ISOLATION AND MOLECULAR CHARACTERISATION OF TOXINS FROM *CAMPYLOBACTER JEJUNI* AND RELATED SPECIES

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Abstract

Campylobacter jejuni is a major cause of food-borne infectious disease in the UK. Last year, approximately 54 000 cases of such diseases caused by *C. jejuni* were reported to the Communicable Disease Surveillance Centre (CDSC) for England and Wales. This level of infection exceeds that of other food poisoning organisms such as *Salmonella.* A significant level of research has been carried out in the last 20 years to try and elucidate the precise pathogenic mechanisms of *C. jejuni*, including the recent completion of the genome sequence of *C. jejuni* NCTC 11168. There is, however, a considerable amount that remains unknown about the mechanisms of disease and transmission of *Campylobacter. Arcobacter* is a newly emergent animal pathogen that is closely related to *Campylobacter* and was originally thought to belong in the genus *Campylobacter.* Very little is known about the pathogenic potential and mechanisms of *Arcobacter* spp. and its possible role as a human pathogen.

Only one toxin, the cytolethal distending toxin, has been purified, sequenced and established as a component of the *C. jejuni* disease process. The production of many other toxins by *C. jejuni* has been reported but their significance in the disease process is unknown. Toxin production is thought to vary in response to various environmental and/or stress conditions and also between different strains of *C. jejuni.* Toxins are often proteinaceous in nature and either cell-associated or secreted from the bacterial cell to the target site.

C. jejuni and *Arcobacter* strains were screened for toxicity using a methyl tetrazolium thiazolyl blue dye (MTT) cytotoxicity assay and haemolysis tests. All the organisms were shown to produce an oxygen-sensitive, cell-associated haemolysin. All *C. jejuni* strains were cytotoxic in the MTT assay, but *C. jejuni* NCTC 11351 demonstrated cytotoxicity from the broadest range of culture conditions. No cytotoxicity was detected in any of the *Arcobacter* strains using the MTT assay. Cytolethal distending toxin genes were detected in *C. jejuni* NCTC 11351 and a 55kDa outer membrane protein (OMP) from this strain also reacted strongly with cholera toxin monoclonal antibody. The cholera-reactive protein was partially purified and N-terminal sequence analysis revealed homology with the major outer membrane protein (MOMP) of *Campylobacter.* LPS complexes probably caused the cross-reactivity of this cytotoxic protein with cholera toxin antibody.

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List of Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BA	Blood Agar
BBE	Bickerstaff's Brainstem Encephalitis
B-FBP	Brucella with FBP (sodium pyruvate, sodium metabisulphite, ferrous sulphate) supplements
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
Caco-2	Human Enterocyte-like Cell Line
CCT	*Campylobacter Cytotonic Toxin
CDSC	Communicable Disease Surveillance Centre
CDR	Communicable Disease Reports
CJT	**C. jejuni* enterotoxin
CLDT	Cytolethal Distending Toxin
CLRT	Cytolethal Rounding Toxin
CLT	*Cholera-like toxin of *C. jejuni*
CHO	Chinese Hamster Ovary Cell Line
CT	Cholera toxin of *Vibrio cholerae*
DNA	Deoxyribonucleic Acid
ECV 304	Human Epithelial Cell Line
ELISA	Enzyme Linked Immunosorbent Assay
ETCC	European Tissue Culture Collection
GBS	Guillain-Barré Syndrome
G+C	Guanine and Cytosine
HeLa	Human Cervical Carcinoma Cell Line
HEp-2	Human Laryngeal Carcinoma Cell Line
INT 407	Human Intestinal Epithelial Cell Line
J774	Mouse Macrophage Cell Line
kb	Kilobases
kDa	Kilodaltons
LOS	Lipooligosaccharide

LPS	Lipopolysaccharide
LT	Labile Toxin of *E. coli*
Mbp	Mega Base Pairs
MFS	Miller Fisher Syndrome
mO_2	Microaerobic
MOMP	Major Outer Membrane Protein
MRC-5	Human Diploid Lung Fibroblast Cell Line
mRNA	Messenger Ribonucleic Acid
MTT	Methyl Tetrazolium Thiazolyl Blue Dye Cytotoxicity Assay
N2a	Mouse Neuroblastoma Cell Line
NCTC	National Collection of Type Cultures
OM	Outer Membrane
OMP	Outer Membrane Protein
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PHL	Public Health Laboratory
RILT	Rat/Rabbit Ileal Loop Test
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SLT	Shiga-like Toxin
Vero	African Green Monkey Kidney Cell Line
VNC	Viable but Non-Culturable
VT	Vero Toxin (toxic to Vero cell line)
WBA	Washed Blood Agar
Y-1	Mouse Adrenocortical Cell Line

* These three definitions all refer to the same toxin.

