) into a unique BglII site in the *spoT* gene on the *C. jejuni* 11168H chromosome. The entire cloning strategy is shown in Figure A2.1.
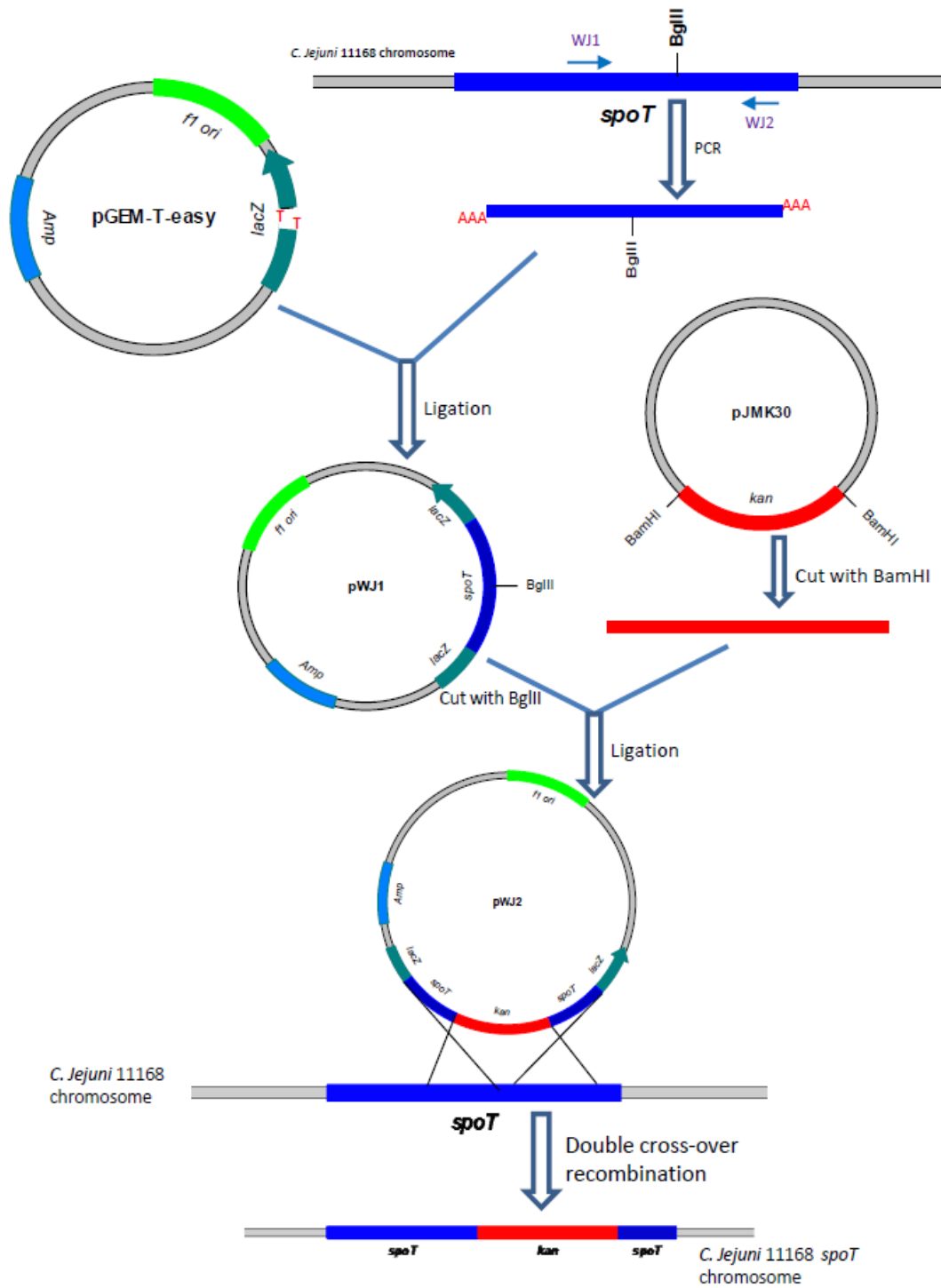
Figure A2.1. Graphical representation of the construction of *C.jejuni* 11168H *spoT* mutant. First, part of the *spoT* gene flanking the *Bgl*II site was amplified and cloned into vector pGEM-T-easy, generating pWJ1. Next the kan[r] cassette was cut from pJMK30 and inserted into pWJ1 at the *Bgl*II site. The resulting plasmid is pWJ2. Finally this vector was transformed into *C. jejuni* NCTC 11168 and the kan[r] cassette was inserted into the *spoT* gene in the chromosome via double cross-over recombination.

