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CHARACTERISATION OF TOXINS PRODUCED BY *CAMPYLOBACTER JEJUNI* AND RELATED SPECIES

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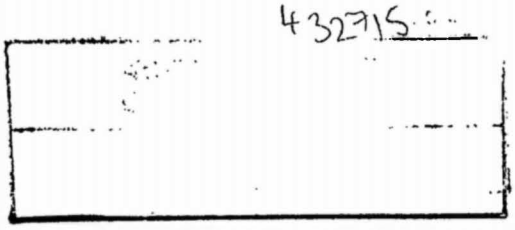
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" A PhD is about finding out more and more about less and less, until one eventually knows everything about nothing."

ANON

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LIST OF ABBREVIATIONS

ADP :	Adenosine Diphosphate
BA :	Blood Agar
BCIP :	5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BFA :	*Campylobacter* Blood Free Agar
BHI :	Brain Heart Infusion
BSA :	Bovine Serum Albumin
Caco-2 :	Human Colonic Adenocarcinoma Cells
cAMP :	Cyclic Adenosine Monophosphate
CAPS :	N-Cyclohexyl-3-Aminopropanesulfonic acid
CCT :	*Campylobacter* Cytotonic Toxin
CDSC :	Communicable Disease Surveillance Centre
CDT :	Cytolethal Distending Toxin
CHO :	Chinese Hamster Ovary Cells
CIAP :	Calf-Intestinal Alkaline Phosphatase
Cj0183 :	*Campylobacter* Putative Haemolysin Domain
CJT :	*Campylobacter jejuni* Toxin
CLRT :	Cytolethal Rounding Toxin
***ClyA* :**	*Campylobacter jejuni tlyA* gene Mutant
***ClyC* :**	*Campylobacter jejuni* Cj0183 Mutant
Compound 48/80 :	Phospholipase C Inhibitor
CT :	Cholera Toxin
DMEM :	Dulbecco's Modified Eagles Medium
DMF :	Dimethyl Formamide
DMSO :	Dimethyl Sulphoxide
DTNB :	5,5'-dithiobis (2-nitrobenzoic acid)
ECACC :	European Collection of Cell Cultures
ECV 304 :	Human Umbilical Cord Endothelial Cells
EDTA :	Ethylene Diamine Tetra Acidic Acid
FCS :	Foetal Calf Serum
FSA :	Food Standards Agency
GBS :	Guillain-Barré Syndrome
GPS :	Glutamine, Penicillin G and Streptomycin
HeLa :	Human Cervical Cancer Cells
Hep-2 :	Human Caucasian Larynx Carcinoma Cells
HlyA :	*Escherichia coli* Alpha Haemolysin
INT-407 :	Human Intestinal Epithelial Cells
KDO :	Ketodeoxyoctonic Acid
LB :	Luria-Bertani Media
LDH :	Lactate Dehydrogenase
LPS :	Lipopolysaccharide
LT :	*Escherichia coli* Heat Labile Enterotoxin
MOMP :	Major Outer Membrane Protein
MTT :	Methyl Tetrazolium Thiazoyl Blue
N2a :	Mouse Neuroblastoma Cells
NBT :	Nitro blue tetrazolium
NCTC :	National Collection of Type Cultures
OM :	Outer Membrane

OMP :	Outer Membrane Protein
OMPLA :	Phospholipase A_1
$PACOCF_3$:	Palmitoyl Trifluoromethyl Ketone
PBS :	Phosphate Buffered Saline
PCR :	Polymerase Chain Reaction
PLA2 :	Phospholiase A_2
PldA :	Phospholipase A Protein
PVDF :	Poly(Vinylidene Fluoride) Membrane
QMC :	Queens Medical Centre, Nottingham
RTX :	Repeat in Toxins
SDS :	Sodium Dodecyl Sulphate
SDS-PAGE :	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
$sPLA_2$:	Secretory Phospholipase A_2
Stx :	*E. coli* Shiga-like Toxin
TEMED :	Tetramethyl Ethylenediamine
Vero :	African Green Monkey Kidney Cells
VNC :	Viable but Non Culturable

ABSTRACT

During the last decade, *Campylobacter jejuni* has become recognised as one of the most common causes of human bacterial gastroenteritis in both the UK and the developing world, with over 46,000 cases of *Campylobacter* infection reported in England and Wales during 2002 alone. The actual number of cases is estimated to be nearer 354,000, and in addition, infection with *C. jejuni* has been linked to more severe conditions such as Guillain-Barré syndrome, reactive arthritis, and inflammatory bowel disease. However, despite nearly 30 years of research, the pathogenic mechanisms used to initiate disease have not been fully documented, and although it is accepted that *C. jejuni* produces a number of toxins, the current understanding is poorly defined and often contradictory. The aim of this research was to investigate and characterise these toxin(s), and to study potential toxins produced by the related pathogen *Arcobacter butzleri*, a recognised veterinary pathogen that recent research indicates may also be an important human pathogen.

Cytotoxic effects were detected when cell free extracts of *C. jejuni* were applied to cultured cells *in vitro*, and it is suggested that most of this cytotoxicity was a result of LPS activity, either acting on its own, or complexed with the major outer membrane protein (MOMP). In addition, both *C. jejuni* and *A. butzleri* produced at least one haemolysin that was able to lyse equine erythrocytes *in vitro*. Characterisation of this activity has shown that the majority of the haemolysis was cell-associated, calcium dependent, partially destroyed by heat, and not regulated by iron. It was proposed that this was due to the activity of phospholipase A (PldA).

A phospholipase A mutant was constructed from *C. jejuni* NCTC 11168 using inverse PCR mutagenesis. The mutant showed a markedly reduced haemolytic activity when tested *in vitro*, although not all of the activity was inhibited. It was demonstrated that PldA was responsible for the majority of the haemolysis detected, and the *pldA* gene was found to be highly conserved amongst a range of *C. jejuni* strains. It is likely that PldA is not the sole haemolytic factor in *C. jejuni*, and the remaining haemolytic activity may be due to a calcium independent, pore-forming toxin.

CHAPTER 1

INTRODUCTION

1.1 *CAMPYLOBACTER* INTRODUCTION AND OVERVIEW

Campylobacter species are recognised as the most common cause of bacterial gastroenteritis or food-borne illness in both the United Kingdom and the rest of the developed world. In 2002, 46,484 laboratory confirmed cases of Campylobacter infection were reported to the Communicable Disease Surveillance Centre (CDSC) in England and Wales, more than three times the number of people infected by the better known pathogen *Salmonella* (see figure 1.1). It is fairly obvious from this data that the incidence of *Campylobacter* is far higher than any of the other presented organisms – the number of *Listeria* and *E. coli* 0157 cases are much lower in comparison, with only a few hundred reported cases each year. The number of *Salmonella* cases, probably one of the best publicised food poisoning organisms, is also in the thousands, but over the last five years or so, the number of people infected with this bacterium has started to decline sharply.

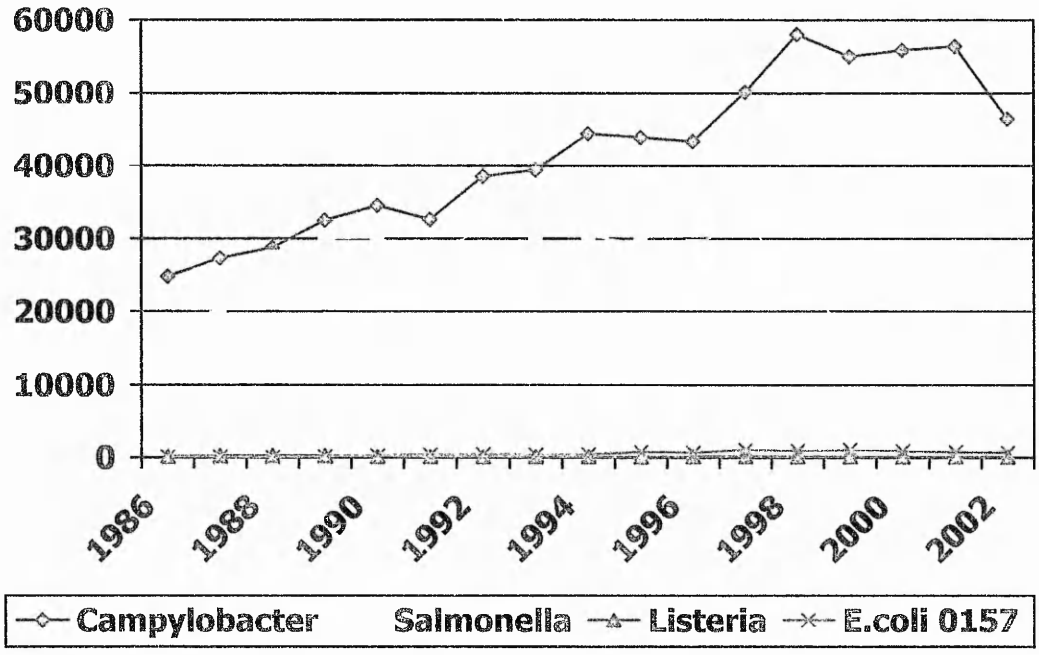


Figure 1.1 – Laboratory reports of commonly isolated foodborne pathogens in England and Wales (1986 – 2002) reported to the Communicable Disease Surveillance Centre (CDSC, www.hpa.org.uk).

Despite a small drop in the *Campylobacter* incidence figures in 2002, which affected all food-borne organisms, the number of *Campylobacter* cases remains high. This decrease in the total number of reported cases of all pathogenic bacteria was likely to be a result of the outbreak of foot and mouth disease, where less meat was purchased and consumed, farm visits were stopped, and animals were kept indoors and away from the public.

The number of *Campylobacter* cases reported to the CDSC is thought to be a gross underestimation of the actual number of cases, as many people with milder food poisoning symptoms do not always seek medical advice, and therefore the disease is under-reported. Wheeler *et al.* (1999), estimated that the true number of cases of *Campylobacter* related food poisoning when compared to the number of cases that are reported each year gives a ratio of approximately 7.6:1, indicating that the actual number of *Campylobacter* related infections in 2002 was closer to 354,000 (Leach 1997; Wheeler *et al.* 1999).

The economic burden due to campylobacter infection is large, and in 1995 it was estimated that the average cost of a case of acute campylobacter infection (excluding chronic effects such as Guillain-Barré syndrome) was an estimated £315 (Roberts *et al.* 2003). Therefore, it is feasible that in 2002, the UK Government spent over £111 million treating *Campylobacter* infection.

The pattern of *Campylobacter* infections seems to show a seasonal variation, with the peak number of cases occurring in late spring to early autumn (Nylen *et al.* 2002; Ure *et al.* 2003). One hypothesis for this unusual pattern is that the seasonal rise in the number of *Campylobacter* cases reported may coincide with barbeque season, where in many cases, the food is charred on the outside, but still raw on the inside. Other possibilities, such as purchasing more young pets during the summer months, and the consumption of bird pecked milk have also been suggested (Adak *et al.* 1995; Frost 2001).

1.1.1 *Campylobacter* as a Food-borne Pathogen

Unlike other enteric pathogens, most *Campylobacter* infections are sporadic and outbreaks are extremely rare with only 50 *Campylobacter* related outbreaks being reported in England and Wales between 1995 and 1999 (Tauxe 1992; Gillespie *et al.* 2003). Of those reported, contaminated chicken was found to be responsible for 35 incidents, and waterborne infection attributed to a further four outbreaks (Godoy *et al.* 2002; Said *et al.* 2003). Therefore, the majority of *Campylobacter* infections are isolated cases, and hence the reason why *Campylobacter* rarely makes the headlines. Although a successful food pathogen, *Campylobacter* spp. are extremely sensitive to hostile environments outside the intestines, which is somewhat surprising especially when compared to other enteric pathogens. For example, *Campylobacter jejuni* does not survive well at low pH conditions or extremes of temperature, and is not able to tolerate desiccation (Blaser *et al.* 1980; Wong 1998).

The optimum growth temperature for thermophilic *Campylobacter* is 42°C, and the organisms cannot grow at temperatures below 30°C. Many domestic animals such as dogs and cats carry *Campylobacter* without displaying any adverse symptoms, and certain birds, in particular poultry, are considered to be a primary reservoir for *C. jejuni*, where the organism is generally considered to be a commensal (Park 2002). It seems little coincidence then, that the internal avian gut temperature of a chicken is 42°C (the optimum temperature of *Campylobacter*), and it is possible that humans may be accidental hosts, with *Campylobacter* generating toxins and other pathogenic mechanisms merely to survive the hostile conditions of the human intestine.

A recent survey carried out by the Food Standards Agency, found that over 50% fresh and frozen retail chickens purchased in the UK tested positive for *Campylobacter* contamination (Food Standards Agency 2003). When it is considered that more than a million tonnes of chicken meat is produced in the UK annually (ACMSF 2002), the importance of research into this food-borne organism cannot be underestimated.

A possible explanation for the success of *C. jejuni* as a food borne pathogen despite its fastidiousness, is that the infectious dose for a healthy human can be quite low (as little as 500 - 800 cells, Black *et al.* 1988), and the contamination levels found in chicken carcasses have been reported to be upwards of 1 million cells recovered from a single rinse of a chicken carcass (Walker *et al.* 1986; Jacobs-Reitsema 2000; Humphrey 2002). Therefore, even if many cells die, there may be enough remaining to cause infection. It is also possible that *Campylobacter* can exist in a state known as 'viable but non culturable' (VNC), whereby the organism remains infectious but can no longer be cultured (Jones *et al.* 1991; Korsak & Popowski 1996; Lazaro *et al.* 1999). The first report of the *Campylobacter* VNC state was published by Rollins and Colwell (1986), who suggested that the organism transforms into a coccoid shape in response to environmental stress, and as such, cannot be cultured using conventional laboratory methods, but is still able to cause infection in a suitable host. Therefore, the organism is able to survive for longer periods in water, foods and food preparation areas, and still remain viable (Moran & Upton 1987).

The existence of this state is currently under debate, as many research groups believe that the reason these bacteria cannot be recovered is simply due to stresses during harsh culturing conditions, and recent improvements in isolation methods mean that it is now possible to recover viable cells in situations where they were previously thought to be non-culturable (Corry *et al.* 1995; Humphrey 2002).

1.2 *CAMPYLOBACTER JEJUNI*

The genus *Campylobacter* currently comprises of 16 species, 12 of which have been associated with human disease (Lastovica & Skirrow 2000; Park 2002). Pathogenic species in the *Campylobacter* family include *C. coli, C. lari* and *C. upsaliensis*, which have all been associated with human illness, but the majority of enteric *Campylobacter* infections (80-90%) are caused by *Campylobacter jejuni* (Ketley 1997).

Campylobacter jejuni, like all the *Campylobacter* family are Gram negative, motile rods, belonging to the ε subgroup of the Proteobacteria (Campylo meaning curved in Greek). The cells commonly measure between 0.2-0.5μm in width by 1.5-5μm in length and are vibrioid in shape, with a single polar flagellum at one or both ends (see figure 1.2), which is responsible for the bacteria's corkscrew-like motility. *C. jejuni* are microaerophilic, requiring reduced levels of oxygen (3-6%) for optimum growth, and thermophilic, with a temperature range between 34-44°C, exhibiting optimum growth at 42°C. The organisms are often found in the intestinal flora of healthy animals (particularly birds and livestock), where they colonise the gut, causing no adverse effects to the host. Research has found that between 20-100% of a flock of chickens can be infected with *C. jejuni* at any one time (Willis & Murray 1997; Corry & Atabay 2001).

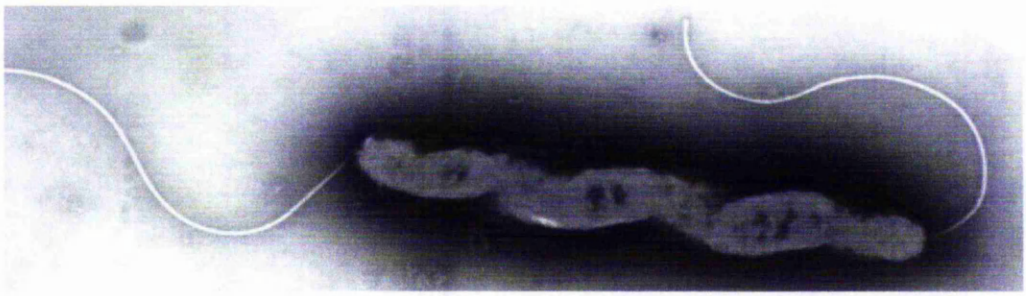


Figure 1.2 - *Campylobacter jejuni* (downloaded from www.foodlink.org.uk/poisonsub3.htm).

Campylobacter have a small AT rich genome of approximately 1.6-1.7 Mbp, comprising a GC ratio of approximately 30%. The genome is unusual in that there are very few insertion sequences or repetitive DNA sequences, but there are many hypervariable sequences predominantly associated with genes responsible for surface structures. This variation occurs mainly in polyG:C tracts, and may be an important survival mechanism whereby the organism can evade host immune responses (Parkhill *et al.* 2000). Extrachromosomal elements such as plasmids and bacteriophages have been reported from some strains, and may be involved with adhesion, antibiotic resistance or toxin production (Taylor 1992; Bacon *et al.* 2000; Gibreel *et al.* 2004).

1.2.1 Transmission and Disease Symptoms

Transmission of *C. jejuni* in humans usually occurs from the consumption of contaminated chicken, though other sources include unpasteurised milk, non-chlorinated water, and contact with infected pets, particularly dogs and cats (Tauxe 1992). Once inside the host, disease symptoms can vary vastly, ranging from a mild watery diarrhoea, most common in developing countries, to a severe, acute inflammatory diarrhoea more commonly found in patients from industrialised nations (Klipstein *et al.* 1985; Walker *et al.* 1986; Ketley 1997; Leach 1997).

The most common clinical manifestation of *Campylobacter* infection in the UK is diarrhoea, which may be watery or inflammatory and can contain blood, mucus and/or leukocytes. Other symptoms include fever, abdominal pain, nausea, headache and muscle pain. Symptoms usually appear between two to five days after ingestion of the bacteria, and generally last for about seven to ten days.

Clinical symptoms in patients from developing countries are usually a lot less severe. The typical clinical presentation is mild watery diarrhoea, usually without any blood, mucus or leukocytes, and is most common in young children (Klipstein *et al.* 1985; Florin & Antillon 1992; Fields & Swerdlow 1999).

Although the mechanisms by which *Campylobacter* causes disease are not fully understood, these very different clinical symptoms suggests that there may be different mechanisms, and possibly different toxins responsible, and that these may be strain related (Ruiz-Palacios *et al.* 1983; Ketley 1997; Park 2002).

1.2.2 Immune Response and Clinical Outcomes of *Campylobacter* Infection

As mentioned in section 1.2.1 above, clinical presentations of patients infected with *Campylobacter* vary, and it is currently unclear as to why infection with the same bacteria can cause so many very different symptoms. One current opinion suggests that the host immune status plays an important role in the disease outcome, and speculates that infection with *Campylobacter* for the first time, particularly in young children or immunocompromised individuals leads to increased susceptibility of developing the inflammatory symptoms of the disease (Konkel *et al.* 2000; figure 1.3 (c)).

After human infection with *C. jejuni*, antibodies can be seen in the serum from about the fifth day of infection, peak within two to four weeks, and then start to decline. Intestinal antibodies are also produced (Black *et al.* 1988; Skirrow & Blaser 2000). When the host is re-challenged with a similar strain, this may result in milder, non-inflammatory symptoms, presenting watery diarrhoea, containing no blood or leucocytes (Wallis 1994; figure 1.3 (b)).

If the host is re-introduced to the same bacterial strain, a protective immune response is initiated, invasion of the bacteria is limited, and little or no symptoms are seen (Ketley 1997; figure 1.3 (a)). Deliberate infection of human volunteers with *C. jejuni* showed that upon first challenge with the bacteria, inflammatory illness was produced, and antibodies were raised against the *C. jejuni* strain. When the same volunteers were re-challenged with the same strain, no disease symptoms were seen, but the bacteria were still able to colonise the mucosa (Black *et al.* 1988).

This theory explains why different patterns of immunity are seen in patients from developed and developing nations, suggesting that in developing countries, recurrent infection with *Campylobacter* leads to the development of protective immunity, resulting in milder disease symptoms (Ketley 1997). Additionally, travellers contracting *Campylobacter* when visiting other countries experience disease symptoms similar to those most common in their country of origin (Scott & Tribble 2000; Van Vliet & Ketley 2001). Whether this hypothesis is confirmed

or not, it is likely that the host immune status plays some role in the pattern of disease symptoms caused by this organism.

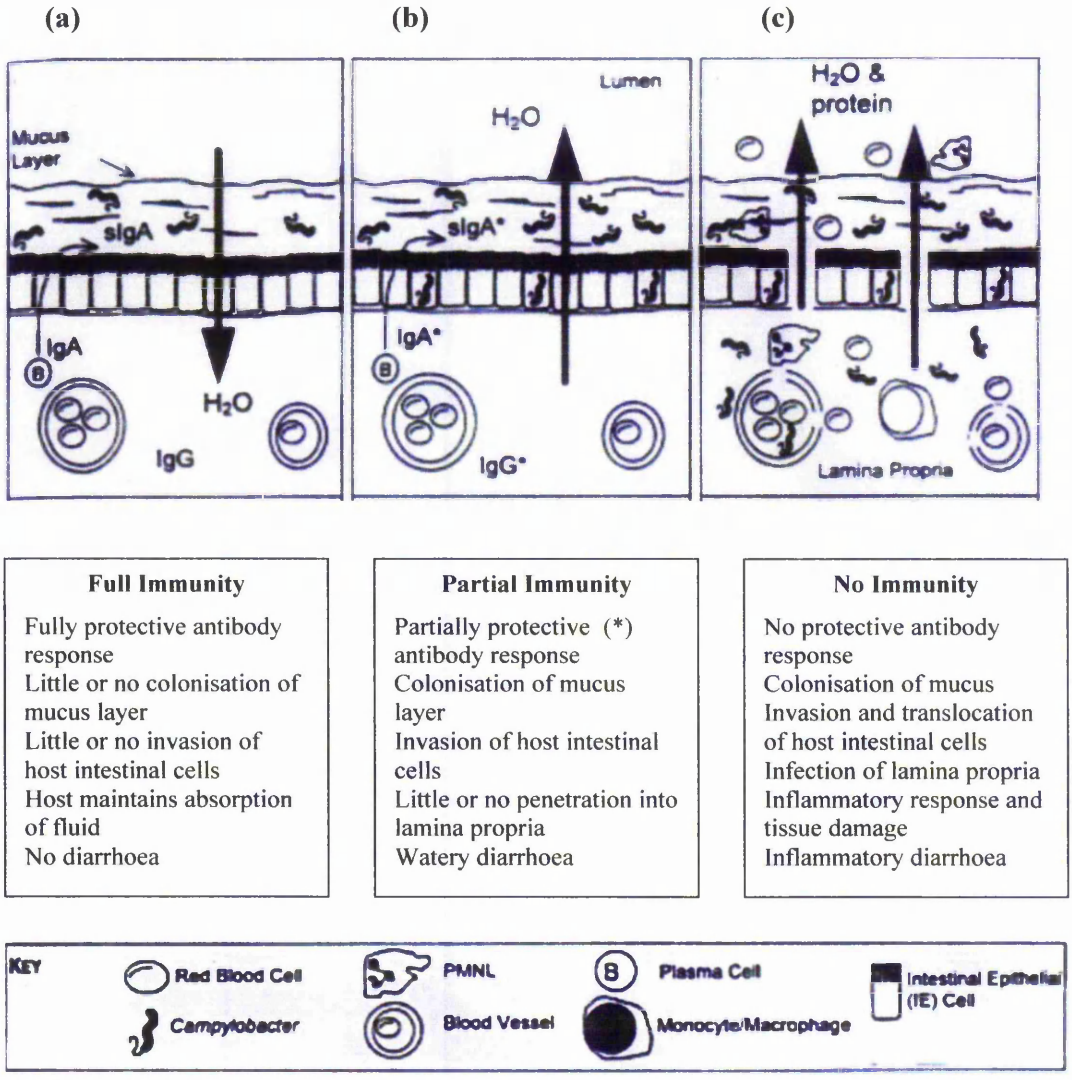


Figure 1.3 – Model to explain the different outcomes of *C. jejuni* infection, where (a) shows infection in a host with protective immunity, (b) the host has been previously infected by a heterologous strain with some shared epitopes, (c) the host has no previous history of *Campylobacter* infection (Reproduced from Ketley 1997).

1.2.3 Chronic Effects of *Campylobacter* Infection

In addition to causing gastritis, *Campylobacter* infection has also been associated with a number of other, more serious illnesses, such as Guillain-Barré syndrome (GBS) (Allos 1998; Hadden & Gregson 2001; Tsang 2002), Miller-Fisher syndrome (Nachamkin 2002), reactive arthritis (Gaston & Lillicrap 2003), and most recently, inflammatory bowel disease and irritable bowel syndrome (IBS) (Rodriguez & Ruigomez 1999; Boyanova *et al.* 2004).

Of these sequelae, the most well studied is GBS (Nachamkin 2002), as although a number of infections have been identified as preceding the development of GBS, *Campylobacter* infection has been attributed to the largest percentage of all cases of GBS studied.

1.2.3.1 Guillain-Barré Syndrome (GBS)

Guillain-Barré syndrome is the most common cause of acute neuromuscular paralysis in the developed world since the eradication of polio (Allos 1998; Haddon & Gregson 2001; Tsang 2002). The disease is an autoimmune mediated disorder, affecting the nervous system, and is characterised by the stripping away of the myelin sheaths around the peripheral nerves, leading to weakness of the limbs and the onset of paralysis, which in severe cases can result in death. GBS is usually preceded by a bacterial or viral infection, most commonly *C. jejuni* enteritis, but other infectious agents include the Epstein-Barr virus, *Mycoplasma pneumoniae*, and the cytomegalovirus.

The first report of a link between *Campylobacter* infection and GBS was published in 1982 (Rhodes & Tattersfield), and since then it has been confirmed that *Campylobacter* is the most common precedent to GBS, with between 30-40% of GBS patients having been infected with *Campylobacter* enteritis in the one to three weeks prior to the disease process. The actual number of cases of *C. jejuni* linked GBS may be a lot higher than this though, as research has shown that methods of culturing *C. jejuni* from stool samples of infected patients is

difficult, and often a negative result is obtained due to limitations in culture techniques and the lag period between the infection with *Campylobacter* and the onset of GBS (Allos 1998; Nachamkin *et al.* 2000).

Symptoms of GBS usually begin with weakness or pain in the legs and arms, spreading to the upper limbs and cranial nerves. This can lead on to facial weakness, and difficulty swallowing and breathing (Hughes *et al.* 1999; Nachamkin *et al.* 2000). Although most patients make a full recovery, up to 20% may be left with severe neurological problems, and roughly 2-3% will die (Hughes & Rees 1997).

The mechanism by which *Campylobacter* is thought to cause the onset of GBS is antigenic mimicry (Hadden & Gregson 2001; Tsang 2002). This may be caused by lipopolysaccharides (LPS) on the surface of *Campylobacter* cells, or sialylated oligosaccharides found in the flagellum, both of which resemble human gangliosides. Gangliosides are the major surface molecules of the peripheral and central nervous system (PNS and CNS), the most common of these being GM1, which is the ganglioside implicated in *Campylobacter* related GBS (Jacobs *et al.* 1997; Linton *et al.* 2000; Haddon & Gregson 2001).

Campylobacter related GBS patients tend to have a worse outcome than other GBS patients (the disease tends to be more severe), and are more likely to have antibodies to ganglioside GM1 (Prendergast *et al.* 1998). Several *C. jejuni* serotypes have been implicated as being more likely to cause GBS than other strains, and it is likely that host factors may additionally play a role in the pathogenesis of the disease. The molecular mimicry theory does not explain why most patients infected with *C. jejuni* do not develop GBS, and additionally how GBS is contracted in patients that have not been exposed to *C. jejuni* enteritis (Allos 1998; Hadden & Gregson 2001; Nachamkin 2002). It is obvious that more research is needed in this area to further understand the mechanisms and molecular pathogenesis of this important disease.

1.3 HISTORICAL SIGNIFICANCE OF *CAMPYLOBACTER* SPP.

In 1886 Theodor Escherich discovered vibrio like bacteria in the colons of children who had died from "cholera infantum" (Kist 1986; Skirrow & Butzler 2000). Escherich was unable to culture these bacteria, and after recording an illustration (figure 1.4) concluded that they had no clinical significance. It is now believed that these spiral bacteria may have been *Campylobacter* spp. and the fastidious nature of these organisms would explain Escherich's failure to grow them on solid media (Kist 1986).

Figure 1.4 – Copy of diagram by T. Escherich (1886) of vibrio bacteria in colonic mucus of a child who died of "cholera infantum" (Kist 1986).

This inability to culture *Campylobacter* led to the organism being overlooked as an important pathogen for many years. It was not until 1913 that McFadyean and Stockman first isolated the bacterium now known as *Campylobacter fetus* from an aborted sheep foetus. Five years later, Smith (1918) discovered microaerophilic spiral shaped bacteria associated with aborted calves, and it was concluded that both these organisms and the bacteria isolated by McFadyean and Stockman were of the same species, and were named *Vibrio fetus* (Vandamme 2000; Moore & Matsuda 2002).

It was not until the 1970's that they were finally recognised as human pathogens and isolated from human stool samples (Dekeyser *et al.* 1972; Butzler *et al.* 1973; Skirrow 1977), although since this date the association of campylobacters with disease has increased annually, and they are now recognised as the leading cause of foodborne disease throughout the developing world (see section 1.1).

1.3.1 Taxonomy and Nomenclature

Sebald and Véron first proposed the genus *Campylobacter* in 1963 (On 2001), which then comprised just two species; *Campylobacter fetus* and '*Campylobacter bubulus*' (now known as *Campylobacter sputorum*). Prior to this, *Campylobacter* had been included with the *Vibrionaceae*, until it was realised that the low G+C content, inability to ferment carbohydrates, and microaerophilic growth requirements of the organism set it apart in its own genus (Moore & Matsuda 2002).

It was not until ten years later that a more comprehensive investigation of the taxonomy of these organisms was published (Vèron & Chatelain 1973), where the *Campylobacter* genus was extended to four distinct species: *Campylobacter fetus, Campylobacter coli, Campylobacter jejuni,* and *Campylobacter sputorum* (Vandamme 2000). This classification system is still in operation today, and is the recognised system in the "Approved Lists of Bacterial Names" (Skerman *et al.* 1980).

Between the early 1970's, when Butzler *et al.* isolated *Campylobacter* from human diarrhoea samples (Butzler *et al.* 1973; Dekeyser *et al.* 1972), and 1988, at least 12 new *Campylobacter* species or subspecies were isolated from a variety of human, animal and environmental sources (On 2001). The advent of 16s rRNA typing in the late eighties (Woese 1987) resulted in the reclassification of many of these bacteria, and in 1989, Goodwin *et al.* proposed that organisms previously identified as *Campylobacter pylori* and *Campylobacter mustelae* should form a new genus named *Helicobacter* (Vandamme 2000; On 2001).

The current genus *Campylobacter* contains 16 species and six subspecies (see figure 1.5), and together with the genera *Helicobacter*, *Wolinella* and *Arcobacter* belongs to a group within the *Proteobacteracae* called the rRNA superfamily VI or the epsilon division. The family *Campylobacteraceae* is a subdivision of this group, and comprises the genera *Campylobacter* and *Arcobacter* (Vandamme 2000; On 2001; Moore & Matsuda 2002).

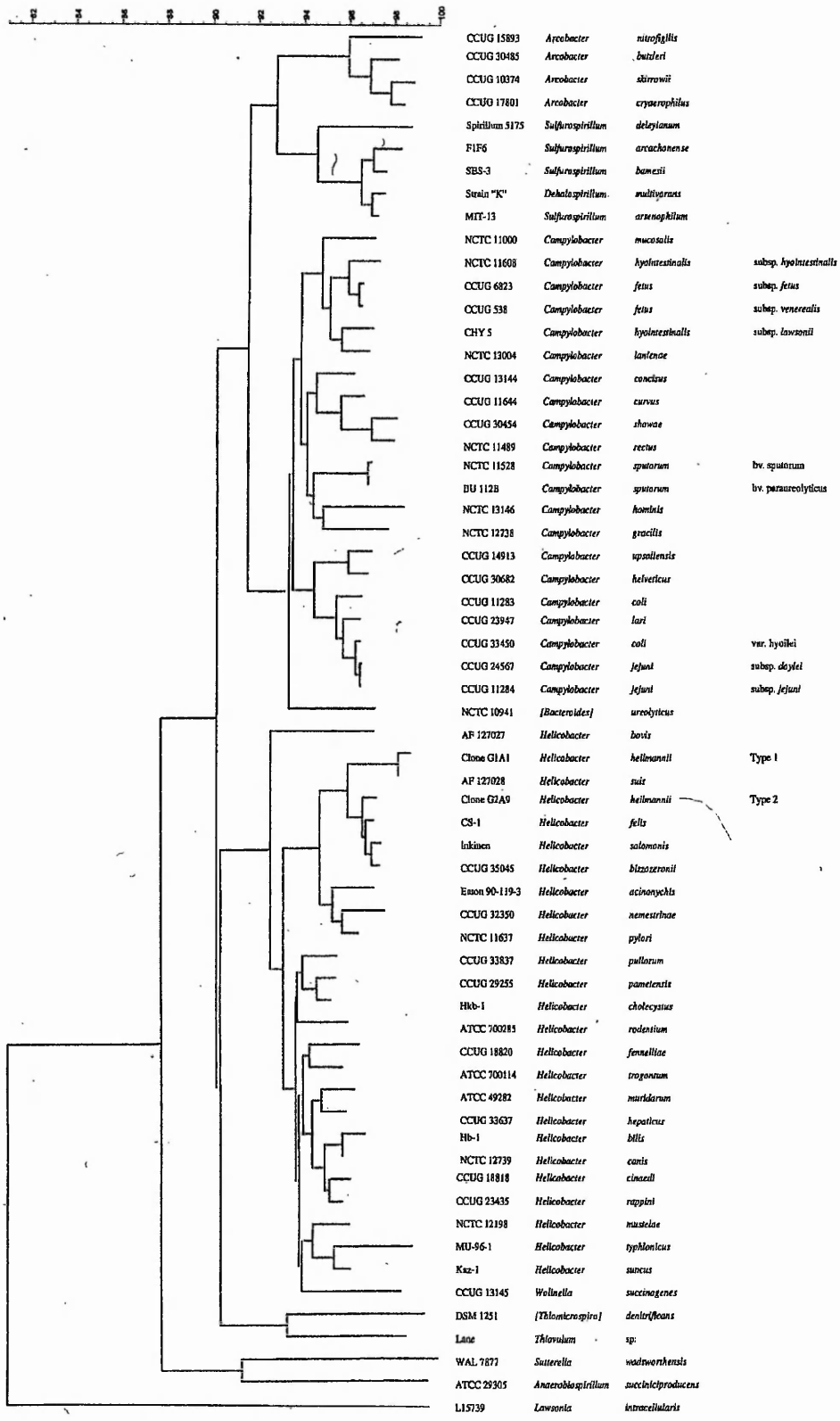


Figure 1.5 – Dendogram showing the relationship between 62 strains of Campylobacter, Arcobacter, Helicobacter and related bacteria, determined by the comparison of 16S rRNA gene sequences and neighbour-joining clustering (On 2001)

1.4 *HELICOBACTER* SPP.

Helicobacter is a spirally curved, Gram negative bacterium with a structure similar to *Campylobacter.* Although only discovered in 1982, there has been an increased interest in *Helicobacter* over recent years, as *Helicobacter pylori* has been shown to be the major cause of chronic gastritis and peptic ulcers in humans (Wilson *et al.* 2002). Infection with *Helicobacter* has also been linked to gastric and liver cancer (Skirrow 1994; Nardone 2000; Garcia *et al.* 2003).

It has been estimated that in the USA more than 80% of adults are infected with *H. pylori*, although the presence of the bacteria does not necessarily mean that the patient will have the disease. *Helicobacter* has also been detected in contaminated water, and it is possible that it can exist in a viable non-culturable state, as has been proposed for *Campylobacter* (Wesley 1997).

Helicobacter pylori shares many phenotypic similarities with *Campylobacter*, but although the two bacteria are closely related, *Helicobacter* is not a food-borne pathogen (Kelly 2001). *Helicobacter* colonises the gastric mucosa rather than the intestinal tract, and is thought to be transmitted person to person via the oral to oral or faecal to oral routes. In order to survive in the stomach, *H. pylori* secretes the enzyme urease, which converts urea to ammonia to protect itself from the acid in the stomach, but may also act as an adhesin, and can also damage the gastric lining. In addition, *H. pylori* secretes protease and some strains express a cytotoxin known as VacA, which further damages the gastric mucosa, leading to the formation of an ulcer. Some strains have been shown to secrete at least one haemolysin (Segal & Tomkins 1994; Drazek *et al.* 1995; Martino *et al.* 2001), and phospholipases that may additionally have haemolytic activity (Ansorg *et al.* 1993; Dorrell *et al.* 1999; Tannaes *et al.* 2001).

1.5 *ARCOBACTER* SPP.

Arcobacter spp. have recently been identified as potential newly emerging pathogens (Mansfield & Forsythe, 2000), and were only recognised as being distinct from *Campylobacter* as recently as 1991 (Vandamme *et al.* 1991). They are currently recognised as veterinary pathogens, and have been implicated in porcine and bovine abortions, but may also be important in human illness (Mansfield & Forsythe 2000). They are genetically and morphologically similar to *Campylobacter*, but *Arcobacter* spp. are more aerotolerant, and capable of growth at lower temperatures than *Campylobacter* spp. (Hilton *et al.* 2001).

There are currently four distinct species of arcobacters; *A. nitrofigilis, A. cryaerophilus, A. skirrowii* and *A. butzleri,* but of these, only the latter three have been recovered from humans and animals. There is also a possible two additional species; one found to be similar to *A. skirrowii,* and the other known as *A. ciberius* (Houf *et al.* 2003; On *et al.* 2003). It is possible that many *Arcobacter* infections have passed undetected, as usual methods of culturing *Campylobacter* spp. are too selective for *Arcobacter*. The most frequent isolations of arcobacters are from abortions in cattle and pigs, as well as contaminated water (Mansfield & Forsythe 2000; On 2002; Diergaardt *et al.* 2004), but recent research has isolated *Arcobacter* from chicken carcasses, suggesting that poultry may also be an important reservoir for this bacteria (Corry & Atabay 2001; Bang *et al.* 2003).

A. butzleri is the most commonly reported human pathogen of the genus and is usually isolated from patients presenting similar symptoms as those with *Campylobacter* infection. The mechanisms of infection of *Arcobacter* spp. are less well known than *Campylobacter* spp., but it is possible that they also produce one or more toxins (Musmanno *et al.* 1997). Recent work has shown that *Arcobacter* produce an entity that was toxic to some cells in culture, but that this toxin was not related to the cytolethal distending toxin (CDT) found in *Campylobacter, Helicobacter* and other related species (Johnson & Murano 2002). Apart from this report, there has only been one other documented case of toxic activity in *Arcobacter*, where alpha-haemolysis was detected by Atabay *et al.* (1998), but to date this has not been repeated.

1.6 DISEASE MECHANISMS OF *CAMPYLOBACTER*

Despite the importance of *Campylobacter* as a human pathogen, its mechanisms of infection/action are still poorly defined, and the understanding of the disease process lags substantially behind that of other enteric pathogens such as *Salmonella* and *Shigella.* Problems with genetic manipulation, culturing from clinical samples, and virulence variability between strains have all been attributed to the lack of progress in uncovering the disease mechanisms (Ketley 1997; Bourke 2002), although the publication of the *C. jejuni* NCTC 11168 genome (Parkhill *et al.* 2000), may help improve research in this area.

1.6.1 Pathogenicity of *Campylobacter*

After ingestion of *Campylobacter*, the bacteria enter the intestine via the stomach, where they initially infect the jejunum and upper ileum. The organisms then colonise the rest of the ileum and colon, where they adhere to the intestinal cell surface, damaging the epithelial cells and disrupting the absorptive capacity of the intestine, either by invasion or initiation of an inflammatory response. In severe cases of *C. jejuni* infection, septicemia may follow colonization (Ketley 1997; Rollins & Joseph 2001).

This infection process is achieved by a combination of several different mechanisms summarised below. The major virulence factors being: - motility, adherence, iron acquisition, invasion and the production of toxins. The clinical manifestations of *C. jejuni* infection depend on many variables, including the strain of *C. jejuni*, the expression of toxins, and the immune status of the infected host (Konkel *et al.* 2000).

1.6.1.1 Motility and Chemotaxis

To cross the lumen of the intestine and reach the mucosal barrier, the bacteria need to be motile. They achieve this by means of the flagellum (see section

1.8.3), which together with their spirally curved cell shape allows the organism to swim in a corkscrew-like manner towards the mucosal barrier, where it can penetrate the mucus layer (Wallis 1994; Wooldridge & Ketley 1997).

The motility of the organism is thought to be directed by chemotaxis (Wallis 1994; Ketley 1997). This is the process whereby the bacterium is able to 'sense' its environment by means of chemical stimuli, and pyruvate, succinate, and components of mucin such as L-fucose and L-serine have all been implicated as chemo attractants although not much is known about the actual process itself (Lee *et al.* 1986; Hugdahl *et al.* 1988; Marchant *et al.* 2002).

1.6.1.2 Adhesion

Once across the mucus layer, *Campylobacter* uses several mechanisms to adhere to the mucosal cells and colonise the intestinal cell surface. This prevents the bacteria from being swept away by peristalsis, and helps to protect from host immune responses such as complement activity and phagocytosis (Hu & Kopecko 2000; Jones *et al.* 2004). Most *C. jejuni* and *C. coli* strains show cell adherence *in vitro*, and a number of *Campylobacter* structures have been identified as adherence factors, including flagella, LPS, and a variety of outer membrane proteins (McSweegen & Walker 1986; Fauchére *et al.* 1989; Kervella *et al.* 1993; Moser & Schroder 1995; Ziprin *et al.* 1999).

Studies involving non-flagellated and various outer membrane protein (OMP) *Campylobacter* mutants show reduced, but not complete inhibition of the adhesion process, suggesting that this process is complex and involves multiple adhesion interactions (Grant *et al.* 1993; Yao *et al.* 1994; Carrillo *et al.* 2004). It has also been suggested that bacterial growth temperature also has an effect on the adherence ability, and a recent study showed that maximum adherence of *C. jejuni* M129 to INT407 cells occurred at 37°C, with a 66% decrease in adherence when the cells were grown at 42°C (Konkel *et al.* 1992; Hu & Kopecko 2000).

1.6.1.3 Iron Acquisition

In order to be a successful pathogen and to be able to compete with other resident bacteria, *Campylobacter* must have suitable mechanisms to scavenge iron from its surrounding environment. In humans, free iron is limited, as most is bound to high affinity iron-binding proteins such as transferrin and haemoglobin, and is therefore not readily available. Although *Campylobacter* do not possess their own siderophores (low molecular mass iron chelators), they have been shown to utilise exogenous siderophores produced by other bacteria in the microflora (Field *et al.* 1986; Richardson & Park 1995). Recent research has shown that *C. jejuni* is able to utilise the siderophores enterochelin and ferrichrome, but not aerobactin, ferritin or lactoferrin (van Vliet *et al.* 2002). In addition, *Campylobacter* is able to produce its own bacterial ferritin, which is involved in iron storage and helps to protect the bacteria from oxidative stress (Wallis 1994; Hu & Kopecko 2000; Palyada *et al.* 2004).

1.6.1.4 Invasion

Results of biopsies of infected patients and experimental animals have shown that intestinal cell invasion can occur in *Campylobacter* infection (Van Spreeuwel *et al.* 1985; Russell & Blake 1994). The occurrence of inflammation and bacteraemia in many cases also suggests that cell invasion is an important pathogenic mechanism (Hu & Kopecko 2000; Monteville *et al.* 2003). The degree to which *C. jejuni* invades appears to be strain dependent, and it has been suggested that to a certain extent, invasiveness can be induced by the presence of other enteroinvasive organisms present in the intestines (Buckholm & Kapperud 1987; Konkel & Joens 1990).

It is likely that flagella may play a role in invasion, as it has been demonstrated that aflagellate *C. jejuni* mutants lose their invasion ability (Yao *et al.* 1994; Szymanski *et al.* 1995). Mutants with insertions to the *flaA* gene demonstrated significantly reduced levels of invasion into cultured cells, which could be only slightly improved by centrifugation of the bacteria onto the host cells. Therefore

motility appears to be required for *Campylobacter* invasion ability (Wassenaar *et al.* 1991; Hu & Kopecko 2000). It has also been hypothesised that *Campylobacter* may secrete certain proteins that stimulate bacterial uptake by altering the host cell signalling mechanisms (Konkel & Cieplak 1992; Konkel *et al.* 1999; Biswas *et al.* 2004).

It has been demonstrated that different *Campylobacter* strains exhibit different invasion efficiencies for the same host cell line when experiments are carried out *in vitro* (Wallis 1994; Ketley 1997; Hanel *et al.* 2004). Alternatively, the same strain can vary in invasion efficiency for different host cell lines, and this appears to be host specific and more likely to invade a human cell line. The most common human cell lines used for invasion experiments are HEp-2 (human caucasian larynx carcinoma), HeLa (human cervical cancer cells), Caco-2 (human colonic adenocarcinoma cells) and INT-407 (human intestinal epithelial cells).

A number of reports suggest that *C.jejuni* invasion involves rearrangement of the host cytoskeleton, possibly via coated pit associated receptors (De Melo *et al.* 1989; Wooldridge *et al.* 1996; Hu & Kopecko 1999). This endocytosis of the bacteria may involve either microtubules or microfilaments or both, but the mechanisms by which this occurs are largely undefined (Kopecko *et al.* 2001; Biswas *et al.* 2003).

1.6.1.5 Translocation

During the infection process, campylobacters have been observed to translocate across an epithelial cell barrier, passing through the enterocyte cell layer to reach the underlying tissues (Everest *et al.* 1992; Grant *et al.* 1993). Translocated bacteria can be found below Caco-2 cell monolayers less than one hour after inoculation above the cells, and can continue to translocate for up to six hours afterwards (Konkel *et al.* 1992; Harvey *et al.* 1999). This mechanism may be due to either vacuole mediated transcytosis (translocation via the cytoplasm), or via

the paracellular route, where the bacteria pass between cells across tight junctions (Konkel *et al.* 1992; Oelschlaeger *et al.* 1993).

1.6.1.6 Interaction with Phagocytes

During infection with *Campylobacter* an inflammatory response is often observed (particularly in patients from developed countries), where phagocytic cells such as leucocytes and monocytes are released into the intestinal epithelium (Ruiz-Palacios *et al.* 1981; Ketley 1997). To resist killing by these phagocytes, it has been shown that *C. jejuni* is able to survive within these macrophages for up to 6-7 days (Kiehlbauch *et al.* 1985). It has also been suggested that *C. jejuni* changed from their usual spiral shape to a coccoid form within four to eight hours of internalisation, but whether this has any significance on bacterial survival rates is unknown (Kiehlbauch *et al.* 1985; Banfi *et al.* 1986).

1.6.1.7 Toxin Production

Although tissue invasion is likely to be responsible for some of the clinical symptoms arising from *Campylobacter* infection, it is probable that toxins may also contribute to the disease process (Wallis 1994; Van Vliet & Ketley 2001). Disruption of normal cell function is the critical outcome of most bacterial diseases, and for many bacteria, toxin production is an important part of this process. The various reported toxins from *Campylobacter* are summarised below.

1.7 TOXIN PRODUCTION BY *C. JEJUNI*

Despite increasing interest in *C. jejuni* over the last 20 years, the pathogenic mechanisms and virulence factors used to initiate disease are still comparatively unknown. It is recognised that toxins are produced as an important factor of the disease, but the current understanding of the mechanisms and relevance to infection is unclear. Much of the literature is contradictory, with some groups

failing to detect any toxins, where others have reported toxin activity (Wassenaar 1997). Different groups use different methods, strains and cell lines, and consequently report conflicting results. Generally, the reported *C. jejuni* toxins can be categorised into two groups according to their mode of action: cytotoxins and enterotoxins. Cytotoxins are generally responsible for causing inflammatory diarrhoea in the host, whilst enterotoxin activity usually results in the production of non-inflammatory, watery diarrhoea, with no evidence of blood or mucus (Ketley 1997). The various toxins detected in *C. jejuni* are summarised in table 1.1 below.