A2.2 Amplification of the *spoT* gene fragment

Nucleotides 968 bp to 2174 bp of the *spoT* gene were amplified from *C. jejuni* 11168H genomic DNA using primers WJ1 and WJ2. This fragment was designated spoT. The PCR product was analysed on an agarose gel as shown in Figure A2.2.
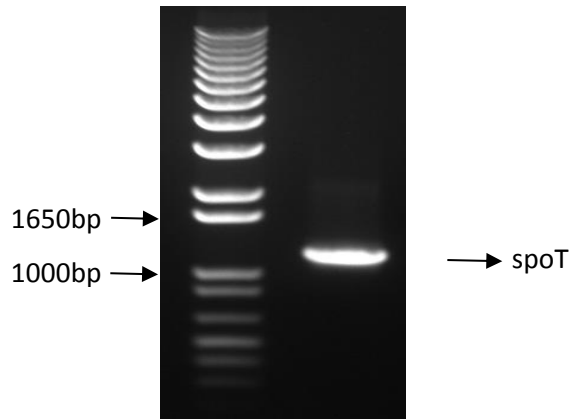
Figure A2.2. Gel electrophoresis of PCR product *spoT*, expected size of product: 1207bp.

A2.3 Cloning of *spoT* into pGEM-T-easy

The PCR product of *spoT* was purified and ligated into vector pGEM-T-easy. The ligation mix was transformed into *E. coli* JM109. Four white colonies were selected and checked for the insertion of *spoT* by PCR using primers WJ1 and WJ2 (Figure A2.3). All the 4 plasmids produced a product of the expected size; one was selected and designated pWJ1.
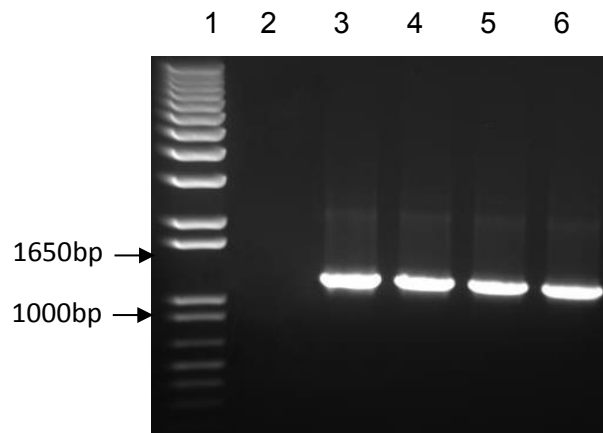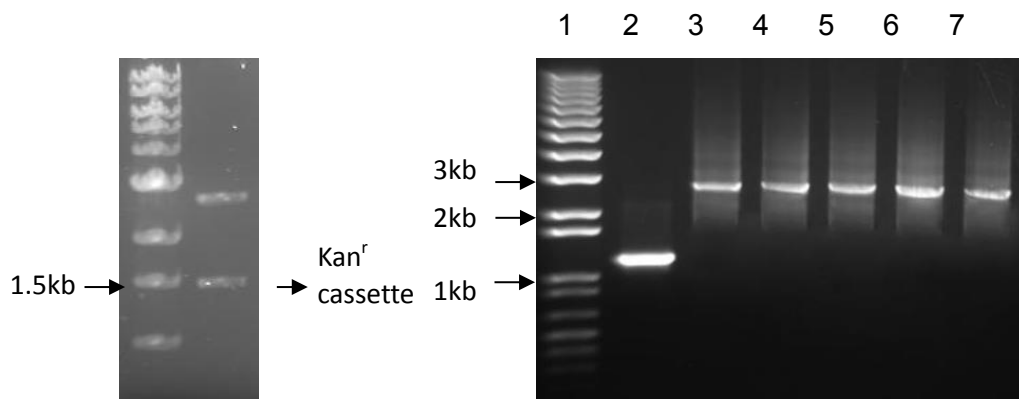
Figure A2.3. Gel electrophoresis of PCR samples amplified pGEM-T-easy transformants. Lane 1, 1 kb marker (New England Biolabs); lane 2, pGEM-T_easy negative control; lanes 3-6, plasmid from transformants. (negative control, the second lane) and purified plasmids (lane 3-6). PCR product from the expected clone is 1207bp.