TOXIN	ACTIVITY	REFERENCE
Cytolethal Distending Toxin (CDT)	Blocks G2 phase of cell division. Causes cell elongation leading to distension and death after 96 hours.	Johnson & Lior, 1988; Pickett 2000
Haemolysin(s)	Lyse red blood cells, releasing haemoglobin.	Hossain *et al.* 1993; Tay *et al.* 1995
Porin-Lipopolysaccharide Toxin	Heat labile, trypsin resistant, cytotoxicity associated with the MOMP. Causes cell rounding in HeLa cells. Only one report to date.	Bacon *et al.* 1999
70kDa Cytotoxin	Heat and Trypsin sensitive, causes cell rounding and death, though not with Vero cells.	Johnson & Lior, 1986; Guerrant *et al.* 1987
Cytolethal Rounding Toxin (CLRT)	Sensitive to both heat and trypsin. Causes cell rounding, with no change in cell size.	Schultze *et al.* 1998; Hanel *et al.* 1998
Shiga-Like Toxin	Cytotoxic to HeLa cells. Only one report to date.	Moore *et al.* 1988
Hepatotoxin	Causes hepatic infection in mice, though only one group has detected this toxin.	Kita *et al.* 1990 & 1992
Enterotoxin	Heat and pH sensitive. Causes elongation of CHO cells, and rounding of Y-1 cells. Cells remain viable after 96 hours.	Ruiz-Palacios *et al.* 1983 Johnson & Lior 1988

Table 1.1 Comparison of toxins detected from *C. jejuni*.

1.7.1 General Cytotoxins

Cytotoxins are described as proteins that kill the target cells, and these can be split into two further categories; cytotoxins which exhibit intracellular activity, such as inhibition of protein synthesis or actin filament formation, and cytotoxins which damage cells by forming pores in the cell membranes.

1.7.1.1 Cytotoxins Acting Intracellularly

Cytotoxins with intracellular activity are generally composed of two subunits, an A subunit which exhibits enzymatic activity, and a B fraction which usually consists of a pentamer of subunits. The B subunit binds to a receptor on the outer membrane of the cell and the A subunit is then proteolytically cleaved, resulting in an active enzyme. There are two main mechanisms by which these toxins act; either by inhibition of protein synthesis of the host cell, or by inhibition of the actin filament formation of the cell cytoskeleton.

Toxins that kill target cells by the inhibition of protein synthesis include the *Shigella dysenteriae* toxin (Shiga toxin), and *E. coli* Shiga-like toxin (Stx or Verotoxin). Shiga toxin inhibits protein synthesis of the host cell, but additionally causes fluid accumulation in the intestine by inhibiting the intestinal absorptive cells (Donohue-Rolfe *et al.* 1991; Mainil 1999). The toxin is structurally similar to cholera toxin, but the mechanisms of the two toxins are very different.

E. coli Shiga-like toxins are closely related to Shiga toxins, both biologically and structurally (Agbodaze 1999; Lord *et al.* 1999). The toxins also share similar mechanisms, inhibiting protein synthesis by depurination of the 28S rRNA, leading to ribosome inactivation and cell death (Reisbig *et al.* 1981; Donohue-Rolfe *et al.* 1991; Balfanz *et al.* 1996). Cell lines commonly used to detect these toxins include green monkey kidney (Vero) cells, and human epithelial cells (HeLa). Chinese hamster ovary cells (CHO) are insensitive to the actions of these toxins, allowing for easier identification (Wassenaar 1997).

Cytotoxins that kill cells by inhibition of actin filament formation include *Clostridium difficile* toxins A and B (Lyerly *et al.* 1988; Lerm *et al.* 2000). Both toxins exert their effects by modification of Rho proteins that regulate the actin cytoskeleton, preventing Rho from binding to its effectors and resulting in the disruption of the cell cytoskeleton (Dillon *et al.* 1995; Wilson *et al.* 2002). These toxins cause rounding of CHO cells before cell death (Wassenaar 1997).

1.7.1.2 Pore-Forming Cytotoxins

This group of cytotoxins exert their effects by the formation of pores in the target cell membranes. Pore-forming cytotoxins are also known as haemolysins, as they can usually be detected as a result of their ability to lyse erythrocytes. This lysis of erythrocytes during the infection process may be a mechanism of iron acquisition by the bacteria, but may also be coincidental, with other cell types such as leukocytes and macrophages being the major targets of the toxin. Killing these cells hamper the hosts immune system, lowering host defences against the bacteria (Braun *et al.* 1991; Bhakdi *et al.* 1998)

In target cells the formation of pores results in a cascade of secondary responses, including the release of cytokines, generation of lipid mediators and dysfunction of the cell cytoskeleton. Examples of pore-forming cytotoxins include *E. coli* alpha haemolysin (HlyA), and other member of a group of toxins known as RTX toxins (repeat in toxins), containing repeats in their primary structure (Boehm *et al.* 1990; Welch *et al.* 1992). In addition to pore-forming, it is suggested that these toxins also cause more subtle, dysfunctional effects on the host cell as experiments using low levels of *E. coli* haemolysin have shown. Concentrations of the HlyA toxin too low to cause lysis of human granulocytes still caused the release of inflammatory mediators such as leukotrienes (Scheffer *et al.* 1985; Welch 1991). Other subgroups of pore-forming toxins include the *Proteus mirabilis* and *Serratia marcescens* haemolysins, and the *Aeromonas* aerolysins (Welch 1991; Bhakdi *et al.* 1998).

1.7.2 Cytotoxins Produced by *C. jejuni*

There are reportedly a number of different cytotoxins produced by *C. jejuni*, although different culture conditions, cell lines, and strains used by each group means that the current situation is confusing and the results are not comparable. Most groups detect the presence of only one type of cytotoxin, suggesting that different conditions may be responsible for differences in toxin activity. Misawa *et al.* (1994) compared different culture conditions and assay systems and their result on cytotoxin production by *Campylobacter jejuni*, finding that different cytotoxins were produced in each system, and also that no toxicity was found when foetal calf serum was added to the growth medium used to culture the various cell lines. This may partly explain why some groups have reported no toxicity with strains known to exhibit toxic activity.

Overall, there appear to be seven different types of cytotoxin produced by *Campylobacter jejuni* (see table 1.1), and these are summarised below.

1.7.2.1 The Cytolethal Distending Toxin (CDT, previously CLDT)

This toxin was first discovered in 1988 by Johnson and Lior and is the most well characterised *C. jejuni* toxin to date. When applied to CHO, HeLa, Hep-2 and Vero cells, CDT causes the cells to slowly distend over a 3-4 day period, leading to eventual cell death. The CDT activity seemed to affect only freshly seeded cells, with no death or distension seen when the toxin was applied to confluent monolayers (Johnson & Lior 1988). The degree of distension and sensitivity of the cell line varies, but cells generally distend to about five times their normal size before disintegrating (See figure 1.6; Pickett & Whitehouse 1999). Johnson & Lior assayed 718 *Campylobacter* strains for CDT, and roughly 40% of each species was found to produce the toxin. They also determined that CDT was heat labile, protease sensitive, and continuously produced by the same strains, even after multiple subculturing (Johnson & Lior 1988; Pickett 2000; Bang & Madsen 2003).

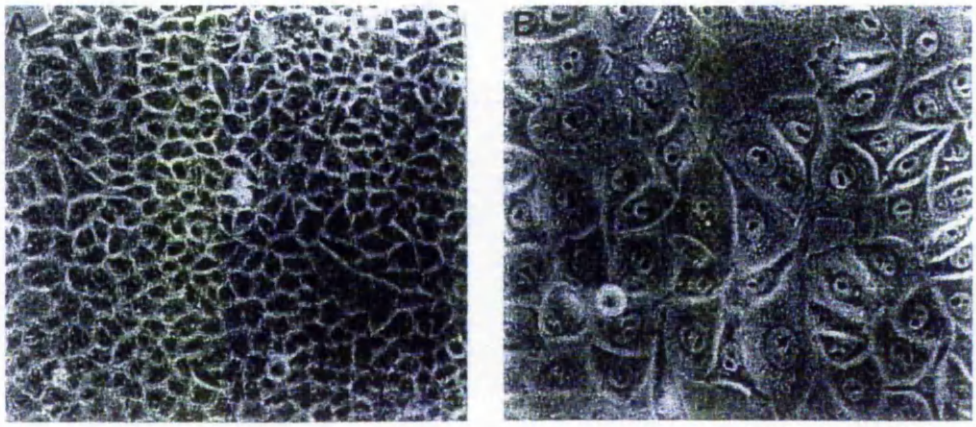


Figure 1.6 – HeLa cell distension caused by cytolethal distending toxin (CDT) (A) = Normal HeLa cells 72hr after addition of control lysates (B) = Distended HeLa cells 72hr after addition of *C. jejuni* CDT. (Reproduced from Pickett & Whitehouse 1999).

The genes encoding CDT have now been identified and cloned (Pickett *et al.* 1996). As with *E. coli* CDT there are three structural genes which compose the cytotoxin: *cdtA, cdtB,* and *cdtC.* These encode proteins predicted to have molecular weights of about 30, 29, and 21 kDa respectively. These genes are similar in size to those of *E. coli*, and when inserted into a non-toxigenic *E. coli* host, have been shown to result in the production of low levels of CDT (Pickett *et al.* 1996). *CdtB* has recently been identified as a nuclease (Lee *et al.* 2003), although little is known about the functions of *cdtA* and *cdtC.* Research has suggested that these subunits may be responsible for the binding of the toxin to the surface of the host cell, and it appears that all three genes must be present to produce an active toxin (Eyigor *et al.* 1999; Purdy *et al.* 2000).

The mechanisms underlying the death of the cells have not been fully elucidated, but it appears that rather than direct killing of the cells, CDT causes damage to the host cell DNA, resulting in the cells becoming blocked during the G2 phase of cell division, leading to cell death (Aragon *et al.* 1997; Whitehouse *et al.* 1998). It has also been suggested that CDT may play a role in the inflammatory response of *Campylobacter* enteritis, and it has been demonstrated that CDT, in addition to blocking the cell cycle also induces the release of the proinflammatory cytokine IL-8 from INT407 cells (Hickey *et al.* 2000).

CDT is not unique to *Campylobacter* spp. and has been detected in many other bacterial pathogens, including *Shigella, Haemophilus ducreyi*, and some species of *Helicobacter* (Pickett 2000; Young *et al.* 2000; Lara-Tejero & Galan 2002). Research indicates that the structure and mode of action of all of these CDT's is similar, and when *cdtB* genes from *Helicobacter* strains were cloned, all were found to share 50-65% homology with *cdtB* genes from *C. jejuni* and *H. ducreyi* (Chien *et al.* 2000; Young *et al.* 2000).

1.7.2.2 General Haemolysins

Haemolysins are cytolytic toxins that lyse erythrocytes, and are a common virulence factor in pathogenic organisms such as *E. coli* and *H. pylori* (Rowe *et al.* 1994; Segal & Tomkins 1994; Reingold *et al.* 1999). For the majority of these toxins, erythrocytes are not the main target, but rather a spurious correlate, and many microbial haemolysins can also lyse leukocytes, thereby enhancing their survival during an immune response (Rowe & Welch 1994).

The mechanisms used by these toxins to cause lysis include enzymatic disruption, or the formation of pores in the membrane of the red blood cell (see section 1.7.1.2). Haemolysins which lyse the cells by enzymatic mechanisms include the α toxin from *Clostridium perfringens*, a phospholipase which cleaves membrane phospholipids into lysophospholipids, lysophosphatides and free fatty acids, leading to membrane lysis (Braun & Focareta 1991; Segal & Tomkins 1994).

Examples of pore-forming haemolysins include the *Listeria monocytogenes* cytolysin, listeriolysin O (LLO), and *E. coli* α-haemolysin (Braun & Focareta 1991). These toxins are usually synthesized as precursor proteins, then covalently modified to yield an active haemolysin that spontaneously inserts into target cell membranes to form transmembrane pores (Braun & Focareta 1991; Glomski *et al.* 2002).

1.7.2.2.1 Haemolysins Produced by *C. jejuni*

Although *Campylobacter* is historically a non-haemolytic organism, several groups have reported evidence of haemolysis produced by *C. jejuni* (Hossain *et al.* 1992; Misawa *et al.* 1995; Akan *et al.* 1998), and it is now generally regarded that some strains of the organism are haemolytic.

Haemolysis was originally detected from *Campylobacter* by growing the bacteria on Blood Agar plates (Arimi *et al.* 1990), but although 92% *C. jejuni* strains and 22% *C. coli* strains tested showed haemolysis, the results were found to be variable, inconsistent and not reproducible. The haemolysin was reported to be heat stable, and observed only with ageing cultures (incubated for at least four days), suggesting that it may be released on cell death and lysis (Arimi *et al.* 1990). Alternatively, Tay *et al.* (1995) detected haemolytic activity from 94% *C. jejuni* and *C. coli* isolates grown on blood agarose plates after only 24 hours incubation.

Misawa *et al.* (1995) reported alpha-haemolytic activity (green zones around the colonies), which was only apparent on blood agar plates when the pH was lowered to 6.0-6.5. To date, however, this has not been repeated. Beta-haemolysis (clear zones around colonies) was observed after prolonged incubation with the same strains reported to produce alpha-haemolysis, and other groups have also detected this type of haemolytic reaction, using both blood agar and blood agarose plates (Tay *et al.* 1995), and BHI plates (Arimi *et al.* 1990).

Tay *et al.* (1995) also demonstrated the use of a microplate assay, reported to be more sensitive than screening blood agar plates. More recently, the contact haemolysis assay has been developed, allowing the blood and bacteria to be in close contact, and further supporting the hypothesis that the haemolysin is cell associated (Wassenaar 1997; Istivan *et al.* 1998).

Pickett *et al.* (1992) tested several *C. jejuni* clinical isolates for their ability to acquire iron from the surrounding media. The ability of pathogenic bacteria to acquire iron from their host has been shown to be of critical importance in

establishing infection, as to be a successful pathogen, the bacteria must be able to utilise iron-containing compounds from the host (see section 1.6.1.3). The results showed that although haemolytic activity was detected from several strains, this did not appear to be iron regulated. This suggests that the haemolytic activity may be constitutively expressed, and may play a role in *C. jejuni* pathogenesis other than, or in addition to iron acquisition (Pickett *et al.* 1992). However, a more recent study carried out by Istivan *et al.* (1998) reported that the haemolysin was iron regulated, and haemolysis decreased upon addition of excess iron to the media.

The published genome sequence of *C. jejuni* NCTC 11168 describes two putative haemolysin domains; a potential haemolysin called Cj0588 or *tlyA*, similar to haemolysin A from *Treponoma hydodysenteriae*, and an integral membrane protein domain known as Cj0183, which has homology to other known haemolysins. These genes are 759 and 1356 base pairs in length, and code for a 29.1 and a 51 kDa protein respectively (Parkhill *et al.* 2000).

Further work is obviously needed into whether *C. jejuni* does produce a haemolysin or not, and if so, its possible role in the pathogenesis of the *Campylobacter* disease process.

1.7.2.3 Porin-Lipopolysaccharide Toxin

Cytotoxic activity associated with the major outer membrane protein of *C. jejuni* (MOMP, see section 1.8.2) has been reported by Bacon *et al.* (1999) and in *C. coli* (Moutinho-Fragoso *et al.* 1998), though to date these are the only two reports linking the MOMP with cytotoxicity.

The toxin was reported to be partially heat stable, resistant to inactivation with trypsin, and shown to cause vacuolation and cell rounding leading to death in Hep-2 and HeLa cells within 24 to 48 hours. Isolation and characterisation of the toxin found it to be a porin-LPS complex with a molecular weight of 45kDa, and N-terminal sequencing revealed a 97% homology of the toxin with *C. jejuni*

MOMP. However, when the carbohydrate component of the LPS was degraded, the porin was still found to exhibit toxicity. It was hypothesised that the LPS was not responsible for the cytotoxic effects, but had a protective role, providing heat and trypsin resistance.

It is not known whether this porin is responsible for some of the activities detected by other groups, but further research is necessary for a fuller understanding of the role of this toxin.

1.7.2.4 70kDa Cytotoxin

This cytotoxin is known as the 70 kDa toxin, as there are several reports finding similar activities produced by a toxin with a molecular weight of around 70 kDa. Most reports suggest that this toxin is both heat and trypsin sensitive, and causes cell rounding and death when applied to CHO, HeLa, HEp-2 and INT407 cells, but has no effect on Vero cells (Goossens *et al.* 1986; Johnson & Lior 1986, Guerrant *et al.* 1987). The toxin cannot be neutralised by antibodies against shiga-like or *Clostridium difficile* toxins, and its expression is lost upon subculturing (Wong *et al.* 1983; McCardell *et al.* 1986).

Overall, there appear to be many reports of a *C. jejuni* cytotoxin of approximately 70 kDa, active against HeLa, CHO, Hep-2 and INT407 cells, and inactive against Vero cells (Pang *et al.* 1987; Daikoku *et al.* 1989) It is likely that many of these reports have detected the same toxin, but its mechanisms of action currently remain unknown.

1.7.2.5 Cytolethal Rounding Toxin (CLRT)

Cytolethal rounding toxin (CLRT) is so named as a result of its ability to cause cell rounding when applied to CHO, HEp-2 and HeLa cells (Schultze *et al.* 1998). The toxin causes a decrease in cell numbers when applied to CHO cells, as is the case when the cells are treated with CDT, but rather than causing the

cells to distend, CLRT treated cells simply appear rounded after 48 hours (Hanel *et al.* 1998). In contrast, when the toxin is applied to Vero cells, the cells develop spindle-shaped cells, growth is again reduced, but followed by cell lysis. The toxin appears to be sensitive to both heat and trypsin, but cannot be neutralised by antibodies against shiga-like or *Clostridium difficile* toxins.

Another toxin known as CLDT/CLRT has also been described by Schultze *et al.* (1998), which produces a mixture of both rounded and distended cells, but it is suspected that this activity is most likely due to a combination of the CDT and CLRT toxins. It is possible that the CLRT toxin is really the same as other toxins described in this review, but as different groups have used different detection mechanisms and the toxin has not been purified, it is impossible to determine whether CLRT is completely distinct from other better characterised toxins.

1.7.2.6 Shiga-Like Toxin

There has only been one report of a shiga-like toxin produced by *Campylobacter* spp. (Moore *et al.* 1988), where the toxin was found to exhibit toxicity to HeLa cells. The toxin was detected from 11 out of 36 strains of *C. jejuni* and *C. coli* and could be neutralized by Stx antitoxin. Despite this cross-reactivity, no homology with shiga-like toxin genes has been found in the *C. jejuni* genome, and no further detection or characterization has been reported since, hence the existence of this toxin is debatable.

1.7.2.7 Hepatotoxin

It has been reported that a possible hepatotoxin is produced by some strains of *C. jejuni*, which induces a severe hepatic infection in infected mice (Kita *et al.* 1990 & 1992). Both infection with hepatotoxin producing strains of *C. jejuni* and intravenous injection with partially purified cell sonicates were shown to cause lesions in the livers of the mice. These results suggest that some strains have the potential to colonize the liver and cause hepatitis by expressing a hepatotoxin,

but further characterization of this toxin is necessary to determine its importance, and to date only one group has detected its presence.

1.7.3 Enterotoxins

Enterotoxins are defined as secreted proteins that have the capacity to bind to a cellular receptor, enter the host cell, and elevate intracellular cyclic AMP (cAMP) levels (Wassenaar 1997). One of the best studied enterotoxins is the cholera toxin (CT) produced by *Vibrio cholerae*. This toxin has an AB_5 structure, where the B subunit of the toxin binds to a receptor on the cell membrane (in this case ganglioside GM_1), and inserts the smaller A subunit into the cell (see figure 1.7). The A subunit possesses a specific enzymatic function that acts intracellularly. After proteolytic activation, the A subunit ADP ribosylates the tissue adenylate cyclase system, resulting in an increase in intracellular cAMP. This then leads to the movement of ions and large quantities of water out of the cells and into the lumen of the gut, causing watery diarrhoea (Gemmell 1984; Merritt & Hol 1995; Ganguly & Kaur 1996).

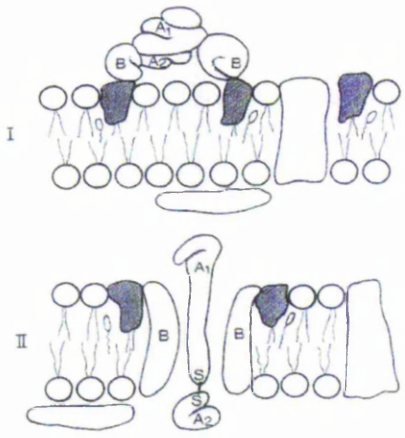


Figure 1.7 – Diagram to show binding to (I) and translocation through (II) the mammalian cell membrane of cholera toxin. A_1, A_2, and B represent subunits of cholera toxin. The shaded proteins represent ganglioside GM_1.

The *E. coli* heat labile enterotoxin (LT) is closely related to CT in both structure and function. It shares about 70% amino acid sequence homology with CT, although LT causes a much milder disease than the severe cramping diarrhoea of *Vibrio cholerae* (Ganguly & Kaur 1996; Popoff 1996). These structural and functional similarities between the two toxins suggest that they may be evolutionarily related. It is possible that the LT gene has been acquired by *E. coli* by gene transfer from *V. cholerae* (Spangler 1992).

1.7.3.1 Enterotoxins Produced by *C. jejuni*

There are numerous reports of an enterotoxin produced by *C. jejuni* (*C. jejuni* toxin, CJT or *Campylobacter* cytotonic toxin, CCT), with a similar activity to cholera toxin, and this is additionally reinforced by the prevalence of watery diarrhoea in many patients infected with *C. jejuni.* The first report of this toxin was demonstrated in 1983 (Ruiz-Palacios *et al.*), when *C. jejuni* culture supernatants applied to a rat ileal loop model induced intraluminal fluid secretion. The same sample, when tested on CHO cells, was found to induce elongation and increase intracellular cAMP levels in a similar manner to CT. The activity of the toxin could be neutralised by cholera antitoxin, and denatured by heat and extremes of pH.

To date, other groups have also demonstrated the presence of an enterotoxin from *C. jejuni* cultures (Klipstein & Engert 1984; Daikoku *et al.* 1990; Collins *et al.* 1992) though many others have failed to find any evidence of enterotoxin activity at all (Wadström *et al.* 1983, Olsvik *et al.* 1984; Coote & Arain 1996). Several groups have detected the loss of toxin activity following storage and subculture, suggesting that the production of CJT is unstable (Daikoku *et al.* 1989; Suzuki *et al.* 1994). Fernandez *et al.* (1999) demonstrated that intraperitoneal passage of injured or non-pathogenic strains through mice or rat gut led to restoration of pathogenic capacities of the bacteria, with enhanced levels of enterotoxin production. This may explain why some groups failed to find evidence of enterotoxin activity.

The reports of enterotoxin activity from *C. jejuni* have found that the toxin causes elongation of CHO cells, and rounding of Y-1 cells. The effects are distinct from the effects of CDT, as although CDT also causes elongation of CHO cells, it has no effect on Y-1 cells, and CJT can be distinguished from CDT by incubating CHO cells for 96 hours rather than the usual 24 (Johnson & Lior 1988). After 96 hours, CHO cells exposed to CDT are found to have extensively distended, with very few cells still viable. In contrast, cells treated with *C. jejuni* enterotoxin remain viable until the end of the 96 hour period.

There have been many reports that this toxin can be neutralized with anti-CT or anti-*E. coli* LT toxin (Ruiz-Palacios *et al.* 1983; McCardell *et al.* 1984; Calva *et al.* 1989; Suzuki *et al.* 1994), though no homology has been found between the genome of *C. jejuni* 11168 and CT or LT genes, casting doubt over the existence of the enterotoxin (Parkhill *et al.* 2000). It is possible that only very small regions show homology at the DNA level, which may explain why the gene has not been detected to date (Baig *et al.* 1986; Calva *et al.* 1989).

Despite the numerous reports detecting *C. jejuni* enterotoxin activity, its presence still remains controversial (Wassenaar 1997). It is clear though, that as with the production of cytotoxins, the growth media, strains and assay conditions do play a large part in the detection or otherwise of the enterotoxin (Goossens *et al.* 1985(b), Suzuki *et al.* 1997). Further work needs to be carried out in this area, and the gene sequence(s) elucidated if the existence of this toxin is to be determined.

1.7.4 Phospholipases

Phospholipases are lipolytic enzymes that facilitate the degradation of phospholipids. Phospholipids are important components of all eukaryotic membranes, and are essential scaffolding proteins, separating the external environment from the inside of the cell. In addition, phospholipids also play an important role in cell signalling. It is therefore hardly surprising that a large number of bacteria have evolved enzymes capable of hydrolysing phospholipids

(Titball 1998). It is recognised that phospholipases can act as bacterial toxins, after *Clostridium perfringens* alpha toxin was found to be a phospholipase C (Macfarlane & Knight 1941; Songer 1997). To date, no toxic activity has been associated with phospholipases in *Campylobacter jejuni,* but there are several reports of phospholipase association with haemolytic activity in *Helicobacter pylori* (Ottlecz *et al.* 1993; Dorrell *et al.* 1999), and *C. coli* (Grant *et al.* 1997). However, the genome of *C. jejuni* has been shown to contain the gene for phospholipase A (*pldA*) a homologue to the *pldA* gene in *C. coli* (Parkhill *et al.* 2000), although it is not known whether the phospholipase activity is A_1 or A_2.

Phospholipases are grouped according to their specificities, and are characterised by their ability to hydrolyse aggregated phospholipids (i.e. substrates located in bilayer membranes, micelles or lipoprotein particles) with a much higher velocity than the same phospholipid in its monomolecular form (Verheij & Dijkstra 1994). Generally phospholipases have two roles;

1. Many are digestive enzymes, and are present in intestinal juices, bacterial secretions, and venoms.
2. They also generate highly active signal molecules or their immediate precursors; e.g. Phospholipase A_2 releases arachidonate, a precursor of signalling molecules such as prostaglandins, leukotrienes and thromboxanes.

All four ester moieties of a phospholipid are susceptible to enzymatic hydrolysis, and therefore phospholipases are categorised into one of four groups – A_1, A_2, C or D, depending on each enzyme's site of cleavage of the phospholipid (see figure 1.8; Brockerhoff & Jensen 1974; Dennis 1983).

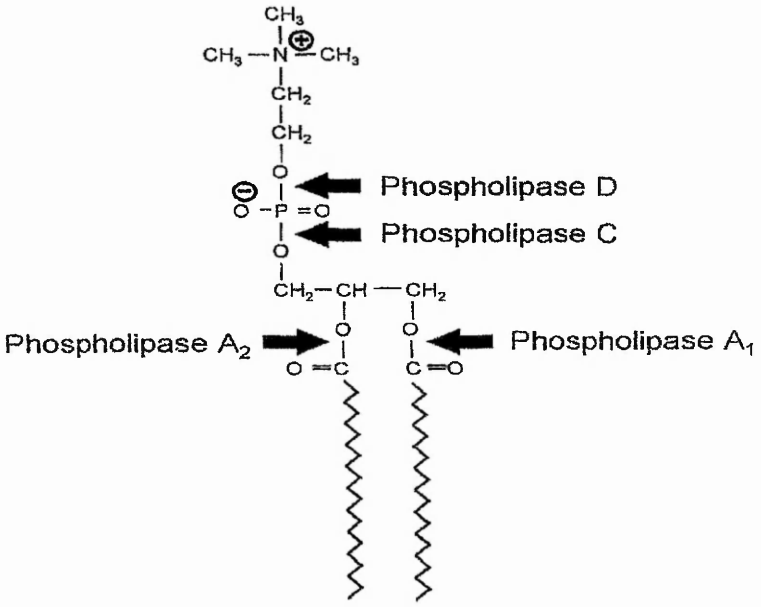


Figure 1.8 – Sites of cleavage of phosphatidylcholine by phospholipases A_1, A_2, C, and D (Titball 1998).

1.7.4.1 Phospholipase A_1

Phospholipase A_1 was first purified in 1971 by Scandella and Kornberg, where it was isolated from *E. coli* (Dennis 1983). Since then, its structural genes have been cloned (Homma *et al.* 1984; Brok *et al.* 1994), and its crystal structure elucidated (Snijder *et al.* 1999). In addition to *E. coli*, PLA1 has been detected in many other enteric bacteria, including *Helicobacter pylori* and *Neisseria meningitidis* (Brok *et al.* 1998; Dorrell *et al.* 1999). The enzyme does not require calcium ions for activation, and is optimally active at a neutral pH.

1.7.4.2 Phospholipase A_2

Phospholipase A_2 (PLA2) is probably the best characterised of all the lipolytic enzymes (Verheij & Dijkstra 1994). Its specificity was determined in 1963 (Van Deenen & De Haas), and since then, its mode of action and crystal structure have been elucidated (see figure 1.9 & Scott *et. al.* 1990).

The enzyme is calcium dependant, and can be found both inside and outside the cell. Intracellularly, PLA2 is prevalent in most mammalian cells where it causes the release of arachidonic acid, an important precursor for signalling molecules in inflammation and allergic reactions (Van den Bosch 1980). Other phospholipase A_2 enzymes have an additional digestive role where they are involved in the breakdown of phagocytosed material (Elsbach & Weiss 1988).

Extracellular PLA2 enzymes are found in mammalian pancreatic juice, where they serve a digestive role (Verheij & Dijkstra 1994). The enzymes are first synthesized in an inactive precursor form, before transportation to the intestine where trypsinolysis occurs, resulting in the active enzyme plus a small peptide. PLA2 enzymes are also found in snake and bee venom, where they have a variety of functions ranging from neurotoxicity to haemolysis (Verheij & Dijkstra 1994).

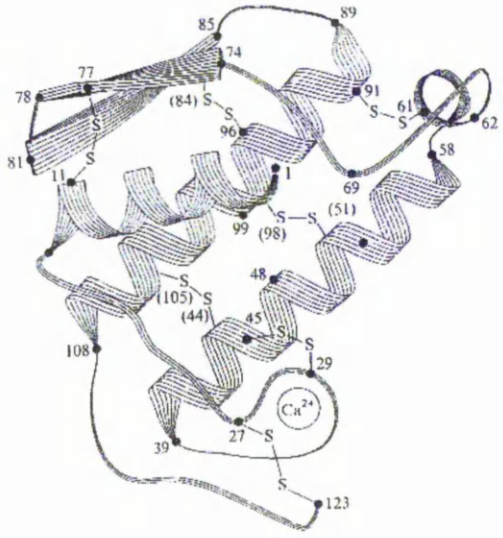


Figure 1.9 – Schematic representation of the three dimensional structure of bovine phospholipase A_2, the first phospholipase enzyme to have its crystal structure determined (Verheij & Dijkstra 1994).

No matter what the function or source of the extracellular PLA2 enzymes, all have similar structure and common sequences. They are all small proteins, between about 13 and 15 kDa, and most contain 6 or 7 disulphide bridges (see figure 1.9). This high number of disulphide bridges most likely explains the stability of these enzymes under denaturing conditions (Verheij & Dijkstra 1994).

1.7.4.3 Outer Membrane Phospholipase A (OMPLA) in *Campylobacter*

Phospholipase A (OMPLA) is one of the few enzymes located in the outer membrane (OM) of Gram-negative bacteria (Dekker 2000). Although the membrane around the enzyme is composed of phospholipids, the expression of OMPLA is tightly regulated to prevent hydrolysis of the surrounding lipids.

OMPLA was first characterised in *Campylobacter* by Grant *et al.* (1997), who detected a gene encoding a 35 kDa protein in *C. coli,* with significant homology to that of the *E. coli* OMPLA. After cloning this gene, and inserting the resultant plasmid into a PldA-deficient strain of *E. coli*, it was found that phospholipase A activity was restored in the mutant, suggesting that this gene does encode an outer membrane phospholipase A in *C. coli.*

The genome of *C. jejuni* has also been shown to contain the gene for phospholipase A (*pldA*), a homologue to the *pldA* gene in *C. coli* (Parkhill *et al.* 2000). To date, the protein has not been characterised, and it is uncertain as to whether the activity is PLA1 or PLA2, or a mixture of both as found in *E. coli* OMPLA (Brok *et al.* 1996).

1.7.4.4 Phospholipase C

Phospholipase C (PLC) was originally purified from bacterial culture supernatant, but has also been extracted from mammalian cells (Dennis 1983). These phospholipases are produced by a wide range of both pathogenic and non-

pathogenic bacteria, and are further grouped according to the nature of their preferred substrate; i.e. phosphatidylcholine, sphingomyelin or phosphatidylinositol. The phosphatidylcholine preferring enzymes are also able to hydrolyse other phospholipids, albeit with lower affinity, but the other two groups have a much narrower substrate specificity (Titball 1998).

Phospholipase C toxins are among the most potent of phospholipase toxins, and include the *C. perfringens* α-toxin and *Listeria monocytogenes* phosphatidylinositol-specific PLC toxins (Jolivet-Reynaud *et al.* 1988; Songer 1997).

Over the last 15 years the genes encoding these enzymes have been cloned, allowing the amino acid sequences of these enzymes to be compared. It was found that enzymes with similar amino acid sequences also shared the same substrate specificities, and on the basis of these sequence similarities, phospholipase C enzymes preferring phosphatidylcholine as a substrate can be further subdivided according to their Gram negative or Gram positive origins (Titball 1998).

It is unlikely that phospholipase C is present as a virulence factor in *C. jejuni*, as sequencing of the genome of *C. jejuni* revealed no gene with homology to PLC genes already characterised in other pathogenic bacteria (Parkhill *et al.* 2000).

1.7.4.5 Phospholipase D

Phospholipase D (PLD) has predominantly been isolated from plant tissue, but it is also found in mammalian systems and bacteria (Dennis 1983). The enzyme requires calcium for optimum activity, but the mechanisms of its activity are not well known. An example of a bacterial phospholipase D toxin is the enzyme produced by *Corynebacterium pseudotuberculosis* (Songer 1997). Again, it is unlikely that genes for a phospholipase D exist in *C. jejuni*, as sequencing of the genome failed to find any genes sharing homology with other characterised PLD genes.

1.8 OUTER MEMBRANE CHARACTERISTICS OF *C. JEJUNI*

1.8.1 The Outer Membrane

The outer membrane (OM) of Gram negative bacteria is composed of lipoproteins, lipopolysaccharides (LPS), and phospholipids (see figure 1.10). As well as serving as a barrier between the bacteria and its host, it can also participate in adherence and invasion into host cells, and confers resistance to bactericidal molecules such as antibiotics, digestive enzymes and detergents. Components of the outer membrane can also help the bacteria with resistance to phagocytosis, and iron sequestration (Logan & Trust 1982).

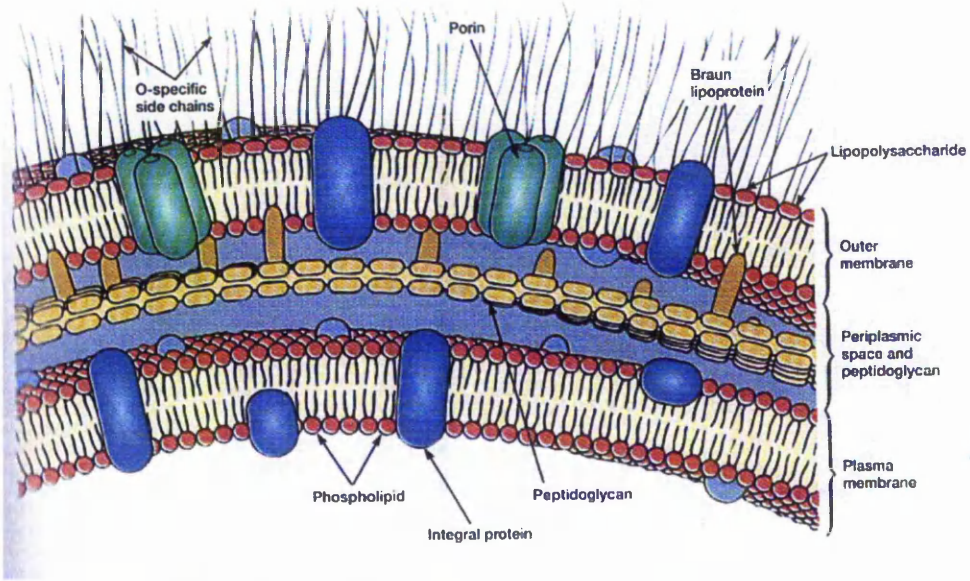


Figure 1.10 The Gram Negative Envelope (Prescott *et al.* 1999)

1.8.2 Outer Membrane Proteins (OMP)

The most abundant protein in the outer membrane is a porin protein of approximately 45kDa and is known as the major outer membrane protein or MOMP (Logan & Trust 1982; Zhang *et al.* 2000). This porin has been reported to have many roles, including adhesion to host cell membranes, nutrient uptake, and there has been one documented case of associated cytotoxicity (Newell *et al.* 1984; Moser *et al.* 1997; Bacon *et al.* 1999; Dé *et al.* 2000; see section 1.7.2.3).

There are reportedly up to 160 other putative membrane proteins of unknown functions discernable from the *C. jejuni* genome sequence (Parkhill *et al.* 2000), though there is little evidence for the presence of any other porin-like outer membrane proteins in *C. jejuni* (Penn 2001).

Other *C. jejuni* outer membrane proteins include Omp18, which appears to be responsible for membrane integrity, and CadF, a potential fibronectin adhesion protein (Penn 2001). Additional OMPs are produced in response to environmental stresses such as iron limited conditions, or an increased temperature (Schwartz *et al.* 1994), and several OMPs have been implicated in host cell adhesion (Ketley 1997).

1.8.3 Flagella

Flagella are defined as thread-like locomotor appendages extending outward from the plasma membrane and cell wall (Prescott *et al.* 1999). The Gram-negative bacterial flagellum is usually several times the length of the bacteria, and is composed of three main parts. The longest part is the filament, which is the outer portion of the flagellum. This is a hollow cylinder, composed of subunits of a protein called flagellin. In *Campylobacter* the filament is composed of two flagellin subunits – FlaA and FlaB, which are about 93% identical to each other (Guerry 1997). The filament is attached to the cell surface by a basal body or granule, which is embedded in the cell membrane. The third part is a short, curved segment known as the hook, which joins the filament to the basal body, and is composed of a series of rings and a rod, located within the cell membrane. Most Gram negative bacteria have four rings connected to a central rod, whereas Gram positive bacteria only have two rings (Guerry *et al.* 2000).

The flagellum is responsible for motility in *Campylobacter*, and is thought to be essential for colonisation of the mucosal barrier (see section 1.6.1.1). It has been suggested that the flagella may play a role in adhesion, and also invasion of the epithelial cells during infection (see sections 1.6.1.2 and 1.6.1.4; Grant *et al.* 1993; Song *et al.* 2004; Konkel *et al.* 2004).

The most unusual feature of *Campylobacter* flagella is that the flagellin subunits are glycosylated (Doig *et al.* 1996; Karlyshev *et al.* 2004). This is a post-translational modification of the flagellin, which has been shown to include a sialic acid residue. This raises the possibility of molecular mimicry of this protein to glycosylated moieties on human glycoproteins, and the flagella has been implicated in the onset of Guillain-Barré syndrome by this mechanism (Tsang 2002; Szymanski *et al.* 1999; section 1.2.2.1). It has additionally been shown that *Campylobacter* can undergo antigenic variation, where the bacterium is able to express flagellins of different antigenicities and different relative molecular weights. This has been demonstrated to be a result of changes in the post-translational modifications of the flagellin (Szymanski *et al.* 1999; Guerry *et al.* 2000). The apparent molecular weight of flagellin as determined by SDS-PAGE electrophoresis has been shown to be 62 kDa, but depending on the posttranslational modification, the flagellin can migrate at relative molecular weights between 59.5 and 62 kDa (Newell *et al.* 1984; Guerry 1997).

1.8.4 Lipopolysaccharide (LPS)

LPS is a major component of the *C. jejuni* outer membrane, and is often referred to as endotoxin, as a result of the cytotoxicity of the lipid A fragment of the molecule (Logan & Trust 1982; Moran *et al.* 2000). The other important part of the molecule is the O antigen, a long carbohydrate chain consisting of repeating sugar units, which is exposed on the surface of the bacterial cell, and is crucial for serotyping to determine the bacterial species (see figure 1.11; Logan & Trust 1982; Moran & Penner 1998). Residues in this outer region often contain sialic acid groups, which mimic GM_1 gangliosides, the receptors for cholera toxin. (Guerry 1997; Tsang 2002). This molecular mimicry is thought to be associated with the occasional onset of Guillain-Barré syndrome after infection with *C. jejuni* (see section 1.2.2.1; St. Michael *et al.* 2002). The third part of the molecule is the oligosaccharide core, which joins the lipid A to the O antigen, and usually consists of two sugars; ketodeoxyoctonic acid (KDO) and heptose (Penner & Aspinall 1997; Fry *et al.* 2000). It has been suggested that *Campylobacter* LPS can also undergo phase variation (the regulation/modification of certain genes) to

change the antigenic properties of its surface structures in a similar way to flagellin (see section 1.2; 1.8.3; Penner & Aspinall 1997; Fry *et al.* 2000; Linton *et al.* 2001).

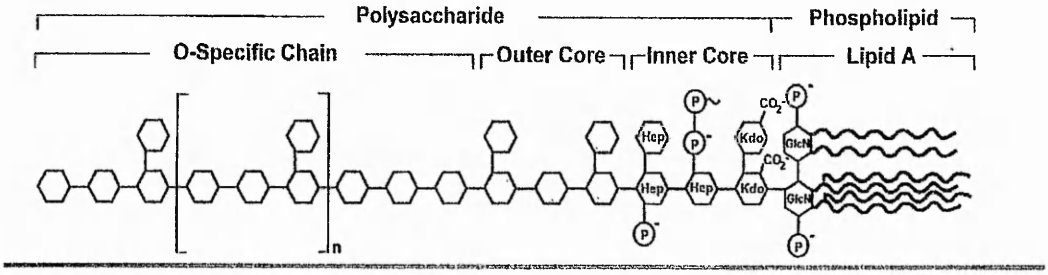


Figure 1.11 General Structure of Lipopolysaccharide (Volker *et al.* 2000).

1.9 BACKGROUND OF THE PROJECT

This review of the current literature shows that *Campylobacter* is a diverse and complicated organism. Despite nearly 30 years of research, we still have very little understanding of how *C. jejuni* causes disease on such a large scale, and our knowledge of its pathogenic mechanisms, particularly the production of toxins, is confused and unclear.

Arcobacter has recently been identified as a newly emerging food-borne pathogen, closely related to *Campylobacter*. Very little is known about the pathogenesis of *Arcobacter* spp. but it is possible that they may produce one or more toxins, using similar mechanisms to *Campylobacter*. *Arcobacter butzleri* is the most commonly reported human pathogen of the genus, therefore, isolates of this strain were included in this investigation, to determine whether *Arcobacter* share any of the pathogenic mechanisms of *Campylobacter*.

Previous research at The Nottingham Trent University (TNTU) has focussed on the characterisation of toxins produced by *C. jejuni*. Previous research students Holmes (2001) and Spears (2002), showed *C. jejuni* NCTC 11351 to have a consistent cytotoxic effect when applied to ECV and N2a cells. The toxin was

semi-purified, and found to be both heat and trypsin stable, with a molecular weight of approximately 50kDa. The protein could be partially neutralised by cholera antitoxin, and N-terminal sequencing of the toxin gave two matches; a *Campylobacter* major outer membrane protein (MOMP) and an amino acid transport periplasmic binding protein.

The initial aim of this project was to enhance and extend this research, continuing the work with ECV and N2a cell lines, but also including Vero cells (sensitive to Shiga and Shiga-like toxins) and Chinese Hamster Ovary cells (CHO) as these are commonly used to test for the Cytolethal Distending Toxin (Wassenaar 1997). In addition to *C. jejuni* NCTC 11351, *C. jejuni* NCTC 11168 was also included, as this strain has recently been sequenced (Parkhill *et al.* 2000), and should toxicity be found from this strain, direct comparisons could be made with the published gene sequence.

Kate Holmes (TNTU) also demonstrated the presence of haemolytic activity from several strains of *C. jejuni*. The toxin was identified as a putative α-haemolysin, but this topic was not studied in any further detail. Since the current understanding of haemolysins produced by *C. jejuni* is limited, it was considered prudent to further investigate this area.

1.10 AIMS

1) To assess the toxicity of *C. jejuni* using mammalian cell culture, with N2a, ECV, Vero and CHO cell lines.

2) To purify and characterise the major outer membrane protein (MOMP) from *C. jejuni* NCTC 11351 and 11168 using SDS-PAGE and N-terminal amino acid sequencing, for comparative homology with the partially purified toxin reported by Holmes (2001) and Spears (2002).

3) To characterise the haemolysin(s) produced by *C. jejuni* and *A. butzleri*, and to use the published genome of *C. jejuni* NCTC 11168 to construct oligonucleotide primers to identify the haemolysin gene(s). These will then be used as probes to isolate haemolysin sequences from southern blots of various strains of *C. jejuni* and *A. butzleri*.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Unless otherwise stated, all chemicals used for buffers, solutions and media were of the highest grade, and purchased from Sigma-Aldrich Company Ltd. (Poole, UK). All DNA manipulation enzymes were purchased from Promega (Southampton, UK).

2.2 MEDIA

2.2.1 Preparation of Blood Agar (BA)

Fourteen grams of Blood agar base No. 2 (Oxoid, UK) was added to 350 ml distilled water and autoclaved (121°C, 15 minutes, Astell-Hearson autoclave). The agar was then cooled to approximately 50°C before adding 25 ml defibrinated horse blood (SROO5OB, Oxoid). After gentle mixing approximately 20 ml was poured into sterile Petri dishes (Sarstedt, UK), allowed to solidify and incubated at 37°C overnight to check for contamination. The plates were then stored at 4°C until required, for a maximum of two weeks.

2.2.1.1 Addition of Desferal Mesylate to BA

When required for iron limitation studies, desferal mesylate was aseptically added to the agar after autoclaving, to a final concentration of 50 μM, and gently mixed before pouring (section 2.2.1).

2.2.2 Preparation of Brain Heart Infusion Broth (BHI)

Eighteen and a half grams of BHI broth base (Oxoid) were added to 500 ml distilled water and stirred to dissolve. The mixture was aliquoted as required and autoclaved as above (section 2.2.1).

2.2.3 Preparation of Luria-Bertani Media (LB)

Ten grams of tryptone (L42, Oxoid), five grams of yeast extract (L21, Oxoid) and five grams of NaCl were added to 900 ml distilled water and mixed until dissolved. The pH was adjusted to 7.0 using 1M NaOH and the media aliquoted and autoclaved as above. To make agar plates, agar bacteriological no. 1 (Oxoid) was added to a final concentration of 1.5%, and plates poured as above.

2.2.3.1 Addition of Ampicillin to LB Agar and Broths

A 50 mg/ml stock solution of ampicillin was made up in sterile distilled water and filter sterilised using a 0.2 μm filter (Microgon, USA). The antibiotic was aseptically added to LB broths and agar just before pouring, to a final concentration of 100 μg/ml.

2.2.3.2 Addition of Chloramphenicol to LB Agar and Broths

A 20 mg/ml stock solution of chloramphenicol was made up in absolute ethanol and filter sterilised using a 0.2 μm filter. The antibiotic was added to LB broths and plates as in 2.2.3.1 to a final concentration of 20 μg/ml.

2.2.4 Preparation of *Campylobacter* Blood Free Agar (BFA)

To make BFA plates, 22.75g *Campylobacter* blood free selective agar base (Oxoid) was added to 500 ml distilled water and autoclaved as in section 2.2.1. The agar was cooled to approximately 50°C, and a vial of CCDA selective supplement (SR 155, Oxoid), was reconstituted in 2 ml sterile distilled water and aseptically added to the agar. The media was then gently mixed before pouring into sterile Petri dishes. The plates were allowed to set, incubated at 37°C as in section 2.2.1, and stored at 4°C. When required, chloramphenicol was added to the plates as above (section 2.2.3.2).

2.3 BACTERIAL STRAINS

The majority of the work presented in this thesis was carried out on two strains of *Campylobacter jejuni*; NCTC 11351 and NCTC 11168 (National Collection of Type Cultures, Colindale, London). *C. jejuni* NCTC 11351 was the strain previously shown by Holmes (2001) and Spears (2002) to produce a cytotoxin, and *C. jejuni* NCTC 11168 was used as a comparison, since its genome has been sequenced and characterised (Parkhill *et al.* 2000).

Arcobacter butzleri NCTC 12481 was originally isolated from a human diarrhoea sample (Kiehlbauch *et al.* 1991).

The additional 21 *C. jejuni* and *A. butzleri* strains were obtained from Dr. S. On, Danish Veterinary Institute, Denmark.

Escherichia coli strain JM109 was purchased from Promega (see table 2.1 for strain details).

Escherichia coli strain TOP10F' was purchased from Invitrogen (see table 2.1 for details).

Escherichia coli strain S-17 was kindly donated by Dr. Phil Hill, University of Nottingham.

2.3.1 Bacterial Growth Conditions

All *Campylobacter* and *Arcobacter* strains were maintained by growth on blood agar plates (section 2.2.1) at 37°C under microaerophilic conditions (10% v/v CO_2, 5% v/v O_2, and 85% v/v N_2) for 48 hours inside a modified anaerobic cabinet (Compact M, Don Whitley Scientific Ltd, UK). Strains were routinely streaked every 72 hours on blood agar plates, and the purity of the cultures checked by Gram's staining.

Escherichia coli strains were grown on LB agar plates at 37°C, or in LB broth in a 37°C shaking incubator (200 rpm). Culture media was supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), or both antibiotics where necessary to select for transformant colonies.

2.3.2 Storage and Recovery of *Campylobacter* and *Arcobacter* Cultures

- The biomass from a 24 hour old confluent plate was aseptically transferred to a vial of Protect Beads (Technical Service Consultants Ltd, UK), creating a thick suspension.
- The vial was capped and inverted several times to mix, then left to stand for one minute, before removing as much of the fluid as possible using a sterile pipette.
- The vial was then stored upright at -20°C until required.
- To recover the cultures from storage, a single bead was aseptically removed from the appropriate vial and streaked across the surface of a blood agar plate.
- The plate was then incubated microaerophilically at 37°C.

2.3.3 Storage and Recovery of *E. coli* Cultures

- An isolated *E. coli* colony was aseptically transferred from an agar plate to 10 ml LB broth using a wire loop, and allowed to grow overnight at 37°C with shaking.
- 2 ml glycerol was added to 8 ml LB broth and mixed and autoclaved.
- 1 ml of this mixture was added to 1 ml overnight culture, gently mixed and transferred to a cryovial and stored at –70°C.
- To recover the *E. coli* from glycerol storage, a cryovial of *E. coli* cells was opened and as quickly as possible, to prevent the cells from defrosting, a sterile tip was scraped across the surface of the frozen culture.

- The cells were then transferred from the tip to a dry LB agar plate, and the cells streaked out aseptically using a wire loop.
- The plate was then incubated overnight at 37°C.

2.4 GROWTH CURVES OF *C. JEJUNI*

- *C. jejuni* strains were grown on blood agar plates (BA) for 48 hours under microaerophilic conditions.
- A loopful of bacteria from the plate was then used to inoculate 10 ml BHI broth in a universal bottle, which was incubated at 37°C microaerophilically for 24 hours, with the cap loosely placed on the bottle.
- This starter culture was used to inoculate 5 further 10 ml BHI broths in universal bottles with 10 µl culture, and the bottles incubated under microaerophilic conditions for 72 hours, with the caps loosely placed on the bottles.
- Absorbance readings were taken every hour for 8 hours, then at 24, 48, and 72 hours, ensuring that each sample was taken from a different tube so as not to affect the volume of each tube too greatly. The absorbance values were read at 650 nm using fresh BHI as a blank, and a growth curve prepared from the results.

2.5 PREPARATION OF *C. JEJUNI* CELL EXTRACT SAMPLES

- The cell material from a two day old, confluent BA plate was aseptically harvested into 1 ml phosphate buffered saline (PBS, Oxoid).
- The solution was shaken to distribute the cells, and 100 µl of the suspension spread onto the surface of each of ten fresh BA plates.
- The plates were incubated under microaerophilic conditions for three days, and each plate was harvested into 1 ml PBS in separate Eppendorf tubes.
- The samples were shaken vigorously to encourage resuspension, and the OD at 650 nm measured and adjusted to 1.3.

- The suspensions were then bath sonicated on ice for 15 minutes, followed by centrifugation in a microfuge (MSE micro centaur, Sussex, UK) at 10 000 g for 30 minutes. The samples were then stored at -20°C until required.

2.5.1 Heat Treatment of Samples for Cytotoxicity Testing

C. jejuni cell extract samples and the PBS controls were boiled at 100°C for 10 minutes and then cooled to room temperature before applying to cell lines.

2.5.2 Protease Treatment of Samples for Cytotoxicity Testing

Trypsin was added to the samples and controls (PBS) to a final concentration of 0.1%, followed by incubation at 37°C for 30 minutes. The reaction was stopped by boiling at 100°C for 10 minutes to denature the trypsin.

As heating the trypsin in this way may also denature proteinaceous toxins, additional samples were denatured by diluting 1:5 with tissue culture growth medium (see section 2.6.1), and adding five times the volume to each sample well. No significant differences were found between the cytotoxicity of the samples using either method.

2.6 TISSUE CULTURE METHODS

Four cell lines were used for cytotoxicity testing. These were Chinese hamster ovary cells (CHO), African green monkey kidney cells (Vero), Mouse neuroblastoma cells (N2a), and Human umbilical cord endothelial cells (ECV 304). All cell lines were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

All procedures were carried out using a class II laminar flow hood (Gelaire BSB 4) to minimise contamination.

2.6.1 Growth Medium

To prepare the growth medium, 50 ml Foetal Calf Serum (FCS) and 5 ml glutamine, penicillin G and streptomycin (GPS, each 2mM) were added to 500 ml Dulbecco's Modified Eagles Medium, with sodium bicarbonate and 4.5 g/L glucose (DMEM).

2.6.2 Culturing Thawed Cells

- A cryovial of frozen cells were retrieved from liquid nitrogen storage, and placed at 37°C until thawed. The contents of the vial were then added to 10 ml warm growth medium (see section 2.6.1) and centrifuged for 5 minutes at 250 g (Labofuge 400e, Heraeus Instruments, Germany).
- The supernatant was carefully poured off, and the cell pellet gently resuspended in 1 ml fresh growth medium by passaging 20 times through a sterile pipette.
- The resuspended cells were then added to a T-25 vented flask (Sarstedt, Nottingham, UK) containing 10 ml fresh growth medium, and incubated at 37°C in a humidified atmosphere of 95% (v/v) air, 5% (v/v) carbon dioxide in a Jouan IG150 incubator.
- After 24 hours, the growth medium was carefully decanted from the cells by pouring down the upper surface of the flask to avoid detaching the cells. A further 10 ml fresh growth medium was gently added, and the cells replaced in the incubator for two to three days, until the cells were between 60 to 80% confluent.

2.6.3 Maintenance of Cell Lines

- CHO, ECV and Vero cells needed to be pre-incubated with trypsin as the cells attach more firmly to the flask, therefore trypsin-EDTA (T4049, Sigma, 10X 200mM) was diluted to a 1X solution in sterile PBS.
- The growth media was gently decanted from the cells down the upper surface of the flask, and 1 ml 1X trypsin-EDTA was added to each flask to rinse any remaining serum from the cells.
- This was poured away, and a further 3 ml 1X trypsin-EDTA was added to the flask, and left for 5 minutes at 37°C until the cells had begun to detach from the surface.
- The flask was gently tapped to ensure all the cells were detached, and the trypsin neutralised by the addition of 10 ml fresh growth medium.
- N2a cells did not need trypsin treatment, and were carefully detached by resuspending gently with 20 passages through a sterile pipette, taking care not to scrape the lower surface of the flask.
- The cells were transferred to a 50 ml centrifuge tube (Sarstedt) and centrifuged at 250 g for 6 minutes. The supernatant was carefully removed, and the cell pellet resuspended in 1 ml growth medium by 20 passes through a sterile pipette, taking care not to froth the suspension.
- A portion of the cell suspension (100 µl) was transferred to a new T-25 flask containing 10 ml fresh growth medium, and the cells incubated at 37°C for a further two to three days, until 60-80% confluent.

2.6.4 Plating out Cell Lines

- Cells were passaged as above, resuspending in 1 ml growth medium after centrifugation.
- A ten fold dilution of each cell suspension was made by adding 20 µl sample to 180 µl trypan blue solution (T8154, Sigma), in an Eppendorf tube and gently mixing to distribute the cells.

- Diluted cells (10 µl) were pipetted into a haemocytometer chamber (Neubauer 1/400 mm^2), and the number of viable cells (those that remained clear), were counted and the number of viable cells/ml was calculated using the following equation;

Viable cells/ml = average number cells per square x 250 000 x dilution factor

- The cell density was adjusted to 50 000 cells/ml for N2a cells, and 25 000 cells/ml for all other cell lines, using growth medium as a diluent.
- 0.5 ml of this solution was added to each well of a 24 well plate (Sarstedt), resulting in 25 000 cells/well for the N2a cell line, and 12 500 cells/well for all other cell lines.
- The plates were then incubated at 37°C for 24 hours to allow the cells to attach and recover, and the remainder of the cell suspension passaged into T-25 flasks.

2.6.5 Applying Samples to Cell Lines

- After 24 hours, the growth medium was removed from each well, taking care not to disturb the cells, and replaced with 0.5 ml fresh medium.
- Samples were applied at concentrations of 5% and 10% of the total volume in each well. i.e. 50 µl of sample was applied to 0.5 ml medium in each well for the 10% sample. Initial experiments also used 1.25% and 2.5% concentrations, but these were found to be inappropriate, so these volumes were not continued. All samples and controls were filter sterilised before testing, using a 0.2µm DynaGard filter (Microgon).
- An equivalent volume of PBS was used as a control, and both the samples and controls were tested in triplicate.
- The plates were then replaced at 37°C and incubated for 48 hours.

2.6.6 MTT Assay

- After the required incubation period, 50 µl of 5 mg/ml methyl tetrazolium thiazoyl blue (MTT) was added to each well without removing the growth medium, and the plates reincubated at 37°C for a further 45 minutes.
- The medium was carefully removed from the wells, and 1 ml dimethyl sulphoxide (DMSO) added to each well.
- The plates were gently agitated for five minutes using a Titertec shaker (Flow Laboratories, UK), to ensure the cells were evenly distributed throughout the well, and 150 µl of each sample aliquoted into a clean 96 well plate (Sarstedt). The absorbance was then read at 570 nm, using a Spectra fluor (Tecan, Reading, UK).
- To calculate the percentage of viable cells in each sample, the triplicate results were averaged, and calculated as a percentage of each control.

e.g. $\frac{\text{10\% sample}}{\text{10\% PBS control}} \times 100 = \text{Viable cells (\%)}$

2.6.7 Fixing and Staining CHO and ECV Cells

- The cells were seeded into 24 well plates as for previous experiments.
- After applying the sample in triplicate and incubation for the required length of time, the cells were fixed at -20°C for 30 minutes in fixing solution (10% methanol in 90% PBS), adding 500 µl per well.
- The fixed cells were then stained at room temperature for 1 minute with Coomassie Blue stain (0.25% Coomassie Blue – R250, 10% (v/v) acetic acid, 40% (v/v) methanol, 50% (v/v) distilled water).
- The wells were washed 3 times with distilled water and left to air dry overnight.
- The cells were viewed with an inverted light microscope (Olympus Optical Company Ltd, UK), noting morphological changes, and counting five fields of vision (approx 100 cells) in each well, with each well in triplicate.

2.6.8 Storage of Cell Lines

- Cells were resuspended and centrifuged as for passaging (section 2.6.3) and the supernatant carefully poured from the pellet.
- The pellet was then resuspended in 1 ml freezing medium (25% (v/v) FCS, 10% DMSO, 65% (v/v) DMEM, containing GPS as in section 2.6.1), by three or four passes through a pipette, taking care not to froth the cell suspension.
- The contents of the tube were then transferred to a cryovial (Sarstedt) and the vial wrapped in tissue paper. The cells were allowed to freeze overnight at -70°C, before transferral to liquid nitrogen for long term storage.

2.7 EXTRACTION OF THE OUTER MEMBRANE PROTEIN (OMP) FROM *C. JEJUNI* (A modification of Bacon *et al.* 1999).

- Confluent 2 day old cell mass from a BA plate was harvested into 5 ml PBS and used to inoculate 50 fresh BA plates (100 µl spread onto each plate).
- These plates were then grown microaerophilically for 3 days. The bacteria were harvested into 50 ml sterile distilled water, and the cell suspension centrifuged at 5000 g for 10 minutes at 4°C.
- The pellet was washed by resuspending in a further 50 ml sterile distilled water, and recentrifuged at 5000 g for 10 minutes.
- The washed pellet was resuspended in 20 ml 0.01M Tris-HCl (pH 7.4) and frozen at -20°C until required, saving a 1 ml portion for analysis of whole cell proteins.
- After defrosting the samples, the cells were sonicated four times on ice for 30 seconds, with 30 seconds rest each time, and the samples kept on ice from this point onwards.
- The samples were then centrifuged twice at 5000 g for 20 minutes at 4°C to remove the cells, and the supernatant retained.
- The resulting supernatant was centrifuged at 100 000 g for one hour at 4°C. The pellet was retained, resuspended in 3 ml sterile distilled water and

added to 20 ml 1% Sarkosyl in 7mM EDTA, and incubated at 37°C for 20 minutes.

- The suspension was then centrifuged at 100 000 g for 2 hours at 4°C, the pellet resuspended in 20 ml 0.01M Tris-HCl, and centrifuged for a further 2 hours at 100 000 g.
- The resulting pellet was resuspended in 1 ml sterile distilled water, and stored at -20°C until required.

2.8 PROTEIN ASSAY OF OMP SAMPLES

- A series of standards were made up, by diluting bovine serum albumin (BSA, Bio-Rad) with distilled water to obtain a range of values from 0 to 1 mg/ml.
- Samples and standards were pipetted into separate wells of a clean 96 well plate in triplicate 5 µl aliquots.
- Reagent A (Bio-Rad) was added to each well (25 µl), followed by 200 µl reagent B. The plate was agitated for a few seconds, then left to develop for 15 minutes.
- The absorbances were read at 750 nm and a calibration curve drawn from the standards, allowing the protein content of each sample to be calculated.

2.9 GEL ELECTROPHORESIS OF OMP SAMPLES

Samples were first separated using a minigel electrophoresis system with a powerpac 3000 (Bio-Rad). For additional separation a Protean II XI system was used (Bio-Rad), enabling larger 20 x 20 gels to be run. Gels were photographed using Grab-It software, and analysed using Phoretix ID software (Phoretix International).

2.9.1 Electrophoresis Buffers

Lower Gel 2X Separating Buffer	Upper Gel 2X Stacking Buffer
0.75M Tris pH 8.8	0.25M Tris-HCl pH 6.8
0.2% SDS	0.2% SDS

Both buffers were made up to 100 ml with deionised water, and stored at 4°C.

10% Resolving Gel	4% Stacking Gel
8.75 ml 2X Lower gel buffer	5 ml 2X Upper gel buffer
5.8 ml 30% Acrylamide solution (Bio-Rad)	1.5 ml 30% Acrylamide solution
2 ml Distilled water	3 ml Distilled water
875 µl 10% APS	500 µl 10% APS
25 µl TEMED	20 µl TEMED

The gel buffer, acrylamide and distilled water were mixed gently in a flask. The APS and TEMED were then added, the solution swirled gently and poured immediately.

2X Loading Buffer
250mM Tris-HCl, pH 6.8
10% Glycerol
20mM DTT
0.01% Bromophenol blue
2% SDS

Running Buffer
25mM Tris base
192mM Glycine
0.1% SDS

Both solutions were made up to volume with distilled water, the 2X loading buffer was then stored at -20°C, and the running buffer at 4°C.

Coomassie Blue Stain	Destain
50% Methanol (BDH)	10% Methanol
10% Acetic acid (BDH)	10% Acetic acid
0.25% Coomassie Blue R-250	

Both stain and destain were made up to volume with distilled water and stored at room temperature.

2.9.2 Gel Assembly and Electrophoresis

- The gel casting apparatus was assembled, and the ingredients for the 10% resolving gel combined. The gel was carefully poured with a syringe, to a level 1 cm below the bottom of the comb.
- This was then overlaid with water, and left to set for 30-45 minutes. When set, the water was removed and the stacking gel cast to the top of the gel plate. The comb was inserted, and the gel left to set for 30-45 minutes.
- Meanwhile, the samples were prepared so that approximately 10 μg protein was loaded into each lane of a small gel, and about 20 μg onto each large gel.
- An equal volume of 2X loading buffer was added to each sample in an Eppendorf tube, and the samples boiled for 5 minutes and pulsed for a few seconds in a microcentrifuge before loading.
- Molecular weight markers (6.5-205 kDa, Sigma) were run alongside the samples. Each sample was loaded into a different lane on the gel, and the tank filled with running buffer.
- The gel was run at 50mA for approximately 40 minutes until the dye front reached the bottom of the tank. The gel was then stained with Coomassie blue stain (see section 2.9.1) for 30 minutes to 1 hour before destaining overnight, or stained using a silver stain kit (Sigma).
- The gels were dried by washing in deionised water and sandwiching between 2 pieces of wet gel drying cellophane (Bio-Rad), ensuring no air bubbles were present. The gel was then left to dry overnight.

2.9.3 Schiffs Carbohydrate Stain

Gels were stained using Schiffs reagent to detect the presence of carbohydrate.

- Samples were run as for previous method (section 2.9.2) but after electrophoresis, gels were soaked in 7.5% acetic acid for 1 hour.
- The gel was then placed in 0.2% aqueous periodic acid (BDH) and left on ice at 4°C for 45 minutes to 1 hour.
- The periodic acid was then replaced with Schiffs reagent and the gel refrigerated for 2 to 3 hours, before destaining at room temperature in 2 to 3 changes of 10% acetic acid.

2.10 GEL ELECTROPHORESIS OF LPS SAMPLES

2.10.1 LPS Extraction from OMP Samples

12.5% Resolving Gel (see section 2.9.1)

8.75 ml 2X Lower gel buffer

7.25 ml 30% Acrylamide solution (Bio-Rad)

500 µl Distilled water

875 µl 10% APS

25 µl TEMED

LPS 2X Sample Buffer

4 ml Glycerol

5 ml 0.5M Tris-HCl pH 6.8

8 ml 10% SDS

2.6 ml Deionised water

400 µl 2-mercaptoethanol

10 mg Bromophenol blue powder

- All the ingredients were mixed together except 2-mercaptoethanol. The buffer was then dispensed into 2.5 ml volumes and stored at -20°C. Just before use, 50 µl 2-mercaptoethanol was added to each 2.5 ml aliquot, which was then stable for 1 week at 4°C.
- Sample buffer (2X, 10 µl) was added to 100 µl of each OMP sample and the samples boiled for 10 minutes in pierced Eppendorf tubes.
- Distilled water (10 µl) and 4 µl proteinase-K (2.5 mg/ml) in 1X sample buffer was added and the samples mixed and incubated at 60°C for 2 to 3 hours.
- Samples were then cooled to room temperature, aliquoted into 20 µl volumes and stored at -20°C until required. Samples were run on a 12.5% resolving gel with 4% stacking, and stained using LPS silver stain.

2.10.2 LPS Silver Stain Reagents

Fixing Solution

200 ml Methanol

25 ml Acetic acid

275 ml Deionised distilled water

Oxidising Solution

0.75 g Periodic acid

100 ml Fixing solution

Silver stain solution

1 g Silver nitrate in 5ml deionised distilled water

28 ml 0.1M Sodium hydroxide (BDH)

2 ml Ammonium hydroxide (BDH)

115 ml Deionised distilled water

The sodium hydroxide was added to a dark reagent bottle, adding the cold ammonium hydroxide whilst mixing. The silver nitrate solution was then added dropwise, the solution mixed and used immediately.

Developing solution

50 mg Citric acid

0.5 ml Formalin (BDH)

The citric acid was dissolved in a little deionised water, before adding the formalin. The solution was mixed well and made up to 1 litre with deionised distilled water.

2.10.3 LPS Silver Stain

- The gel was placed in a clean staining tray, covered with fixing solution and gently agitated at room temperature overnight.
- The fixer was then discarded and the gel covered with oxidising solution and shaken for 5 minutes.
- The oxidiser was then discarded and the gel washed 3 times in deionised distilled water with gentle shaking (15 minutes each).
- The silver stain was prepared during the last wash step and poured into a fresh staining tray. The gel was then transferred to the stain and gently mixed for 10 minutes.
- The stain was then discarded, and the gel washed with deionised distilled water as before.
- The gel was then placed in developing solution and gently agitated over a light box until the bands appeared. The reaction was stopped with copious deionised distilled water.

2.11 IMMUNOBLOTTING OF OMP SAMPLES

Nitrocellulose and Polyvinylidenefluoride (PVDF) membranes were used for Western blotting the OMP samples. Nitrocellulose was used for standard blotting with antibodies and PVDF was used to transfer the proteins for sequencing. The transfer method was generally the same in both cases, with the exception of the buffers and stains required.

2.11.1 Transfer of Proteins to Nitrocellulose Membrane for Immunoblotting

Transfer Buffer pH 8.0
20mM Tris base
15mM Glycine
20% Methanol

The tris base and glycine were added to 700 ml distilled water. The pH was adjusted to 8.0, 20% methanol added, and made up to 1 litre with distilled water.

- The samples were separated by electrophoresis using a 10% resolving polyacrylamide gel (see previous method), though no stacking gel was required.
- The gel was then removed and allowed to equilibrate in transfer buffer for a few minutes.
- A piece of nitrocellulose membrane was cut to the size of the gel, as were 12 pieces of filter paper, and these were also soaked in transfer buffer.
- The top and bottom electrodes of a semi-dry blotter (Bio-Rad) were moistened with transfer buffer, and 6 pieces of wet filter paper placed on the bottom electrode, ensuring no air bubbles were caught between any of the pieces.
- The membrane was placed on top of these, again ensuring no air bubbles were trapped, and the gel then placed on top of the membrane.

- The remaining 6 pieces of filter paper were placed on top, and a glass rod rolled across the surface to remove trapped air.
- The blotter was run for an hour at 150mA. To ensure that transfer had occurred, the membrane was stained with 0.05% copper phthalocyanine for a few seconds, then destained in 12mM NaOH.
- The membrane was then washed in distilled water, allowed to dry between 2 pieces of filter paper, and stored at 4°C.

2.11.2 Transfer of Proteins to PVDF Membrane for Sequencing

CAPS (3-(Cyclohexylamino)-1-Propane Sulphonic Acid) Transfer Buffer

10mM Caps

10% Methanol

The buffer was made up with distilled water and the pH adjusted to 11.0 using NaOH.

- The same procedure as above was followed, using 0.1μm PVDF or immobilon membrane, which was pre-wet in methanol for 10 seconds before use.
- The procedure for semi-dry transfer was the same, except CAPS buffer was substituted for transfer buffer.
- To check proteins had transferred, the membrane was soaked in amido black stain for 1 minute.
- The membrane was then destained by washing with distilled water, speeding up the process with the addition of methanol if required.
- The membrane was stored briefly at 4°C, before being taken to the Queens Medical Centre (QMC, Nottingham) for sequence analysis.

2.11.3 Immunoblot of OMP Samples

- The membrane filter was incubated at room temperature overnight in 3% BSA (Bio-Rad) in PBS, followed by a 2 hour incubation with the primary antibody in BSA/PBS (anti-cholera toxin antibody, raised in rabbit, Sigma) at a dilution of 1: 500.
- Unbound antibody was washed off in 3 x 20 minute washes in 1% tween-20 in PBS.
- The membrane was then incubated with the secondary antibody (anti-rabbit Ig G, Sigma) at a dilution of 1:1000 in BSA/PBS, for 2 hours.
- The membrane was washed again for 3 x 20 minutes, and then rinsed in substrate buffer for 2 minutes (0.75M Tris pH 8.5).
- The blot was then developed in the following substrate mix;

33 µl BCIP (50 mg/ml in N,N–Dimethylformamide (DMF)
44 µl NBT (Nitro blue tetrazolium, 75 mg/ml in DMF)
20 ml Substrate buffer

- The membrane was left in the substrate mix until bands could be seen (between 30 minutes and 2 hours).
- The reaction was stopped by washing in distilled water, and the membrane blotted dry on filter paper. The blot was then photographed and stored at 4°C.

2.12 CONTACT HAEMOLYSIN ASSAY OF *C. JEJUNI* AND *A. BUTZLERI*

2.12.1 Preparation of 1% Horse Blood

- A vial of 25 ml sterile defibrinated horse blood (SROO5OB, Oxoid) was centrifuged at 3000 g for 10 minutes at 4°C.
- The supernatant was then removed and replaced with sterile PBS (4°C). The centrifugation and removal of supernatant was repeated at least twice more until the supernatant was clear.
- Then 2 ml of PBS was added to the washed blood, and mixed in with the packed cells. A plain glass capillary tube was placed in the blood, which was drawn into the tube by capillary action.
- The end of the tube was sealed using a Hawksley Cristaseal (Hawksley and Sons, Sussex), and the tube centrifuged in a micro-haematocrit centrifuge (Hawksley and Sons, Sussex) for 2 minutes.
- The packed cell density was read using a Hawksley haematocrit reader. This percentage of packed cells was then used to make a 1% solution of blood, by diluting with sterile PBS (i.e. where the concentration of blood cells was 85%, 1 ml blood was added to 85 ml PBS).

2.12.2 Contact Haemolysin Assay (Modification of Istivan *et al.* 1998)

- Confluent 3 day old bacterial cultures were harvested from BA plates into sterile PBS and the cell density adjusted to 10^{10} cells/ml (OD = 1.3 at 650 nm, usually about 1 plate/ml PBS).
- 500 µl of this suspension was placed into a 10 ml sterile centrifuge tube (in triplicate) and 1 ml of 1% washed horse blood added.
- For positive controls, the bacterial suspension was replaced with 500 µl sterile distilled water, and for the negative controls, the suspension was replaced with PBS.

- The samples were centrifuged at 3000 g for 10 minutes at 4°C, then left for 5 hours at 37°C. Preliminary studies used an incubation period of 30 hours, but subsequent experiments proved this prolonged incubation period unnecessary.
- After incubation, the samples were vortex mixed and centrifuged at 3000 g for 10 minutes to remove intact cells. The absorbances of the supernatants were read at 540 nm, averaged, and the percentage haemolysis calculated using the controls.

$$\text{i.e.} \quad \frac{\text{sample average} - \text{negative control average}}{\text{positive control average}} \times 100\% = \% \text{ haemolysis}$$

2.12.2.1 Contact Haemolysin Assay with Varying Biomass

- The same procedure as in section 2.12.2 was followed, but in addition to adjusting the cell density to 10^{10} cells/ml, the sample was halved and the same volume of PBS added to one half to produce a cell density of 5×10^9 cells/ml.
- This sample was then halved, and an equal volume of PBS added to one half to produce a cell density of 2.5×10^9 cells/ml.
- 500 µl each sample was then placed into a 10 ml sterile centrifuge tube (in triplicate) and 1 ml of 1% washed horse blood added.
- The same procedure as in 2.12.2 was then followed for the rest of the experiment.

2.12.2.2 Sonication of Samples

- The same procedure as in section 2.12.2 was followed to obtain a cell suspension containing 10^{10} cells/ml.
- 1ml of this suspension was aliquoted into 1.5ml centrifuge tubes, and bath sonicated on ice for 15 minutes, then centrifuged in a microcentrifuge for 30 minutes at 10 000g as in section 2.5.

- The cell free supernatant was retained, and 500 µl added to the haemolysin assay (in triplicate) as in section 2.12.2.
- The cell pellet was resuspended in 1ml sterile PBS, and 500 µl this suspension added to the haemolysin assay as above.
- Additional cell suspension samples of the same cell density that had not been sonicated were also centrifuged for 30 minutes at 10 000g. The supernatant was retained, and 500 µl added to each of a further three tubes in the haemolysin assay.

2.12.2.3 Heat Treatment of Samples

- The same procedure as in section 2.12.2 was followed to obtain a cell suspension containing 10^{10} cells/ml.
- *C. jejuni* and *A. butzleri* suspensions, and PBS controls were boiled at 100°C for 10 minutes and then cooled to room temperature before 500 µl each sample was added (in triplicate) to the haemolysin assay as in section 2.12.2.
- In addition to the heat treated samples, unheated samples of each strain of bacteria were also included in the assay as comparator tubes.

2.12.2.4 Addition of Desferal Mesylate to the Contact Haemolysin Assay

- Bacteria were grown on BA plates containing 50 µM desferal mesylate (see section 2.2.1.1). A 500 µM stock solution of desferal mesylate was made up using sterile PBS, and 150 µl of this was added to each assay tube in triplicate.
- Negative controls were included in the assay as comparator tubes, by adding 150 µl of the stock solution of desferal mesylate to each of 3 tubes containing PBS rather than the bacterial suspension. The absorbance readings from these tubes were averaged and this value used as the negative control average to calculate the percentage haemolysis as in section 2.12.2.

- In addition to these samples, suspensions of each strain of bacteria grown under the same conditions as for 2.12.2 were included in the assay as comparator tubes.

2.12.2.5 Addition of EDTA (Ethylenediaminetetraacetic Acid)

- A stock solution of 100 mM EDTA was made up using sterile distilled water, and where required, 150 µl was added to each assay tube in triplicate, to produce a final concentration of 10 mM.
- Controls were prepared by adding the same concentration of EDTA to each of 3 tubes where the bacterial suspension had been replaced by sterile PBS (see section 2.12.2.4).
- In addition to the EDTA treated tubes, untreated samples of each strain of bacteria were also included in the assay as comparator tubes.

2.12.2.6 Addition of Dextran Sulphate (Dextran 5000)

- A stock solution of 300 mM dextran sulphate was made up using sterile PBS. Where required in the assay, 150 µl of the stock solution was added to each tube in triplicate, resulting in a 30 mM final concentration.
- Controls were prepared by adding the same concentration of dextran sulphate to each of 3 tubes where the bacterial suspension had been replaced by sterile PBS (see section 2.12.2.4).
- In addition to the dextran sulphate treated tubes, untreated samples of each strain of bacteria were also included in the assay as comparator tubes.

2.12.2.7 Addition of Phospholipase C Inhibitor (Compound 48/80)

- A stock solution of 10 mg/ml compound 48/80 $(C_{11}H_{15}NO(monomer)^2$, Sigma) was prepared in sterile PBS.
- Various concentrations of the inhibitor were added to 1% red blood cells (as in section 2.12.2), and the highest concentration of inhibitor that did not cause lysis was found to be 4 µg.
- Therefore, when phospholipase C inhibitors were included in the assay, either 2 µl (2 µg) or 4 µl (4 µg) of the stock solution was added to the assay tubes before incubation.

- **2.12.2.8 Addition of Phospholipase A_2 Inhibitor Palmitoyl Trifluoromethyl Ketone ($PACOCF_3$)**

- The $PACOCF_3$ (Sigma), was reconstituted using absolute ethanol to produce a 10 mg/ml stock solution. Various concentrations of the inhibitor were applied to 1% red blood cells, and the highest concentration of inhibitor that did not cause lysis of the blood cells was found to be 128 µM.
- Therefore, in the contact assays where a phospholipase inhibitor was included, either 3 µl (64 µM) or 6 µl (128 µM) of the stock solution was added to each assay tube in triplicate, before incubation.

2.13 PHOSPHOLIPASE A_2 ASSAY

The phospholipase A_2 assay was carried out using a kit produced by Assay Designs, Inc., USA, for the detection of secretory phospholipase A_2 ($sPLA_2$). The kit uses a specific substrate for $sPLA_2$ that is converted into a sulfhydryl molecule, which can be detected colorimetrically using Ellman's reagent DTNB (5,5'-dithiobis, 2-nitrobenzoic acid). This forms a yellow coloured product with the sulfhydryl, and is measured at 405 nm.

- Samples were prepared as for the haemolysin assay (section 2.12), and 50 µl each sample pipetted into a separate well of a microtitre plate in duplicate.
- The PLA_2 inhibitor $PACOCF_3$ was added to the remainder of each sample using the same concentrations as in section 2.12, and 50 µl of each sample + inhibitor was then added to a further two wells of the microtitre plate.
- $SPLA_2$ standards were pipetted into duplicate wells of the plate and the assay was carried out according to instructions provided with the $sPLA_2$ assay kit.
- When the assay had been completed, the optical density of each sample was read at 405 nm, each set of values averaged, and the average value for the blank standard subtracted from the other results.
- A standard curve was plotted using the values obtained from the $sPLA_2$ standards, and the $sPLA_2$ concentration of each sample calculated using the equation determined from the standard curve.
- *Streptomyces violaceoruber* phospholipase A2 (Sigma) was used as a positive control.

2.14 DNA EXTRACTION

- Overnight BA cultures of *C. jejuni* and *A. butzleri* were harvested into 1 ml PBS and centrifuged at 13 000 g for 2 minutes until a rice grain sized pellet was obtained (typically a large loopful of bacteria would achieve this).
- DNA was then extracted from the cells using either the Wizard Genomic DNA Purification Kit (Promega) or the Dneasy Tissue kit (Qiagen), and the final pellet resuspended in 50 µl sterile distilled water or TE buffer (10 mM Tris-HCl ,pH 7.5, 1mM EDTA).
- The concentration of DNA in each sample was measured at 260 nm using a UV spectrophotometer (Philips), and the purity of the DNA measured by dividing the absorbance value at 260 nm by the absorbance reading at 280 nm. A value of about 1.8 indicated pure DNA.

2.15 PCR OF *CAMPYLOBACTER* GENOMIC DNA USING PRIMERS FOR THE PHOSPHOLIPASE A GENE

2.15.1 Primer Design

Primers for the phospholipase gene were designed with the help of Professor M. Darlison (TNTU), using the published sequence of *C. jejuni* NCTC 11168 and the identified phospholipase A gene (*pldA)* from *C. coli* (Grant *et al.* 1997). BamH I sites (underlined and in red) were built into the 5' end of each primer to allow for cleavage of the product at a later date.

Primer pldA1 : 5' TAAGGATCCCTTATGTGGCT 3'
Primer pldA2 : 5' GATGGATCCTTGAGTGGAGT 3'

Primers were ordered from Sigma-Genosys, reconstituted in Tris-EDTA (TE buffer) to a 50 μM stock solution, and stored at –20°C.

2.15.2 PCR of Phospholipase A Gene (*pldA*)

The following reagents were added to a 0.5 ml PCR tube on ice;

5 μl Pfu polymerase 10X buffer
1 μl each dNTP (10 mM each)
0.5 μl Pfu polymerase (3 u/μl)
Sterile distilled water up to a final volume of 50 μl

The tube was gently flicked and the following reagents added:

DNA template (to final concentration of 100 ng)
1 μl forward primer (50 μM stock)
1 μl reverse primer (50 μM stock)

A control tube was also run alongside the *pldA* tube, containing all the reagents, but replacing the DNA template with sterile distilled water. This was to ensure that no contamination arose during the PCR.

The tubes were placed in the PCR thermocycler (Techne genius, SLS) and incubated under the following conditions;

- Pre cycle 94°C, 3 minutes
- 35 Cycles 94°C, 1 minute (denaturation)
  50°C, 1 minute (annealing)
  72°C, 3 minutes (extension)
- Soak 4°C, indefinitely

A 10 µl aliquot of the product was analysed by agarose gel electrophoresis, and the remainder of the sample stored at -20°C until required.

2.16 AGAROSE GEL ELECTROPHORESIS

TAE (Tris-Acetate) 50X Buffer
242 g Tris base
57.1 ml Glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)

The pH was adjusted to pH 7.2 and the volume brought to 1 litre with distilled water.

- The gel casting tray was assembled, ensuring the bottom was level by adjusting the feet. The comb was inserted, allowing approximately 0.5-1 mm space between the bottom of the comb teeth and the casting tray.
- The required amount of agarose (SeaPlaque, Flowgen, FMC BioProducts) was weighed into a beaker and the appropriate volume of 1X TAE buffer

added. For example, to make a 1% gel, 1 g agarose was added to 100 ml buffer.

- The beaker was swirled to mix the contents, and then heated in a microwave oven (Matsui) on high power until all the agarose had dissolved, interrupting at regular intervals to swirl the solution, and ensuring that the contents did not boil over.
- The beaker was removed from the oven, gently swirled again, and allowed to cool to approximately 50-60°C.
- Prior to casting, ethidium bromide was added to the mixture to a final concentration of 0.5 μg/ml, and the solution poured into the casting tray to a height of about 3-4 mm (approximately 25 ml solution for a mini gel system, Bio-Rad).
- The gel was then left to set for approximately 40 minutes.
- Once the gel was set, the comb was carefully removed and the gel placed in an electrophoresis chamber (Bio-Rad).
- The wells were gently flushed out with 1X TAE buffer, and the chamber filled with 1X TAE buffer (running buffer) until the buffer reached 3-5 mm above the surface of the gel.
- 1 μl 6X loading buffer was added to each 5 μl of DNA sample, and these were then loaded on the gel alongside a 1Kb DNA ladder (Promega) for size determination, or a 1Kb hyperladder (Bioline) for quantitative analysis.
- The gel was run at 1-5V/cm (i.e. for a mini gel system, 100V for 40 minutes), using a powerpac 3000 (Bio-Rad).
- After electrophoresis, the gel was removed from the chamber and placed on a UV transilluminator (UVP, San Gabriel) for image recording using Grab-It software.

2.17 EXTRACTION OF PCR PRODUCT FROM AGAROSE GEL

- The remainder of the PCR product (40 µl) was loaded onto a 1% SeaPlaque agarose gel (Flowgen), and the gel run as above.
- The product was cut out of the gel using a nuclease free scalpel wiped in ethanol, and the gel slice weighed, ensuring that the total weight was less than 300 mg.
- The gel slice was heated at 70°C until completely melted and the DNA purified from the agarose using the wizard PCR preps DNA purification system (Promega) and the vac-man Jr laboratory vacuum manifold (Promega).

2.18 ETHANOL PRECIPITATION OF PCR SAMPLE

- 0.1 volume 3M potassium acetate was added to the DNA sample and mixed.
- 3 volumes absolute ethanol were then added, and the mixture left on ice for 10 minutes.
- The sample was then centrifuged at 13 000 g for 30 minutes in a microcentrifuge, and the supernatant removed.
- The pellet was then washed with 500 µl cold 70% ethanol and centrifuged at 13 000 g for a further 5 minutes.
- The supernatant was removed, and the pellet left to air dry for 10-15 minutes, before resuspending in an appropriate volume of sterile distilled water.

2.19 AMPLIFICATION OF PCR PRODUCT FOR SEQUENCING

The following reagents were added to 2 sterile PCR tubes on ice;

4 µl Big Dye (ABI)
0.5 µl primer (50 µM stock, one direction)
10 µl template DNA (200 ng)
0.5 µl sterile distilled water

The tubes were flicked to mix, and placed in the thermocycler under the following conditions for 25 cycles;

96°C 30 seconds
50°C 15 seconds
60°C 4 minutes

The pellets were concentrated by ethanol precipitation (see section 2.18) and taken to the QMC for DNA sequence analysis.

2.20 RESTRICTION DIGEST OF GENOMIC DNA

The following reagents were added to a sterile 0.5 ml centrifuge tube;

Restriction enzyme 10X buffer	2 µl
Sterile distilled water	13 µl
Bovine serum albumin (BSA, 1mg/ml)	2 µl
DNA (1 µg/µl)	1 µl
Bam HI (10 u/µl)	2 µl
	20 µl

The tube was gently mixed, pulsed in a microcentrifuge, and left overnight at 37°C. The samples were stored frozen until required, after the addition of 4 µl 6X loading buffer.

2.21 SOUTHERN BLOTTING OF *C. JEJUNI* AND *A. BUTZLERI* DNA

2.21.1 Southern Blotting using a Radioactive Probe

The genome of *C. jejuni* 11168 contains two haemolysin domains in addition to a phospholipase A gene. To date, these genes have not been fully investigated, therefore southern blot analysis was carried out on all strains of *C. jejuni* and *A. butzleri* to determine whether these genes were ubiquitous in all the strains, or unique to *C. jejuni* 11168.

2.21.1.1 Southern Blotting onto Hybond-XL Membrane

- *C. jejuni* and *A. butzleri* restriction digest samples (10 µl each, see section 2.20) were loaded onto a 1% agarose gel, ensuring that the gel was cast no thicker than 4 mm, and that as soon as the gel was set it was submerged into the buffer to prevent a skin forming on the surface.
- The gel was run for 40 minutes at 100V, and photographed as quickly as possible, to minimise exposure of the DNA to UV.
- The gel was then washed briefly in distilled water, and could be stored for up to 1 day wrapped in clingfilm (Alchem, UK) at 4°C before proceeding to the depurination step.

<u>Depurination</u>

- The gel was placed in depurination buffer (22 ml conc. HCl in 1 litre distilled water) for 10 minutes with gentle agitation until the bromophenol blue in the loading buffer turned yellow. Care was taken to ensure that the gel was not overdepurinated, as this can lead to fuzzy bands.
- The gel was then gently rinsed with distilled water 2-3 times, before denaturation.

Denaturation

- The gel was incubated in denaturation buffer (87.66 g NaCl, 20 g NaOH in 1 litre distilled water) for 30 minutes with gentle agitation until the bromophenol blue in the samples turned back to blue.
- The gel was then rinsed 2-3 times with distilled water.

Neutralization

- The gel was then placed in neutralization buffer (87.66 g NaCl, 60.5 g Tris base in 1 litre of distilled water, and adjusted to pH 7 with conc. HCl), for 30 minutes with constant agitation.
- Meanwhile, a sheet of Hybond-XL membrane (Amersham Life Sciences, Amersham, UK), was cut with clean sharp scissors to the exact size of the gel, ensuring that the membrane was only handled with gloved hands.
- A large dish was half filled with transfer buffer (20X SSC – 88.23 g Tri-sodium citrate, 175.32 g NaCl in 1 litre of distilled water, ensuring the pH was between 7 and 8).
- A sponge (ASDA, UK), slightly larger than the gel was soaked in transfer buffer, and placed in the dish so that about half was submerged in the buffer.
- Three pieces of blotting paper were cut to the same size as the sponge, saturated in transfer buffer and placed on top of the sponge.
- The neutralized gel was placed onto the paper (with the underside of the gel uppermost) and any air bubbles removed by rolling a glass rod over the surface.
- Four pieces of saran wrap were cut to fit around the gel, just overlapping the edges of the gel and covering the paper and sponge, to prevent the buffer from short circuiting around the gel.
- The membrane was then placed on top of the gel, again ensuring no air bubbles were present, and avoiding moving the membrane once it had touched the gel.
- Three further pieces of blotting paper were cut to the same size as the gel, and placed on top of the membrane.

- A stack of absorbent towels or 10 sheets of quick draw paper (Sigma) were placed on top of the gel, to at least 5 cm high, to act as a wick.
- A glass plate was placed on top of these sheets, and a flask containing 350 ml water balanced on top of the plate to act as a weight.
- The transfer was allowed to proceed overnight, for at least 16 hours, checking the towels every so often and replacing with more if saturated.
- When transfer was complete, the membrane was marked in the top left hand corner using a pencil, and the DNA was fixed to the membrane by baking at 80°C for 2 hours between 2 sheets of filter paper.
- Once dry, the membrane could be stored between 2 sheets of filter paper for several months under desiccation.

2.21.1.2 Dot Blotting *C. jejuni* and *A. butzleri* DNA onto Hybond-XL Membrane

- Small pieces of Hybond-XL membrane were cut as in section 2.21.1.1, large enough to ensure that six or seven samples could be spotted onto each piece.
- The concentration of DNA for each organism was determined (see section 2.14), and the volume required to load 500 ng calculated.
- All samples were made up to 5 µl with sterile distilled water, and small crosses were drawn on the membrane about 2 cm apart to ensure that samples did not cross contaminate.
- The samples were spotted onto the membrane in 2.5 µl aliquots, allowing each spot to dry before adding the second aliquot.
- Once dry, the membranes were baked at 80°C for 2 hours between 2 sheets of filter paper, and stored under desiccation until required.

2.21.1.3 Probe Labelling using ^{32}P

Radiolabelled probes were produced using the megaprime DNA labelling system (Amersham Life Sciences), incorporating dATP radiolabelled with ^{32}P (Redivue deoxyadenosine 5'[$\alpha^{32}P$]- triphosphate triethylamine salt, Amersham Life Sciences) to about $1x10^6$ cpm/ng. 25 ng template DNA was used for each reaction, and the probe was used immediately to minimise reduction in sensitivity caused by probe decay.

2.21.1.4 Southern Hybridisation using a ^{32}P Labelled Probe

Hybridisation buffer

0.5% w/v SDS

5X SSC (Transfer buffer)

5X Denhardts solution (2 g BSA, 2 g Ficoll, 2 g Polyvinylpyrrolidone in 1 litre distilled water makes 100X)

- The radiolabelled probe was produced as in section 2.21.1.3.
- The required volume of hybridisation buffer was preheated to 65°C, and the blot placed into the buffer, allowing 125 µl buffer per cm^2 membrane.
- The blot was allowed to prehybridise for 30 minutes at 65°C with constant agitation.
- The probe was added (1 µl per ml buffer) to the hybridisation buffer and hybridised overnight at 65°C with gentle agitation.
- Meanwhile, the stringency wash solutions were prepared, allowing an excess of solution to wash the membrane (at least 1-5 ml/cm^2 membrane).
- After hybridisation the blot was washed in a low stringency wash solution (2X SSC, 0.1% w/v SDS), incubating twice for 5 minutes each.
- The blot was then either taken for exposure, or washed at higher stringency. This entailed following the low stringency washes with 15 minutes incubation in a medium stringency wash (1X SSC, 0.1% w/v SDS), and a

further two 10 minute incubations in a high stringency wash solution (0.1X SSC, 0.1% w/v SDS).

- After the last wash, the blot was removed from the relevant solution, drained, and sealed in a plastic bag to keep the membrane moist.
- The membrane was exposed to X-ray film (Hyperfilm MP, Amersham Life Sciences) between 5 hours and overnight, ensuring that the blot was kept moist if reprobing was required later.
- After the required exposure time, the film was developed using a hyperprocessor (Amersham Life Sciences).

2.21.1.5 Reprobing of Southern Blots

- After exposure to X-ray film, the membrane was placed in a tray and covered in a boiling solution of 0.1% w/v SDS which was allowed to cool for 30 minutes.
- The blot was rinsed briefly in 2X SSC, and to ensure complete removal of the probe, the blot was exposed to X-ray film overnight.
- If the film was clear, the blot was rehybridized overnight in appropriate conditions, or dried and stored under desiccation until required.

2.21.2 Southern Blotting using a Non Radioactive Probe

These experiments were carried out using similar methods to the radioactive blotting, but the probe was non-radioactive, and therefore safer to use.

2.21.2.1 Dot Blotting *C. jejuni* and *A. butzleri* DNA onto Hybond N+ Membrane

- Small pieces of Hybond N+ membrane were cut as in section 2.21.1.1, large enough to ensure that six or seven samples could be spotted onto each piece.

- The concentration of each sample was determined, and the volume required to load 500 ng calculated.
- Samples were made up to 5 µl with sterile distilled water, and dotted onto the membrane as in section 2.21.1.2.
- Once dry, the membranes were baked for 2 hours at 80°C, and stored under desiccation until required.

2.21.2.2 Probe Labelling using the Random Prime Labelling System

- Non radiolabelled probes were produced using the *Gene Images* random prime labelling module (Amersham Life Sciences), yielding a fluorescein labelled probe of between 6 to 8 ng/µl.
- When the probe was not being used immediately, EDTA was added to a final concentration of 20 mM, and the probe frozen in the dark for up to 6 months.

2.21.2.3 Southern Hybridisation using a Fluorescein Labelled Probe

Hybridisation buffer

5X SSC (Transfer buffer, see section 2.21.1.1)

0.1% w/v SDS

5% w/v Dextran sulphate

20-fold dilution of liquid block (Amersham Life Sciences)

The reagents were combined and made up to volume with distilled water, whilst gently heating to dissolve the dextran sulphate. Once dissolved, the buffer was stored at -20°C for up to 12 months.

- The labelled probe was produced as in section 2.21.2.2.
- The required volume of hybridisation buffer was preheated to 60°C, and the blot placed into the buffer, allowing 0.3 ml buffer per cm^2 membrane.

- The blot was allowed to prehybridise for 30 minutes with constant agitation.
- The required amount of probe was defrosted, and denatured by boiling for 5 minutes and snap cooling on ice.
- The probe was added to the hybridisation buffer (1 µl per ml buffer), and left to hybridise overnight at 60°C with gentle agitation.
- After hybridisation, the blot was washed in an excess of wash solution (1X SSC, 0.1% w/v SDS) at 60°C for 15 minutes, followed by a further 15 minutes at 60°C in a lower stringency wash solution (0.5X SSC, 0.1% w/v SDS), before detection.

2.21.2.4 Southern Hybridisation using the *Gene Images* Random Prime Labelling Module (Amersham Life Sciences)

Buffer A
100 mM Tris-HCl
300 mM NaCl

The required amount of buffer was made up in distilled water and autoclaved in 25 ml aliquots. These were stored at -20°C, and used within a day of opening.

- After washing off the unbound probe, the blot was blocked by incubation with gentle agitation for an hour at room temperature in a 1/10 dilution of liquid blocking agent (Amersham Life Sciences) in buffer A (1 ml/cm^2 blot).
- Meanwhile, anti-fluorescein-AP conjugate 5000 (Amersham Life Sciences) was diluted 5000 fold in freshly prepared 0.5% w/v BSA in buffer A.
- After blocking, the blot was incubated for a further hour at room temperature in the solution above (0.3 ml/cm^2)
- Unbound conjugate was removed by washing for 3 x 10 minutes in an excess volume of 0.3% v/v tween 20 in buffer A, at room temperature with gentle agitation.

- After washing, the blot was drained, and placed sample side up on a piece of cling film.
- Detection reagent (Amersham Life Sciences) was pipetted onto the blots, allowing 30-40 $\mu l/cm^2$, and left for 3 minutes.
- Excess reagent was drained off by touching the corner of the blot on a piece of cling film, and the blot wrapped in a fresh piece of cling film to keep the blot moist.
- The membrane was exposed to X-ray film (Hyperfilm MP) between 10 minutes and an hour, and the film developed using a hyperprocessor (Amersham Life Sciences).

2.22 PCR OF *CAMPYLOBACTER* GENOMIC DNA USING PRIMERS FOR THE *tlyA* AND *Cj0183* (*clyC*) GENES

2.22.1 Primer Design for the *tlyA* Gene

Primers were designed using the published sequence of *C. jejuni* NCTC 11168 and the putative haemolysin gene (*tlyA)* from Parkhill *et al.* 2000. BamHI sites (underlined and in red) were built into the 5' end of each primer to allow for cleavage of the product at a later date.