A2.4 Insertion of kan^r cassette into pWJ1

A 1.4 kb BamHI fragment carrying the kan^r gene *aphA-3* was cut from plasmid pJMK30 (Figure A2.4). The 1.4 kb DNA fragment was gel-purified and ligated to *Bgl*II-digested pWJ1. The ligation mix was transformed into *E. coli* JM109. Five transformants were selected and analysed by PCR using primers WJ1 and WJ2. The PCR product generated by WJ1/WJ2 from the vector containing the kan^r cassette is ~ 2.6 kb (right). All five clones showed a band ~ 2.6kb, indicating that *aphA-3* had been inserted. A second PCR was used to identify clones that contained *aphA-3* in the same transcriptional orientation as *spoT* (data not shown). The plasmid with the insertion of kan^r cassette was designated pWJ2.

Figure A2.4. Gel electrophoresis of pJMK30 BamHI digests. B. Lane 1 contains a 1 kb ladder (New England Biolabs) PCR samples amplified from lane 2, control pWJ1; lanes 3-7, and purified plasmids (lane 3-7). PCR product from the expected clone is ~2.6kb.

A2.5 Integration of kan^r cassette into *C. jejuni* 11168H chromosome

Plasmid pWJ2 was electroporated into *C. jejuni* 11168H. Genomic DNA was purified from kanamycin resistant transformants, and analyzed by PCR using primers WJ1 and WJ3 to check the insertion and orientation of kan^r cassette. WJ3 is located in the kan^r cassette and is 500bp and 900bp to each end. The expected mutants with kan^r cassette inserted in a right direction will be able to generate a PCR product around 1.1kb (600bp of *spoT* and 500bp of kan^r gene). Figure A2.5 shows the amplified PCR product from genomica DNA of five transformants. All the five transformants displayed the expected DNA bands at about 1.1kb. This strain was named *C. jejuni* 11168H *spoT* mutant.
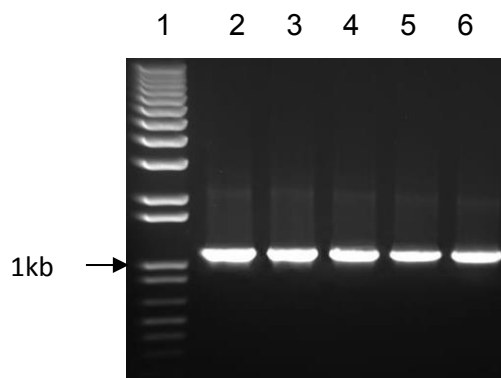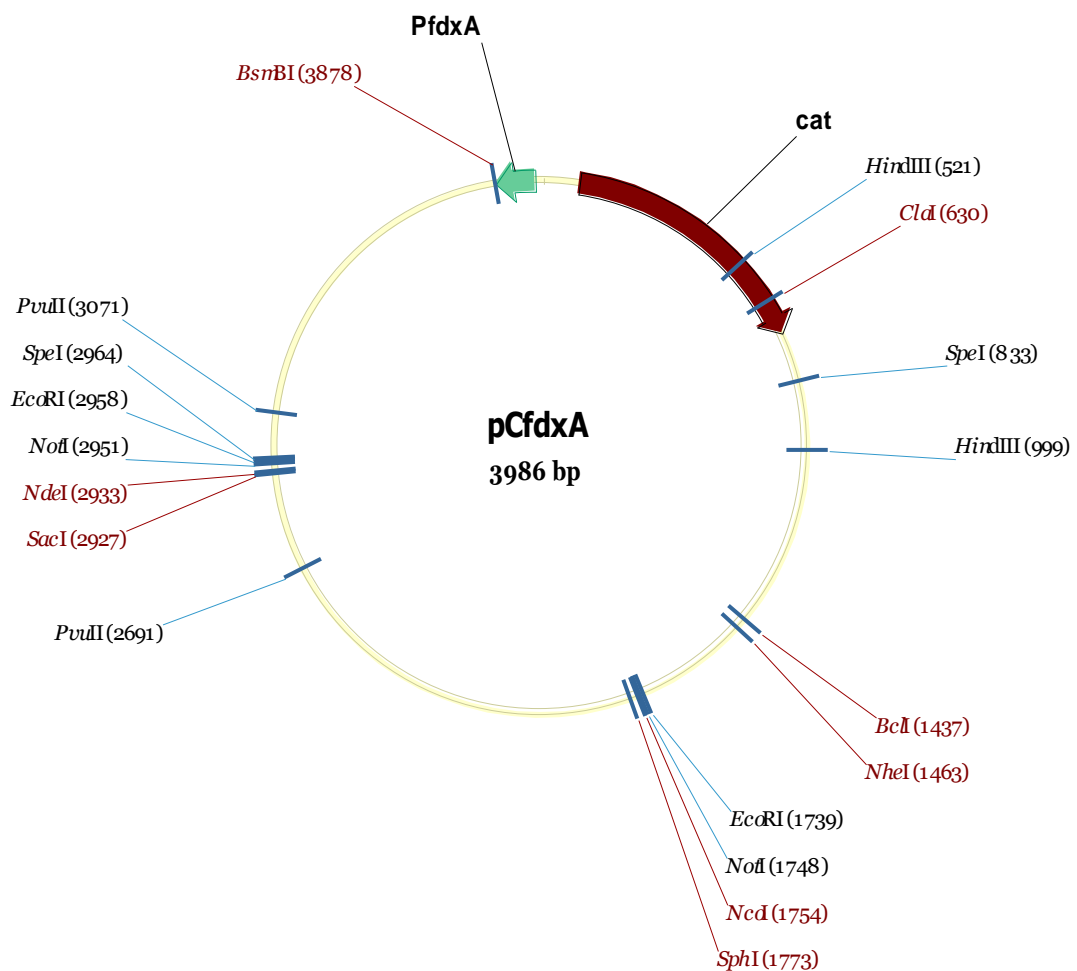