Primer tlyAF : 5' TGAGGATCCTATTTGGTGGT 3'
Primer tlyAR : 5' CATGGATCCAAAACAACCTA 3'

2.22.2 Primer Design for the *Cj0183* Gene (*clyC*)

Primers were designed using the published sequence of *C. jejuni* NCTC 11168 and the putative haemolysin gene (*Cj0183*, named *clyC* for ease), from Parkhill *et al.* 2000. BamHI sites were built into the ends of the primers as above (section 2.22.1).

Primer clyCF : 5' AAAGGATCCAACTATCACTC 3'
Primer clyCR : 5' CGAGGATCCAATAGATGATG 3'

2.22.3 PCR of *tlyA* and *clyC* Genes

The same PCR conditions as those used for the *pldA* primers (section 2.15.2) were used for both the *tlyA* and *clyC* primers, as both sets of primers were designed to be 20 bases long with similar melting temperatures.

The resulting product was excised from the gel, and extracted as in sections 2.16-2.17. The purified DNA was then used as a probe for southern blotting as in section 2.20.

2.23 CONSTRUCTING THE *pldA* MUTANT

STRAIN/PLASMID	CHARACTERISTICS	SOURCE
Bacteria		
C. jejuni NCTC 11168	Sequenced wild type strain	Parkhill *et al.* 2000
E. coli TOP10F'	F' {*lacI*^{q}, Tn10(*Tet*R)}, *mcr*A, Δ(mrr-hsdRMS-mcrBC),Φ80lacZM15, Δ*lac*74, *rec*A1,*deo*R, *ara*D139Δ(ara-leu)7697, *gal*U, *gal*K, *rps*L (*Str*R), *end*A1, *nup*G	Invitrogen
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (rk-, mk+),*rel*A1, *sup*E44, Δ(*lac-pro*AB),[F', *tra*D36, *pro*AB, *lacI*^{q}ZΔM15]	Promega
Plasmids		
PCR®T7/NT-TOPO®	Cloning vector, Ampicillin resistance	Invitrogen
pSJM01	PCR®T7/NT-TOPO® containing the *pldA* gene from *C. jejuni* NCTC 11168 (0.98Kb) and flanking DNA (0.786Kb)	This study
pSJM02	pSJM01 containing half the *C. jejuni* NCTC 11168 *pldA* gene sequence	This study
pSJM03	pSJM02 plus a 1kb chloramphenicol resistance cassette	This study
PRY109	Plasmid containing a 1kb chloramphenicol resistance cassette	Yao *et al.* 1993

Table 2.1 – Bacterial strains and plasmids used to construct the *pldA* mutant

2.23.1 Primer Design

Primers were designed with the help of Dr. Karl Wooldrige (QMC, Nottingham), and the published sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.* 2000).

Primer pldAF : 5' ATCTAGCCGTATGGGACAAG 3'
Primer pldAR : 5' GTTATGATTTCTAGTTGAGTGGAG 3'

2.23.2 PCR using *pldA* Primers

Taq polymerase was used for the PCR rather than Pfu polymerase, as Taq adds a single deoxyadenosine to the 3' ends of PCR products, which is necessary for cloning into the PCR®T7/NT-TOPO® plasmid. Therefore, the PCR conditions were adjusted by shortening the extension time from 3 minutes to 2 minutes.

The following reagents were added to a 0.5 ml PCR tube on ice;

5 µl Taq Polymerase buffer 10X buffer
3 µl Magnesium Chloride (25 mM)
1 µl each dNTP (10 mM each)
0.3 µl Taq polymerase (5 u/µl)
Sterile distilled water up to a final volume of 50 µl

The tube was gently flicked and the following reagents added:

DNA template (to final concentration of 100 ng)
0.5 µl primer pldAF (100 µM stock)
0.5 µl primer pldAR (100 µM stock)

A control tube was also run alongside the tube (see section 2.15.2).

The tubes were placed in the PCR thermocycler and incubated under the following conditions;

- Pre cycle 94°C, 3 minutes
- 30 Cycles 94°C, 1 minute (denaturation)
  55°C, 1 minute (annealing)
  72°C, 2 minutes (extension)
- Soak 4°C, indefinitely

A 10 µl aliquot of the product was analysed by agarose gel electrophoresis, and the remainder of the sample stored at -20°C until required.

2.23.3 Cloning *pldA* into PCR®T7/NT-TOPO® Cloning Vector

The cloning reaction was carried out using the PCR®T7 TOPO®TA expression kit (Invitrogen), for the direct insertion of the Taq polymerase amplified *pldA* product into the PCR®T7/NT-TOPO® plasmid cloning vector (see table 2.1 for details of plasmids), resulting in pSJM01 plasmid.

2.23.4 Transformation of pSJM01 into Competent *E. coli* Cells

- The pSJM01 plasmid was transformed into chemically competent *E. coli* TOP10F' cells according to methods supplied by Invitrogen.
- 50 µl transformation mixture was spread onto LB plates in duplicate, containing 100 µg/ml ampicillin, and incubated overnight at 37°C.
- Transformant colonies were picked off and used to inoculate 10 ml LB broth supplemented with 100 µg/ml ampicillin.
- These broths were allowed to grow overnight at 37°C in a shaking incubator at 200 rpm, before plasmid purification using either the miniprep method (section 2.23.5) or the Quiaprep spin kit (Quiagen).
- The resulting clones were checked by PCR analysis (see section 2.23.2) using the original pldAF and pldAR primers to ensure that the transformant colonies were carrying the correct insert.

2.23.5 Plasmid Miniprep Purification

- Bacterial cells were harvested by centrifuging 1 ml of the culture at 13 000 g for 3 minutes in a 1.5ml microfuge tube four times, until 4 ml of the culture had been pelletted.
- The cells were then resuspended in 200 µl solution I (25 mM tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 50 mM glucose).

- 200 µl solution II was added and the solution mixed gently (made fresh; 60 µl 5M NaOH, 1305 µl sterile distilled water, 132 µl 10% SDS), and incubated for 5 minutes.
- 200 µl 3M potassium acetate was added, and the solution centrifuged at 13 000 g for 10 minutes.
- The supernatant was removed to a fresh tube, and 900 µl isopropanol added.
- The mixture was then centrifuged at 13 000 g for 15 minutes.
- The pellet was washed in 1 ml 70% ethanol and re-centrifuged for 4 minutes at 13 000 g.
- The supernatant was poured off, and the pellet allowed to dry on the bench top for 10 minutes.
- Once dry, the pellet was resuspended in 25 µl sterile distilled water and stored at –20°C until required.

2.23.6 Design of Primers for Inverse PCR

Primers were designed with the help of Dr. Karl Wooldrige (QMC, Nottingham), and the *C. jejuni* NCTC 11168 pldA gene.

Primer pldA-M1: 5' GCGAGATCTCATTTTAACTTGCTTTTAAATTTTTC 3'
Primer pldA-M2: 5' TCTCCTTTTAGAGAAACCAACTATC 3'

Primer pldA-M1 was designed to incorporate a 5' Bgl II site (underlined and in red), and a GCG clamp to aid digestion. No enzyme sites were included in primer pldA-M2 as there is a Bgl II site located within the *pldA* gene.

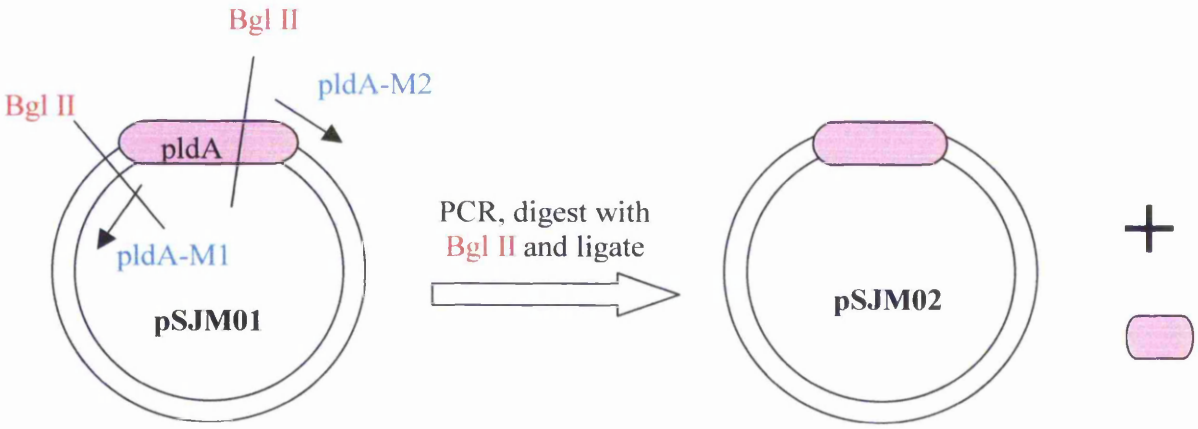


Figure 2.1 - Diagram to show inverse PCR of the pSJM01 plasmid

2.23.7 Inverse PCR of pSJM01 Plasmid

- The appropriate enzymes, primers and reagents were assembled in a 0.5 ml PCR tube on ice, as in section 2.23.2, using 5 ng plasmid pSJM01 as the template DNA.
- The tubes were placed in the PCR thermocycler and incubated under the following conditions;

  - Pre cycle 94°C, 3 minutes
  - 35 Cycles 94°C, 1 minute (denaturation)
    55°C, 1 minute (annealing)
    72°C, 4.5 minutes (extension)
  - Soak 4°C, indefinitely

A 10 µl aliquot of the product was analysed by agarose gel electrophoresis, and the remainder of the sample stored at -20°C until required.

2.23.8 *Bgl* II Digest of Inverse PCR (IPCR) Product

The same procedure as in section 2.19 was followed, using 2 µl *Bgl* II (10 u/µl), 2 µl 10X buffer D, and 1 µg plasmid. The tube was gently mixed, pulsed in a microcentrifuge, and placed in a 37°C waterbath overnight.

2.23.9 Ligation of Digested IPCR Products

The following reagents were added to a 0.5 ml PCR tube;

4 µl PCR product digest (100 ng)
1 µl 10X ligation buffer
3 µl sterile distilled water
2 µl T4 DNA ligase
10 µl

The contents were gently mixed, pulsed in a microcentrifuge, and incubated overnight at 4°C, to create the resultant plasmid pSJM02.

2.23.10 Making Competent *E. coli* JM109 Cells

- 30 ml LB broth was inoculated with 0.3 ml overnight culture of *E. coli* JM109 cells, and incubated in a shaking incubator at 37°C for 2 hours until an OD of about 0.4 was reached at 600 nm.
- The cells were harvested by centrifugation at 4000 rpm for 5 minutes at 4°C.
- The supernatant was discarded, and the cells resuspended in 6 ml ice-cold $CaCl_2$ solution. As soon as the cells were resuspended, the tube was placed on ice for 10 minutes, and kept cold for the remainder of the procedure.

- The centrifugation was repeated, and the supernatant discarded. The cells were resuspended in another 6 ml ice-cold $CaCl_2$ and placed on ice for a further 10 minutes.
- The centrifugation was then repeated for a third time, and the supernatant discarded once again. The cells were resuspended in 1.2 ml ice-cold $CaCl_2$ solution, and incubated on ice for a further 20 minutes.
- The competent cells were aliquoted into chilled Eppendorf tubes, quick frozen by partial submersion into dry ice and transferred to a -70°C freezer.

2.23.11 Transformation of *E. coli* JM109 Cells with pSJM02

- Competent *E. coli* JM109 cells were recovered from storage at –70°C and thawed on ice.
- Between 2 and 5 µl plasmid DNA was added to 100 µl aliquots of the competent cells and gently mixed before leaving on ice for at least 30 minutes.
- The cells were heat shocked at 42°C for 90 seconds, and returned to the ice for a further 2 minutes.
- 800 µl cold LB broth was added to the cells, and they were allowed to recover by incubation at 37°C for 1 hour.
- After an hour, 100 µl aliquots of the transformed cells were spread onto LB plates containing 100 µg/ml ampicillin, and the plates incubated at 37°C overnight.
- The following day several transformant colonies growing on the plates were selected and each was aseptically transferred to fresh 10 ml LB broths containing 100 µg/ml ampicillin.
- These were incubated overnight at 37°C in a shaking incubator, and the plasmid extracted and re-purified using the miniprep method as in section 2.23.5.

2.23.12 Digestion of pSJM02 with *Bgl* II

The purified plasmid was digested with 2 µl *Bgl* II as in section 2.23.8.

2.23.13 Dephosphorylation of pSJM02

- The *Bgl* II digested plasmid was treated with calf alkaline phosphatase (CIAP) to prevent the ends from re-ligating during the ligation process.
- The following reagents were added to a sterile 0.5 ml centrifuge tube;

  10 µl plasmid DNA
  2 µl CIAP
  2 µl 10X reaction buffer
  6 µl Sterile distilled water

- The tube was left at 37°C for 30 minutes.
- After half an hour, 2 µl more CIAP was added to the tube, and left for a further 30 minutes at 37°C.
- Then 130 µl sterile distilled water was added to the tube, followed by an equal volume of phenol:chloroform:isoamyl alcohol (150 µl), and the tube vortex mixed.
- The tube was centrifuged for 15 minutes at 13 000 g.
- The top aqueous phase was removed to a fresh tube, and 150 µl chloroform added. The tube was then spun for a further 10 minutes at 13 000 g.
- The top aqueous layer was again removed to a clean tube, and 15 µl potassium acetate, and 450 µl absolute ethanol added to the tube.
- This was left on ice for 10 minutes, and centrifuged for 30 minutes at 13 000 g.
- The supernatant was gently poured away, and the pellet washed with 200 µl 70% ethanol for 4 minutes at 13 000 g.
- The supernatant was carefully removed, and the pellet left to air dry for 10 minutes at room temperature.

- The dried pellet was resuspended in 5 µl sterile distilled water and stored at –20°C until required.

2.23.14 Ligation of a Chloramphenicol Resistance Cassette into pSJM02

In addition to disruption of the *pldA* gene by inverse PCR mutagenesis, a chloramphenicol resistance gene was inserted into the disrupted *pldA* gene in order to further knock out the gene, and to allow for selection by chloramphenicol resistance after cloning. After the plasmid and cassette insert had been prepared for ligation, the concentration of each was determined by running an aliquot on an agarose gel alongside molecular weight standards (see section 2.16). The optimum ratio of vector : insert was determined using the following equation;

$$\frac{\text{ng vector} \times \text{kb size insert}}{\text{kb size vector}} \times \frac{3}{1} = \text{ng insert}$$

- The chloramphenicol resistance gene was obtained from plasmid pRY109, kindly donated by Karl Wooldridge (Yao *et al* 1993, QMC, Nottingham).
- The plasmid was extracted and purified using methods from section 2.23.5, and digested with Bam HI as in section 2.19, incubating for 4 hours at 37°C.
- The pRY109 digest was loaded onto a 1% seaPlaque agarose gel and run as in section 2.16.
- The 1Kb chloramphenicol resistance gene (Cm^r) was excised from the gel as in section 2.16 and ethanol precipitated (see section 2.18) before ligation into the plasmid.
- The following reagents were added to a 0.5 ml PCR tube;

50 –100 ng plasmid
100 – 200 ng insert
1 µl 10X ligation buffer
2 µl T4 DNA ligase
sterile distilled water to a final volume of 10 µl

The contents were gently mixed, pulsed in a microcentrifuge, and incubated overnight at 4°C, creating plasmid pSJM03.

2.23.15 Transformation of *E. coli* JM109 Cells with pSJM03 Plasmid

- Competent *E. coli* JM109 cells were recovered from storage and transformed with the constructed pSJM03 plasmid as in section 2.23.11.
- Aliquots of the transformed cell mixture (100 µl) were spread onto LB agar plates supplemented with 100 µg/ml ampicillin, and 20 µg/ml chloramphenicol.
- The plates were incubated overnight at 37°C.
- Transformants were selected and overnight cultures grown as in section 2.23.11, in LB broth supplemented with 100 µg/ml ampicillin and 20 µg/ml chloramphenicol.
- The plasmids were extracted and purified as in section 2.23.5.

2.23.16 Natural Transformation of pSJM03 into *C. jejuni* NCTC 11168 Cells

- A fresh log phase culture of *C. jejuni* 11168 was produced by diluting 9 ml fresh BHI media with 1 ml *C. jejuni* overnight culture, and incubating to an OD of about 0.4 at 600 nm.
- Sterile blood free agar was added to each well of a 24 well plate in 1 ml aliquots, and allowed to solidify before 250 µl of the above culture was pipetted on the top of each agar filled well.
- Between 2 and 5 µl pSJM03 plasmid DNA was added to the suspension in each well, and the plate incubated overnight under microaerophilic conditions.
- The cells were harvested from the interface by pipetting, and spread onto blood free agar plates supplemented with 20 µg/ml chloramphenicol.
- The plates were incubated microaerophilically for 2 to 3 days.

2.23.17 Transformation of pSJM03 into *C. jejuni* NCTC 11168 Cells using Conjugation

- Competent *E. coli* S17 cells were recovered from storage and transformed with the constructed pSJM03 plasmid as in section 2.23.11.
- Aliquots of the transformed cell mixture (100 µl) were spread onto LB agar plates supplemented with 100 µg/ml ampicillin and 20 µg/ml chloramphenicol, and transformant colonies selected as in section 2.23.15.
- 1 ml of an overnight culture of the S17 cells carrying the pSJM03 plasmid was used to inoculate 9 ml fresh LB broth supplemented with antibiotics (as above), and incubated for 4 hours at 37°C in a shaking incubator.
- Meanwhile, a 3 day old confluent plate of *C. jejuni* 11168 was spread onto a fresh blood free agar plate, 50 µl S17 culture pipetted on top, and the 2 strains mixed together.
- The plate was incubated under microaerophilic conditions for 2 to 3 days, until transformant colonies were seen.
- One of these colonies was then picked off, and restreaked on a fresh blood free agar plate supplemented with 20 µg/ml chloramphenicol.
- The resulting mutant was then Gram stained to check its purity, and confirm that it was *C. jejuni*. Negative controls were carried out by growing the wild type *C. jejuni* on additional plates supplemented with the same antibiotics to confirm that the bacteria wasn't naturally resistant to both antibiotics.

CHAPTER 3

CYTOTOXICITY AND THE OUTER MEMBRANE PROTEIN (OMP)

3.1 GROWTH CURVES OF *C. JEJUNI*

Growth curves were determined for both *C. jejuni* NCTC 11351 and NCTC 11168 in order to determine and compare the growth patterns of the bacteria. This was to establish the time points at which the different phases of growth occurred, and to ensure that similar amounts of biomass would be harvested from the cells at the same growth phase.

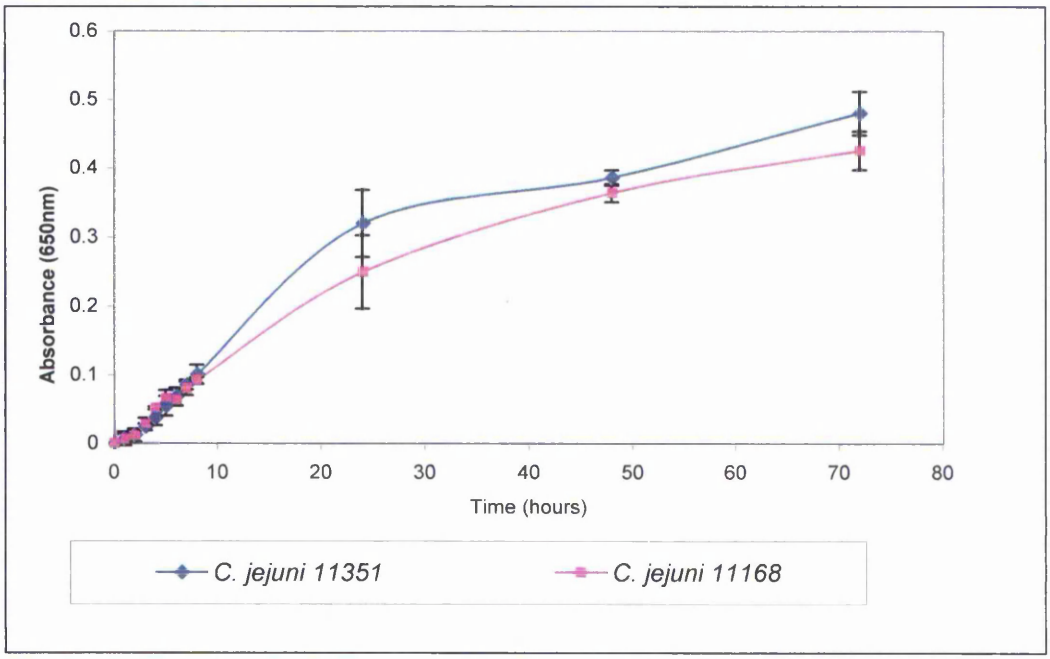


Figure 3.1 – Growth curves of *C. jejuni* NCTC 11351 and 11168 grown in BHI broth over 72 hours. Error bars indicate standard deviation of the data (n=3).

Figure 3.1 illustrates that both *C. jejuni* strains 11168 and 11351 exhibit comparable growth rates when grown over the same time scale in BHI broth. Both strains were in the lag phase between time 0 and 2 hours, while the cells took time to adapt to the new medium. The bacteria then entered the exponential phase between about 2 and 24 hours, where the cells were growing and dividing at their maximum rate. After 24 hours the growth started to slow, and the absorbance value continued to steadily increase up to 72 hours. The bacteria were likely to be in the stationary phase between 24 and 72 hours, where the number of cell deaths was beginning to equal the amount of new cells, causing the population to stabilise.

3.2 CYTOTOXICITY TESTING OF *C. JEJUNI* SAMPLES

Previous research by former TNTU PhD students Kate Holmes and Kevin Spears showed *C. jejuni* NCTC 11351 to have a consistent cytotoxic effect when cell extract supernatants were applied to mouse neuroblastoma (N2a) and human umbilical cord endothelial cells (ECV 304) *in vitro* (Holmes 2001). Therefore, for initial cytotoxicity experiments, these already established cell lines were used in addition to two others; Chinese hamster ovary cells (CHO) and Vero (African green monkey kidney) cells. Both CHO and Vero cell lines have been frequently used by other research groups for the detection of *C. jejuni* toxins. CHO cells are commonly used to test for the *C. jejuni* cytolethal distending toxin (Johnson & Lior 1988, Wassenaar 1997), and Vero cells are reported to be sensitive to shiga and shiga-like toxins produced by other Gram negative bacteria (Johnson & Lior 1984, Florin & Antillon 1992). As the characteristics of the detected toxin(s) are uncertain, it was considered that these cell lines would be a good starting point.

3.2.1 Cytotoxicity Testing of *C. jejuni* Cell Extract Samples on N2a Cells

Previous investigations had already established the optimum conditions required for cytotoxin production from *C. jejuni,* using three day old cultures grown on BA under microaerophilic conditions. At this point the bacteria were in the stationary growth phase. Therefore, samples were taken from bacteria grown under these same standardized conditions (see section 2.5).

In addition to testing *C. jejuni* NCTC 11351, samples were also taken from another strain, *C. jejuni* NCTC 11168. The genome of this strain has been sequenced, and is readily available on the internet (Parkhill *et al.* 2000). Therefore it was considered prudent to study cytotoxicity from this strain, as direct comparisons may then be made with the published gene sequence.

Initial cytotoxicity assays were carried out using a range of sample concentrations to determine the dose-response relationship. Initial experiments involved centrifuging the bacteria after harvesting into PBS, to pellet the cells

before removing the supernatant. The cell free supernatant was then added to the cultured cell lines but no significant toxicity was detected (data not shown). Therefore, the harvested cell suspensions were bath sonicated before centrifugation, as it was suspected that the potential toxin(s) may be membrane bound or intracellular, and if so, were likely to be released upon sonication. These sonicated cell free supernatants were then referred to as cell extract samples throughout this report.

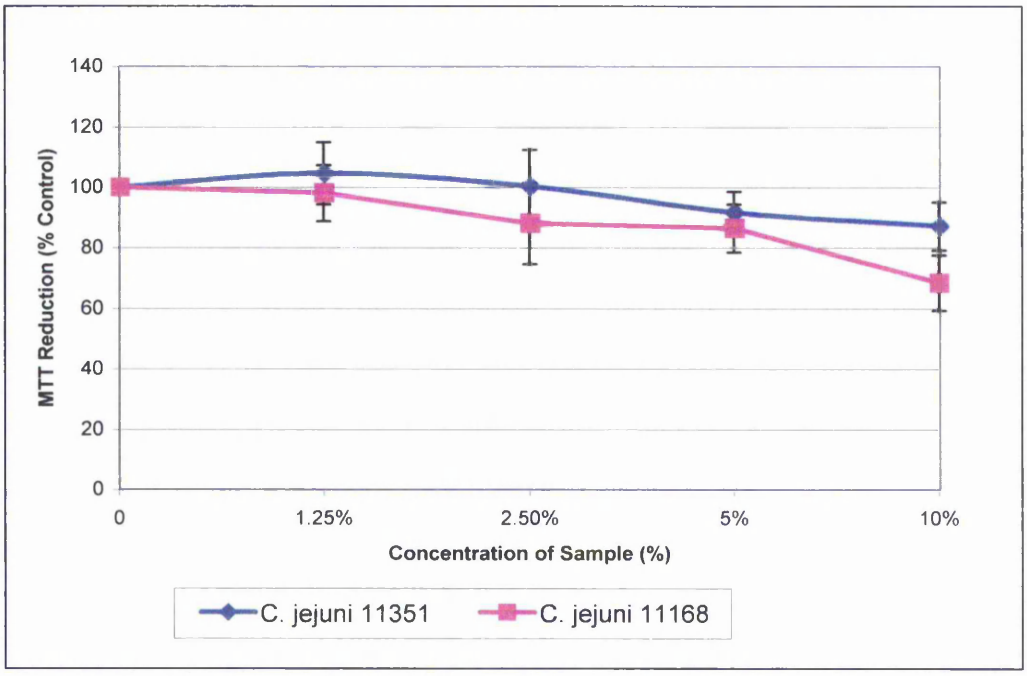


Figure 3.2 – MTT reduction assay of *C. jejuni* NCTC 11351 and 11168 cell extract samples applied to N2a cells. Error bars represent the standard deviation of the data (n=15). Paired t-tests were carried out on the data with 95% confidence limits.

Figure 3.2 shows that *C. jejuni* cell extract samples from both 11351 and 11168 exhibited some cytotoxicity when applied to N2a cells. Generally *C. jejuni* 11168 samples appear to have caused the least amount of MTT reduction, and therefore a higher amount of cell death than the *C. jejuni* 11351 samples. A paired t-test was carried out on the data, and the samples from *C. jejuni* 11168 were shown to be statistically significant when compared to the PBS controls (see section 2.6.5), at both the 5% ($P<0.036$) and 10% ($P<0.023$) concentrations. The *C. jejuni* 11168 2.5% sample was not statistically significant, although the value was only just outside the 95% confidence level ($P=0.064$).

Statistical analysis of the *C. jejuni* 11351 samples showed that both the 10% and 5% cell extract samples were significantly cytotoxic, with P values of less than 0.047 and 0.032 respectively. There was no significant cytotoxicity from the 1.25% or 2.5% samples which was not surprising, as figure 3.2 shows no MTT reduction from either of these two samples.

To ensure that similar amounts of sample were added in each experiment, in addition to standardising the absorbance values of each strain, a protein assay was carried out to determine the amounts of protein present in each sample. It was found that the amount of protein present correlated very closely with the absorbance value, and on average, the protein concentration of each sample measured between 82 and 85µg/ml.

From these results it was decided to continue using both strains of *C. jejuni*, as different effects may be seen with other cell lines. Experiments using 1.25% and 2.5% samples were discontinued, as these concentrations were too low to produce significant cytotoxicity, and were excluded from further experiments to save time.

3.2.2 Cytotoxicity Testing of *C. jejuni* Cell Extract Samples on ECV, Vero and CHO Cells

These experiments show that both *C. jejuni* strains did exhibit slight, though significant cytotoxicity to ECV cells when applied at a concentration of 10% ($P<0.032$, *C. jejuni* 11351; $P<0.0051$, *C. jejuni* 11168; see figure 3.3). Neither *C. jejuni* samples 11168 or 11351 were found to be significantly cytotoxic at the 5% concentration, but despite the large error bars, showed significant cytotoxicity when a t-test was carried out on the 10% samples. These large error bars were caused by variations in cell culture growth between experiments, but as each sample had its own experimental controls as a comparison, when the data was analysed statistically, the values were shown to be significant.

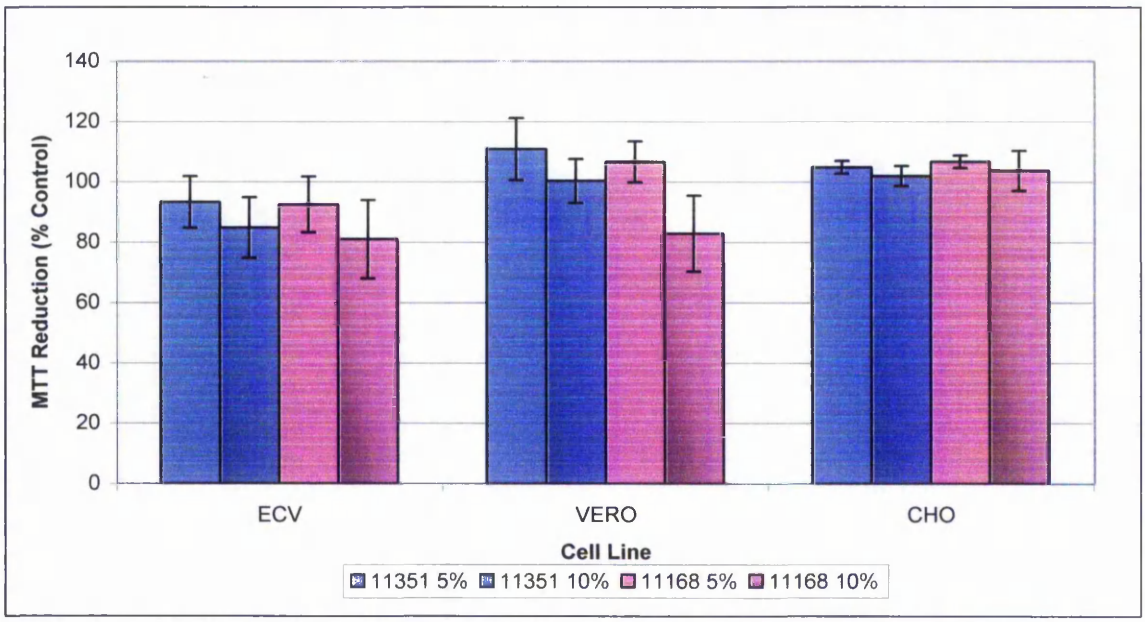


Figure 3.3 – MTT reduction assay of *C. jejuni* 11351 and 11168 cell extract samples applied to ECV, Vero and CHO cells. Error bars represent the standard deviation of the data (n=15). Paired t-tests were carried out on the data with 95% confidence limits.

The *C. jejuni* 11351 samples had no effect on the Vero cell line, and although the *C. jejuni* 11168 data also showed no significant values, the 10% cell extract sample was only just outside the 95% confidence limit, and was significant using 90% confidence limits ($P<0.062$). Neither bacterial extract showed any MTT reduction when applied to CHO cells ($P>0.169$, see figure 3.3). Therefore it does not seem likely that the cytotoxicity produced from samples from either strain is caused by the cytolethal distending toxin (see section 1.7.2.1).

A CytoTox 96 cytotoxicity assay (Promega) was carried out alongside the MTT assay, as it was possible that the MTT assay was too insensitive to detect low changes in cell numbers. The CytoTox 96 assay measures lactate dehydrogenase (LDH), a cytosolic enzyme released on cell lysis, rather than the MTT dye reduction assay, which uses methyl tetrazolium thiazoyl blue dye (MTT) and changes colour from yellow to blue by the metabolic activity of live cells. No significant differences were found between the two assays (data not shown), and so it was decided to retain the MTT assay for future experiments.

In conclusion, figures 3.2 and 3.3 show that N2a cells were the most susceptible cell line to *C. jejuni* cell extract samples, and that samples taken from *C. jejuni* 11168 were more cytotoxic than those taken from *C. jejuni* 11351 at the 10% concentration.

3.2.3 Cytotoxicity Testing of Heat and Trypsin Treated *C. jejuni* Samples

Having established the presence of cytotoxin(s) in the *C. jejuni* cell extract samples, further experiments were carried out to determine whether the toxin(s) present were proteinaceous or endotoxic. Samples were applied to all the cell lines, but only experiments using N2a cells have been presented, as these repeatedly showed the most sensitivity to the toxin samples.

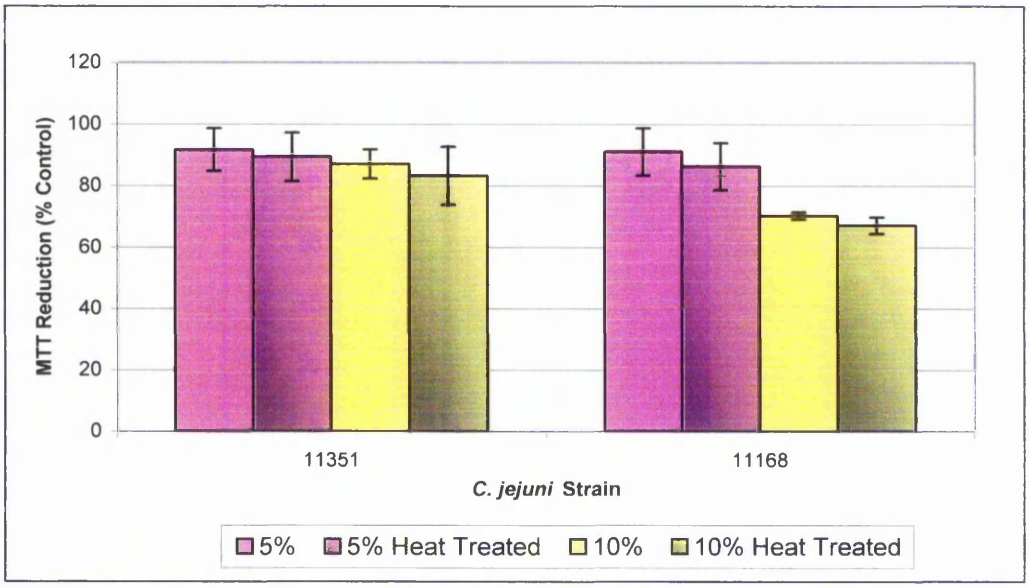


Figure 3.4 - MTT Reduction assay of *C. jejuni* 11351 and 11168 cell extract samples applied to N2a cells, with and without heat treatment. Error bars represent the standard deviation of the data (n=9). Paired t-tests were carried out on the data using 95% confidence limits. Heat treated extracts from both 11351 and 11168 showed significant toxicity to N2a cells at the 10% concentration ($P<0.004$), but no significant difference was determined between the heat treated and untreated samples ($P>0.32$).

Figure 3.4 shows that heating the bacterial cell extracts does not destroy their cytotoxicity, and additionally there was a slight but not significant increase in toxicity ($P>0.32$). Trypsin treatment of the *C. jejuni* samples showed similar

trends to the heat treatment experiments (see table 3.3). No significant reduction in cytotoxicity was detected when the samples were treated with protease. Therefore, it does not seem feasible that the toxin is proteinaceous, or if it is a protein, it is likely to be both heat and trypsin resistant.

3.3 MORPHOLOGY STUDIES

3.3.1 Morphology Studies of CHO Cells

Although cytotoxicity was demonstrated when applying *C. jejuni* cell extract samples to N2a, ECV and Vero cell lines, no significant changes in cell viability were detected when applying *C. jejuni* samples to CHO cells (see figure 3.3). Therefore, further experiments were carried out to determine whether the samples could be producing sub lethal effects to the general cell morphology of this cell line, which would not necessarily be detected by the MTT assay.

The cells were incubated for a further 48 hours in addition to the usual 48 hour incubation after application of the *C. jejuni* samples, in order to distinguish between different toxins affecting the CHO cells, as reported by Wassenaar (1997). MTT assays were also carried out in parallel to both the 48 hour and 96 hour experiments, and as in previous experiments, no cytotoxicity was found (data not shown). The different cell morphologies are defined below (figure 3.5).

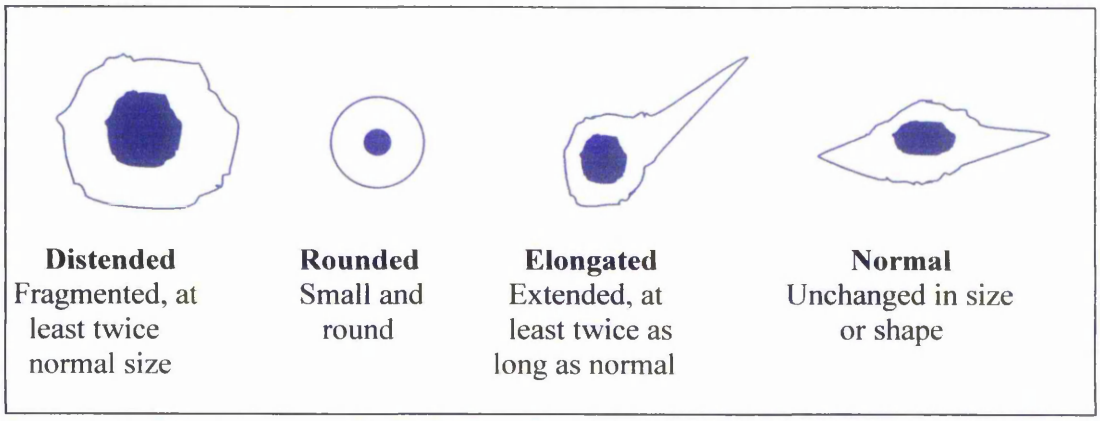


Figure 3.5 – Different cell types identified during morphology studies of CHO cells.

(a)

Strain	% Change in Cell Type (48 h)			
	Distended	Rounded	Elongated	Normal
C. jejuni 11351	*3.8	0.9	1.5	-6.4
C. jejuni 11168	*3.5	*6.4	0.6	*-10.6

(b)

Strain	% Change in Cell Type (96 h)			
	Distended	Rounded	Elongated	Normal
C. jejuni 11351	*3.4	**17.8	0.8	*-21
C. jejuni 11168	**6.6	**24.3	1.9	*-32

Table 3.1 – Morphology studies of CHO cells (a) 48 hours after treatment and (b) 96 hours after treatment with 10% *C. jejuni* 11351 and 11168 cell extract samples. Paired t-tests were carried out on the data using 95% confidence limits (* indicates statistically significant changes from the controls (10%PBS), where* indicates P<0.05, ** indicates P<0.01).

After 48 hours incubation with the *C. jejuni* samples, significant increases were seen with two of the cell morphologies when compared to the PBS control (table 3.1a). The distended cell type showed the highest significant increase in both 11351 and 11168 samples ($P<0.05$). The number of rounded cells was also higher in the wells treated with the *C. jejuni* 11168 samples, and although an increase was seen in the 11351 sample wells, this was not statistically significant ($P>0.4$). The number of elongated cells also appeared to increase on addition of cell extracts from both *C. jejuni* strains, though this was not determined significant in either case.

After 96 hours incubation, the number of distended cells was increased further in both cell extracts from *C. jejuni* 11351 and 11168, and again, this was more pronounced in samples from 11168 (table 3.1b, figure 3.6 a-c). Both strains caused significant increases in rounded cell types ($P<0.02$), but neither resulted in significant changes in elongated cell types. The number of unchanged or normal cell types was found to be significantly less with cell extracts from both

strains when compared with the PBS control, particularly with the *C. jejuni* 11168 cell samples (P<0.00005).

These results indicate that exposure to *C. jejuni* cell extracts caused changes in the morphology of CHO cells, and that these changes must be sub-lethal, due to the lack of cytotoxicity detected by the MTT assay. This suggests that there may be more than one toxin produced by *C. jejuni,* and that the expression of these toxins may vary between strains.

3.3.2 Morphology Studies of ECV Cells

As significant changes in morphology were observed when microscopically studying CHO cells exposed to *C. jejuni* cell extract samples, similar experiments were carried out using ECV tissue culture cells. This would determine whether the toxin(s) present in the cell extract caused an effect in more than one cell type, and if so, whether the effects were the same in each case.

(a)

Strain	% Change in Cell Type (48 h)			
	Distended	Rounded	Elongated	Normal
C. jejuni 11351	-1.79	-2.6	**17.45	**-14.45
C. jejuni 11168	1.29	0.21	**16.78	**-19.68

(b)

Strain	% Change in Cell Type (96 h)			
	Distended	Rounded	Elongated	Normal
C. jejuni 11351	-3.75	-3.82	**25.66	**-18.09
C. jejuni 11168	*4.6	0.7	**25.65	**-30.94

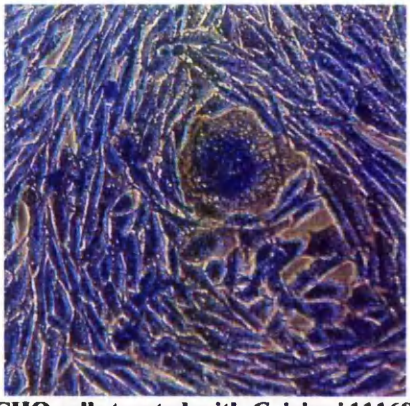
Table 3.2 – Morphology studies of ECV cells (a) 48 hours after treatment and (b) 96 hours after treatment with 10% *C. jejuni* 11351 and 11168 cell extract samples. Paired t-tests were carried out on the data using 95% confidence limits (* indicates statistically significant changes from the controls (10%PBS), where* indicates P<0.05, ** indicates P<0.01).

After 48 hours incubation of the ECV cells with the *C. jejuni* cell extract samples, definite changes could be seen in the cell morphologies (table 3.2a, figure 3.6 d-f). The number of elongated cells in each well was significantly higher than the control cell wells, and this was true for both *C. jejuni* 11168 ($P<0.01$) and *C. jejuni* 11351 samples ($P<0.0008$). The decrease in the amount of normal or unchanged cells was significantly lower than the control wells, but this was most likely due to a combination of the large increase in the elongated cell type, and the cytotoxic effects of the cell extract sample, as it was noted that the total number of cells was less in the sample wells than the control wells.

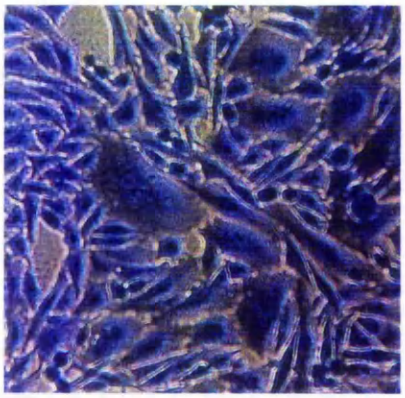
Incubation for an additional 48 hours did not really affect the numbers of elongated cells (table 3.2b), although there was a small, though significant increase in the number of distended cells in the wells treated with *C. jejuni* 11168 cell extract samples. Therefore it seems that whatever substance was present in the *C. jejuni* samples had exerted its effect during the first 48 hours of incubation with the cells.

These results were in contrast to those of the previous experiment where significant changes were detected in the amount of distended and rounded cells, but not in the number of elongated cells. In addition, the changes in numbers of cell types in the CHO experiments were only just significant after 48 hours, requiring an additional 48 hours incubation for the data to be significant using 99% confidence limits,

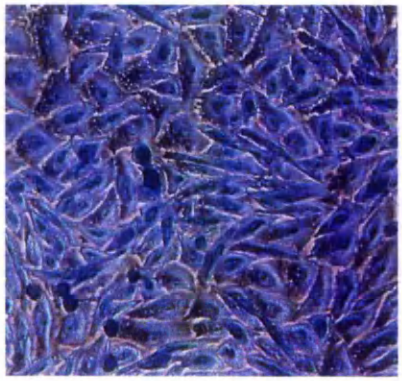
Overall, the results of these experiments suggest that morphological changes have occurred in both cell types, and that the changes were different for each cell line. In the CHO cell line, these morphology changes are sub-lethal, as no cytotoxicity could be detected using the MTT assay, whereas the changes to the ECV cell line may or may not be directly related to the cytotoxicity detected.



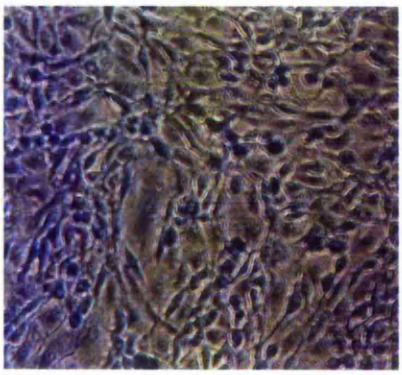
(a) CHO cells treated with *C. jejuni* 11168 cell extract samples



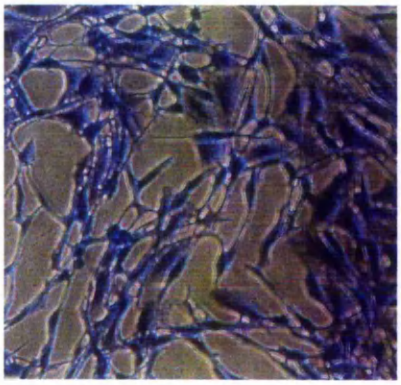
(b) CHO cells treated with *C. jejuni* 11351 cell extract samples



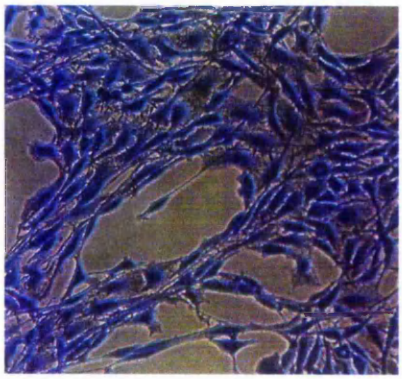
(c) CHO cells treated with PBS



(d) ECV cells treated with PBS



(e) ECV cells treated with *C. jejuni* 11168 cell extract samples



(f) ECV cells treated with *C. jejuni* 11351 cell extract samples

Figure 3.6 – Coomassie blue stained CHO and ECV cells after 96 hours incubation with (a & e) 10% *C. jejuni* 11168 cell extract samples, (b & f) 10% *C. jejuni* 11351 cell extract samples (c & d) 10% PBS samples.

3.4 SUMMARY OF *C. JEJUNI* TOXICITY RESULTS

Table 3.3 shows that although both strains of *C. jejuni* caused cytotoxic and morphological effects on various cell lines, samples from *C. jejuni* 11168 produced slightly greater toxic effects overall than those from *C. jejuni* 11351, the strain previously studied by Holmes (2001) & Spears (2002). The N2a cell line was found to be the most susceptible to cytotoxic effects of the samples, and both heating and trypsin treating the cell extract samples had no effect on cytotoxicity.

Cell Line	*C. jejuni* Strain	
	11351	11168
Cytotoxicity		
N2a	++	+++
heat treated	++	+++
trypsin treated	++	+++
ECV	+	++
Vero	-	+/-
CHO	-	-
Sublethal Effects CHO morphology	Significant increase in distended and rounded cells	Greater significant increase in distended and rounded cells
ECV morphology	Significant increase in elongated cells	Significant increase in elongated and distended cells

Table 3.3 – Summary of toxicity of *C. jejuni* cell extract samples (10%) applied to various cell lines.

KEY;

- = 90-100% viable cells remaining, no significant cytotoxicity
+/- = 80-90% viable cells remaining, no significant cytotoxicity
\+ = 80-90% viable cells remaining, significant cytotoxicity
++ = 70-80% viable cells remaining, significant cytotoxicity
+++ = Less than 70% viable cells remaining, significant cytotoxicity

3.5 SDS-PAGE OF *C. JEJUNI* OUTER MEMBRANE PROTEINS

Previous research by Holmes & Spears (TNTU) identified a potential cholera-like enterotoxin produced by *C. jejuni* NCTC 11351. The toxin was found to have a molecular weight of about 50kDa, and an N-terminal sequence homologous to the *C. jejuni* major outer membrane protein (MOMP). Therefore outer membrane protein (OMP) samples were extracted from stationary phase bacterial cultures and separated by gel electrophoresis to determine the molecular weights of the major proteins contained in the outer membrane of *C. jejuni.*

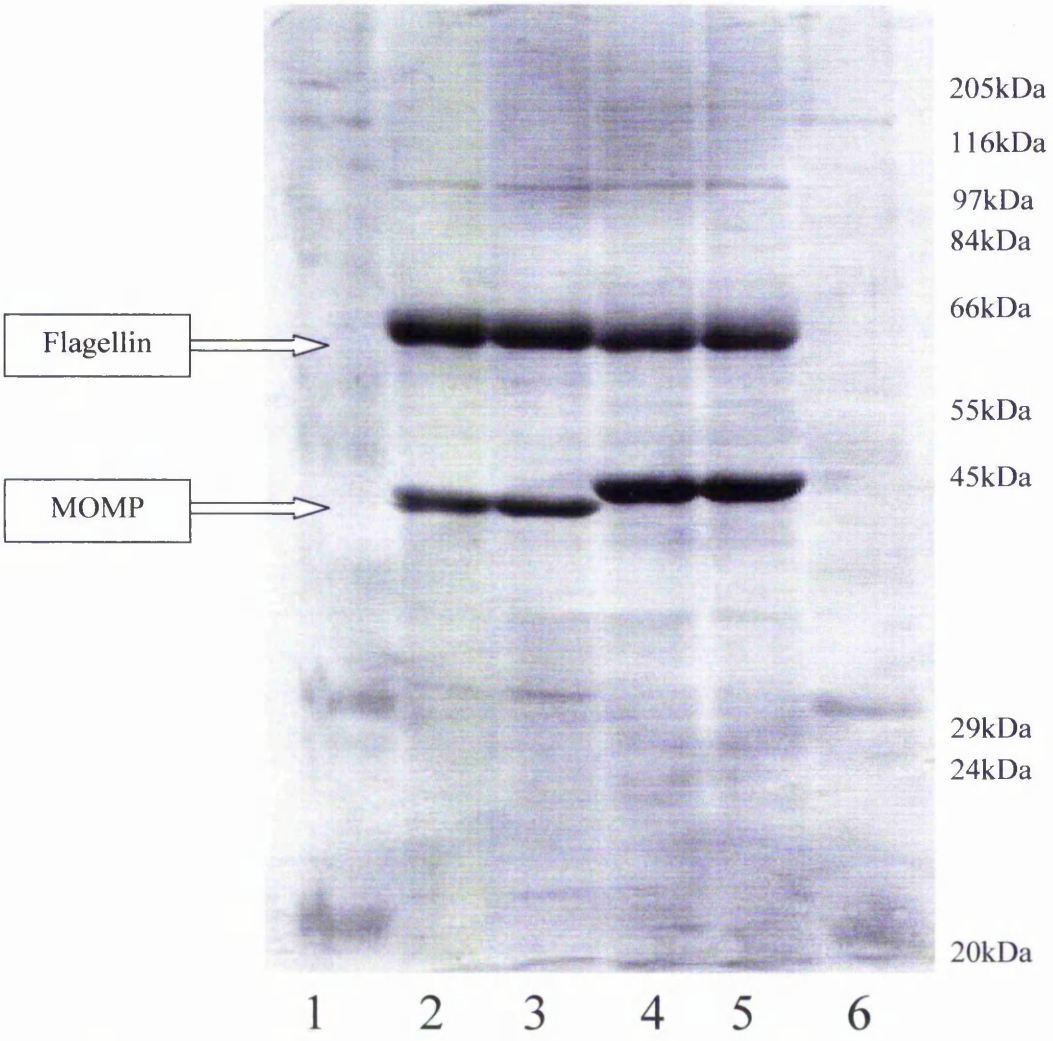


Figure 3.7 - Coomassie Blue stained SDS-PAGE of *C. jejuni* OMP samples.

Lane 1 and 6 = 10µl wide range protein molecular markers (Sigma)
Lane 2 and 3 = 20µg *C. jejuni* 11351 OMP extraction
Lane 4 and 5 = 20µg *C. jejuni* 11168 OMP extraction

The extracted OMP samples were first run on a mini gel system (data not shown) and then to obtain better separation, electrophoresed on a 20x20cm gel using a large gel system.

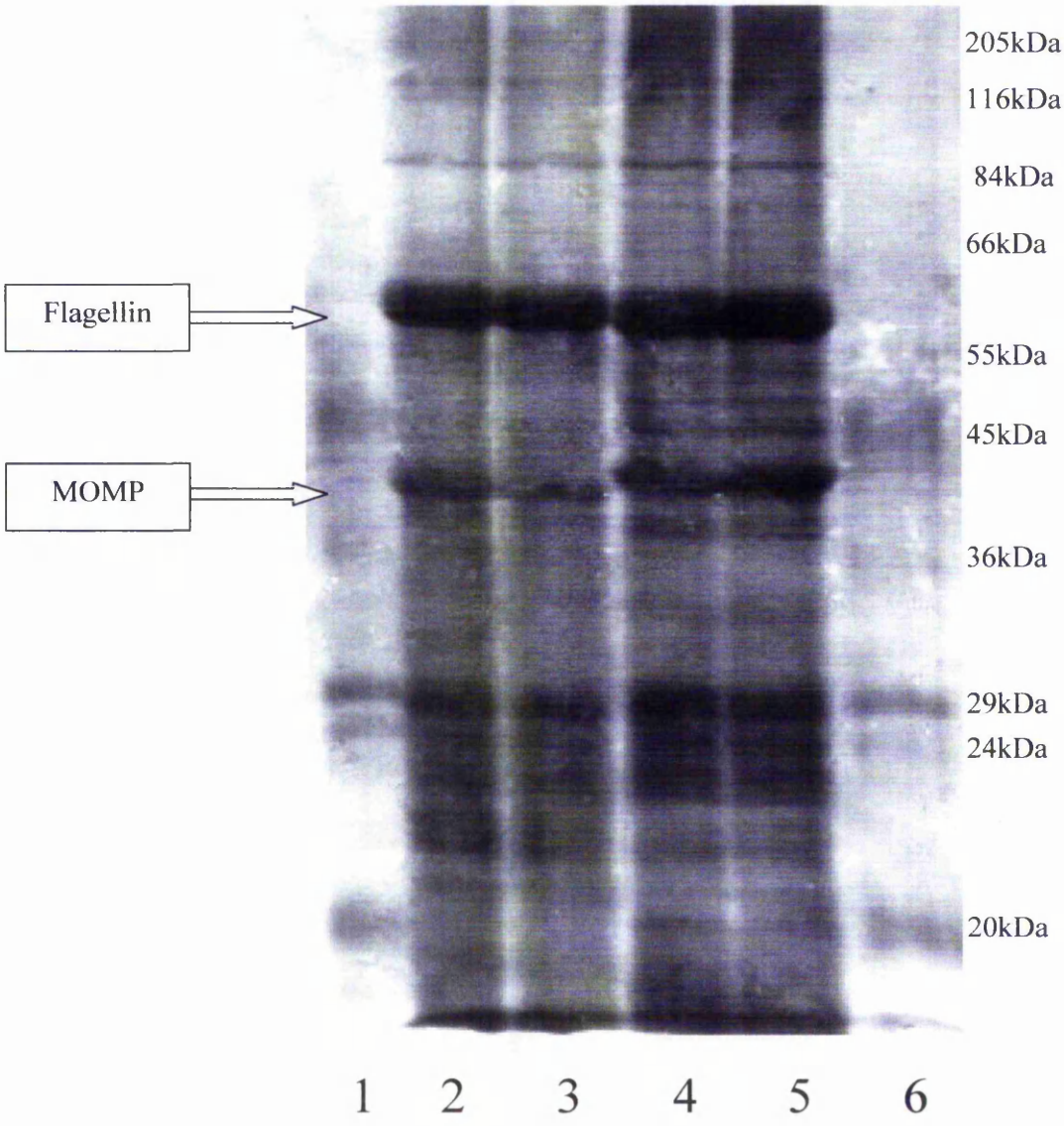


Figure 3.8 - Silver stained SDS-PAGE of *C. jejuni* OMP samples.

Lane 1 and 6 = 10µl wide range protein molecular markers (Sigma)
Lane 2 and 3 = 20µg *C. jejuni* 11351 OMP extraction
Lane 4 and 5 = 20µg *C. jejuni* 11168 OMP extraction

Figure 3.7 is a 10% SDS-PAGE of an OMP extraction from *C. jejuni* 11351 and 11168 cultures, stained with Coomassie Blue. The dark band at approximately 62kDa was presumed to be the flagellin protein (Logan & Trust 1982; Schwartz *et al.* 1994), and the other prominent band in the region of 40 to 45kDa was identified as the major outer membrane protein (MOMP), (Bolla *et al.* 1995). The identity of both bands was confirmed by sequence analysis (section 3.6).

The gel in figure 3.8 is identical to that of 3.7, but has been stained with silver stain rather than Coomassie blue. The same major bands can be identified on both gels, although the silver stain is more sensitive, and additional bands of low concentration proteins can also be visualised.

An unexpected result can be seen between the two different strains of *C. jejuni* in that the MOMP bands migrate at slightly different molecular weights. The MOMP band of *C. jejuni* 11351 (lanes 4 & 5 figures 3.7 and 3.8) had an apparent molecular weight of 40kDa, while the molecular weight of the *C. jejuni* 11168 MOMP band (lanes 2 & 3 figures 3.7 and 3.8) was approximately 43kDa. Repeat SDS-PAGE gels of separate OMP extractions gave consistently similar results, confirming that this difference is due to variations between the two strains rather than experimental conditions.

3.6 N-TERMINAL SEQUENCING OF *C. JEJUNI* MOMP

In order to confirm the identity of the major protein bands from the OMP extraction, a duplicate gel was blotted onto PVDF membrane and sequenced by Kevin Bailey at the Queens Medical Centre (see section 2.11 for detailed methods). Both the 62kDa band and the 40-43kDa bands from each strain of *C. jejuni* were sequenced.

Table 3.4 illustrates that the N-terminal sequences of the 62kDa bands of both *C. jejuni* 11351 and 11168 were found to be identical, and homologous to the N-terminal sequence of *C. jejuni* 11168 flagellin A (Parkhill *et al.* 2000).

N- terminal sequence of *C. jejuni* 11351 & 11168 62kDa band	G F R I X X X V A A
N-terminal sequence of *C. jejuni* 11168 Flagellin A protein (Swiss prot entry no. P56963)	M G F R I N T N V A A L N A

Table 3.4 – N-terminal sequence of flagellin protein from *C. jejuni* 11351 & 11168. X = uncallable amino acid (sequencing machine failed to inject at these residues).

Research group	Molecular Weight	Amino acid sequence
N- terminal sequence of *C. jejuni* 11351 & 11168	40 - 45kDa	T P L E E A I K D V D V
N-terminal sequence of *C. jejuni* 11168 MOMP (Swiss prot entry no. P80672) Parkhill *et al.* 2000).	43kDa	T P L E E A I K D V D V S G V
N-terminal sequence of semi purified toxin (Holmes & Spears TNTU).	50kDa	T P L E E A I K D
Toxin from *C. coli* (Moutinho-fragoso *et al.*1998)	45-50kDa	T P L E E A I K D I D V S G V
Porin toxin from *C. jejuni* (Bacon *et al.*1999)	45kDa	T P L E E A I K D V D V S G V

Table 3.5 – N-terminal sequence of MOMP from *C. jejuni* 11351 & 11168.

Both MOMP bands from *C. jejuni* 11351 and 11168 shared the same sequence (table 3.5), which is analogous to the *C. jejuni* MOMP (Parkhill *et al.* 2000). The

approximate molecular weight of this protein is 43kDa, which agrees with the molecular weights of the bands obtained from the gels. As the N-terminal sequences from both strains of *C. jejuni* are the same, the slight difference in molecular weights between the two MOMP bands must be due to differences elsewhere in the amino acid sequence or possibly post translational modifications.

The protein sequence obtained from N-terminal sequencing was also found to match with several other reports of a potential toxin from *C. jejuni* and *C. coli* (see table 3.5). This further suggests that the MOMP may play an important part in cytotoxicity.

It was also confirmed that the N-terminal sequence of the MOMP was an exact match with the semi purified toxin previously detected by Holmes & Spears (TNTU), although the molecular weights were not the same.

3.7 APPLICATION OF OMP SAMPLES TO TISSUE CULTURE CELL LINES

If the reports in the literature are correct, the MOMP may have cytotoxic properties (Moutinho-Fragoso *et al.* 1998; Bacon *et al.* 1999). This evidence, combined with Holmes & Spears findings and the results of the cytotoxicity assays (see section 6.2), suggests that it may be pertinent to further investigate the MOMP and its contribution to pathogenesis. Therefore, the OMP extraction was repeated until enough was obtained to apply to the tissue culture cell lines tested with the *C. jejuni* cell extract samples. ECV, Vero and N2a cells were selected for this experiment, as it was time consuming and expensive to purify large volumes of the OMP preparation, and CHO cells showed no cytotoxic effects when treated with the crude cell extracts.

Figure 3.9 shows the results of the MTT assays, when OMP extracts from *C. jejuni* 11351 and 11168 were applied to ECV, Vero and N2a cells at 10% concentrations. Clearly there was no cytotoxicity produced by samples from

either strain on any of the cell lines, and there was little point carrying out any statistical tests on the data, as it is obvious that none of the samples show any reduction below 100%, and the error bars are relatively small, therefore the data is quite consistent. This was quite disappointing, as it was hoped that extracting and purifying the OMP would lead to a greater MTT reduction when applied to the cell lines, and therefore, greater cytotoxicity when compared to the crude cell extract samples.

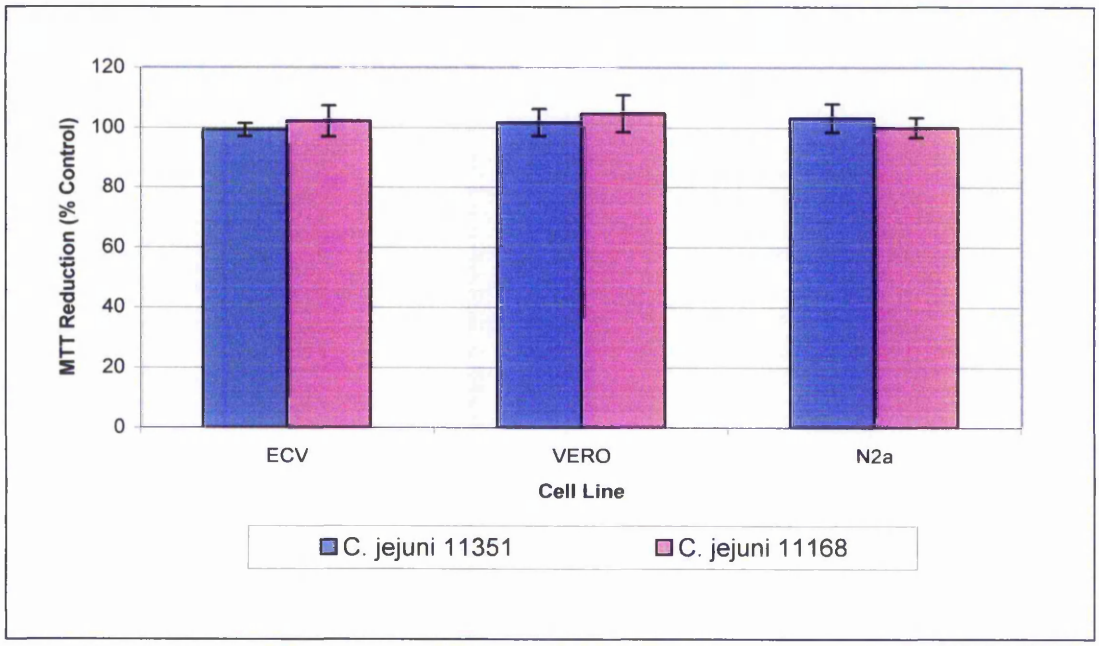


Figure 3.9 - MTT reduction assay of *C. jejuni* 11351 and 11168 OMP samples applied to ECV, Vero and CHO cells at a 10% concentration (about 44µg protein added per well). Error bars represent the standard deviation of the data (n=9).

It is possible that the OMP needs to be cleaved or activated in some way before it becomes cytotoxic, or that the extraction process may have denatured the protein, and therefore this could explain the lack of cytotoxicity when purified. Consequently, further experiments will focus on the cross reactivity of the toxin with anti-cholera toxin, as reported by Holmes & Spears, and the detection of LPS from the OMP extraction.

3.8 IMMUNOBLOT OF *C. JEJUNI* OMP USING ANTI-CHOLERA TOXIN ANTIBODIES

The toxin semi-purified by Holmes (2001) & Spears (2002), was reported to be partially neutralised by cholera antitoxin. Therefore, the OMP extracts were western blotted to determine whether an anti-cholera toxin antibody would bind to the samples.

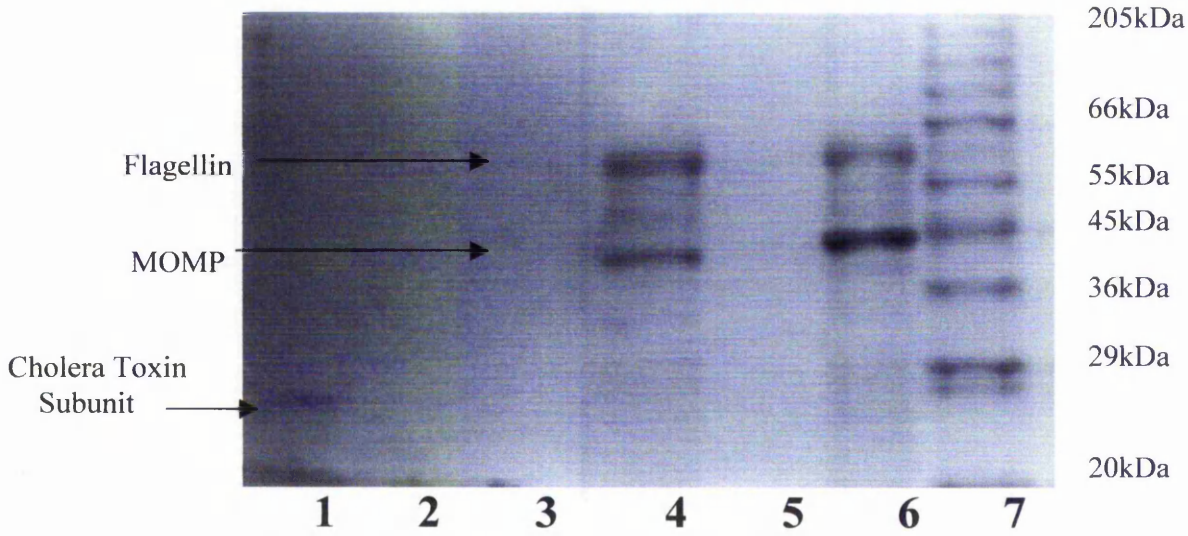


Figure 3.10 – Coomassie Blue stained SDS-PAGE of *C. jejuni* OMP and cholera toxin.

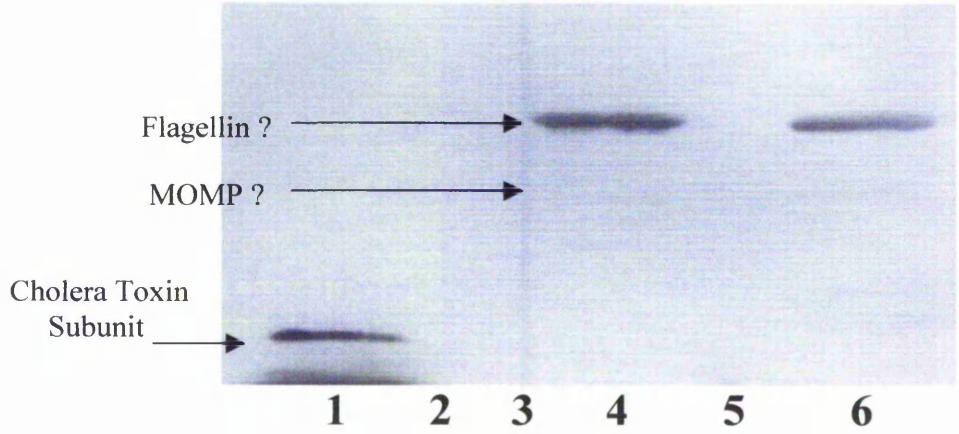


Figure 3.11 - Immunoblot of *C. jejuni* OMP and cholera toxin probed with anti cholera toxin antibodies.

Lane 1 = 15µg Cholera toxin (C8052, Sigma)
Lane 2 ,3 & 5 = blank
Lane 4 = 8µg *C. jejuni* 11351 OMP extraction
Lane 6 = 8µg *C. jejuni* 11168 OMP extraction
Lane 7 (figure 3.10 only) = 5µl wide range molecular markers

Two bands were found to cross-react with the anti-cholera toxin in the *C. jejuni* OMP lanes, though only one band cross-reacted strongly (lanes 4 & 6, figure 3.11). It is impossible to confirm the identity of the bands as there are no molecular weight markers on the blot, but it is likely that the more prominent band is the 65kDa flagellin protein and the weakly cross-reacting band the MOMP.

Unfortunately, the cholera toxin controls cannot be easily visualised, although a faint band can be seen at approximately 25kDa in the cholera toxin lane (lane 1, figures 3.10 & 3.11). The cholera toxin is a protein of approximately 84kDa, but when denatured and run on SDS-PAGE the disulphide bonds between the subunits are broken, and the protein dissociates to fragments between 5 and 27kDa (Sigma). Therefore, it is unlikely that the lower fragments would be detected (below ~11kDa), but the 27kDa fragment is most likely to be the identified band.

This binding of the cholera toxin to the flagellin band was most likely to be non-specific, as there is no reason why the cholera toxin should bind to the flagellin protein. The anti-cholera toxin antibody was polyclonal, as this was the only anti-cholera toxin antibody available without ordering one to be specifically manufactured, and so it was not as specific as a monoclonal antibody, although in hindsight, the blot could have been washed with another non-specific antibody, to see if the same effect was produced. There is also evidence to suggest that the flagellin protein is glycosylated (Szymanski *et al.* 1999), which would explain the binding of the antibody to this band, as glycosylated proteins contain sialic acid groups, which mimic GM1 – the cholera toxin binding site. Sialic acid groups are also present in LPS, and it is possible that the OMP preparation contains LPS, and that this is binding the anti-cholera toxin.

3.9 SDS-PAGE OF LPS EXTRACTED FROM *C. JEJUNI* OMP

As a final experiment on this topic, an LPS extraction from the OMP samples was carried out, as it is possible that LPS is present in the OMP extraction, and may be responsible for some of the toxic effects seen when the *C. jejuni* samples and partially purified enterotoxin are applied to the cell lines. LPS was extracted from the OMP samples, run on a polyacrylamide gel, and silver stained using methods detailed in section 2.10. Unfortunately protein markers are inappropriate for LPS gels, and hence the molecular weight of LPS cannot be determined from the gel. Therefore, lane 6 was loaded with *Salmonella* Typhimurium as an LPS control, as it produces a distinct LPS banding pattern.

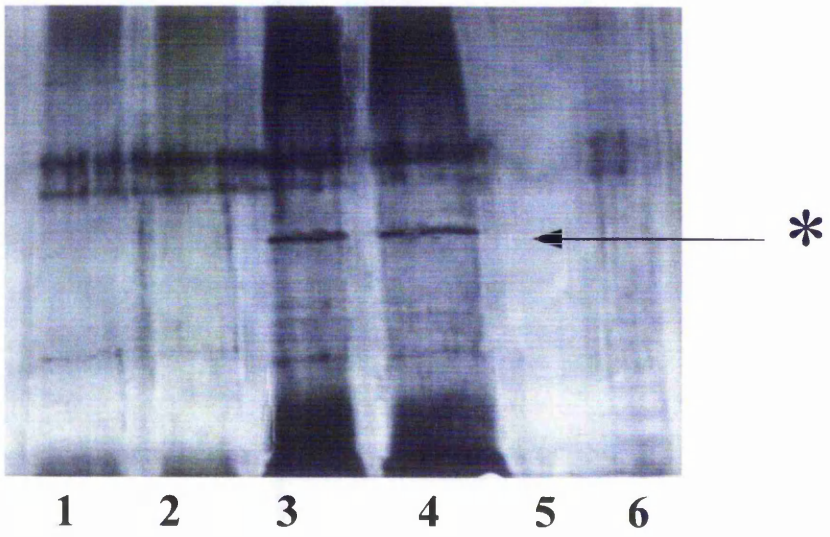
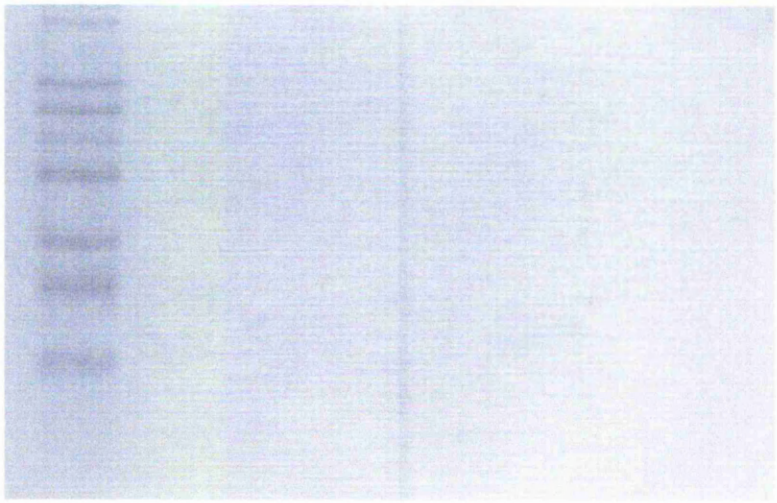


Figure 3.12 – Silver stained SDS-PAGE of LPS extraction from *C. jejuni* OMP samples.

Lane 1 and 2 = LPS extraction from 10µg *C. jejuni* 11351 OMP
Lane 3 and 4 = LPS extraction from 10µg *C. jejuni* 11168 OMP
Lane 5 = blank
Lane 6 = 5µl *Salmonella* Typhimurium

Figure 3.12 shows that LPS was present in the OMP samples, of both low and high molecular weight, and interestingly, that the *C. jejuni* 11168 LPS sample had an extra band (marked with *, figure 3.12) about halfway down the gel that was not present in 11351. As lipopolysaccharide is a potent endotoxin in a

number of Gram negative bacteria, and both heat and trypsin stable, it is likely that LPS could be responsible for the cytotoxicity seen in earlier experiments. It is also possible that if cytotoxicity is produced by the MOMP, the presence of this additional band in *C. jejuni* 11168 and not 11351 could explain the increased cytotoxicity seen in samples produced from the type strain. Additionally, if this extra band is usually complexed with the MOMP, this may explain the different molecular weights of the MOMP in figures 3.7 and 3.8.



1 2 3 4 5 6 7

Figure 3.13 – Coomassie stained SDS-PAGE of LPS extraction from *C. jejuni* OMP samples.

Lane 1 = 5µl wide range protein molecular markers (Sigma)
Lane 3 and 4 = LPS extraction from 10µg *C. jejuni* 11351 OMP
Lane 6 and 7 = LPS extraction from 10µg *C. jejuni* 11168 OMP

Figure 3.13 shows a duplicate gel which has been stained with Coomassie blue to detect any protein present which may have interfered with the LPS staining (see figure 3.12). This gel clearly shows that, as far as the limits of the stain allow, there is no detectable protein present in the LPS preparation, and therefore all the bands present in figure 3.12 must be LPS. Similar gels were also stained with Schiffs stain to detect carbohydrate, but these results proved inconclusive (data not shown).

This area clearly requires further investigation, but it looks likely from these results that the cytotoxic effects seen in previous experiments are a result of the activity of LPS. As LPS has been well characterised in the literature, and is not a novel toxin, further investigations into this area were halted to allow time for more significant experiments to be undertaken.

CHAPTER 4

HAEMOLYSINS

4.1 LINEARITY STUDIES OF *C. JEJUNI* AND *A. BUTZLERI* HAEMOLYTIC ACTIVITIES

4.1.1 Contact Haemolysin Assays of *C. jejuni* and *A. butzleri* over Time

The detection of haemolytic activity from bacterial cultures is a commonly used test to determine whether a particular bacterium displays cytolytic activity. When testing a bacterial exotoxin for the first time, it is common to first test for lytic function using an erythrocyte haemolysin assay. Lysis of red blood cells does not necessarily mean that the red blood cells are the target cells of the bacterial toxin, but can serve as a simple visible assay to determine the pathogenicity of the organism. For example, many cell membrane damaging cytolysins that cause damage to a variety of eukaryotic cells will also lyse erythrocytes in addition to their target cells, releasing haemoglobin that can be measured in the supernatant spectrophotometrically. Therefore, by using a series of haemolysin assays, these cytolysins can be easily quantified.

Haemolytic activity has been previously identified as a potential virulence factor of *Campylobacter* (Wassenaar 1997; Akan *et al.* 1998). Therefore, it was considered prudent to study the effects of the two strains of *C. jejuni* (NCTC 11351 and 11168) on red blood cells to determine whether they exhibit haemolytic activity. In addition, *A. butzleri*, a related pathogen (see section 1.5) was included in the assays, to compare their activity, as little is known about the pathogenic mechanisms of this related emergent pathogen (Mansfield & Forsythe 2000).

The method commonly used to detect haemolysis is to grow the organism on an agar plate containing 5% (v/v) defibrinated blood (Rowe & Welch 1994). In preliminary studies, neither *C. jejuni* or *A. butzleri* were found to be haemolytic, or showed inconsistent haemolysis using this method, even after washing the blood used, and growing the bacteria on agarose plates rather than agar. Therefore further experiments were carried out using the contact haemolysis assay (see section 2.12 for methods).

Further haemolysin experiments concluded that there was no difference in bacterial haemolytic activity whether the contact assays were incubated under microaerophilic conditions or aerobic conditions (data not shown). Hence, for ease, further experiments were conducted under aerobic conditions. Horse blood was used in the contact assays as previous experiments by Holmes (2001) had shown that the results obtained using this blood were more consistent than when other blood sources (e.g. sheep blood) was included in the assays.

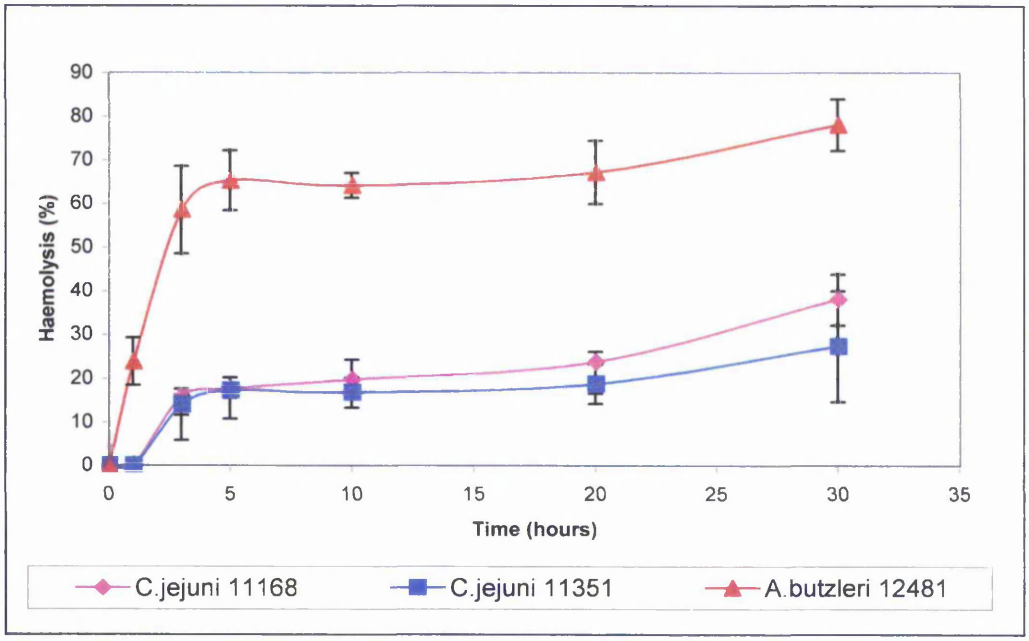


Figure 4.1 – Contact haemolysin assays showing haemolytic activity over time for *C. jejuni* 11168, *C. jejuni* 11351 and *A. butzleri* 12481 added to 1% red blood cells at a concentration of $1x10^{10}$ cells/ml. Error bars represent the standard deviation of the data (n=9). Paired t-tests were carried out on the data at the 30 hour time point, with 95% confidence limits.

Previous contact haemolysin experiments (Istivan *et al.* 1998) used a 20 hour incubation period to determine haemolysis, but it was not known why this endpoint was originally chosen. Therefore, experiments on all three organisms were assayed after one, three and five hours, and then again at 10, 20, and 30 hours, to determine the kinetics of the haemolysis reaction.

Figure 4.1 shows that the majority of the haemolytic activity of all three organisms takes place over the first 5 hours. Therefore, a prolonged contact time of 20 hours, used by previous researchers is unnecessary, and future haemolysis experiments will have an endpoint of 5 hours.

It can also be seen that although all 3 strains produced a haemolytic substance, the amount of haemolysis varied demonstrably between the strains. *A. butzleri* 12481 was by far the most haemolytic, lysing nearly 80% of the red blood cells after 30 hours (see figure 4.1). In contrast, both *C. jejuni* strains showed similar amounts of lysis, with *C. jejuni* 11168 exhibiting slightly higher haemolysis at just below 40%, whereas *C. jejuni* 11351 lysed almost 30% of the red blood cells in the assay. Statistical t-tests were carried out on the data, and all three strains showed significant amounts of haemolysis when compared to the PBS controls. *A. butzleri* showed extremely high significance ($P<0.000025$), though both *C. jejuni* 11168 and 11351 strains were also found to produce significantly high haemolysis with P values of 0.000354 and 0.0191 respectively.

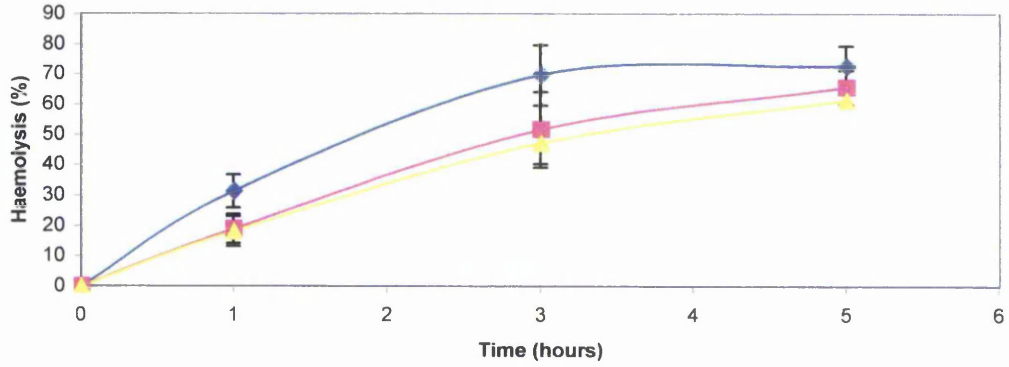
In conclusion, all 3 organisms tested with the contact haemolysis assay showed significantly high amounts of haemolytic activity, with the *A. butzleri* strain lysing more than twice the percentage of red blood cells than the *C. jejuni* strains.

4.1.2 Contact Assays of *C. jejuni* and *A. butzleri* with Varying Biomass

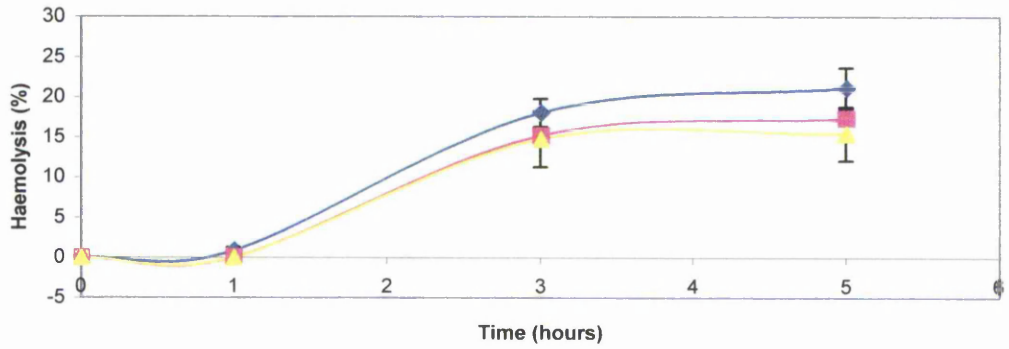
In order to further investigate the haemolysis reaction, it was necessary to determine the haemolytic activity with respect to linearity with time and cell biomass. Therefore, experiments were carried out with all three organisms, to determine the effect of varying the biomass on the haemolytic reaction.

Reducing the biomass (see figure 4.2), as expected, reduced the amount of haemolytic activity, though the rate of haemolysis was not directly proportional to the amount of biomass in the assay. These experiments were carried out over a time period of 5 hours, as figure 4.1 had shown that most haemolysis takes place within the first 5 hours of the contact assay. These experiments demonstrate that reducing the biomass by half does not reduce the amount of haemolysis by half as one might expect. Therefore, the large difference in the percentage haemolysis seen between both *C. jejuni* strains and the *A. butzleri* strain cannot simply be explained by the suggestion that the *A. butzleri* sample may have contained a larger biomass.

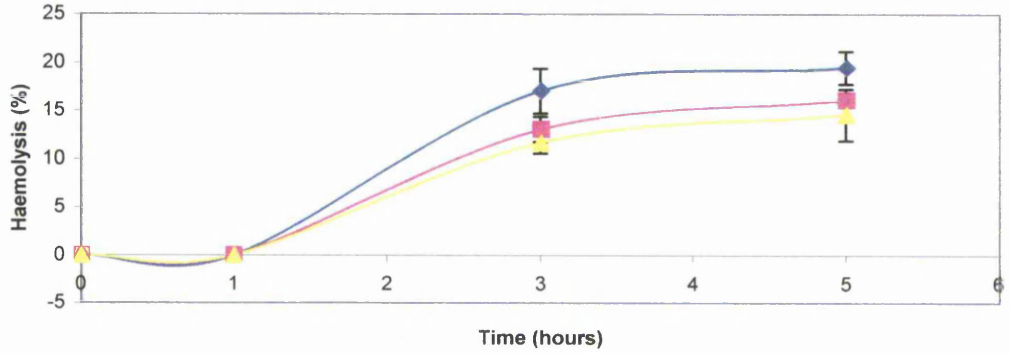
(a)



(b)



(c)



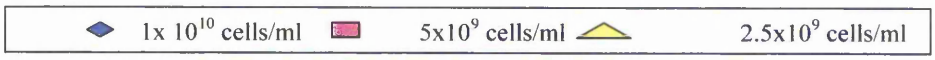


Figure 4.2 – Contact haemolysis assays showing haemolytic activity over time with differing biomass of (a) *C. jejuni* 11168 (b) *C. jejuni* 11351 (c) *A. butzleri* 12481. Error bars represent the standard deviation of the data (n=9).

4.2 HAEMOLYSIN ASSAYS OF SONICATED *C. JEJUNI* AND *A. BUTZLERI* SAMPLES

To determine whether the haemolysin(s) are membrane bound or released into the supernatant, the cells were resuspended, centrifuged and the supernatant separated from the pellet. Additional samples were sonicated as for the cell cytotoxicity assays in the previous chapter, as sonication of the bacteria before removing the supernatant led to greater cytotoxicity in the cell culture assays. Additionally, Daw *et al.* (1991) reported that sonicated preparations of *H. pylori* cells contained phospholipase C, which exhibited haemolytic activity. All samples were tested for cytolytic activity using the contact assay.

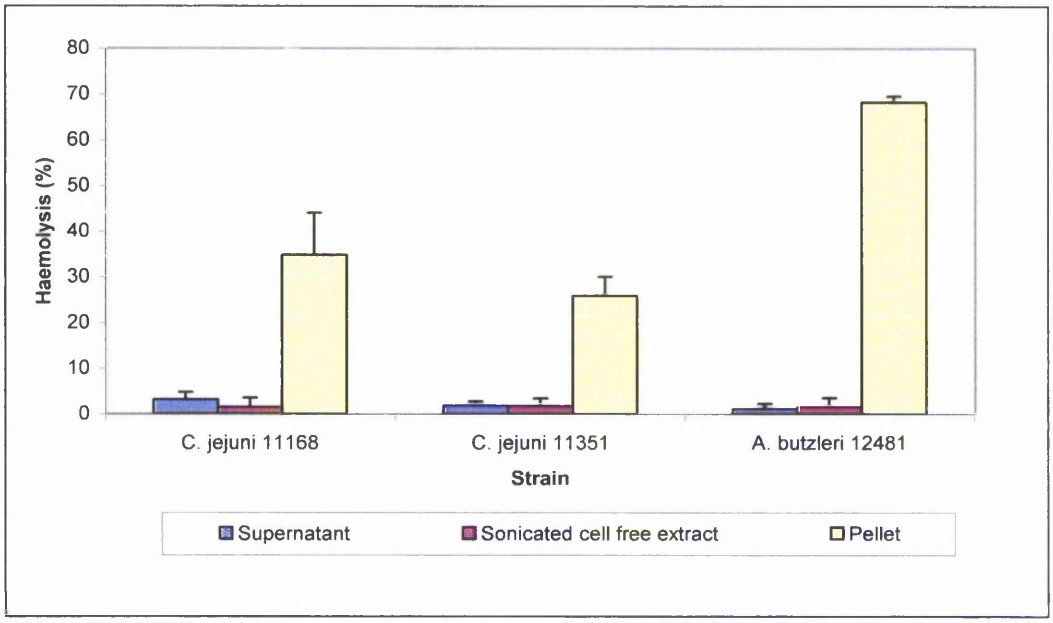


Figure 4.3 – Contact haemolysin assays showing haemolytic activity of cell pellets, supernatants, and sonicated cell free extract samples from *C. jejuni* 11168, *C. jejuni* 11351, and *A. butzleri* 12481 (Original cell concentration before centrifugation was $1x10^{10}$ cells/ml).

Figure 4.3 shows that for all three strains, after centrifugation of the samples, the majority of the haemolytic activity was present in the bacterial cell pellet, and there was very little activity released into the supernatant. Cell free extracts were prepared by sonicating and centrifuging the bacterial suspensions (see section 2.12.2.2 for methods), but these extracts also showed very little haemolytic activity when compared to the cell pellet samples.

Therefore, this experiment shows that even after sonication, whatever was responsible for the lysis was not released into the supernatant, and so is likely to be a cell-associated haemolysin, and distinct from the cytotoxin detected in the cell free supernatant during cell cytotoxicity experiments in chapter 3.

4.3 HAEMOLYTIC ACTIVITY OF VARIOUS STRAINS OF *C. JEJUNI* AND *A. BUTZLERI*

In order to investigate the diversity of *C. jejuni* and *A. butzleri* haemolysins, a total of 21 *C. jejuni* strains from a range of different isolates, and 2 strains of *A. butzleri* were screened using the contact haemolysis assay. Sources of the strains included human, bovine, chicken, porcine and water isolates (see table 4.1). The additional strains of *C. jejuni* were supplied by Dr. S. On (Danish Veterinary Institute), and the sources of the strains were not revealed until the experimental work had been completed.

Table 4.1 shows the sources of the *C. jejuni* and *A. butzleri* strains tested using the contact haemolysis assay, arranged in order of lowest to highest haemolytic activity. From this table it was apparent that all *C. jejuni* and *A. butzleri* strains were found to cause lysis to red blood cells in the contact assay, though the amount of haemolysis varied demonstrably between strains. This was not due to experimental differences, as each strain was assayed in triplicate, averaged, then repeated on at least two further occasions.

The haemolysis produced by the *A. butzleri* strains was interesting, as to date there has only been one report of haemolytic activity from *Arcobacter* strains (Atabay *et al.* 1998). It was also interesting to note that the two strains had very different haemolytic activities. Further experiments using a wider selection of *A. butzleri* strains may be important in the characterisation of this emerging human pathogen.

STRAIN	HAEMOLYSIN ACTIVITY	SOURCE
C. jejuni		
5025	LOW	HUMAN
10938	LOW	CHICKEN
992	LOW	CHICKEN
380-827	LOW	CHICKEN
5784	LOW	HUMAN
1425	LOW	CHICKEN
5826	MEDIUM	HUMAN
11351	MEDIUM	CHICKEN
5795	MEDIUM	HUMAN
5785	MEDIUM	HUMAN
835-770	MEDIUM	CHICKEN
11168	MEDIUM	HUMAN
5809	MEDIUM	HUMAN
5803	MEDIUM	HUMAN
5819	MEDIUM	HUMAN
4039	MEDIUM	CATTLE/HUMAN
3141	MEDIUM	PIG/HUMAN
1099	MEDIUM	CHICKEN/HUMAN
5141	HIGH	WATER/HUMAN OUTBREAK
5836	HIGH	HUMAN
5832	HIGH	HUMAN
A. butzleri		
1714	LOW	HUMAN
12481	HIGH	HUMAN

Table 4.1 – Sources of *C. jejuni* and *A. butzleri* strains tested using the haemolysin contact assay, arranged in order of haemolytic activity. Strains were designated low haemolytic organisms if red blood cell lysis was below 25%, medium for strains lysing 25-50% red blood cells, and high for strains lysing over 50% of the red blood cells in the assay.

It is quite difficult to determine whether there is any link between the origin of the strain and its activity, so the data is presented in a histogram in figure 4.4 below. The *C. jejuni* strains that were isolated from humans are coloured pink, strains found only in chickens are blue, and strains found in both animals and humans, and responsible for outbreaks are coloured red. The two *A. butzleri* strains are also included as a comparison and coloured yellow.

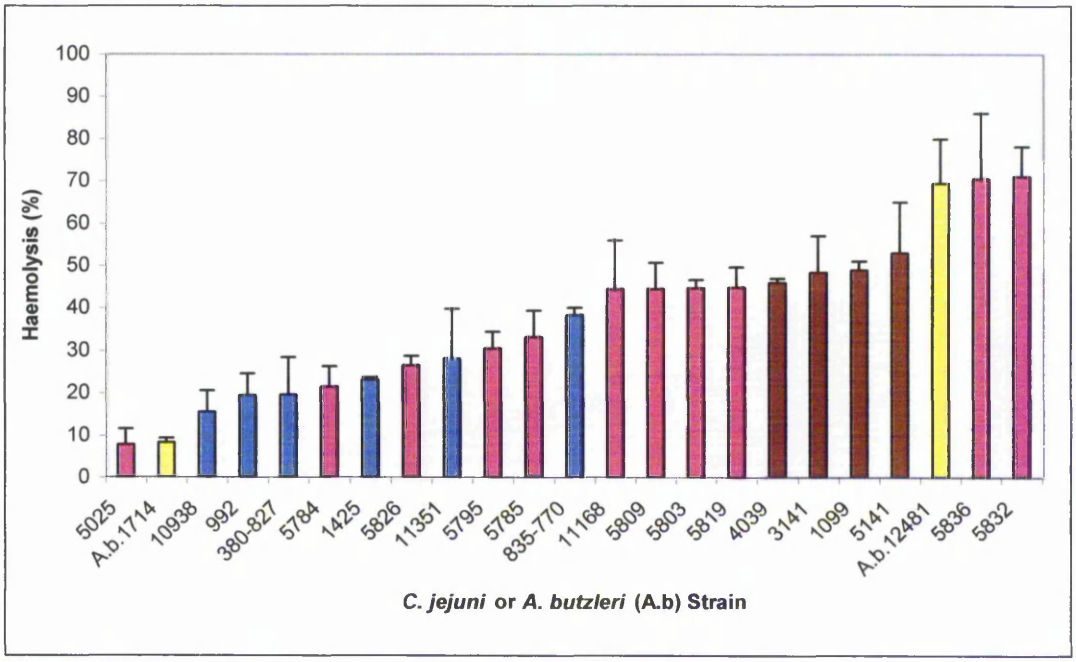


Figure 4.4 – Contact haemolysis assays showing haemolytic activity of 21 *C. jejuni* strains and 2 *A. butzleri* strains from a variety of sources. The *C. jejuni* strains isolated from humans are coloured pink, strains found only in chickens are coloured blue, and strains found in both humans and animals and responsible for outbreaks are red. *A. butzleri* strains are coloured yellow. Error bars represent the standard deviation of the data (n=9).

The data presented in this histogram (figure 4.4) seems to suggest that there may be some sort of pattern between the source of the strain of *C. jejuni* and its virulence in the haemolysin assay. The strains found only in humans tended to produce higher amounts of haemolysis than those isolated from chickens, although there were a few exceptions (strains 5025, 5784 and 5826). Those strains found in both humans and animals and/or responsible for gastroenteric outbreaks were all at the higher end of the haemolytic scale, and, although this data is not conclusive, there is potentially an association between strain origin and haemolytic virulence, and it would be interesting to test more strains to determine whether similar results are obtained.

4.4 CONTACT HAEMOLYSIN ASSAYS OF *C. JEJUNI* AND *A. BUTZLERI* STRAINS WITH VARYING CONDITIONS

From figure 4.4, four strains of *C. jejuni*, and the 2 strains of *A. butzleri* showing a range of haemolytic activity were chosen as representative samples and screened for activity against a range of inhibitors to try to further characterise the haemolysin(s).

C. jejuni 11168 and 11351 had been used throughout this study, so they were chosen as representatives producing medium amounts of lysis (25-50%). *C. jejuni* 5025 was selected as it produced less than 25% haemolysis, and *C. jejuni* 5832 was chosen as a highly haemolytic organism (above 50% lysis of red blood cells). The two strains of *A. butzleri*, acted as both low and highly haemolytic organisms respectively.