Figure A2.5. Gel electrophoresis of PCR samples amplified from chromosome DNA of five

Appendix 3

## A3.1 Complementation of the *Cj0511* mutant using pC46*fdxA*

The *C. jejuni* 11168H *Cj0511* mutant was complemented by insertion of a functional *Cj0511* gene into pseudogene *Cj0046* under control of the *fdxA* promoter by using plasmid pC46*fdxA* (Figure A3.1).

Figure A3.1. pC*fdxA* vector used for insertion of wildtype gene *Cj0511.* Expression is controlled by P*fdxA* to allow over expression of Cj0511.

## A3.2 Amplification of the *Cj0511*gene

The *Cj0511* gene (~1.3 kb) was amplified from *C. jejuni* 11168H genomic DNA. To enable formation of complementary ends for ligation the restriction site BsmBI was added to the 5' ends: Figure A3.2 shows a gel image of the PCR product (lane 3), which is at the expected size of 1.3 kb.
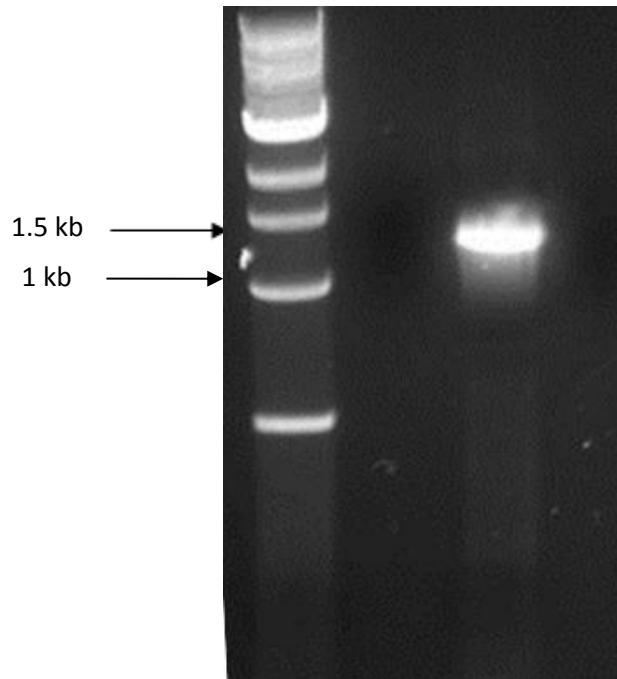
Figure A3.2. Gel electrophoresis of PCR of *Cj0511,* expected size of the product is 1346 bp