4.4.1 Contact Haemolysin Assays Using Heat Treated Bacterial Samples

Figure 4.5 shows the percentage haemolysis produced by the four strains of *C. jejuni* and two strains of *A. butzleri* after heat treatment. The samples were boiled at 100°C in order to denature any proteins present, and as this figure shows, some but not all of the activity was lost after boiling the samples for 10 minutes.

Statistical analysis showed that boiling the samples resulted in significant loss of activity in all strains, with P values ranging from 0.030 for *C. jejuni* 11168, to 0.000003 for *C. jejuni* 5832.

These results suggest that the majority of haemolysis is caused by a proteinaceous toxin, but there is also some residual activity remaining, particularly in the most haemolytic strains. This gives rise to the possibility that as well as a heat labile haemolysin, there may also be a heat stable toxin present in the highly haemolytic strains (*C. jejuni* 5832 and *A. butzleri* 12481).

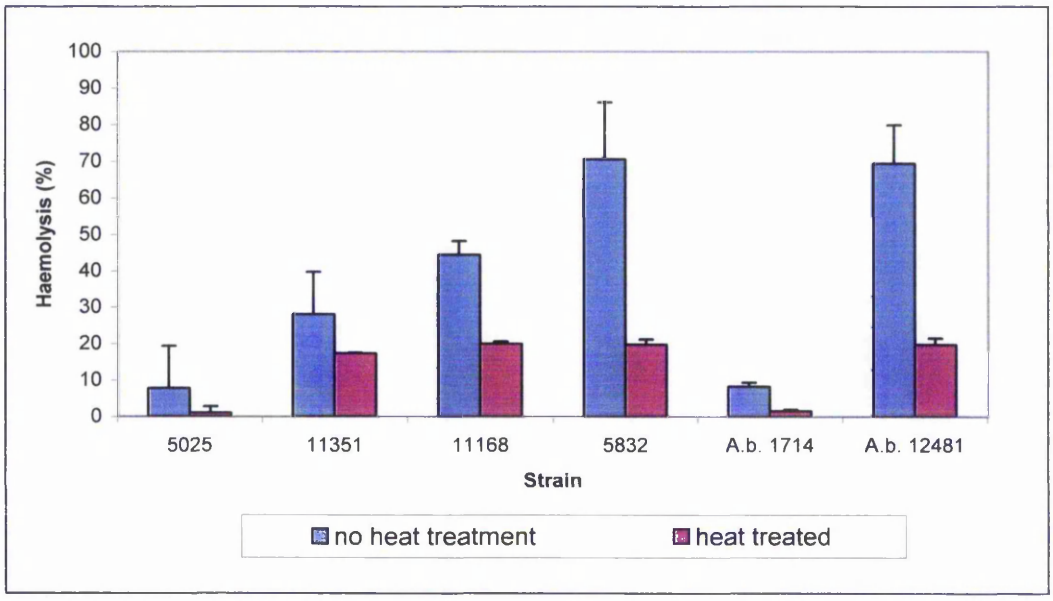


Figure 4.5 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains before and after boiling samples at 100°C for 10 minutes. Error bars represent the standard deviation of the data (n=9).

4.4.2 Contact Haemolysin Assays Under Iron Limiting Conditions

Many haemolysins are reported to be regulated by iron (Pickett *et al.* 1992; Segal & Tompkins 1994), therefore, an iron chelator was included in both the growth media and the haemolysin assay to determine the role of iron on the haemolytic activity produced by the bacterial strains.

Iron limitation experiments were carried out by both growing and assaying the bacteria in the presence of 50 μM desferal mesylate (see section 2.12.2.4. for methods; Segal & Tomkins 1994; personal communication with K. Holmes, Institute of Food Research, Norwich).

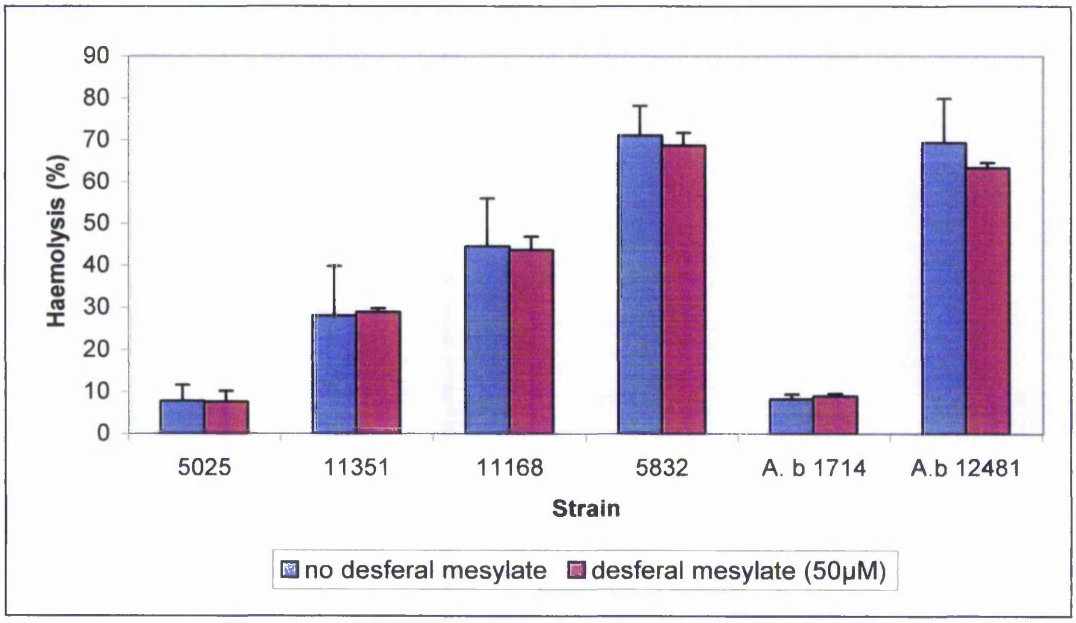


Figure 4.6 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains grown and assayed in the presence and absence of 50 μM desferal mesylate. Error bars represent the standard deviation of the data (n=9).

Figure 4.6 shows that the addition of the iron chelator desferal mesylate to both the growth media and the contact assay appears to have no effect on the haemolytic activity of any of the strains tested. This suggests that the haemolysin(s) may be constitutively expressed, and that iron acquisition is not a major role of haemolysin activity in these strains.

4.4.3 Contact Haemolysin Assays in the Presence of EDTA

Since the majority of characterised haemolysins are dependent upon calcium for their activity, 10 mM EDTA was added to the contact assay to chelate any available calcium present (Welch 1991; Rowe *et al.* 1994; Segal & Tompkins 1994).

Figure 4.7 shows that in the presence of EDTA the haemolytic activity was greatly reduced in all strains of *C. jejuni* and *A.butzleri*, although not completely inhibited, implying that the majority of haemolysis in these strains is calcium dependent. This reduction was significant in all strains with P values ranging

from P=0.01 (*C. jejuni* 11168) to P=0.0000058 (*A. butzleri* 12481). Further experiments using higher concentrations of EDTA showed no increased reduction in haemolytic activity, demonstrating that all the available calcium had been chelated (data not shown). Therefore the residual haemolytic activity seen, particularly in the most haemolytic strains, suggests that these strains may also possess another haemolysin, and that this toxin may be calcium independent.

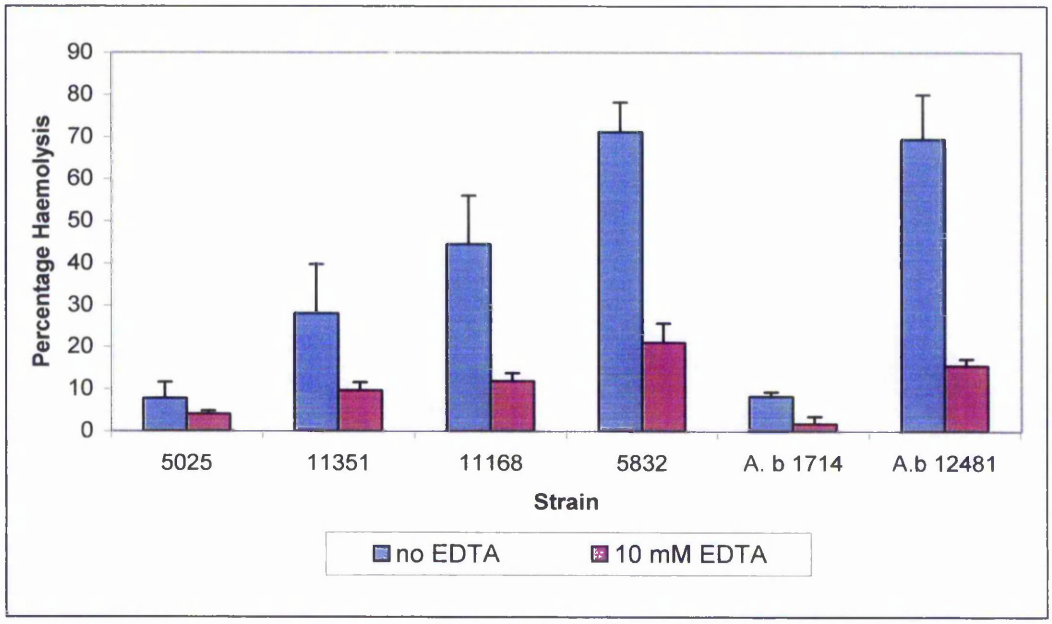


Figure 4.7 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of 10 mM EDTA. Error bars represent the standard deviation of the data (n=9).

4.4.4 Contact Haemolysin Assays in the Presence of Dextran Sulphate

Dextran sulphate (also known as dextran 5000), is a small molecule which can inhibit RTX/pore forming cytolytic toxins by inserting into the pore formed in the host cell membrane by the toxin, and inhibiting lysis (Martino *et al.* 2001). Therefore, to try to further characterise the haemolysin(s), dextran sulphate was added to the contact assays at a concentration of 30 mM.

Figure 4.8 shows that dextran sulphate did not reduce the amount of haemolysis of the 4 lower haemolytic organisms, but interestingly, in both the highly

haemolytic *C. jejuni* 5832 and *A. butzleri* 12481, haemolysis is significantly reduced (P=0.0015 and P=0.003 respectively). This suggests therefore, that the haemolysin detected from *C. jejuni* strains 5025, 11351, 11168 and *A. butzleri* 1714 is not a pore-forming toxin. However, there may an additional haemolysin produced by *C. jejuni* 5832 and *A. butzleri* 12481, and the reduction in haemolysis in the presence of dextran sulphate suggests that this additional haemolysin may be a pore-forming toxin.

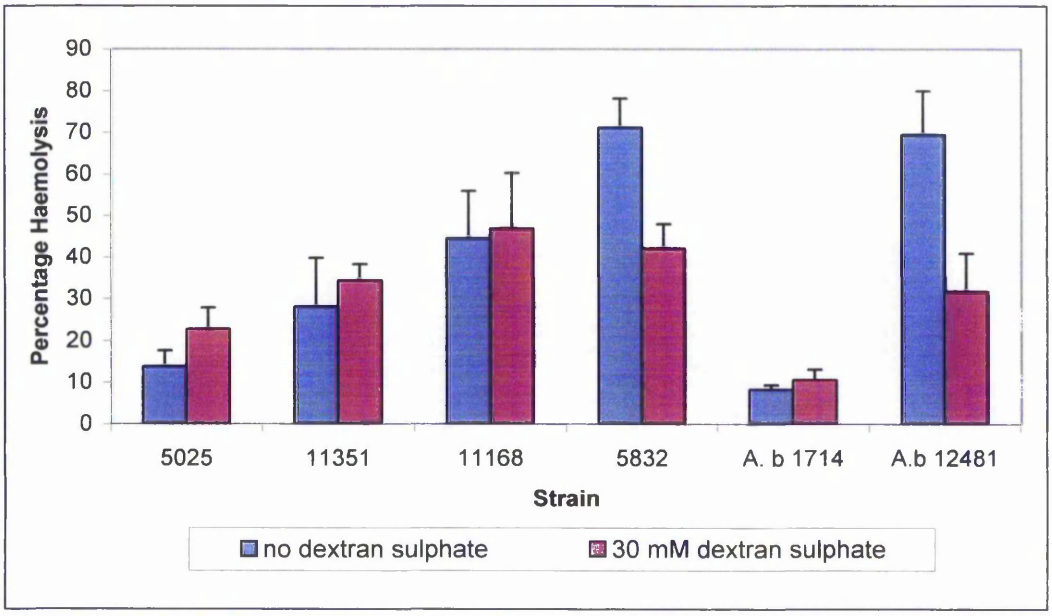


Figure 4.8 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of 30 mM dextran sulphate. Error bars represent the standard deviation of the data (n=9).

4.4.5 Contact Haemolysin Assays in the Presence of a Phospholipase C Inhibitor

Phospholipase C is produced by a wide range of bacteria, both Gram positive and Gram negative (see section 1.7.4.3). The main activity of phospholipase C is degradation of the phospholipids in eukaryotic membranes, but many phospholipases have also been identified as haemolysins (section 1.7.4).

Phospholipase C activity has been reported in *H. pylori* (Ansorg *et al.* 1993; Nilius & Malfertheiner 1996; Bode *et al.* 2001), therefore it was considered

prudent to include compound 48/80, a phospholipase C inhibitor, in the contact assay, to determine whether this would have any effect on the haemolytic activity.

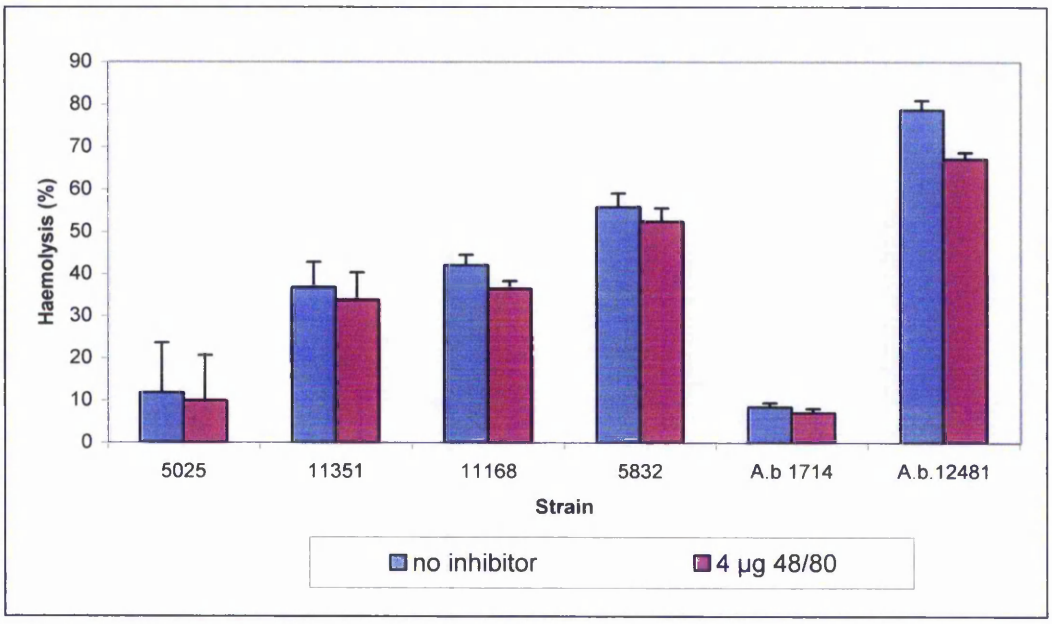


Figure 4.9 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of 4 µg compound 48/80. Error bars represent the standard deviation of the data (n=9).

The phospholipase C inhibitor compound 48/80 appeared to have little effect on the haemolytic activity of any of the strains. There was a slight reduction in the percentage of red blood cells lysed, but this was not significant (see figure 4.9, P>0.107). Unfortunately, it was not possible to determine whether a higher concentration of inhibitor would increase the slight reduction of haemolysis detected, as 4 µg compound 48/80 was the highest concentration of inhibitor that could be used without causing red blood cell lysis. From these results however, it would appear that phospholipase C was not responsible for haemolysis in *C. jejuni* or *A. butzleri*.

4.4.6 Contact Haemolysin Assays in the Presence of a Phospholipase A Inhibitor

The genome of *C. jejuni* 11168 has been shown to contain the gene for phospholipase A (*pldA*), a homologue to the *pldA* gene in *C. coli* (section 1.7.4.1 & 1.7.4.2; Grant *et al.* 1997; Parkhill *et al.* 2000). However, the gene has not been well characterised and it is not determined whether the phospholipase activity is A_1 or A_2. It has been reported that phospholipase A may also act as a haemolytic toxin in *H. pylori* and *C. coli* (Grant *et al.* 1997; Dekker 2000; Xerry & Owen 2001). Therefore, haemolysin assays were repeated in the presence of palmitoyl trifluoromethyl ketone ($PACOCF_3$) a phospholipase A_2 inhibitor. Preliminary experiments established 64µM as the most suitable inhibitor concentration to use.

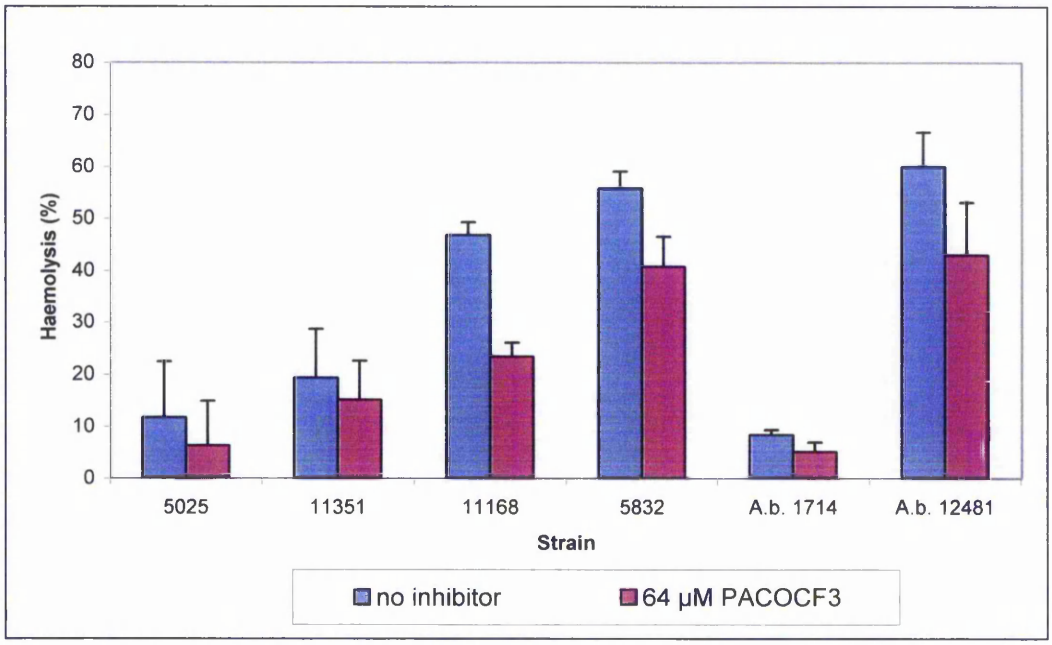


Figure 4.10 – Contact haemolysis assay of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of 64 µM $PACOCF_3$. Error bars represent the standard deviation of the data (n=9).

Figure 4.10 shows that $PACOCF_3$ significantly reduced the haemolytic activity of all the *Campylobacter* and *Arcobacter* strains (P=0.036, *C. jejuni* 11168; P=0.0026 *A. butzleri* 12481), but did not completely prevent haemolysis,

suggesting that only some of the haemolytic activity is a result of phospholipase A. Unfortunately, as for the phospholipase C experiments, adding a higher concentration of inhibitor to the assay resulted in spontaneous lysis of the red blood cells. Therefore, it cannot be determined whether the addition of more inhibitor may result in a further reduction of haemolysis, or whether this reduction is a result of blocking all the phospholipase A present.

Additionally, the inhibitor $PACOCF_3$ inhibits phospholipase A_2 activity only, whereas it has been shown in other pathogens that the phospholipase A gene codes for both the phospholipase A_1 and A_2 forms of the enzyme (Brok *et al.* 1995; Xerry & Owen 2001). Currently there are no inhibitors commercially available to inhibit phospholipase A_1, therefore, it is impossible to determine from these results whether the lack of inhibition was simply because the enzyme was in a higher concentration than the inhibitor, or whether the majority of the detected lysis was a result of other haemolysin(s) activity.

4.5 PHOSPHOLIPASE A_2 ASSAY

As it was uncertain whether the PLA_2 inhibitor was inhibiting the enzyme sufficiently at the highest concentration possible in the contact assay, a secretory phospholipase A_2 ($sPLA_2$) assay kit was purchased. This kit specifically measures the amount of phospholipase A_2 produced by each sample, using a colorimetric reaction that can be measured using a spectrophotometer. The inhibitor $PACOCF_3$ was added to each sample, to determine whether all of the $sPLA_2$ activity detected could be inhibited.

Figure 4.11 shows that all strains produced phospholipase A_2 (PLA_2). The amounts of PLA_2 produced by each strain did seem to generally correlate to a certain extent with the percentage haemolysis produced by each strain, however further experiments would be necessary to prove this connection.

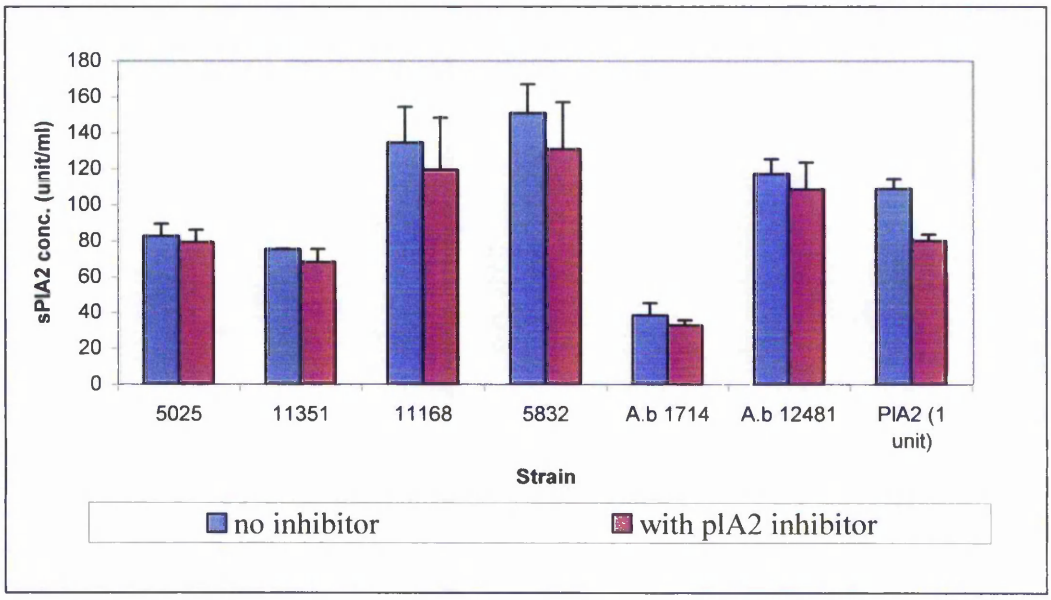


Figure 4.11 – Phospholipase A_2 assay of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of 64 µM $PACOCF_3$. Error bars represent the standard deviation of the data (n=6).

This data also shows that when the phospholipase A_2 inhibitor was added at the same concentration as for the contact assays, no significant inhibition was detectable. There was some reduction in the activity of the pure phospholipase, but this was not statistically significant. Therefore, it is likely that when the inhibitor was added to the contact assay at this concentration, it did not actually inhibit much of the activity, and this could explain why some, but not all of the haemolytic activity was reduced when $PACOCF_3$ was added to the contact assay.

4.6 SOUTHERN BLOTTING OF *C. JEJUNI* AND *A. BUTZLERI* DNA

The published sequence of *C. jejuni* 11168 (Parkhill *et al.* 2000), contains the gene sequences encoding two possible haemolysin domains, in addition to a phospholipase A gene. These are a putative haemolysin called cj0588 or *tlyA*, which is similar to haemolysin A produced by *Treponema hydodysenteriae*. The other possible haemolysin gene is a probable integral membrane protein haemolysin domain designated Cj0183, which has been shown to have similarity to reported haemolysins of other organisms. The position of these three genes on the genome, and the neighbouring genes is shown in figure 4.12.

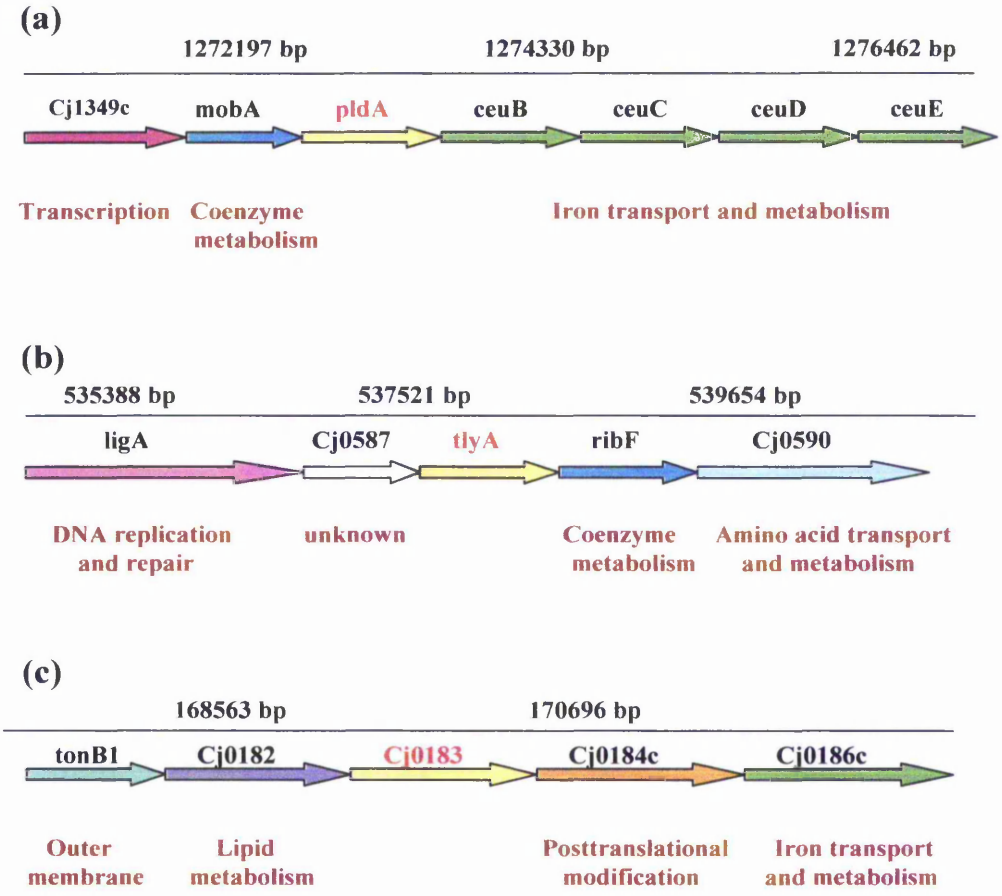


Figure 4.12 – Position of *pldA* (a), *tlyA* (b), and *Cj0183* (c), in the *C. jejuni* NCTC 11168 genome, and the neighbouring genes and predicted functions (Intergenic DNA is not illustrated for diagrammatical purposes).

A Blast search using the NCBI sequence alignment program (Altschul *et al.* 1997) revealed the *pldA* gene sequence to have a 73% identity with the *pldA* reported haemolysin from *C. coli*, and a 26% identity with the *pldA* gene from *H. pylori* J99. The *tlyA* gene showed homology to the *tlyA* haemolysins from *H. pylori* (43% identity), and *T. hyodysenteriae* (32% identity). Whereas the Cj0183 gene sequence showed 49 and 32.5% identity to a haemolysin from *H. pylori* and the *tlyC* gene from *T. hyodysenteriae* respectively.

To date, these three potential haemolysin genes have not been fully investigated. Therefore it was decided to study these genes further, starting with Southern blot analysis. This would determine not only if these genes exist in other strains of *C. jejuni*, or are unique to 11168, but also whether they can be found in *A. butzleri*.

4.6.1 Southern Blotting of *pldA*, the Phospholipase A Gene

The full sequence of the *pldA* gene, and position of the primers can be found in the Appendix (figure 8.1). Primers were designed as given in section 2.15.1. Originally, the genomic DNA was digested, run on an agarose gel and transferred to the membrane by capillary transfer, but the results were found to be inconsistent, therefore 500 ng DNA was spotted onto the membrane instead. Dot blots of all 21 strains of *C. jejuni* and 2 strains of *A. butzleri* tested in the contact assay were carried out, and the *pldA* probe was labelled with ^{32}P as in section 2.21.1.3.

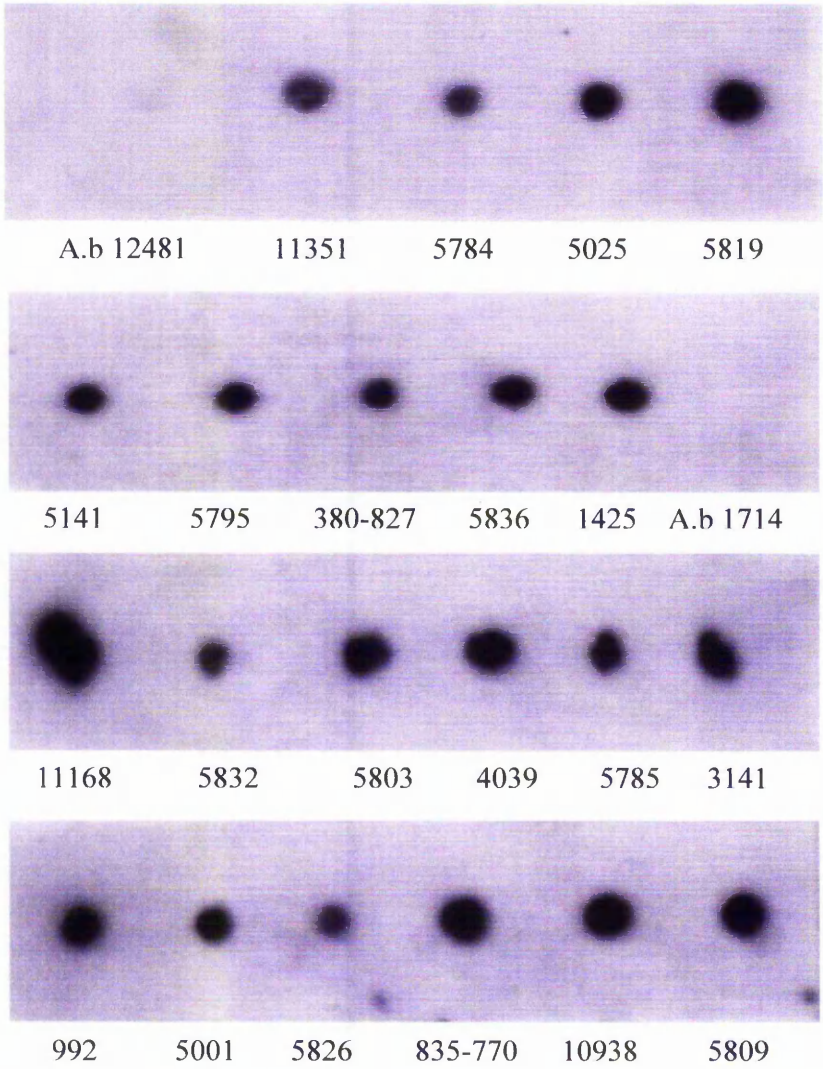


Figure 4.13– Dot blots of 21 strains of *C. jejuni* and 2 strains of *A. butzleri* (A.b) from a range of isolates (see table 4.1), using a probe designed from the *pldA* gene of *C. jejuni* 11168.

As can be seen from figure 4.13, all strains of *C. jejuni* tested showed the presence of the *pldA* gene, and even at high stringency washes, the probe remained bound to all strains, suggesting that the *pldA* gene is ubiquitous in all strains of *C. jejuni*, and appears to be highly conserved. Neither *A. butzleri* strain showed any hybridisation with the probe, even at low stringency washes (see section 2.21.1.4), and as the probe was made from the entire *C. jejuni pldA* gene, it would be expected to show some hybridisation if there were any sequence similarities.

Therefore, it can be concluded that the *pldA* gene is ubiquitous in all *C. jejuni* strains tested, but cannot be found in either *A. butzleri* strain tested. Unfortunately, there is no easy way of determining whether the gene sequence does exist in *Arcobacter,* as its genome has not yet been sequenced.

4.6.2 Southern Blotting of *tlyA* Putative Haemolysin Domain

As with the *pldA* blots (figure 4.13), all the *C. jejuni* strains showed the presence of the *tlyA* gene (see figure 4.14), and again, the probe remained hybridised even after high stringency washing. No hybridisation was seen with either *A. butzleri* strain, but this does not necessarily mean that the gene is not present, it may just have a modified structure, and therefore not complimentary enough for the probe to hybridise sufficiently.

There is no easy way of determining whether either gene sequence exists in *A. butzleri*, short of screening a gene library, as the *A. butzleri* genome has not yet been sequenced.

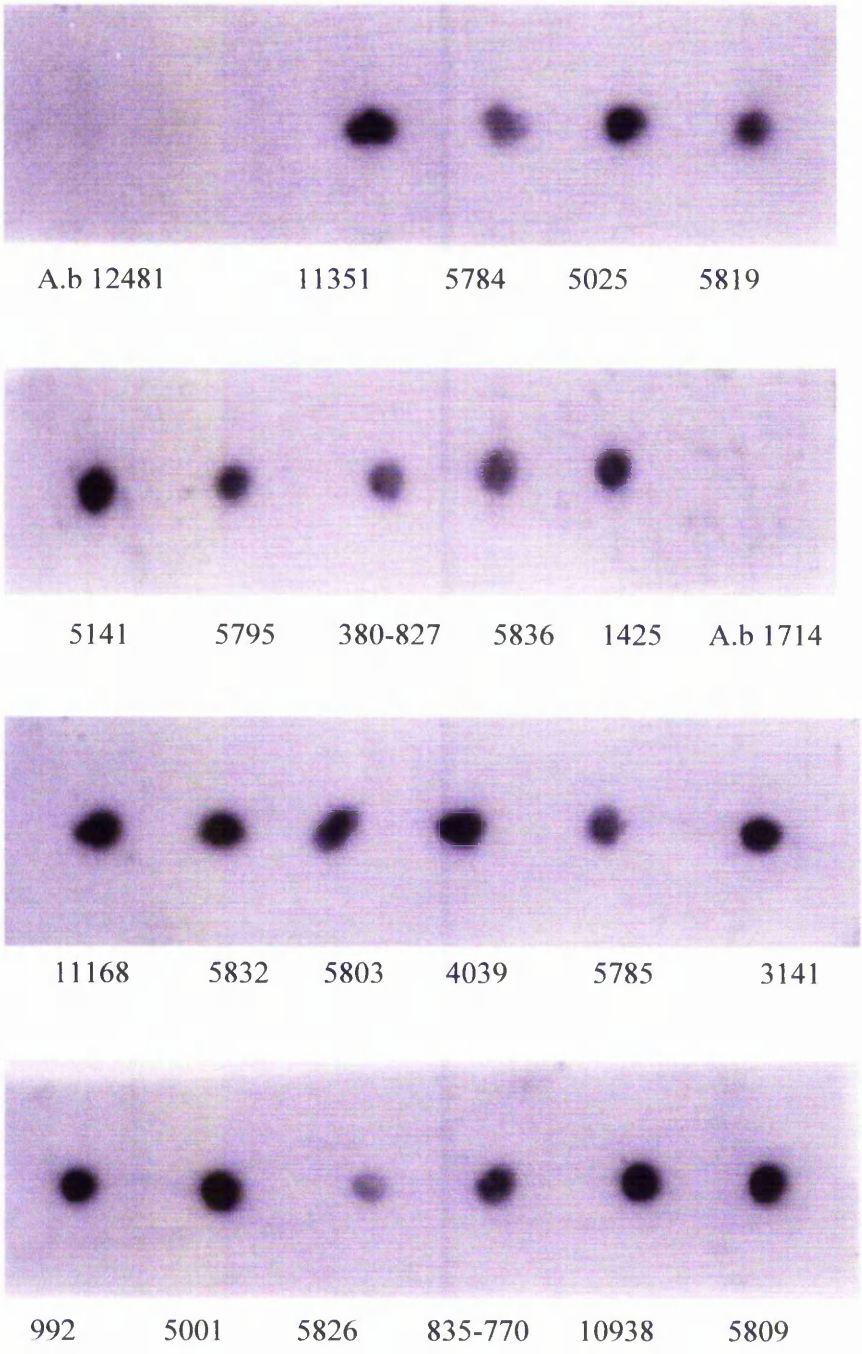


Figure 4.14 – Dot blots of 21 strains of *C. jejuni* and 2 strains of *A. butzleri* (A.b) from a range of isolates (see table 4.1), using a probe designed from the *tlyA* gene of *C. jejuni* 11168.

4.6.3 Southern Blotting of Cj0183 Putative Haemolysin Domain

These experiments were carried out using similar methods to the two previous southern blotting experiments, except that these blots were carried out using non-radioactive methods (see section 2.21.2).

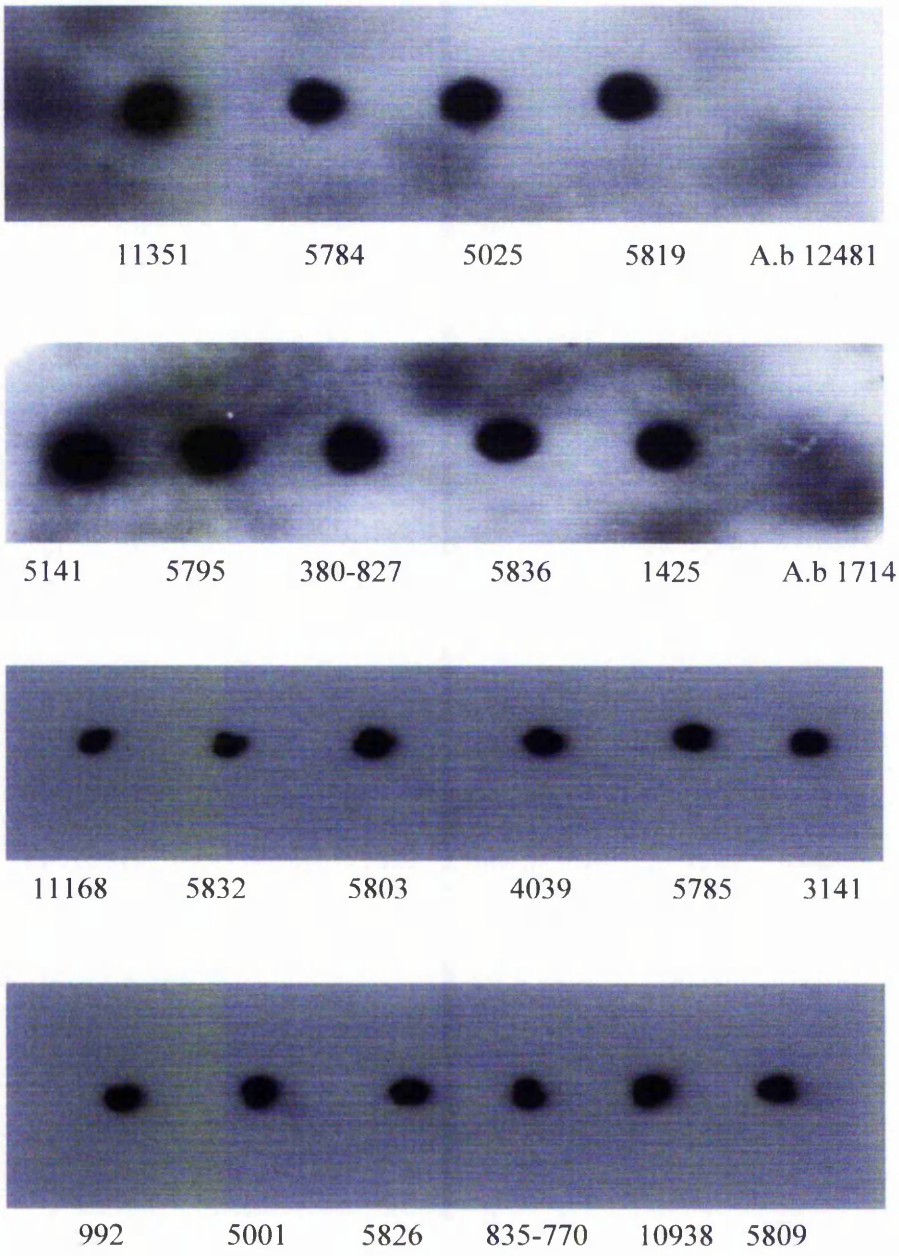


Figure 4.15 – Dot blots of 21 strains of *C. jejuni* and 2 strains of *A. butzleri* (A.b) from a range of isolates (see table 4.1), using a probe designed from the Cj0183 gene of *C. jejuni* 11168.

Figure 4.15 shows that similar results as with the two previous experiments were obtained. All the *C. jejuni* strains showed the presence of the Cj0183 gene, even after high stringency washing. Neither of the *A. butzleri* strains showed any hybridisation with the probe, as with the two previous genes.

In summary, the results in this chapter suggest that phospholipase A may play a role in the haemolysis reaction. Therefore the next logical step seemed to be the construction of a phospholipase A gene mutant from *C. jejuni* NCTC 11168.

CHAPTER 5

THE PHOSPHOLIPASE A GENE

5.1 CONSTRUCTION OF THE *C. JEJUNI* PHOSPHOLIPASE A MUTANT

The phospholipase A gene (*pldA*) was found to be ubiquitous in all *C jejuni* strains southern blotted using the *pldA* gene as a probe, and inhibitors for the phospholipase A_2 protein were found to reduce haemolysis when added to the contact haemolysin assay. Therefore, the next step was to construct a *C. jejuni* phospholipase A mutant to confirm that phospholipase A was responsible for the lytic activity seen when *C. jejuni* strains were in close contact with erythrocytes.

During the course of the haemolysin experiments in chapter 4, it was discovered that Dr. Karl Wooldridge and Dr. Dlawar Ala'Aldeen (Queens Medical Centre (QMC) Nottingham) had constructed mutants of the *tlyA* and Cj0183 genes, using *C. jejuni* NCTC 11168 as the parent strain, in separate studies to those reported here. These mutants were called *clyA* and *clyC* respectively, and constructed to investigate the role of these genes in chicken colonisation. As a result of this, Karl Wooldridge very kindly agreed to help with many of the methods used in this chapter.

5.1.1 PCR of *C. jejuni* 11168 using Primers for the *PldA* Gene

The gene encoding the *pldA* protein was amplified from *C. jejuni* 11168 using primers designed to incorporate about 0.8 kb of flanking DNA upstream of the gene (see section 2.23.1). These primers were designed with the help of Dr. Karl Wooldridge, as the primers previously used to amplify the *pldA* gene for the southern blotting were located too close to the start of the gene to allow for efficient mutagenesis. The *pldA* gene itself is 0.989 kb, and with an additional 0.786 kb flanking DNA, the PCR product should be 1.745 kb in size.

The gel in figure 5.1 shows the results of the PCR, with a major band between 1500 and 2000 base pairs, which corresponds to the predicted size of the *pldA* gene together with the flanking DNA.

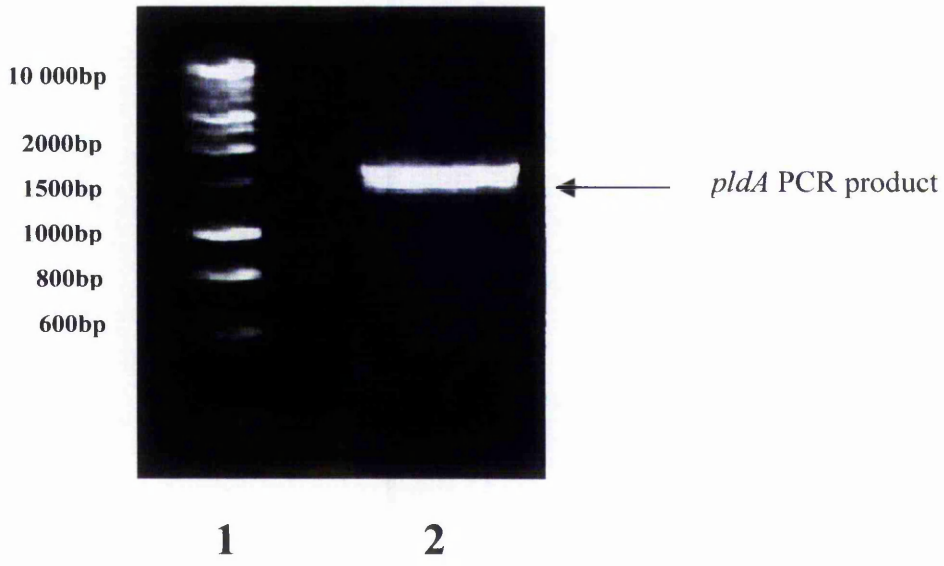


Figure 5.1 – Agarose gel electrophoresis showing amplified *pldA* PCR product from *C. jejuni* NCTC 11168 on a 1% SeaPlaque agarose gel.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lane 2 = 40µl *pldA* PCR product

5.1.2 Cloning into T7/NT – TOPO Plasmid and Transformation

The *pldA* gene product was excised from the gel, purified (see sections 2.17 & 2.18), and cloned into the T7/NT-TOPO vector to construct plasmid pSJM01. This was then transformed into TOP 10F' *E. coli*, typically giving rise to approximately 25 transformant colonies per experiment. Six of these clones were selected and grown in LB broth. The plasmids from each clone were then extracted, purified and screened by PCR analysis using the original pldAF and pldAR primers for the *pldA* gene (section 2.23.1), to ensure that the transformants were carrying the correct plasmid and gene insert.

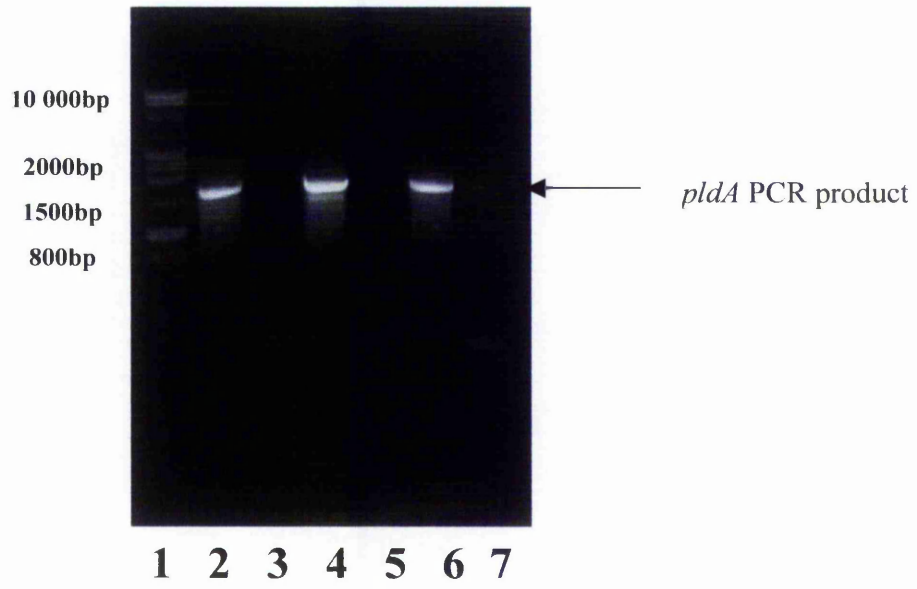


Figure 5.2 – Agarose gel electrophoresis of plasmid extraction from transformant clones using primers for the *pldA* gene and the same PCR conditions as in section 5.1.1, on a 1% agarose gel.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lanes 2-7 = 10µl PCR product of plasmid extractions from six different transformant colonies

Figure 5.2 shows that the transformant colonies in lanes 2, 4 and 6 are all carrying the plasmid pSJM01, containing the correct insert of the 1.7 kb *pldA* gene. Therefore, the clones from lanes 2, 4 and 6 were selected for further experiments.

5.1.3 Inverse PCR Mutagenesis of the *pldA* Gene

The plasmid pSJM01 was used as a template for inverse PCR mutagenesis. Purified plasmids selected from the previous section were amplified using inverse PCR primers (see section 2.23.6 &2.23.7). This removed a section of about 0.5 kb to inactivate the *pldA* gene (for diagram see figure 2.1, and figure 8.5). The primers also incorporated a *Bgl*II site, to enable digestion of the plasmid after amplification. The resulting products were analysed using agarose gel electrophoresis to determine the success of the inverse PCR.