## A3.3 Cloning of *Cj0511* into pC*fdxA*

The PCR product (amplified *Cj0511*) was digested with BsmBI, mixed with similarly digested pC*fdxA* and ligated. The ligation mix was transformed into *E. coli* JM109. For confirmation that the *Cj0511* gene was inserted into pC*fdxA*, plasmids were purified from chloramphenicol resistant *E. coli* transformants, and digested with BsmBI generating two fragments. One fragment of ~3.9 kb was of the plasmid backbone and the other a fragment of ~1.3 kb (Figure A3.3).
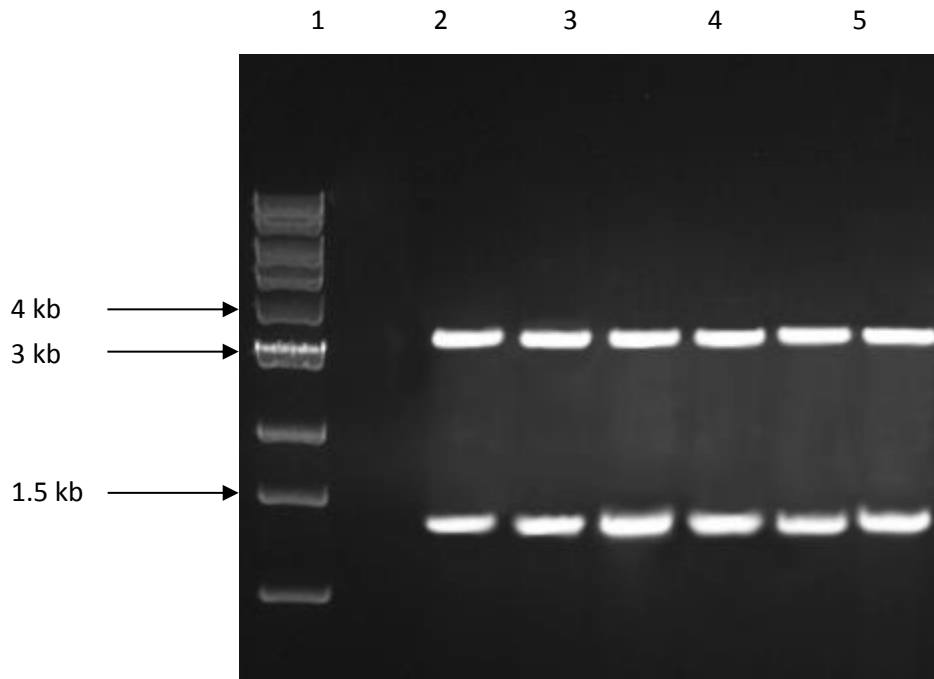
Figure A3.3. Gel electrophoresis of BsmBI restriction digest of *Cj0511* cloned into pC*fdxA*, expected digestion pattern: two fragments of 3926 bp and 1328 bp. Lane 1: 1kb DNA ladder (NEB). Lane 2: negative control. Lanes 3-8: Digested pC*fdxA-Cj0511* five individual transformants.

A3.4 Orientation of the *Cj0511* insertion

To determine the orientation of the *Cj0511* gene insertion in pC*fdxA* was correct with respect to P*fdxA* promoter, PCR amplifications from the pC*fdxA-Cj0511* plasmids were done using a forward primer specific for the *fdxA* promoter on Pc*fdxA* facing the BsmBI cloning site (PcfdxA F) and a reverse primer specific to the 3' end of the *Cj0511* gene (WJ10 R) as shown in Figure A3.4.
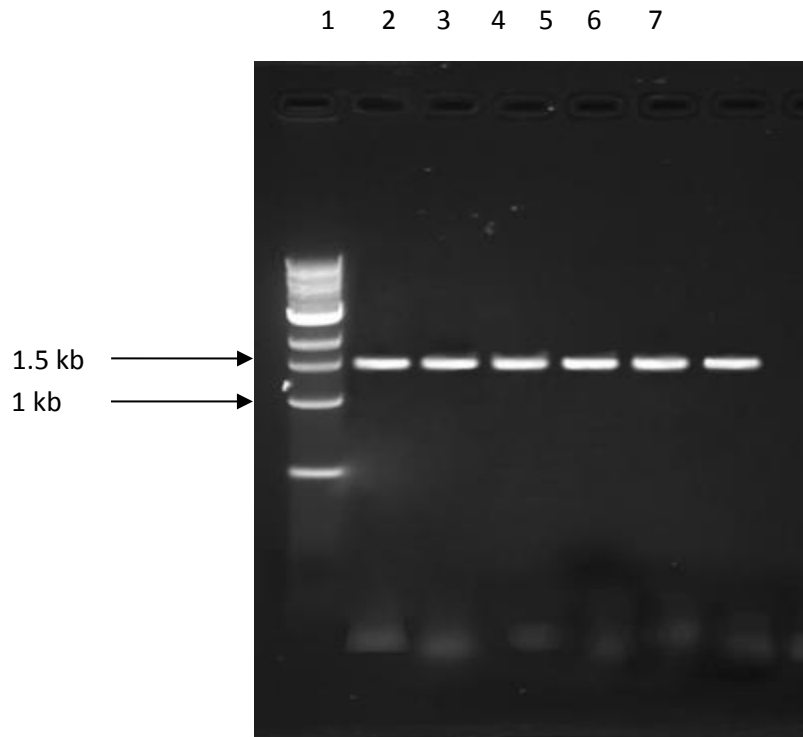
Figure A3.4. Gel electrophoresis ofPCR products amplified from pC*fdxA-Cj0511*
transformants. Lane 1: 1kb DNA ladder (NEB). Lane 2-7: pC*fdxA-Cj0511* fragments.
Expected product product size 1427 bp.

The plasmid with *Cj0511* gene inserted in the correct orientation was
designated pWJ4 and subsequently electroporated into the *C. jejuni* 11168H
Cj0511 mutant. The insertion of the promoter-gene fragment into *Cj0046* was
confirmed by PCR using primers specific for *Cj0511* (WJ10F and WJ10R) in
combination with primers specific for sequences flanking *Cj0046* in the
genome (WJ11 and WJ12) (Figure. A3.5).
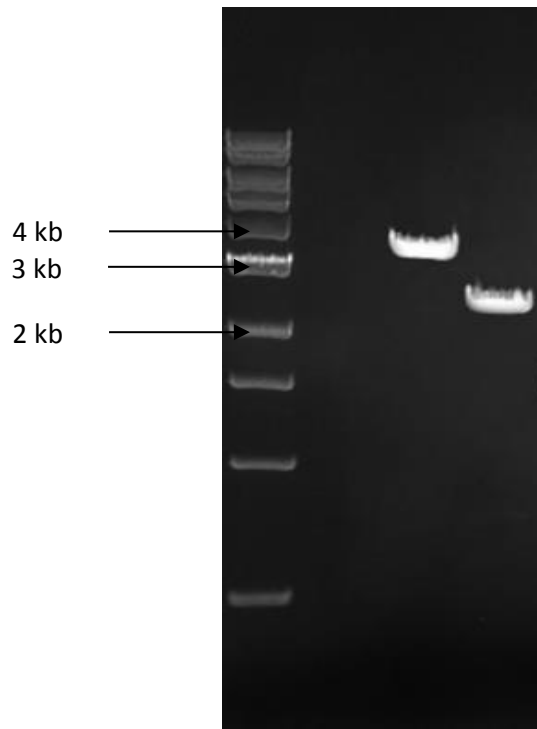
Figure A3.5. Gel electrophoresis of PCR products. Lane 1: 1kb DNA ladder (NEB). Lane 2: negative control. Lane 3: PCR product of WJ12 and WJ10R generate a product of 3255 bp. Lane 4: PCR product of WJ11 and WJ10F generate a product of 2396 bp.

Appendix 4

A4.1 Cj0511 recombinant protein expression

Genomic DNA from *C. jejuni* 11168H was used to amplify gene *Cj0511* using primers CJ1AF and CJ1AR (Figure A4.1). The amplified fragment was directionally cloned into the pET151/D-TOPO® vector expression system (not shown). The vector adds an N-terminal His_6 tag, a V5 epitope and a tobacco etch virus (TEV) protease cleavage site to the expressed recombinant protein. Correct cloning was verified by sequencing the DNA construct obtained by re-amplifying *Cj0511* from the ligated pET151/D-TOPO vector using standard primers for T7 promoters (data not shown).
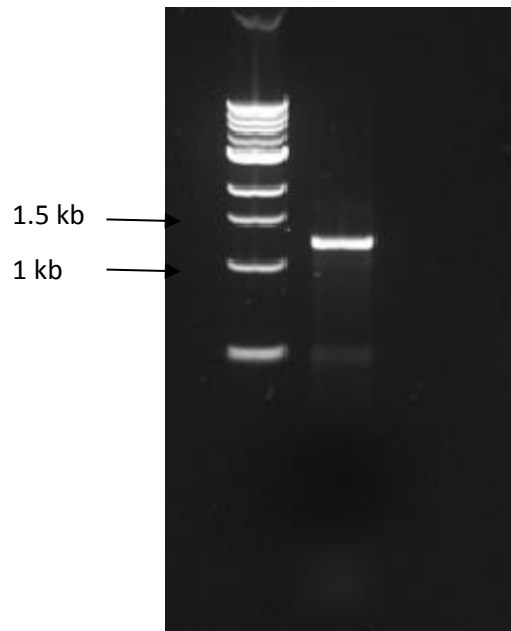