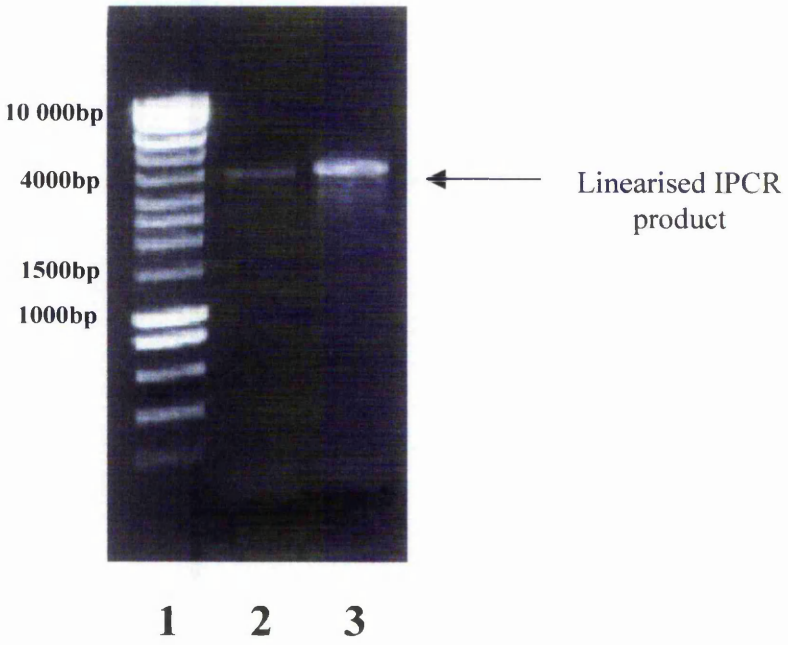


Figure 5.3 – Agarose gel electrophoresis of transformant plasmid pSJM01 after IPCR mutagenesis, on a 1% agarose gel.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lanes 2&3 = 10µl PCR product of pSJM01 after IPCR mutagenesis

Figure 5.3 shows the linearised plasmid pSJM01 after inverse PCR using primers shown in section 2.23.6. The product seen in lanes 2 and 3 is of the expected size (2.87 kb plasmid + 1.245 kb remaining *pldA* gene = 4.115 kb), just above the 4kb band in the marker lane. Both PCR products were then digested using *Bgl*II, the ends religated to each other using T4 DNA ligase, and then transformed into JM109 *E. coli* cells to amplify the resulting vector (pSJM02).

5.1.4 Insertion of an Antibiotic Resistance Cassette into pSJM02

To allow for selection of the mutant after construction, and to ensure that the *pldA* gene was completely disabled, an antibiotic resistance cassette was inserted into the centre of the gene, using the integral *Bgl*II restriction site. Two plasmids were kindly supplied by Dr. Karl Wooldridge; pJMK30 (van Vliet *et al.* 1998), containing a kanamycin resistance cassette, and pRY109 (Yao *et al.* 1993), containing a chloramphenicol resistance cassette. The plasmids were extracted

from their host using methods from section 2.23.5, and quantified using agarose gel electrophoresis (figure 5.4a). Both plasmids were then digested using *BamH*I, to separate the fragments containing the respective antibiotic resistance genes from the remainder of the plasmids.

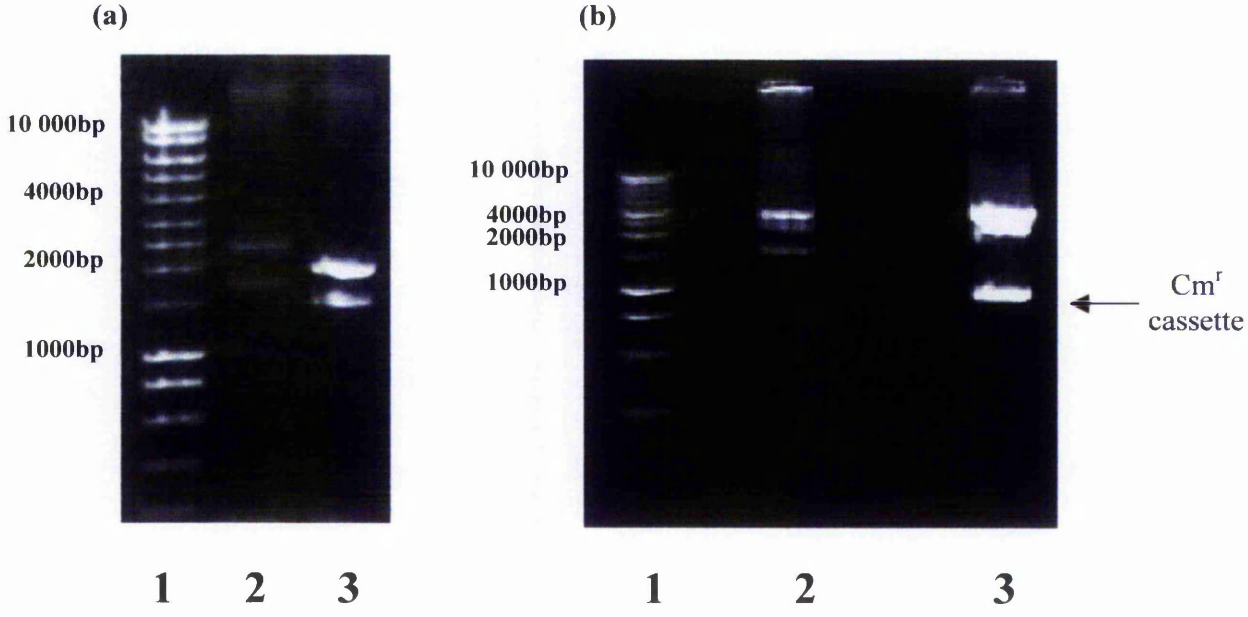


Figure 5.4 – Agarose gel electrophoresis on a 1% SeaPlaque agarose gel, of pJMK30 (lane 2) and pRY109 (lane 3) before (a), and after (b), digestion with *BamH*I.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lane 2 = 2µl plasmid pJMK30 before digestion (a) and 10µl pJMK30 digestion (b)
Lane 3 = 2µl plasmid pRY109 before digestion (a) and 10µl pRY109 digestion (b)

The 1 kb band obtained when pRY109 was digested with *BamH*I was identified as the chloramphenicol resistance cassette (Cm^r, see figure 5.4 (b), lane 3), and the 1.5 kb band obtained when pJMK30 was digested with the same enzyme was identified as the kanamycin resistance gene (Km^r, figure 5.4(b), lane 2). The chloramphenicol resistance cassette from plasmid pRY109 was selected for insertion into plasmid pSJM02, as this fragment was of a higher concentration, and easier to extract and purify from the gel than the cassette from pJMK30 (figure 5.4 (b)). Therefore, the 1 kb restriction fragment containing the

chloramphenicol gene was extracted and purified from the gel, followed by ethanol precipitation to concentrate the DNA (see section 2.23.14).

The plasmid pSJM02, containing the digested and religated IPCR product was digested with *Bgl*II, dephosphorylated to prevent the ends from re-ligating (see section 2.23.13), and run on a 1% agarose gel next to the undigested pSJM02 plasmid (figure 5.5) to ensure that the digestion had been successful.

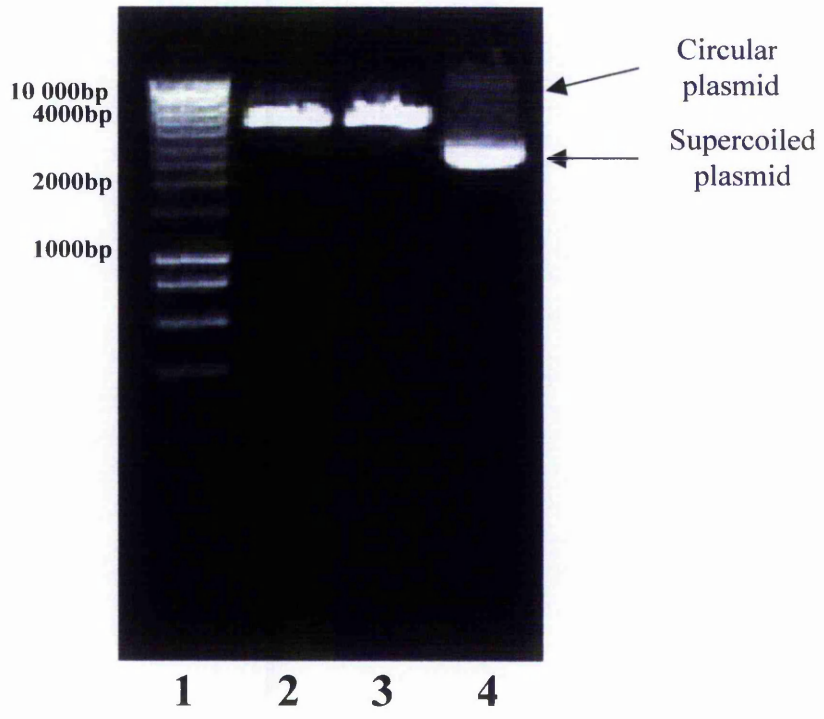


Figure 5.5 – Agarose gel electrophoresis of digested (lanes 2 and 3) and undigested (lane 4) pSJM02, after digestion with *Bgl*II, on a 1% agarose gel.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lane 2 & 3 = 5µl *Bgl*II digest and dephosphorylation of pSJM02
Lane 4 = 5µl undigested pSJM02

Lane 4 of figure 5.5 shows the undigested pSJM02 plasmid, with a major band at about 3 kb, and a faint band above the linearised plasmid in lanes 2 and 3, although it is difficult to determine the size of this band, as the markers have not separated well at the top end of the gel. The lower band is the circular pSJM02 plasmid in its supercoiled state, where the DNA is tightly wound together, and as a smaller tightly coiled molecule, can travel further down the gel than the

linearised plasmid in lanes 2 and 3. The minor band in lane 4, above the supercoiled DNA, is likely to be the plasmid in its open circular conformation. This occurs when one of the DNA strands becomes nicked, and the plasmid then returns to its relaxed circular state. Most plasmids usually exist in the supercoiled state, but the more that the DNA is manipulated, i.e. during extraction methods, the more plasmids will become damaged and revert to the circular form.

Lanes 2 and 3 show the pSJM02 plasmid after digestion with *Bgl*II, and when compared to lane 4 containing the undigested plasmid, demonstrate that the digestion has been successful.

After pSJM02 and the chloramphenicol resistance cassette had been prepared for ligation, the concentration of each was quantified, and the optimum ratio of vector : insert was determined using the equation given in section 2.23.14. The plasmid and insert were then ligated to each other, resulting in the formation of plasmid pSJM03.

5.1.5 Transformation of JM109 Cells with pSJM03

The plasmid pSJM03 was used to transform competent *E. coli* JM109 cells. Aliquots of the transformed cell mixture were spread onto LB plates supplemented with ampicillin and chloramphenicol (see section 2.23.15) and incubated overnight. Only the JM109 cells that had taken up the pSJM03 plasmid would be able to grow on these selective plates, and transformant colonies were selected and grown as overnight cultures in LB broth containing ampicillin and chloramphenicol. The resulting cells were then harvested, and the plasmids extracted and purified.

Figure 5.6 shows plasmid extractions of pSJM03 from four of the transformant colonies (lanes 2-5), compared with pSJM02 (lane 6), which does not contain the additional 1 kb chloramphenicol cassette. This gel clearly shows that all four plasmids in lanes 2-5 are about 1 kb larger than pSJM02 in lane 6, and therefore,

it seems that all of these plasmids are pSJM03, and the ligation of an antibiotic resistance cassette into plasmid pSJM02 has been successful.

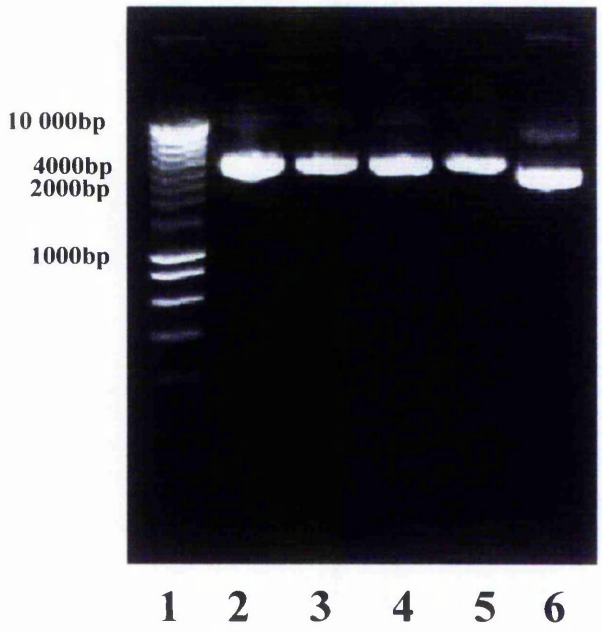


Figure 5.6 – Agarose gel electrophoresis of pSJM03 plasmid extractions from overnight cultures, compared with plasmid pSJM02, on a 1% agarose gel.

Lane 1 = 5µl 1kb Hyperladder (Bioline)
Lane 2-5 = 5µl pSJM03 plasmid extractions
Lane 6 = 5µl pSJM02 plasmid extraction

5.1.6 Transformation of *C. jejuni* 11168 with pSJM03

Transformation of the pSJM03 plasmid back into *C. jejuni* proved to be more difficult than anticipated. Natural transformation methods were unsuccessful (see section 2. 23.16) and yielded no transformant colonies despite several attempts. Electroporation would have been the best method to use, however, the equipment required is expensive, and this technique is not currently used at the University. At the time of this research, Liu Xinghai, another Ph.D student working in the group at NTU was using a conjugation method to transfer genetic material between strains of *E .coli*, so it was decided to try out this method with *C. jejuni*.

The method involves transformation of *E. coli* S17 cells with pSJM03, then using a conjugation method (see section 2.23.17) to transfer the plasmid into *C. jejuni* 11168. Once the plasmid has been transferred, the homology between the complete *pldA* gene and the inactivated *pldA* gene should allow for a double recombination event, where the mutated *pldA* gene integrates into the genome. Consequently, the cell loses the remainder of the pSJM03 plasmid, and therefore the bacteria is not resistant to ampicillin, as this gene was carried on the plasmid. The kanamycin resistance gene, however, should be incorporated within the genome, as it is part of the mutated *pldA* gene.

This method proved successful, and resulting *C. jejuni* 11168 double crossover mutants were selected for by growth on agar containing only chloramphenicol. No difference in size or morphology was noticed between these transformant colonies and the wild type *C. jejuni* strain. Gram's staining proved that the mutants were pure *Campylobacter* cultures, and negative controls showed that the wild type *C. jejuni* 11168 was unable to grow on either ampicillin or chloramphenicol supplemented agar. Unfortunately the mutant was not sequenced, as this technique is costly, and the University does not currently possess a DNA sequencer. However, confirmation of the mutant by PCR analysis was carried out as a secondary check (Figure 5.6)

5.2 Haemolysis Assay of *C. jejuni pldA*, *clyA* and *clyC* Mutants

After construction of the *C. jejuni pldA* mutant, it was tested for haemolytic activity using the contact haemolysin assay, alongside the *clyA* and *clyC* mutants, kindly donated by Dr. Karl Wooldridge.

Figure 5.7 shows that the *C. jejuni pldA* mutant demonstrates a significant reduction in haemolysis when compared to the wild type ($p=0.0003225$), with a haemolytic activity of less than 40% of the amount produced by the original *C. jejuni* NCTC 11168 strain. Therefore, it does seem likely that haemolysis produced by *C. jejuni* 11168 is mainly due to the activity of the phospholipase A gene.

In contrast, neither the *clyA* nor the *clyC* mutant had any effect on the haemolytic reaction produced by *C. jejuni* NCTC 11168, suggesting that these genes do not have a role in the haemolysis reaction. However, recent experiments by Dr. K Wooldridge have shown that both *clyA* and *clyC* appear to be essential for efficient colonisation of chickens (Wooldridge *et al.* 2003).

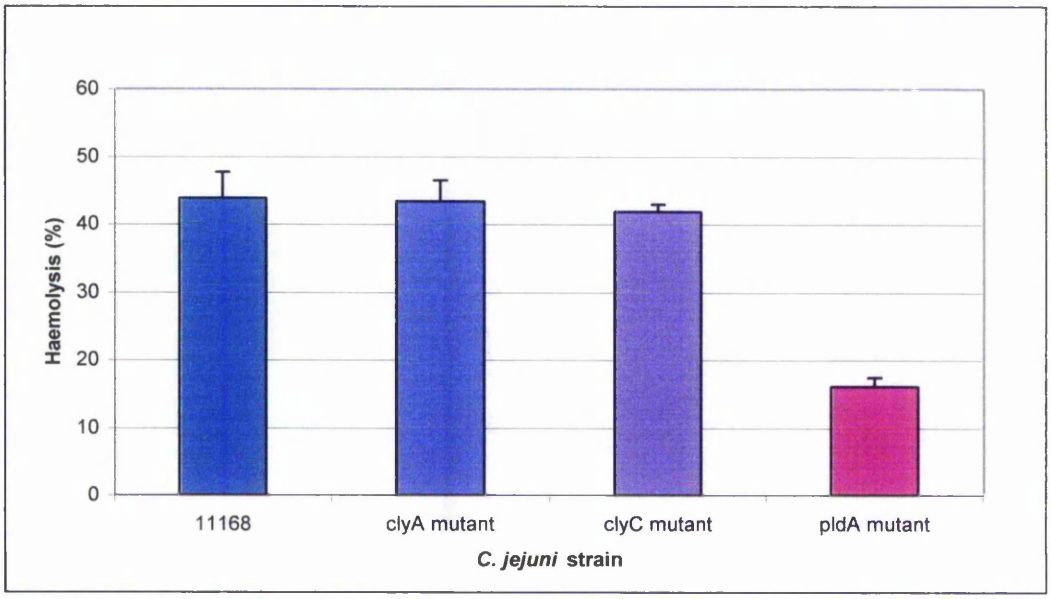


Figure 5.7 Contact haemolysin assays of *C. jejuni* 11168, the *pldA* mutant, and the *clyA* and *clyC* mutants kindly donated by Dr. K. Wooldridge. Error bars represent the standard deviation of the data (n=9).

5.3 Phospholipase A_2 Assay of *C. jejuni pldA*, *clyA* and *clyC* Mutants

The *C. jejuni* 11168 *pldA* mutant has been shown to cause significantly lower amounts of red blood cell lysis in the contact haemolysin assay than the wild type *C. jejuni* NCTC 11168 (see figure 5.7). However, knocking out the *pldA* gene did not completely remove all haemolytic activity from the bacteria. It is possible that haemolytic activity is a result of the activity of another gene in addition to *pldA*, and as the *clyA* and *clyC* genes have been identified as possible haemolysin domains, it was considered prudent to include the *clyA* and *clyC* mutants in the phospholipase A_2 assay, to determine whether they would have any reduced PlA_2

activity. All three mutants were assayed for phospholipase activity using a secretory phospholipase A_2 assay kit (see section 2.13).

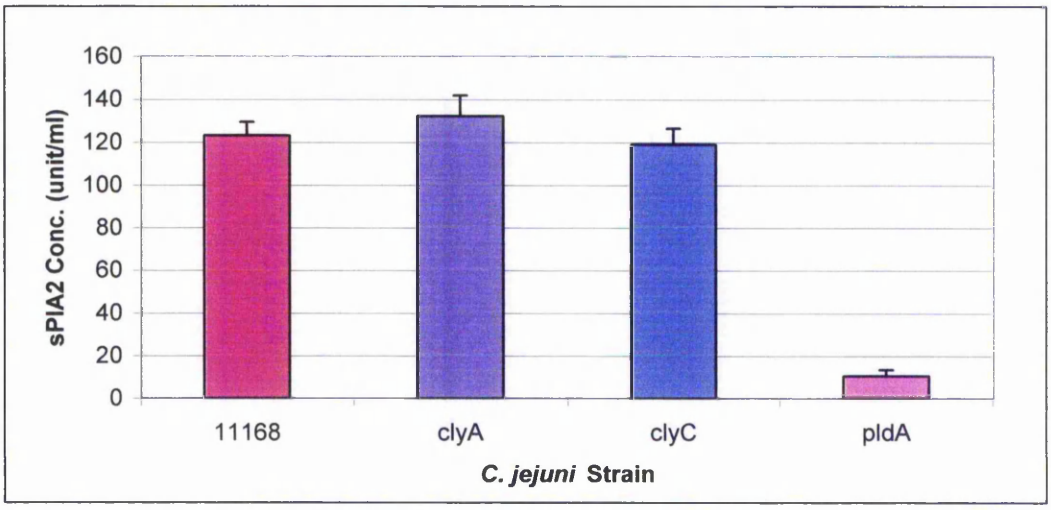


Figure 5.8 Secretory phospholipase A_2 assay of *C. jejuni* 11168, the *pldA* mutant, and the *clyA* and *clyC* mutants kindly donated by Dr. K. Wooldridge. Error bars represent the standard deviation of the data (n=6).

Figure 5.8 demonstrates that the *clyA* and *clyC* mutants produce similar amounts of phospholipase A_2 when compared to the *C. jejuni* 11168 wild type. The phospholipase A_2 activity of the *pldA* mutant however, is greatly reduced, proving that the *pldA* mutagenesis has worked, and the majority of the phospholipase activity has been removed. This assay proves that the reduction in haemolysis seen when the mutant is applied to the contact assay, is due to mutation of the *pldA* gene, and not a result of an unrelated mutation elsewhere in the genome.

CHAPTER 6

DISCUSSION

6.1 GROWTH CURVES

The growth curves shown in figure 3.1 demonstrated that both strains of *Campylobacter jejuni* exhibited comparable growth in liquid media, and the time points of each growth phase were elucidated. *C. jejuni* replicates much slower than other food-borne pathogens such as *E. coli*, and so these experiments were important to become accustomed to the fastidious nature of this pathogen. Both strains were shown to be in the lag phase for 2 hours after inoculation, followed by the exponential phase up to about 24 hours. After this point, the growth began to slow, and the cells entered the stationary phase.

6.2 CYTOTOXICITY TESTING OF *C. JEJUNI* SAMPLES

These experiments demonstrated that cytotoxicity was present in *C. jejuni* cell supernatants from both 11168 and 11351 strains, but only after sonication of the cells. This indicates that the toxin(s) detected were released into the culture supernatant after lysis of the cells, and suggests that the cytotoxic effect was caused by a toxin(s) that is membrane bound or intracellular, and not secreted into the growth media whilst the bacteria was actively growing and dividing. Additionally, it was found that during all of the experiments, cell extract samples from *C. jejuni* 11168 were consistently more cytotoxic than samples prepared from *C. jejuni* 11351.

N2a cells showed the most sensitivity to both *C. jejuni* 11351 and 11168 samples, with ECV cells exhibiting slightly less, but still significant cytotoxicity in response to cell extract samples from both strains (see figures 3.2 and 3.3). Vero cells also showed a slight decrease in cell viability when samples from *C. jejuni* 11168 were applied, though t-tests determined that this reduction was not significant (figure 3.3). CHO cells showed no significant sensitivity to cell extracts from either strain of *C. jejuni,* in contrast to previous experiments carried out by Holmes and Spears (TNTU), and reports in the literature (Johnson & Lior 1988; Pickett 2000).

Neither heat nor trypsin treatment of the *C. jejuni* samples caused a decrease in cytotoxicity, suggesting that the toxin(s) is not proteinaceous. This is unusual, as most reported toxins are proteins, and would be destroyed under such conditions. It is also possible that heat or protease treatment of a particular toxin could result in cleavage of a particular component or subunit, resulting in an active toxin. This may then mask any cytotoxicity lost by other toxin(s) in the extract that may have been destroyed by the treatments. As the samples applied to the cell lines are crude bacterial extracts, it is feasible that more than one toxic entity may be present, and at least one of these toxins may be both heat and trypsin resistant.

Morphology studies of CHO cells after treatment with samples from both *C. jejuni* strains (table 3.1) showed that sub-lethal changes had occurred. A significantly large number of distended cells were detected, particularly after the 96 hour incubation. This implies the presence of the cytolethal distending toxin (Pickett & Whitehouse 1999). However, no significant changes in MTT reduction were detected, suggesting that either the toxin concentration was too low to cause detectable changes in the MTT assay, or that the toxin causing the cell distortion is the controversial cholera-like enterotoxin, which is reported to cause distension without cell death (Ruiz-Palacious *et al.* 1983; Wassenaar 1997). These experiments also showed a significant amount of rounding of the CHO cells, but when the same samples were applied to ECV cells, the majority of the cells changed to the elongated morphology, and MTT assays showed significant cell death. These effects were detected after just 48 hours, in contrast to the CHO experiments where effects were more pronounced after 96 hours incubation. This further suggests the presence of more than one toxin, and would help to explain the different effects seen on the two cell lines.

These results indicate that the cytotoxic activity detected from *C. jejuni* is not due to the activity of a cytolethal distending toxin (CDT), as this toxin causes CHO cells to distend over a period of 3-4 days, leading to cell death. Neither the morphology studies nor the cell cytotoxicity assays on CHO cells showed evidence of this toxic effect. It is known that *C. jejuni* 11168 does contain genes for the CDT in its genome (Parkhill *et al.* 2000), however, it has been shown that

although all *C. jejuni* strains to date possess the genes, only about 40% of strains express the toxin (Johnson & Lior 1988; Bang & Madsen 2003).

It is also unlikely that these experiments have detected a 70 kDa toxin, as this toxin causes rounding and death when applied to CHO and Vero cells. Although rounding was detected in CHO morphology studies, no cell death was apparent, and Vero cells only exhibited slight cytotoxicity to the cell samples from one strain. It is possible that the rounding effects were due to the cytolethal rounding toxin, although the lack of significant cytotoxicity when the samples were applied to the Vero cell line suggests otherwise, as this cell line is commonly used for the detection of this toxin (Schultze *et al.* 1998). Additionally, there was no evidence of a shiga-like toxin or a hepatotoxin, but as the cell extracts were not purified, it is impossible to say for certain that any of these reported toxins were definitely present or absent.

Another possibility for these cytotoxic effects could be that the porin-lipopolysaccharide toxin was detected. This toxin is reported to be trypsin stable, and cytotoxic to Hep-2 and HeLa cells, though it has not been studied on CHO, ECV, Vero or N2a cell lines. The toxin is reported to cause cell death following cell rounding, and share homology with the *C. jejuni* MOMP. It is also possible that LPS on its own could be responsible for the toxicity seen, as LPS is a potent endotoxin released from Gram-negative bacterial membranes upon cell lysis, and is both heat stable and trypsin resistant, as it is not a protein (see section 1.8.4).

In conclusion, it appears that there is at least one toxin present in the unpurified *C. jejuni* cell extract samples that causes cell death and/or sublethal changes to the cell lines. *C. jejuni* produces a number of different toxins, which may vary either between strains or within the same strain under different growth conditions. It is known that *Campylobacter* is able to regulate its gene expression depending on its environment (Penner & Aspinall 1997; Fry *et al.* 2000), and it is possible that repeated laboratory sub-culturing of the bacteria may result in the loss of expression of some of its virulence genes. This may explain why the current understanding of toxin production by *C. jejuni* is so limited, and why some groups are unable to detect toxins whereas others have reported toxic

activity. It is clear that further research is needed in this area to fully understand the disease process of this important pathogen.

6.3 SDS-PAGE AND N-TERMINAL SEQUENCING OF *C. JEJUNI* MOMP AND FLAGELLIN

Two major bands were identified on the OMP gel (Figure 3.7 & 3.8). The higher band migrated at approximately 62kDa and was identified as flagellin (Logan & Trust 1982; Schwartz *et al.* 1994). This was confirmed by N-terminal sequence analysis (Table 3.4). The second band varied between 40 and 43kDa depending on the *C. jejuni* strain, and was identified as the major outer membrane porin (MOMP).

N-terminal sequencing of both the lower bands confirmed that they were *C. jejuni* MOMP, though there were no differences in the sequence that could explain the difference in molecular weights (table 3.5). The major outer membrane of *C. jejuni* is the most highly expressed outer membrane protein of the bacteria, representing up to 90% of the total OMP (De *et al.* 2000; Penn 2001). It has recently been shown that this protein exhibits variation between isolates, with molecular weights ranging from 41 to 46kDa (Penn 2001). The reason for this polymorphism is unknown, although it is possible that this difference in molecular mass is due to phase variation, as it has been shown that *C. jejuni* is able to modify certain genes, such as those for flagellin and LPS, in order to evade host immune responses (Parkhill *et al.* 2000; Linton *et al.* 2001). It has also been reported that *H. pylori* is able to modify its outer membrane phospholipase A (OMPLA) genes using the same mechanisms, according to the pH of its surroundings (Tannaes *et al.* 2001). Therefore, a mechanism may exist whereby *C. jejuni* is able to modify its MOMP genes in the same way.

The N-terminal sequence was found to match the sequence data of Holmes (2001) and Spears (2002) putative enterotoxin, though the apparent molecular weights are slightly different. The toxin detected by Holmes & Spears was reported to have a molecular weight of approximately 50kDa, whereas the

MOMP has a molecular weight between 40 and 45kDa (Bacon *et al.* 1999). This discrepancy may be because the toxin described by Holmes & Spears was only partially purified, and possibly includes an additional bound entity, which would explain the increased molecular weight. In addition, protein molecular weight standards can be notoriously inaccurate, and such a discrepancy is not unusual. The MOMP protein sequence was also found to match with other reports of a potential porin toxin from *C. jejuni* (Bacon *et al.* 1999) and *C. coli* (Moutinho-Fragoso *et al.* 1998), implying that the MOMP may play a role in *C. jejuni* pathogenesis.

6.4 APPLICATION OF OMP SAMPLES TO CELL LINES

It was hoped that this experiment would show that the MOMP was cytotoxic to cultured cell lines, when purified from the bacterial cell and supernatant. Unfortunately, no cytotoxicity was seen in any of the cell lines when purified OMP extractions from both *C. jejuni* strains were applied to ECV, Vero and N2a cells at a 10% concentration.

There are several reports in the literature suggesting that the MOMP may have cytotoxic properties when complexed with LPS (Moutinho-Fragoso *et al.* 1998; Bacon *et al.* 1999). It is possible that the cytotoxicity detected in previous experiments (section 3.2 and section 6.2) was due to a porin-LPS toxin, but the toxin may have been denatured by the extraction process, or needs to be cleaved in some way to become active. The extraction process may also have removed the LPS that it is reported to form a complex with. Alternatively, there may be something else in the bacterial cell extract that is responsible for the cytotoxicity seen.

6.5 IMMUNOBLOT OF *C. JEJUNI* OMP USING ANTI-CHOLERA TOXIN ANTIBODIES

Probing the OMP samples with anti-cholera toxin antibodies (figure 3.11) showed strong cross reactivity with one band, though another faint band could be seen just below. When compared to the Coomassie Blue stained gel in figure 3.10, the prominent band appears to be the flagellin protein (65kDa) and the faint band the MOMP (40-45kDa).

The anti-cholera toxin antibody used for the immunoblot was polyclonal, therefore increasing the chance of non-specific binding. In addition, it has been well documented that LPS and glycosylated proteins contain sialic acid groups, which mimic GM_1 gangliosides, the receptors for cholera toxin. (Suzuki *et al.* 1994; Tsang 2002). It has been demonstrated that the *C. jejuni* flagellin is glycosylated (Guerry 1997; Syzmanski *et al.* 1999; Karlyshev *et al.* 2004), therefore, it is feasible that the anti-cholera toxin recognises either LPS or N-acetylneuramic acid (sialic acid) groups in the flagella or MOMP, resulting in non-specific binding. Further experiments using a monoclonal antibody against cholera toxin would clarify this situation, but it is likely that the neutralisation of the toxin found by Holmes and Spears was a false positive reaction due to the toxin containing similar epitopes to the binding site for cholera toxin. In addition, the genome of *C. jejuni* contains no sequence homology to cholera toxin, and to date, no cholera like toxin or enterotoxin has been shown to produce cytotoxic effects on mammalian cell lines. The cholera toxin has also been demonstrated as being sensitive to trypsin and heat, so it is unlikely that the toxin detected by Holmes and Spears, and the cytotoxin present in crude cell extract samples, is a cholera-like toxin.

6.6 SDS-PAGE OF LPS EXTRACTED FROM *C. JEJUNI* OMP

LPS was detected from both *C. jejuni* 11351 and 11168 OMP samples, of both high and low molecular weight (Figure 3.12). Interestingly, it was also shown that the *C. jejuni* 11168 OMP contained an additional LPS band that was not

present in *C. jejuni* 11351. The reason for this is unknown, but it could explain the increased toxicity found from *C. jejuni* 11168 samples when compared with those from *C. jejuni* 11351. If LPS is attached to the MOMP, as proposed by Bacon *et al.* (1999), it is possible that size differences between LPS moieties could affect the molecular weight of the MOMP when separated on SDS-PAGE, and would explain the difference in molecular weights between the MOMP bands of the two *C. jejuni* strains. This may also explain the slight discrepancy between Holmes and Spears toxin and the MOMP band in the OMP extractions (Figures 3.7 & 3.8).

In conclusion, it seems likely that the cytotoxic effects seen in the cell culture assays and the partially purified toxin detected by Holmes and Spears may be due to the activity of LPS, whether on its own, or complexed with MOMP as reported in the literature (Bacon *et al.* 1999). LPS has been detected in the OMP extractions, and the presence of an additional LPS band in *C. jejuni* 11168 (Figure 3.12), may answer questions such as why the bacterial MOMPs migrate at different molecular weights when run on the same SDS-PAGE, or why *C. jejuni* 11168 is consistently more cytotoxic than *C. jejuni* 11351. As LPS has been well characterised and reported in the literature, and is not a novel toxin, there seemed little point in continuing further work in this area. Cytotoxicity and cell morphology experiments had shown the presence of at least one other toxin in the bacterial cell extract (Table 3.3), and so future work will concentrate on the elucidation of less well characterised toxin(s).

6.7 CONTACT HAEMOLYSIN ASSAYS OF *C. JEJUNI* AND *A. BUTZLERI*

The usual method to determine whether a pathogen exhibits haemolytic activity is to grow the bacteria on agar plates containing 5% (v/v) defibrinated blood (Row & Welch 1994). Neither *C. jejuni* nor *A. butzleri* were found to be consistently haemolytic using this method, despite reports suggesting otherwise (Misawa *et al.* 1995; Tay *et al.* 1995). It is possible that something in the blood or media may be acting as an inhibitor against the bacteria, but even after

washing the blood and using agarose plates rather than agar, no increase in haemolytic activity was detected (data not shown). This could help to explain the inconsistency of reported haemolytic activity from *C. jejuni* and *A. butzleri* in the current literature.

However, when the bacteria were tested for haemolytic activity using the contact assay, all strains were found to exhibit some haemolysis, though the amount of haemolysis produced varied demonstrably between strains (see figure 4.4). Therefore, close contact between the bacteria and the blood cells is obviously necessary for optimum haemolysis production.

Early experiments comparing the *C. jejuni* 11351 and 11168 strains with *A. butzleri* showed *C. jejuni* 11168 to be more haemolytic than 11351 (see figure 4.1). This correlates with the cell cytotoxicity assays, where cell extract samples from *C. jejuni* 11168 were consistently more toxigenic than samples from *C. jejuni* 11351, suggesting that *C. jejuni* 11168 is a more virulent strain. The haemolytic activity from *A. butzleri* was notable, as the production of toxins from this organism is not well documented. The amount of haemolysin produced by *A. butzleri* 12481 was approximately three times that produced by *C. jejuni* 11168, suggesting that haemolytic activity may be an important virulence factor in *Arcobacter* infection.

Time course experiments showed that most haemolysis occured over the first five hours of the assay (figure 4.1). Therefore, future experiments were carried out over this shorter time period, as the long 20 hour incubation time is obviously unnecessary, and may lead to inaccurate results. The experiments with varying bacterial biomass (figure 4.2) showed that the rate of haemolysis was not directly proportional to the amount of biomass in the assay, and confirms that the large difference in percentage haemolysis seen between the *C. jejuni* and *A. butzleri* strains cannot be a result of additional biomass in the *A. butzleri* sample.

Experiments to determine whether the haemolysin(s) were membrane bound or released into the supernatant showed that for all three strains, the majority of the haemolytic activity was detected from the bacterial cell pellet, and very little

activity was released into the supernatant (figure 4.3). Sonicating the cells before removing the supernatant did not release any more of the haemolysin from the cell pellet, and suggests that whatever is responsible for the lysis of the blood cells is likely to be cell-associated and not released into the supernatant or growth media. This would explain why the bacterial cells and blood need to be in close contact, and it would appear that this haemolysin is distinct from the cytotoxin found in the cell free supernatants during tissue culture experiments in chapter 3.

Screening a wide range of *C. jejuni* strains from different sources showed that there was a great deal of variability of haemolysin produced from different strains (table 4.1). Figure 4.4 shows that there may be some sort of pattern between the source of *C. jejuni* and haemolytic activity. Poultry strains tended to be less haemolytic than those found in human disease. The strains responsible for outbreaks were all highly haemolytic organisms, and it would be interesting to test more strains from a wider geographic distribution to test this hypothesis. A recent paper (Guevremont *et al.* 2004), characterised over 100 *C. jejuni* and *C. coli* strains from healthy pigs and spontaneous diarrhoea in humans, and found that the genetic profiles from the porcine isolates were considerably different from the profiles obtained from the human isolates. They suggested that the hazard of contamination of humans by *Campylobacter* associated with pigs is low, as they found no epidemiological relationship between the two groups of isolates.

The data in figure 4.4 shows that from the strains tested, the human strains are more haemolytic than those found in chickens, where the chickens are healthy and show no signs of the disease. If it is true that different isolates have different toxicity levels, and infect different hosts, then haemolysis could be an important pathogenic detection mechanism. Miyamoto *et al.* (1969) reported that the production of a thermostable haemolysin by *Vibrio parahaemolyticus* is strongly correlated with its human pathogenicity, and a recent paper by Gilbert and Slavik (2004) suggests that *C. jejuni* isolates from poultry exhibit lower toxicity than those isolated from humans. Therefore it is possible that the production of

haemolysins by *C. jejuni* in some way correlates with its pathogenicity for humans and animals.

6.8 CONTACT HAEMOLYSIN ASSAYS OF *C. JEJUNI* AND *A. BUTZLERI* WITH VARYING CONDITIONS

It has been reported in the literature that many haemolytic toxins are proteinaceous, and hence destroyed by heat (Bhakdi *et al.* 1998; Hossain *et al.* 1993). Therefore, bacterial samples were boiled to denature any proteins and determine whether activity was lost. Figure 4.5 shows that some, but not all of the haemolytic activity was lost upon boiling the samples at 100°C for 10 minutes, although in the weakly haemolytic strains the majority of the activity was destroyed. Tay *et al.* (1995) reported that two different haemolysins were detected from different strains of *C. jejuni* clinical isolates. The first haemolysin was heat labile and inactivated at 100°C, whereas the other showed a decrease in haemolytic activity upon boiling, but was not inactivated and still produced moderate amounts of haemolysis. It is possible that there may be two different haemolysins present in the isolates tested in figure 4.5, with the majority of the activity caused by a heat-labile toxin, though there may also be a heat-stable toxin present, particularly in the more haemolytic strains.

Many bacterial haemolysins are involved in iron acquisition (Stoebner & Payne 1988; Braun *et al.* 1991) and there has been one report to date suggesting that *C. jejuni* haemolysin may be iron regulated (Hossain *et al.* 1993). It has also been reported that the *H. pylori* haemolysin is involved in iron acquisition (Segal & Tompkins 1994), and so an iron chelator (desferal mesylate) was added to both the growth media of the bacteria, and the contact assay. Figure 4.6 shows that the addition of desferal mesylate had no effect on the haemolytic activity produced by *C. jejuni* or *A. butzleri*, and it can be concluded that the detected haemolysin(s) do not appear to be iron regulated, suggesting that haemolysin does not play a major role in iron acquisition. Pickett *et al.* (1992) tested *C. jejuni* strains for their ability to acquire iron from heme-containing compounds using a plate assay, and concluded that haemolytic activity was detected, but did not

appear to be iron regulated, and was likely to be constitutively expressed. Similar experiments with *E. coli* haemolysins suggested that *E. coli* does not use haemolysins as a mechanism to acquire iron either (Law *et al.* 1992). Therefore, it is likely that the *C. jejuni* haemolysin may have another role – possibly the lysis of epithelial host cell membranes, or vesicle membranes for intracellular survival. *E. coli* haemolysin causes the release of inflammatory mediators at sublytic concentrations (Welch 1991) so it is possible that the *C. jejuni* haemolysin could have a similar role.

The majority of characterised haemolysins are calcium dependent (Braun *et al.* 1991; Welch 1991), and it has been reported that the addition of 10 mM EDTA, a calcium chelator, significantly reduces the amount of haemolysis produced by *H. pylori* (Segal & Tompkins 1994). When EDTA was added to the contact assay, haemolytic activity was greatly reduced in all strains, implying that the majority of haemolysis produced from *C. jejuni* and *A. butzleri* was calcium dependent (figure 4.7). Experiments with increasing amounts of EDTA showed that haemolysis could not be completely inhibited, suggesting that there may be another haemolysin present, particularly in the highly haemolytic strains, that is calcium independent.

Pore forming cytolysins have been shown to be sensitive to the presence of sugars such as dextran sulphate (dextran 5000). These sugars can inhibit RTX or pore forming toxins by inserting into the pore formed in the target cell membrane and inhibiting cell lysis (Martino *et al.* 2001). Figure 4.8 shows that dextran sulphate does not reduce the amount of haemolysis produced by the four lower haemolytic strains, therefore, it is not likely that the detected haemolysin has a pore forming mechanism of action. However, the two highly haemolytic strains of *C. jejuni* (5832) and *A. butzleri* (12481) showed significantly reduced haemolysis after dextran sulphate was added to the assay. This indicates that there may be an additional haemolysin in these two strains, and that this haemolysin may be an RTX or pore forming toxin.

Phospholipase C has been detected from a wide range of Gram positive and Gram negative bacteria (Songer 1997; Titball 1998). These enzymes are among

the most potent of phospholipase toxins, and have been reported from related pathogens such as *H. pylori* (Ansorg *et al.* 1993; Nilius & Malfertheiner 1996; Bode *et al.* 2001). However, Hossain *et al.* (1993) tested *C. jejuni* strains for phospholipase C using the egg yolk lecithin test, and concluded that this enzyme was not responsible for haemolysis. This agrees with the data shown in figure 4.9, which demonstrates that the addition of a phospholipase inhibitor showed no significant reduction in haemolysis, even when the highest concentration possible without lysing the cells was added. Therefore, it is unlikely that phospholipase C is responsible for the haemolytic reaction produced by *C. jejuni* and *A. butzleri.*

Phospholipase A has been reported to have haemolytic properties, and has been demonstrated in a variety of Gram negative organisms including *E. coli, H. pylori* and *C. coli* (Brok *et al.* 1995; Grant *et al.* 1997; Dekker 2000). A recent paper by Istivan *et al.* (2004), detected PLA_2 activity in *C. concisus*, and suggested that PLA_2 is a potential virulence factor in this bacterium. Figure 4.10 shows that the addition of the phospholipase A_2 inhibitor $PACOCF_3$ to the contact assay significantly reduced the haemolytic activity of all strains of *C. jejuni* and *A. butzleri*, but did not completely prevent haemolysis. Unfortunately it was impossible to determine whether all the phospholipase A_2 activity had been inhibited or not, as the concentration of the inhibitor could not be increased as it caused spontaneous lysis of the blood cells. Additionally, the phospholipase inhibitor only inhibits phospholipase A_2 activity, and there is currently no inhibitor available for phospholipase A_1 activity. It has been shown in *E. coli*, that phospholipase A_1 activity is 6 times higher than phospholipase A_2 (Horrevoets *et al* 1989). Therefore it is likely that phospholipase A_1 activity may be responsible for the residual activity.

When the *C. jejuni* and *A. butzleri* strains were applied to the $sPLA_2$ assay kit in the same bacterial concentrations as in the contact haemolysin assay, all strains were found to produce $sPLA_2$ (figure 4.11). The amount of $sPLA_2$ produced by each strain did seem to correlate with the percentage haemolysis usually produced by that strain i.e. the highly haemolytic strains such as *C. jejuni* 5832 and *A. butzleri* 12481 produced the most $sPLA_2$. This data also shows that the highest concentration of phospholipase A_2 inhibitor used in the contact assay did

not significantly inhibit $sPLA_2$ activity from any of the bacterial strains, or the pure phospholipase A_2. Therefore, it is possible that phospholipase A_1 or A_2 may be responsible for the haemolysin produced by the bacteria.

In conclusion, all strains of *C. jejuni* and *A. butzleri* have been shown to produce a haemolysin(s) that can lyse horse erythrocytes *in vitro*. Characterisation of the haemolysin has shown that it is membrane bound or cell-associated, predominantly calcium dependent, not regulated by iron, and some of its activity can be inhibited by dextran 5000, indicating the presence of both a pore-forming and a non pore-forming toxin. This correlates with work carried out by Grant *et al.* (1997), who detected a calcium dependent, haemolytic toxin in *C. coli*, which was identified as a phospholipase A gene, and was found to have reduced haemolytic activity when the *pldA* gene was inactivated.

It is likely that more than one haemolysin was detected by these assays, as the haemolytic activity was only partially destroyed by heating the toxin, suggesting that the activity may be due to both a heat stable, and a heat labile entity. This agrees with Tay *et al.* (1995), who reported the presence of both a heat stable and a heat labile haemolysin from different *Campylobacter* strains, and the dextran sulphate data suggests the presence of a pore forming toxin in addition to another haemolysin. The toxin was not found to have phospholipase C activity, but haemolytic activity was reduced upon addition of a phospholipase A_2 inhibitor. From this data it can be concluded that at least one haemolysin is produced by both *C. jejuni* and *A. butzleri*, and that one of these toxins may have phospholipase A activity.

6.9 SOUTHERN BLOTTING OF *C. JEJUNI* AND *A. BUTZLERI* DNA

Southern blotting of all strains of *C. jejuni* showed that the *pldA*, *tlyA* and cj0183 genes were ubiquitous in all strains of *C. jejuni* tested, even under high stringency conditions, where a low salt concentration in the hybridisation and wash solutions was used to reduce binding of the probe to non-complimentary sequences of genomic DNA. In addition, no reduction in dot intensity was seen

after high stringency washing of the blots, suggesting that these three genes are not only ubiquitious in *C. jejuni*, but also highly conserved. This indicates that all of these genes have an important function, as they seem to all have high sequence homology with the probe. However, the presence of the gene does not necessarily mean that the gene is expressed, or that the expression of the gene is the same for all strains.

Neither of the two strains of *A. butzleri* tested showed any hybridisation with any of the gene probes, despite using low stringency washes where sequences that were not entirely complimentary or contained a lot of mismatches could anneal. From these results it seems unlikely that the two strains of *A. butzleri* carry either *pldA*, *tlyA* or cj0183 genes that share homology with the same genes in *C. jejuni*. However, these genes may be present in *A. butzleri*, but may be insufficiently complimentary, or have only small sections of the gene that share homology with the *C. jejuni* probes.

6.10 THE *C. JEJUNI* PHOSPHOLIPASE A MUTANT

The phospholipase A mutant was constructed from *C. jejuni* NCTC 11168, using inverse PCR mutagenesis and insertion of a chloramphenicol antibiotic resistance cassette. When the *pldA* mutant was tested for haemolytic activity in the contact haemolysin assay, haemolytic activity was reduced by over 60% when compared to the activity of the wild type *C. jejuni* 11168 (see figure 5.7). The amount of haemolytic activity remaining when the mutant was tested in the contact assay (around 16%) was a similar level to the amount of activity remaining after 10mM EDTA was added to the contact assay to remove all available calcium (less than 12%, see figure 4.7). This was also comparable to the residual haemolysis after heat treatment of the *C. jejuni* 11168 samples (less than 20% haemolytic activity, see figure 4.6). This suggests that the majority of haemolysin produced by *C. jejuni* 11168 is a result of phospholipase A activity, and that this enzyme is heat labile and calcium dependent.

The literature states that in general, phospholipase A_1 does not require calcium ions for activation, and therefore is calcium independent (see section 1.7.4.1). In contrast, phospholipase A_2 has been shown to be calcium dependent (section 1.7.4.2) therefore it seems likely that the haemolytic activity detected is due to phospholipase A_2 activity. In agreement with this, the $sPLA_2$ assay of the *pldA* mutant (see figure 5.8) shows that almost all of the $sPLA_2$ activity of the mutant has been destroyed, demonstrating that the mutation of the *pldA* gene has worked, and suggesting that phospholipase A_2 is responsible for the majority of haemolysis produced by *C. jejuni* 11168. Unfortunately there is no assay for phospholipase A_1 activity, and since the *pldA* gene of other pathogens such as *E. coli* has been reported to comprise both PLA_1 and PlA_2 activity (Horrevoets *et al.* 1989), it is possible that the phospholipase activity may be a result of the activity of both enzymes. This agrees with the work of Grant *et al.* (1997), who identified a *pldA* gene in the related strain *Campylobacter coli.* Mutation of this gene was shown to reduce haemolysis, although *E. coli* cells expressing the purified protein did not demonstrate increased haemolytic activity. Similar work on the *E. coli pldA* gene failed to find a function for the phospholipase enzyme (Homma *et al.* 1984; Brok *et al.* 1994)

Although it has been demonstrated that phospholipase A contributes to the majority of the haemolytic reaction in *C. jejuni*, there is also a residual amount of haemolysis that does not appear to be reduced by the mutation of the *pldA* gene. Therefore, it seems likely that there is at least one more haemolysin present in *C. jejuni*. This haemolysin appears to be calcium independent and cell associated, and potentially heat stable. It is possible that there is more than one additional haemolytic toxin, and if so, the mechanism of action of one of these haemolysins may be pore-formation, as dextran sulphate did seem to reduce haemolysis in the highly haemolytic strains. Research into the haemolytic activity of *E. coli* has shown that there are at least three different genes responsible for haemolysis (Reingold *et al.* 1999). Two of these that have been identified as coding for RTX or pore-forming toxins, but the other has not yet been characterised. The related pathogen *H. pylori* is thought to have six haemolysin genes; two with homology to RTX cytotoxins, a phospholipase A, and a tlyA protein similar to the tlyA cytotoxin from *Salmonella hyodysenteriae*. The remaining two putative genes

have not been characterised (Martino *et al.* 2001). Therefore, it is not implausible that *C. jejuni* has more than one haemolysin domain.

The *clyA* and *clyC* genes do not appear to play any part in the haemolysis reaction, but may be responsible for colonisation of the bacteria in chickens. A *H. pylori pldA* mutant was shown to have reduced colonisation in mice in addition to demonstrating reduced haemolytic activity (Dorrell *et al.* 1999; Xerry & Owen 2001). Therefore it would be interesting to determine whether the *pldA* mutant from *C. jejuni* has any effect on colonisation. The *tlyA* gene in *H. pylori* has been shown to be partially responsible for haemolytic activity by pore formation (Martino *et al.* 2001; Zhang *et al.* 2002), but the *C. jejuni tlyA* mutant showed no such effect. More experiments need to be carried out on these genes to determine their function, but it is evident that neither gene plays a role in the haemolytic reaction of *C. jejuni.*

CHAPTER 7

CONCLUSIONS

Campylobacter jejuni is an important food-borne pathogen, responsible for the majority of food related illnesses worldwide The main aim of this research was to investigate the area of pathogenesis of *C. jejuni*, with particular emphasis on the production of toxins from this important pathogen, as the current understanding is poorly defined and the literature is confused and contradictory. In some of the later experiments, the related pathogen *Arcobacter butzleri* was included as a comparator. *A. butzleri* is a recognised veterinary pathogen, but recent research has demonstrated that its importance in human food-borne illness may be underestimated, and its disease mechanisms are currently unknown.

Cytotoxicity towards certain *in vitro* cell lines was detected from cell extracts of the two strains of *C. jejuni* used in this study. The detected toxin was determined to be either cell associated, and released from the outer membrane, or intracellular, and released upon lysis of the cells. In addition, the results showed that cell extract samples from *C. jejuni* 11168 were consistently more cytotoxic than samples from *C. jejuni* 11351.

Mouse neuroblastoma cells (N2a) and human endothelial cells (ECV 304) were found to be the most sensitive to the effects of the cytotoxin(s). Alternatively, chinese hamster ovary cells (CHO), commonly used to test for the *C. jejuni* cytolethal distending toxin (CDT), were not found to be significantly sensitive to cell extracts from either *C. jejuni* strain, despite reports to the contrary (Johnson & Lior 1998; Pickett 2000). However, morphology studies of CHO cells after treatment with the same concentrations of *C. jejuni* cell extract samples showed that sub-lethal changes had occurred inside the cells, despite the lack of cytotoxicity. The majority of the cells were distended in size to over twice that of the normal cells, suggesting the presence of the controversial cholera-like enterotoxin, which can be distinguished from the cytolethal distending toxin as it causes distension without cell death (Ruiz-Palacious *et al.* 1983; Wassenaar 1997).

Morphology studies were also carried out on ECV cells, where the same samples were applied, but different results were observed. Rather than the distension seen with the CHO cells, the majority of the ECV cells became elongated, leading to

significant cell death. These effects were detected within 48 hours, in contrast to the CHO experiments, where effects were most pronounced after 96 hours.

Overall, these results suggest that there is potentially more than one toxin in the *C. jejuni* crude cell extract samples. Heat and trypsin treatment of the samples before application to the cell lines did not significantly decrease the cytotoxicity, suggesting that the potential toxin(s) in the cell extract sample is not proteinaceous. It is unlikely that the toxin(s) detected is the cytolethal distending toxin, or the 70 kDa toxin reported in the literature. There was also no evidence of either shiga-like toxin or hepatotoxin activities.

These experiments suggest that the majority of the cytotoxic effects seen when *C. jejuni* cell extracts are applied to susceptible cell lines is a result of lipopolysaccharide (LPS) activity. However, it is unlikely that LPS is responsible for all the cytotoxicity observed. LPS is a potent endotoxin, released by Gram negative bacterial membranes upon cell lysis, and would be released during sonication of the cells. In addition, LPS is both heat and trypsin stable, as it is not a protein. It is possible that this LPS is complexed in some way with a porin protein, as several reports in the literature have detected a porin-lipopolysaccharide toxin that contains LPS, and shares homology with the *Campylobacter* MOMP (Moutinho-Fragoso *et al.* 1998; Bacon *et al.* 1999). This toxin is reportedly trypsin stable, and cytotoxic to Hep-2 and He-La cells, although has not been tested on any of the cell lines used in this study.

These experiments suggest that *C. jejuni* produces a number of different toxins that may vary either between strains, or within the same strain under different growth conditions. It is likely that repeatedly sub-culturing the bacteria may cause loss of expression of certain virulence genes, and it is recognised that *C. jejuni* is able to regulate its gene expression according to its environment (Penner & Aspinall 1997; Fry *et al.* 2000). This may explain why the current understanding of pathogenicity and toxin production by *Campylobacter* is limited and confusing, and it is clear that further research is needed in this area to clarify and fully understand the disease process of this important pathogen.

As a consequence of these results, outer membrane protein extractions were carried out on both strains, and the resulting proteins separated by SDS-PAGE. Two major bands predominated over all the others, and these were identified as the major outer membrane protein (MOMP) and the flagellin protein. The major outer membrane proteins from each strain were found to migrate at slightly different molecular weights, although no differences in the N-terminal sequence could be found to explain this discrepancy. It is known that *C. jejuni* is able to modify certain outer membrane genes in order to evade host immune responses, and so it is possible that the bacterium is able to modify its MOMP genes using similar mechanisms.

The N-terminal sequence of the MOMP was found to match that of the putative enterotoxin semi-purified by Holmes & Spears, suggesting that some of the effects seen in the cell culture assays could be a result of this toxin. The sequence was also a direct match with the porin toxin reported from *C. jejuni* by Bacon *et al.* (1999), and only one amino acid different from a sequence of a toxin found in *C. coli* (Mountinho-Fragoso *et al.* 1998), reinforcing the hypothesis that the MOMP may play an important role in the pathogenesis of *C. jejuni.* Unfortunately, when the purified *C. jejuni* OMP preparations were applied to cultured cells *in vitro*, no cytotoxicity was observed, suggesting that the MOMP is not cytotoxic to ECV, Vero and N2a cells. It is possible that the protein may have been denatured by the extraction process, losing its cytotoxicity as a result, or it may need to be cleaved before activation. Alternatively, it is likely that another entity in the bacterial cell extract is responsible for the cytotoxicity observed in earlier experiments.

Western blot experiments of the same OMP preparations, using an anti-cholera toxin antibody as a probe, did show strong cross reactivity with one of the bands on the blot, although this was identified as the flagellin protein. It is probable that since the flagellin protein is glycosylated, and may contain cholera toxin epitopes, that this cross reactivity was due to non-specific binding and not a result of sequence homology. Faint cross reactivity could be seen where small amounts of the cholera toxin had bound to the MOMP band, but again this was

likely to be due to non-specific binding. Therefore, if the MOMP is a toxin, it does not appear to share homology with cholera toxin.

Lipopolysaccharide was detected from both *C. jejuni* OMP samples, though an additional band was observed in the *C. jejuni* 11168 OMP sample that was not present in the *C. jejuni* 11351 sample. This could possibly explain the higher toxicity detected from *C. jejuni* 11168, and could also suggest why the MOMP bands migrate at different molecular weights on SDS-PAGE gels. These experiments further suggest that LPS may be responsible for the cytotoxic effects seen when *C. jejuni* cell extract samples are applied to eukaryotic cells *in vitro*, whether on its own, or complexed with the MOMP.

In addition to tissue culture experiments, both *C. jejuni* strains, and the related pathogen *A. butzleri* were found to exhibit haemolytic activity when in close contact with horse erythrocytes. Mirroring earlier experiments, *C. jejuni* 11168 was determined to be more haemolytic than *C. jejuni* 11351, suggesting that *C. jejuni* 11168 is a more pathogenic strain. However, *A. butzleri* 12481 was found to be approximately three times more haemolytic than *C. jejuni* 11168, implying that haemolytic activity may be important in *Arcobacter* pathogenesis.

In response to these experiments, a wide range of *C. jejuni* strains from various sources were screened using the haemolysin assay. The results indicate that there may be a connection between strain origin and haemolytic activity, as the strains isolated from humans and/or responsible for outbreaks tended to be more haemolytic than those isolated from chickens, although more strains would need to be tested to determine whether this observation is correct.

Further characterisation of the haemolysin(s) revealed that the toxin(s) was cell-associated, and not released into the supernatant or growth media even after sonication. This suggests that the haemolysin is attached to the bacterial cell wall and not actively secreted, therefore it is unlikely to be the same as the cytolysin detected in earlier experiments.

The majority of the haemolytic activity could be destroyed by heat, suggesting that the toxin was proteinaceous. However, some residual activity remained in the highly haemolytic strains, implying that there may be an additional heat stable toxin present in these strains. The haemolysin(s) does not appear to be iron regulated, suggesting that it does not play a major role in iron acquisition. In addition, the majority of the haemolytic activity is calcium dependent, but not all haemolysis was inhibited by a calcium chelator, further indicating that there may be more than one haemolysin produced by both *C. jejuni* and *A. butzleri*.

The addition of dextran sulphate to the bacteria had no effect on the haemolytic reaction in four of the strains implying that the mechanism of action of the haemolysin(s) is not by pore formation. However, the two highly haemolytic strains of *C. jejuni* (5832) and *A. butzleri* (12481), showed a significant reduction in haemolytic activity after the addition of dextran sulphate, suggesting that there may be an additional haemolysin in these strains which is likely to be an RTX or pore-forming toxin.

As the majority of the haemolytic activity detected was not due to a pore-forming toxin, it was postulated that a phospholipase enzyme might be responsible. Experiments using a phospholipase C inhibitor had no effect on haemolysis, although a phospholipase A_2 inhibitor was found to significantly reduce haemolysis. All strains were found to produce secretory PLA_2, although the amount of this enzyme released by each bacterium varied, and was found to correlate with the percentage haemolysis produced by each strain. From these experiments it can be concluded that haemolytic activity is produced by all strains of *C. jejuni* and *A. butzleri* tested, that this activity is a result of multiple toxins, one of which may be phospholipase A.

Southern blot experiments were carried out on all *C. jejuni* and *A. butzleri* strains tested in the contact assay. These showed that the *pldA, tlyA* and *cj0183* genes were ubiquitous in all strains of *C. jejuni* tested, and fairly highly conserved, even after high stringency washing. No hybridisation was seen with any of the three probes and *A. butzleri* DNA, suggesting that *pldA, tlyA* and *cj0183* genes in *C. jejuni* do not share homology with genes in *A. butzleri*.

The results of the haemolysin assays suggested that phospholipase A might be responsible for haemolytic activity in *C. jejuni*. Therefore, a phospholipase A mutant was constructed from the type strain *C. jejuni* NCTC 11168. The mutant was found to have significantly reduced haemolytic activity of over 60% when compared to the wild type *C. jejuni* 11168. This reduction in haemolysis correlated to the amount of reduction observed when a calcium inhibitor was added to the assay, and was similar to the amount of activity lost upon boiling the samples. This suggests that the majority of the haemolytic activity produced by *C. jejuni* NCTC 11168 is a result of phospholipase A activity, and that this enzyme is heat labile and calcium dependent. In addition to the haemolytic activity attributed to phospholipase A, it is likely that there is at least one other haemolysin present in *C. jejuni*. This toxin(s) appears to be calcium independent, cell associated, and heat stable. It is also possible that it may have a pore-forming mechanism of action.

In summary, *C. jejuni* has been shown to possess cytotoxic activity when cell extract samples from both *C. jejuni* 11168 and 11351 samples are applied cultured cells *in vitro*. The toxin was shown to be both heat and trypsin stable, and not due to the activity of a cytolethal distending toxin, a shiga-like toxin, a hepatotoxin, or the 70 kDa toxin reported by other groups. It is also likely that more than one toxin is present in these crude extracts, and that most or all of the activity is caused by lipopolysaccharide activity. In addition to this cytotoxin, both *C. jejuni* and *A. butzleri* also possess at least one haemolysin. The majority of the haemolytic activity from *C. jejuni* is a result of phospholipase A activity, but there is likely to be at least one other haemolysin which may be a pore-forming or RTX toxin. The *clyA* (*tlyA*) and *clyC* (*cj0183*) genes do not appear to have a role in the haemolysis reaction of *C. jejuni*, but further experiments have shown that they may be responsible for colonisation in chickens (Wooldridge *et al.* 2003).

Unfortunately, due to time constraints, the research had to conclude at this point. Planned future work included the application of the *pldA* mutant to *in vitro* cultured cell lines, expression and purification of the pldA protein, and colonisation studies of the mutant in chickens, as several reports suggest that

phospholipase A may play a role in colonisation (Dorrell et al. 1999; Ziprin et al. 2001). It is clear that further research is needed into the mechanisms of pathogenesis of this bacterium, and attention should be paid to the related pathogen *A. butzleri*, which may be more important than current opinion suggests.

Despite nearly 30 years of research since *Campylobacter* was first detected, and the publication of the *C. jejuni* genome, there is still very little understanding as to how *C. jejuni* causes disease on such a large scale. Reducing the numbers of *Campylobacter* in chicken carcasses, and educating the public to pursue better cooking methods will help to reduce the spread of the disease, but determining the mechanisms of infection of this important pathogen may lead to complete eradication of the disease. This study represents one of the first attempts to characterise haemolytic activity in both *C. jejuni* and *A. butzleri*, although further investigation is necessary to determine the importance and role(s) of this potentially pathogenic property.

CHAPTER 8

APPENDIX

60421 tgcttccttt tttagaacaa aactataaaa taatcatagc aaaaacccct ttgcataaac

60481 atcccttatg tggcttttat gacagctctt tagctcaaac ttgtaaaaat ttcttagaaa

60541 aaaatgaaca aaaaatagga cttttatttt ctgagattaa aaccaaattt gtagaatttg

60601 aagatgaaga tgctttttta aatcttaatt tttatgaaga atatgaaaaa tttaaaagcc

60661 agttaaaatg agaaaaattg ctttattttt aagcttatgc gtttttatat gggctagtga

60721 tttacaacaa gctttagagt atgaaaaaca aggcgattat aaaaaagcta tggaaattta

60781 caaaaaactc gctttaaaaa attcatctgt tttgatttct caagaacaaa ataactcaag

60841 tcaagcaaca caaacgcaaa attctatcac tataaaaaaa gaagaaaaac aagacttttc

60901 tcgcttagct ttggctaatt atcttggtga aaatgaaagc tttaatcccc ttggcattag

60961 ctcttataag atgaattatt ttttaccttt tgcttatagt tttaattctt taggagtaaa

61021 taataataag agtgaggcga aattccaact tagtgttaaa aaaagacttt ttgaaaattt

61081 actaggacta gatgaaaaat attatatagc ctatactcaa acttcttggt ggcaaattta

61141 tgagcattct tctccttttа gagaaaccaa ctatcaacca gaatttttta tagatcttcc

61201 tttatatctt aaagattatg aatttttttaa caatctacgc gtaggtatat tgcatgaaag

61261 caatggcaag ggagatgaga atttacaatc tcgctcatgg aatagaattt atgtatctac

61321 tgctatttta tataataaat ttttatttgt tccacgcctt tggtatagaa ttcctgaaaa

61381 caaaaaagat gatgataatc ctgccatctt gcattatatg ggaaattttg atgtgaattt

61441 agcgtattta ggtgatgatt attttatcaa tcttatgcta agaaataatc ttaaattcca

61501 caataacaaa ggcgctatac aagttgatct aggatatgat atttttaata acggaattta

61561 ttggtattta caatacttta atggctatgg agaaagcctt atagactata acaaacactt

61621 gcaaagactt tccactggat ttttgatttc ttactaaaat atcctttata gttttatcat

61681 ttcttaaaac aaatttcatt acaatttcat ttttgataat tattattact ataaggaaag

61741 atttgttttt taagcatata ttgagtttaa aagtgcttat agctttactt ttattctttg

61801 gaatgataag tttatttata ggagttatca gtatcaatgt aaaagatatt cttaatctta

61861 actccactca actagaaatc ataactctca caagaattcc tagacttata gcgattttac

Figure 8.1 - *C. jejuni* NCTC 11168 *pldA* complete sequence (yellow), with primers for southern blot probe shown in pink, and primers for inverse mutagenesis shown in blue. Where primers are within the gene sequence, the sequence is shown in light blue.

234061 ttagagaaat ttttaaaaaa aatgcttttg aaataggtat ttggtggttt tctaaagaaa

234121 aagaagactt agaaaaaaga ataaatgaga gtttaaaatg agatttgatt tttttgtttc

234181 aaagcgttta aatatcagta gaaataaagc cttagagctt atagaaaatg aagagatttt

234241 acttaatggt aagagtttta aagcttcttt tgatgtgaaa aatttttag aaaatttaaa

234301 aaaaacgcaa gatttaaatc ctgaagatat acttttagcc aatgagttaa aattggatct

234361 tttaagtgaa atttatgttt caagagcagc tttgaaatta aaaaaatttt tagaagaaaa

234421 tgatattgaa ataaaacata aaaattgtct tgatatagga tctagtacgg gcggttttgt

234481 tcaaatttta cttgaaaatc aggctttaaa aatcactgct cttgatgtgg gtagtaatca

234541 actccatcca agtttaagag taaatgaaaa aattatcttg catgaaaata cagatcttag

234601 agcctttaaa agtgaagaaa aatttgaact tgttacttgt gatgtgagtt ttatttctct

234661 tattaattta ctttattata ttgataattt agctttaaag gaaattattt tacttttaa

234721 acctcagttt gaagtgggaa aaaatatcaa aagagataaa aaaggtgttt taaaagatga

234781 taaggcaatc ttaaaggcaa gaatggattt tgaaaaagca tgtgctaaat tgggttggct

234841 tttaaaaaat acgcaaaaat caagcattaa aggaaaggaa ggtaatgttg aatatttta

234901 ctactatatc aaaaattaat attacctctt tagccatagg ttgttttgat ggtatgcatt

Figure 8.2 - *C. jejuni* NCTC 11168 *tlyA* (*clyA*) complete sequence (yellow), with primers for southern blot probe shown in pink.

179101 ttttagtatt tttatcacaa actggacaac tatcactcaa cttagaagta tatataagcg
179161 tttaaaagaa tttgagaaaa atatttctta taagtcttag ttttttctta taaaatacaa
179221 gaaaaaattc aaatattaaa ttactttta attgtgtata ataaggttta ataatatttt
179281 atttcttaag gagcttcatt ggaccccagt caggttttgg atttaaacca aacttctaca
179341 gcatcttttg atgcaggata ttctatactt atggttgttg ttgcacttgc tctagtgttt
179401 ttaaatggtt tttttgtttt gtctgaattt agtattgtta aagtacgtcg ttccaagctt
179461 gaagagatgg taaaagaaaa aaaagctggt gccaagaaag ctttggaggt tacttcaaga
179521 cttgatactt atcttagtgc ttgtcaatta ggaatcactt taagttctct agctcttggt
179581 tggataggtg agcctgctat tgcaaaaatg ctagaaattc cgcttattaa tcttggtttt
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179701 catgtggttt taggagaact tgtgccaaaa agtatagcta ttgcagttgc agataaagcg
179761 gttttattta tagctagacc gcttcattgg ttttggatgc tctttttgcc ttgtattaaa
179821 attttcgatt ttttagcagc tataagtttg aaacttttttg gaataaaacc tgctaaagaa
179881 agcgagctaa ctcatagcga agaagaaatt aaaatcatag caagtgagag tcaaaaaggt
179941 ggagttttag atgaatttga aacagagatc atacgcaatg ctgttgattt ttcagatact
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180061 tacgaagaaa atatgcaaat catttgtgag cataaacata ctcgctttcc ttatattgat
180121 ggctctaaag atactatttt aggtatgata cacatacgag atataataca aaatgaatta
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180541 cctatggtag gagatcgcat tgaagatgaa ctttgttact atgaagttaa aaaaatggat
180601 gggaattcta tagagcgtgt taaggtggtt aaaaaaacaa ataaagatga agaataagcc
180661 tctttaggct tatttctttt tgtaaatttg gtttttaatt tctatacaca ctcaaaacct
180721 ttttcatcta gtgtaactac gcgtaaatat ccaccatcat ctattttacc ctcgcataaa

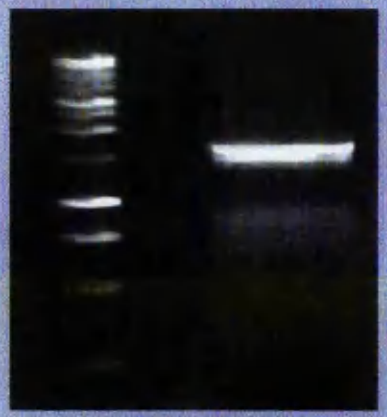
Figure 8.3 - *C. jejuni* NCTC 11168 Cj0183 (*clyC*) complete sequence (yellow), with primers for southern blot probe shown in pink.

Mutant Construction

- *pldA* gene ~ 1 kb
- Primers designed from published genome to incorporate ~ 0.8 kb flanking DNA

PldAF: 5′ ATCTAGCCGTATGGGACAAG 3′

PldAR: 5′ GTTATGATTTCTAGTTGAGTGGAG 3′



Cloned into T7/NT-TOPO vector

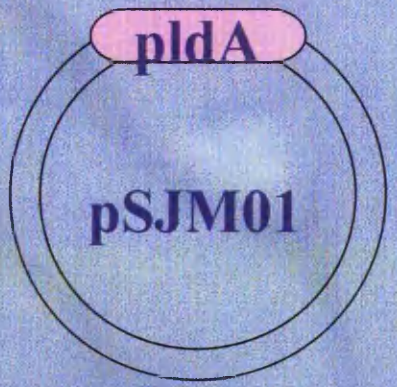


Figure 8.4 – Construction of pSJM01, the plasmid carrying the *pldA* gene from *C. jejuni* NCTC 11168

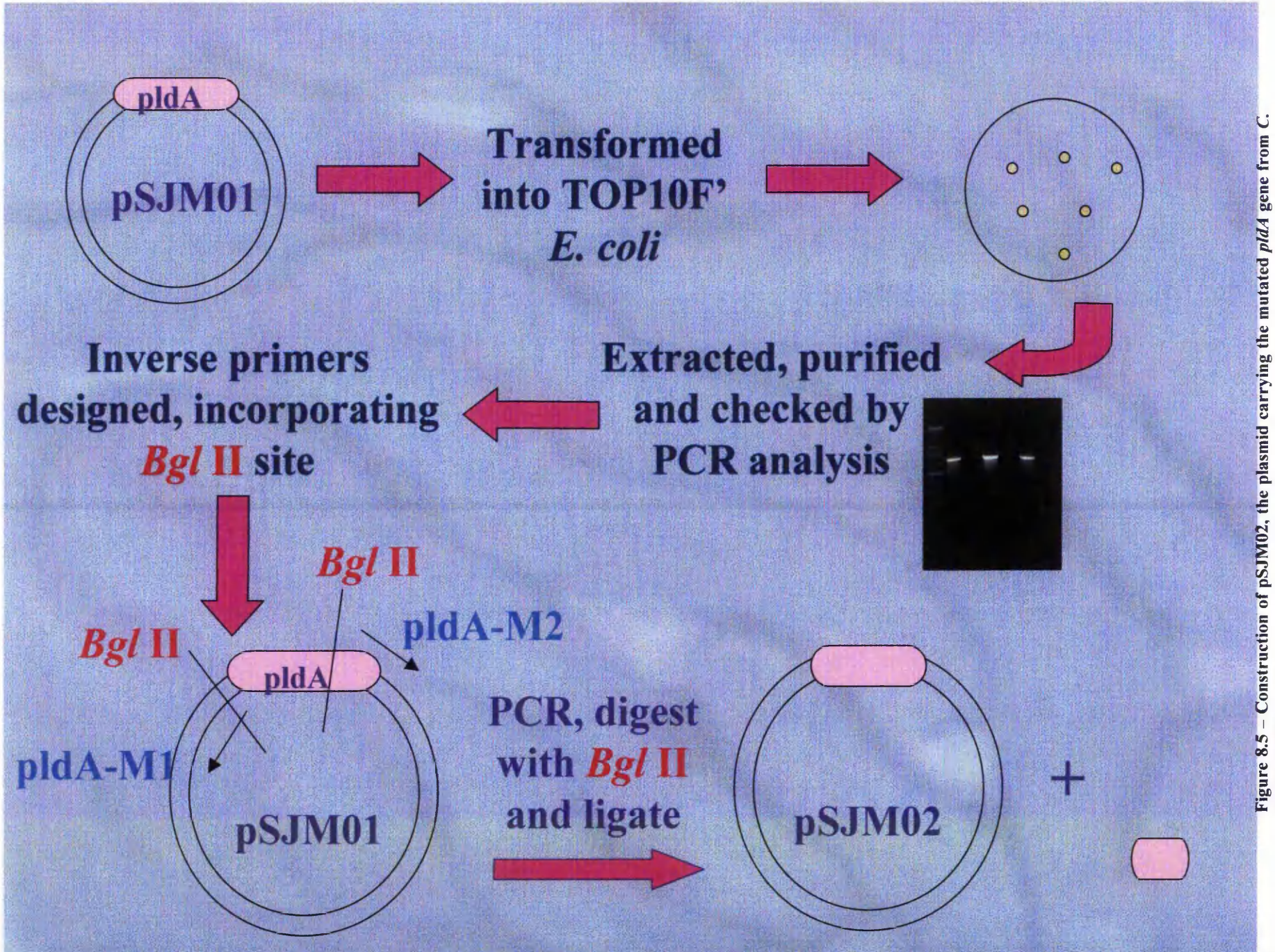


Figure 8.5 – Construction of pSJM02, the plasmid carrying the mutated *pldA* gene from *C. jejuni* NCTC 11168

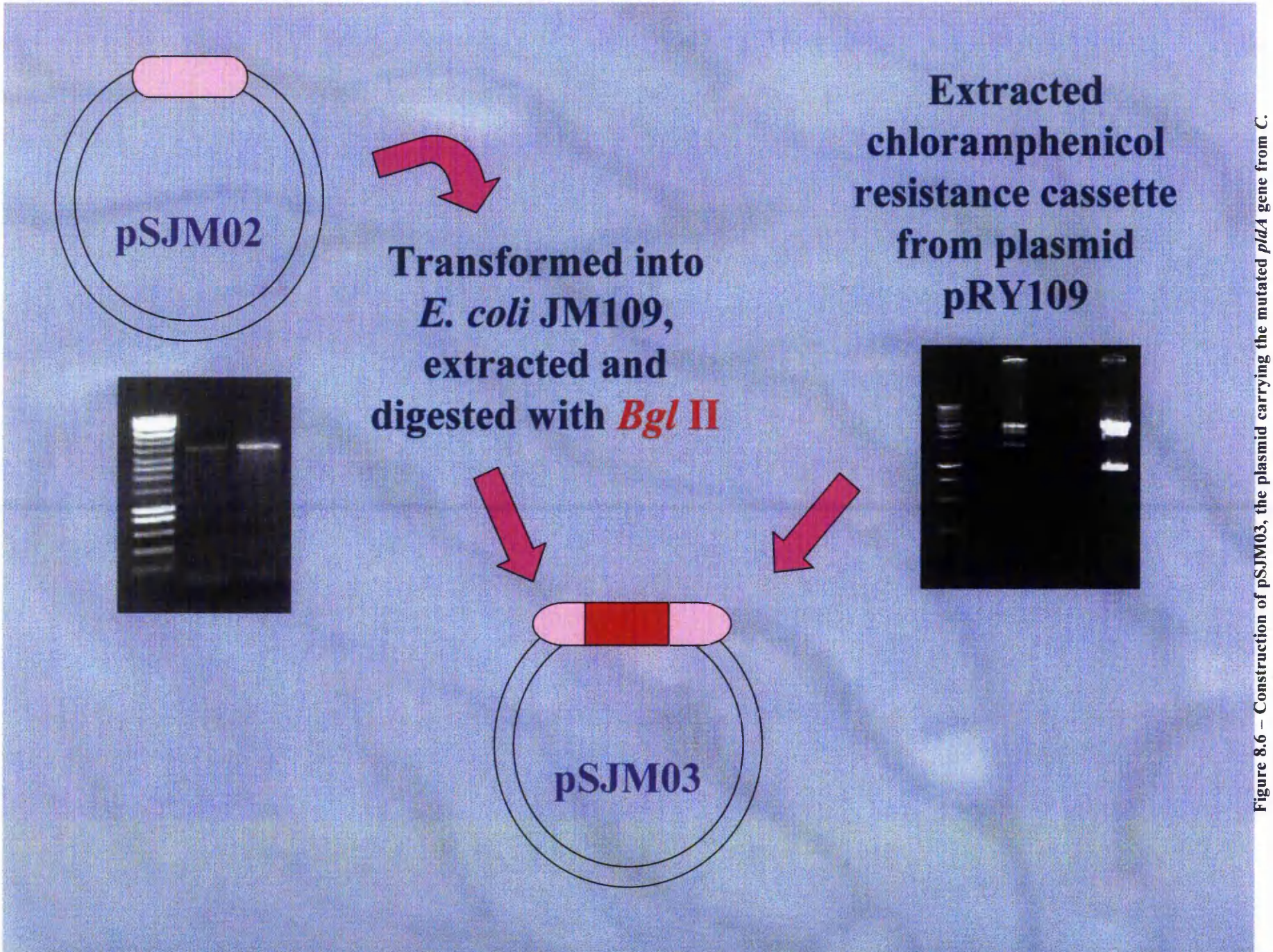


Figure 8.6 – Construction of pSJM03, the plasmid carrying the mutated *pldA* gene from *C. jejuni* NCTC 11168 and a chloramphenicol resistance cassette.

Investigation and Characterisation of Haemolytic Activity from *Campylobacter* and *Arcobacter* spp.

Samantha J.S. Morris, A. J. Hargreaves and S.J. Forsythe.
The Nottingham Trent University, Nottingham, NG11 8NS.

INTRODUCTION

- *Campylobacter jejuni* has been recognised as a major cause of human gastroenteritis in both industrialised and developing countries.
- A major virulence factor of campylobacter enteritis is the production of one or more toxins. It is recognised that toxins may be produced during the disease process, but the current understanding of the mechanisms and relevance to infection is unclear, and much of the literature is contradictory (Wassenaar 1997).
- *Arcobacter* spp. are both genetically and morphologically similar to campylobacter, and have recently been identified as newly emergent veterinary and potential human pathogens (Mansfield & Forsythe 2000).
- *A. butzleri* is the most reported human pathogen of the genus, and is commonly isolated from patients presenting similar symptoms as those with campylobacter infection, but is not yet accepted as an important human pathogen.
- There are several reports of potential haemolysin activity from *Campylobacter* spp. but little attempt has been made to characterise the toxin(s) further. To date, haemolytic activity has not been investigated in *Arcobacter* spp.

AIM

The aim of this study was to investigate the haemolytic reaction from a range of different isolates of *Campylobacter* and *Arcobacter* spp. and to further characterise the haemolysin(s) detected from these important pathogens.

METHODS

Strains

- A range of 23 *C. jejuni* and *A. butzleri* isolates from humans, cattle, chicken, pigs and water were screened for haemolytic activity, and 4 *C. jejuni* and 2 *A. butzleri* strains selected as a representative sample for further characterisation experiments.
- All strains were grown on blood agar plates (BA) for 3 days at 37°C under microaerobic conditions. For iron limitation studies, the bacteria were grown in the presence of 50μM desferal mesylate.

Contact Haemolysin Assay

- Aliquots of 0.5ml washed 1 x 10^9 bacterial suspension were added to 1ml washed horse blood in a sterile centrifuge tube
- Where stated, $PACOCF_3$, compound 48/80 (phospholipase C inhibitor), dextran sulphate, EDTA, and desferal mesylate were included in the assay at the appropriate concentrations.
- Control tubes were set up by replacing the bacterial suspension with either PBS (negative control) or sterile distilled water (positive control). The samples were centrifuged at 3000g for 10 mins and incubated at 37°C for 5h.The tubes were then vortexed, recentrifuged, and the absorbances read at 540nm and calculated as a percentage of the positive controls

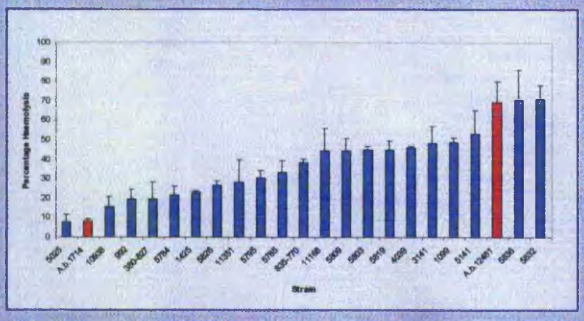


Figure 1 – Contact assays showing haemolytic activity of 21 *C. jejuni* strains and 2 *A. butzleri* strains from a range of sources

- Twenty one strains of *C. jejuni* and two strains of *A. butzleri* were screened for haemolytic activity.
- All strains were found to cause lysis to red blood cells in the contact assay, though this varied demonstrably between strains (figure 1).
- Four strains of *C. jejuni* and the two strains of *A. butzleri* showing differing haemolytic activity were chosen as representative samples and screened for activity against a range of inhibitors to try to further characterise the haemolysin(s).

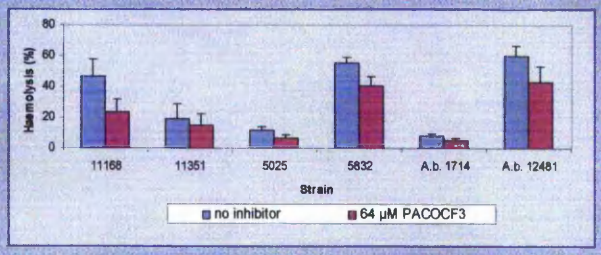


Figure 2 – Contact haemolysin assays of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of $PACOCF_3$

- The phospholipase A_2 inhibitor ($PACOCF_3$) significantly inhibited some of the haemolytic activity from all *C. jejuni* and *A. butzleri* strains tested (figure 2).
- The concentration of $PACOCF_3$ could not be increased further, as the inhibitor causes lysis itself at higher levels.
- It is likely that at least some of the haemolytic activity produced by *C. jejuni* and *A. butzleri* strains is a result of phospholipase A_2 activity
- The assay was also carried out in the presence of phospholipase C inhibitors, although no significant inhibition of haemolysis was determined (data not shown).

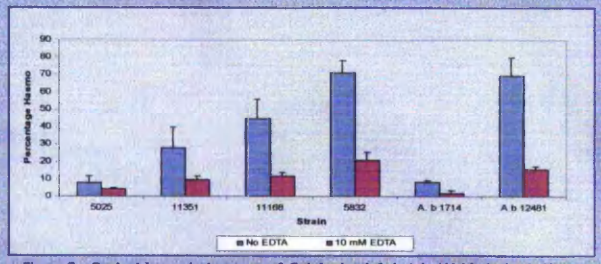


Figure 3 – Contact haemolysin assays of *C. jejuni* and *A. butzleri* (A.b) strains in the presence and absence of EDTA

- The addition of EDTA to the contact assay drastically reduced (up to 75%) haemolytic activity in all strains tested (figure 3).
- Therefore, it is likely that at least one haemolysin is calcium dependent.
- Growing and assaying cultures in the presence of the iron chelator desferal mesylate (50 μM, data not shown), had no effect on the haemolytic activity, suggesting that haemolysis does not play a major role in iron acquisition.
- Dextran sulphate (30 mM), an inhibitor of pore forming/RTX toxins, only inhibited the haemolysis of *C. jejuni* 5832 and *A. butzleri* 12481, the strains with high intrinsic haemolytic activity (data not shown).

CONCLUSIONS

- All *C. jejuni* and *A. butzleri* strains tested were haemolytically active, though this varied demonstrably between strains.
- The activity was found to be at least partially calcium dependent.
- Inhibitor studies have shown that the haemolytic activity does not appear to be due to the actions of a phospholipase C, but is partly a result of phospholipase A_2 activity. There are currently no inhibitors to block phospholipase A_1 activity.
- Dextran sulphate slightly inhibited activity in the two highly haemolytic strains. This suggests that there may be more than one haemolysin detected, and at least one of these may be an RTX toxin.
- Growing and assaying the strains in the presence of an iron chelator has no effect on haemolytic activity, suggesting that haemolysis does not play a major role in iron acquisition.
- The presence of haemolytic activity in all strains tested, suggests that haemolysis may be an important virulence factor in *Campylobacter* and *Arcobacter* pathogenesis.

ACKNOWLEDGEMENTS

We would like to thank Professor Stephen On for kindly donating many of the *C. jejuni* strains used during the course of this work. Also thanks to Dr. Karl Wooldridge and Professor Diawer Ala'Aldeen for their help and assistance following on from this research.

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Figure 8.7 – Poster P29, presented at CHRO, Aarhus, Denmark, September 2003

The University of Nottingham

MBIG

Two toxin-related genes of *C. jejuni* have a role in colonisation of the chicken

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Stephen Forsythe[3] & Dlawer Ala'Aldeen[1]

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Introduction

Haemolytic activity has been described in campylobacter species and, in *C. coli*, the *pldA* gene (phospholipase A) has been shown to mediate the majority of haemolytic activity[1]. In *C. jejuni*, *pldA* has been shown to be required for efficient colonisation of chickens[2]. *C.jejuni* 11168 posesses two additional genes: Cj0588 and Cj0183 (here called *clyA* and *clyC*), with homology to reported haemolysins of other organisms. We demonstrate that neither gene appears to have a role in haemolysis but that both are essential for efficient colonisation of the chicken.

ClyA and ClyC are predicted to localise to the cytoplasmic membrane. Bioinformatic analyses predict that ClyA and ClyC are both localised in the cytoplasmic membrane (Fig 1).

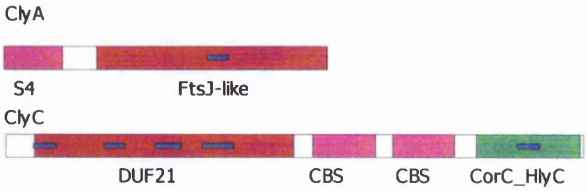

Fig 1. Schematic diagram of ClyA and ClyC. Pfam domains are indicated and predicted transmembrane domains are depicted in blue. S4 and FtsJ-like domains are implicated in RNA methylation. The role of DUF21 is unknown but it usually occurs with 2 or more CBS domains, which are thought to have a regulatory role. CorC_HlyC is thought to be involved in ion transport modulation.

Cloning, expression and mutagenesis. ClyA and ClyC were expressed, purified and used to raise antisera. Expression of *clyA* and *clyC* could not be detected in immunoblots of whole cell extracts of *C. jejuni* 11168 or several clinical isolates (not shown). A very faint band of the expected size was detected in a cytoplasmic membrane-enriched fraction of *C. jejuni* 11168 (Fig. 2). *clyA*, *clyC* and *pldA* genes of *C. jejuni* 11168 were disrupted by insertion of either a chloramphenicol (*clyC*, *pldA*) or kanamycin (*clyA*) resistance cassette.

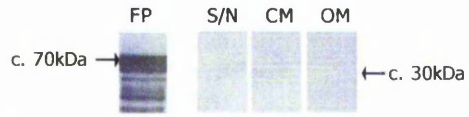

Fig 2. Subcellular localisation of ClyA.Concentrated supernatants (S/N) and fractions enriched for cytoplasmic membrane proteins (CM) or outer membrane proteins (OM) were probed with anti-ClyA (maltose-binding protein fusion; ClyA-MBP). The ClyA-MBP protein probed with the same serum is also shown (FP)

***pldA* but not *clyA* or *clyC* contributes to haemolytic activity.** The haemolytic activity of the *pldA* mutant was approximately 40% of wild type. The activity of the *clyA* and *clyC* mutants, however, was not reduced compared to wild type cells (Fig. 3).

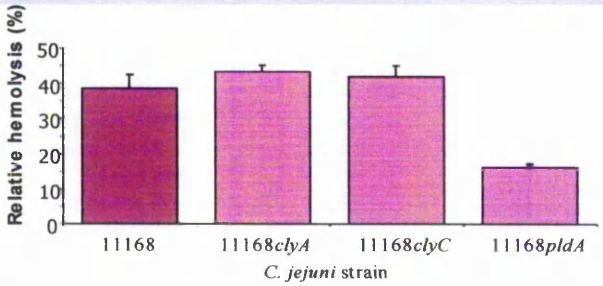

Fig 3. Haemolytic activity of *C. jejuni* 11168 and isogenic *clyA*, *clyC* and *pldA* mutants. Cells of each strain were tested in an assay for haemolytic activity using horse erythrocytes. Activity is expressed as a percentage of total haemolysis.

***clyA* and *clyC* mutants are unable to efficiently colonise the chicken gut.** Wild type cells colonised all birds within 3 weeks and colonisation was maintained throughout a 6-week period. A low level of initial colonisation by the *clyA* mutant was observed but by week 3 no further bacteria could be detected. The *clyC* mutant also showed a marked reduction in its ability to colonise chickens (Fig. 4).

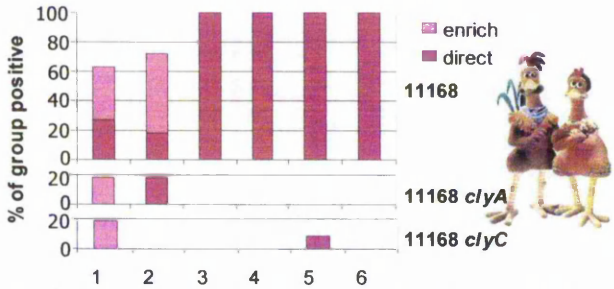

Fig 4. Chicken colonisation experiment comparing wild-type *C. jejuni* strain 11168 and its isogenic *clyA and clyC* mutants. Detection by direct culture (maroon) or after enrichment (pink) is indicated. Each group consists of 20 birds.

Conclusions and Discussion

clyA and *clyC* play an essential role in efficient colonisation of the chicken by mechanisms that remain to be determined. Haemolytic activity of *C. jejuni* 11168 was shown to be mainly due to the activity of the *pldA* gene. *clyA* or *clyC* were annotated as putative haemolysins due to similarity with the reported haemolysins of *Brachyspira hyodysenteriae*. The haemolysin of this organism has been shown to be distinct from those genes, however, and it has been suggested that they may play a regulatory role[4]. The predicted location and the presence of putative regulatory domains within ClyA and ClyC also argues against a role as haemolysins.

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Figure 8.8 – Poster I-43, presented at CHRO, Aarhus, Denmark, September 2003

"The successful clone" revealed: genetic and phenotypic characteristics distinguish "low" and "high"-risk *Campylobacter jejuni* strains

Stephen L. W. On[1*], Nick Dorrell[2], Lise Petersen[3], Dang D. Bang[4], Samantha Morris[5], Stephen Forsythe[5], David W. Ussery[6], and Brendan W. Wren[2]

Danish Veterinary Institute, [1]Copenhagen and [4] Aarhus, Denmark; London School of Hygiene and Tropical Medicine, London, UK[2]; [3]Bioinformatics Centre, University of Copenhagen, Denmark; [5]Nottingham Trent University, Nottingham, UK; [6]Centre for Biological Sequence Analysis, Lyngby, Denmark. *Presenting author. E-mail sto@vetinst.dk

INTRODUCTION

Some molecular epidemiological studies of *C. jejuni* have suggested that not all animal isolates are equally pathogenic to humans and may be animal host-adapted, unable to infect humans.

To determine the validity of this hypothesis, we critically compared genetic, survival and toxigenic properties of strain populations considered to be "low" and "high" risk to human health.

METHODS- 1

Strains. Twelve Danish strains of diverse origin were examined. By molecular epidemiological studies (On et al. 1998, On and Harrington 2001, Siemer et al. 2002), 7 were considered to be "high-risk" and 5 "low-risk" to human health.

Whole-genome microarray analysis. Hybridisations were performed in triplicate using the PCR-clone library array derived from NCTC 11168 as described previously (Dorrell et al., 2001). Subsequent lists of genes not detected in field strains were interrogated by a program written in PERL.

METHODS - 2

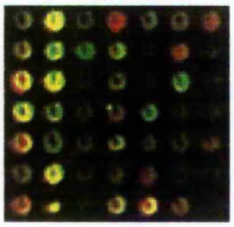
Prediction of expression potential. The degree of flexibility for each annotated gene in NCTC 11168 was calculated according to Satchwell et al. (1986) as described previously. Values below 0.15 were considered to represent genes that are highly expressed.

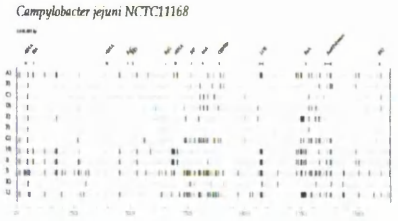
Survival at room temperature in air. Standardised (OD 1.0 at 450 nm) suspensions of four-day old cultures were made in nutrient broth, kept at room temperature under aerobic conditions, and examined for viability for up to six days. Trends in cell death were determined using duplicate analyses in Graphpad Prism v. 3.0.

Cytolethal distending toxin production. CDT activity was quantitated in Vero, chicken embryo and colon 205 cell lines as described previously (Bang et al. 2001).

Haemolysin activity. A contact haemolysin assay based upon the method of Istivan et al. (1998) was used. A 1:2 ratio of bacterial cell suspension (10^{10} cfu/ml) to washed blood cells was incubated at 37°C for 5 h and % of haemolysis determined by comparing ODs pre- and post-incubation.

RESULTS



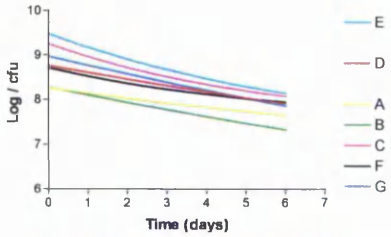


Genomic diversity among field strains by microarray analysis

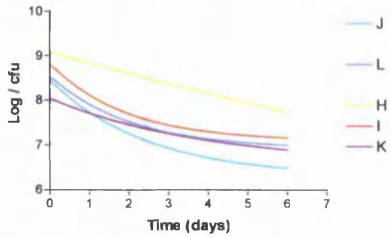
Comparative genetics: are there genes "missing" in certain groups of strains?
Chicken strains only: 0
Turkey strains only: 0
Cattle/pig strains only: 0
≥ 3 "High risk" strains, ≤2 "Low risk" strains: 1

≥ 3 "Low risk" strains, ≤2 "High risk" strains: 94

"Low risk" strains differ substantially in gene content or composition compared with NCTC 11168



Rate of cell death, "high-risk" strains



Rate of cell death, "low-risk" strains

Strain code	Source	CDT titre	Haemolysin activity	Mean rate of cell death	Rate of cell death, first 24 hrs
A	Bovine	1:100	46	0.191	0.125
B	Bovine	1:80	8	0.160	0.158
C	Bovine	1:80	41	0.200	0.306
D	Bovine	1:80	41	0.148	0.361
E	Chicken	1:80	45	0.111	0.132
F	Chicken	1:100	23	0.125	0.097
G	Chicken	1:80	22	0.191	0.204
H	Chicken	1:80	38	0.231	0.251
I	Chicken	1:100	19	0.270	0.714
J	Turkey	1:80	ND	0.313	0.729
K	Bovine	1:100	ND	0.198	0.335
L	Turkey	1:10	ND	0.271	0.641

Table 1. Sources, CDT and haemolysin activities and survival characteristics of strains examined

CONCLUSIONS

Microarray analysis shows a substantial gene-level difference between "low risk" strains and NCTC 11168

No genetic basis for host specificity of strains was found

Both low- and high risk strains may exhibit high and low CDT and haemolysin activities: most "high risk" strains survive aerobic conditions better than "low-risk strains

Thus the health risk potential of *C. jejuni* strains has a demonstrable genetic and phenotypic basis

WHERE ARE THE MAJOR "HIGH-RISK" MARKERS?

74 genes are distributed in 12 major clusters

At least seven of these gene clusters seem to be surface-associated (structure, transport), including LOS and surface polysaccharide loci

3 of 12 groups contain hypothetical proteins, or genes of unknown function

Fla A/B and sugar metabolism genes comprise the remaining major groups

Eight of the clusters contain genes with *RpoD* promoters (Petersen et al., 2003)

Only 9 genes are predicted to be highly expressed: most (except *Fla A/B*) are widely distributed in the genome

ACKNOWLEDGEMENTS

This work was supported by grants from the Danish Directorate for Food and Agribusiness, the Danish Research Council, the Wellcome Trust and BBSRC.

Figure 8.9 Poster I-32, presented at CHRO, Aarhus, Denmark, September 2003

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