Figure A4.1. Gel electrophoresis of PCR products. Lane 1: 1kb DNA ladder (NEB). Lane 2: PCR product of CJ1AF and CJ1AR generate a product of 1355 bp

A4.2 Expression and Purification

The resulting plasmid pWJ3 was transformed into *E. coli* BL21 Star[TM] (DE3) One Shot®. Figure A4.2 shows SDS-PAGE analysis of different protein factions; in lane seven the over expressed 48 kDa recombinant Cj0511His protein band can be seen.
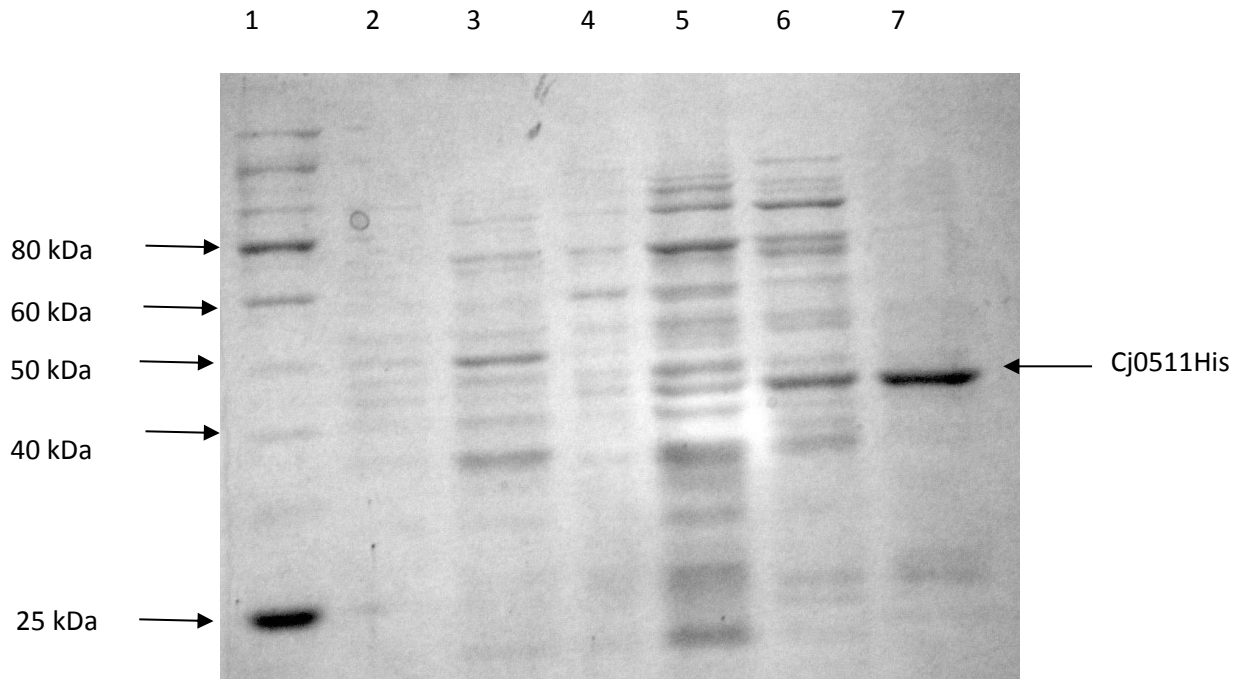
Figure A4.2. Coomassie stained 10% SDS PAGE. Lane 1: 10-250 kDa ladder (NEB). Lane 2: Unexpressed initial fraction. Lane 3: Unexpressed wash fraction. Lane 4: Unexpressed eluted fraction. Lane 5. Expressed initial fraction. Lane 6: Expressed wash fraction. Lane 7: Expressed eluted fraction containing purified recombinant Cj0511 of 48 kDa.