Chapter 1 – Introduction

1.1 The Biology and Taxonomy of *Campylobacter* spp.

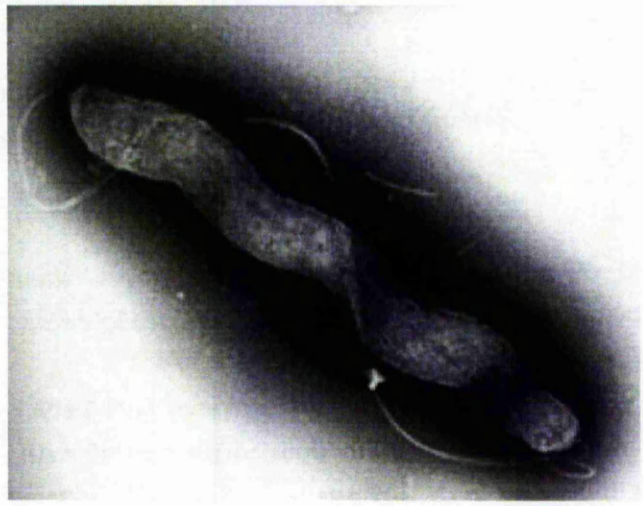
Campylobacter jejuni is the most common cause of bacterial diarrhoea worldwide (Ketley 1997, Leach 1997, Wassenaar 1997, Fields and Swerdlow 1999, van Vliet and Ketley 2001). Campylobacters are slender, spirally curved, Gram negative rod-shaped or vibrioid cells 0.2-0.5μm wide and 0.5-5μm long (Fig. 1). They are non-sporeforming and the cells may become coccoid in old cultures. They have a single, unsheathed, polar flagellum at one or both ends of the cell which may be 2-3 times the length of the cell and provide the cell with a characteristic corkscrew-like motility. Campylobacters are microaerophilic with a respiratory type of metabolism requiring an oxygen concentration of 3-15% and a carbon dioxide concentration of 3-5%. The optimum oxygen concentration for growth is 3-6%. Campylobacters are chemoorganotrophs so do not utilise carbohydrates. They are found in the reproductive organs, intestinal tract and oral cavity of humans and animals (Holt *et al.* 1994). Campylobacters have a small genome which is approximately 1.6-1.7 Mbp of AT-rich DNA, this is half the size of the *E. coli* genome, with a GC ratio of approximately 30% (Ketley 1997, Fields and Swerdlow 1999, Parkhill *et al.* 2000). Conjugative plasmids and bacteriophages are extrachromosomal elements which have been reported in *Campylobacter* spp. (Taylor 1992a and 1992b, Ketley 1997).

All species of *Campylobacter* have been found in man and most are found in other animals although not all are associated with disease. The most important group in terms of pathogenesis are the so-called thermophilic campylobacters, consisting principally of *C. jejuni* and *C. coli*, but also including *C. lari* and *C. upsaliensis* (Fricker and Park 1989, Skirrow 1994). *C. jejuni* and *C. coli* produce the same clinical syndrome but *C. jejuni* is more commonly isolated from human specimens than *C. coli*, representing about 90% of all human diarrhoeal isolates (Ketley 1997, Fields and Swerdlow 1999). It is for this reason that *Campylobacter jejuni* will be the species focussed upon during this study.

The genus *Campylobacter*, together with the new genera *Arcobacter* and *Helicobacter*, form a new division within the Proteobacteria called rRNA Superfamily VI or the epsilon subdivision (Griffiths and Park 1990, Vandamme

and Goossens 1992, Skirrow 1994, Fields and Swerdlow 1999, On 2001). The group also includes *Wolinella succinogenes*. All members of the superfamily are spiral or curved, Gram negative, non-sporing and possess one or more flagella which impart a high degree of motility. The family *Campylobacteraceae* exists within Superfamily VI and consists only of the genera *Campylobacter* and *Arcobacter* (Skirrow 1994, Nachamkin 1995).

Fig. 1 *Campylobacter jejuni* Morphology



Reproduced from http://www.medmicro.mds.qmw.ac.uk/campylobacter/

Campylobacters were originally included in *Vibrionaceae* on the basis of morphology. *Campylobacter fetus* (originally *V. fetus*) is the type species of the genus *Campylobacter* which was first isolated from a sheep foetus in 1913 by McFadyean and Stockman. For many years abortion in cattle and sheep was the only known disease of any importance caused by campylobacters and they were not cultured from humans associated with gastrointestinal disease until 1946 (Skirrow 1994, Fields and Swerdlow 1999). It was not until 1963 that Sebald and Veron designated the new genus *Campylobacter* due to their different G+C ratio and certain other differences from *Vibrio* spp. (Neill *et al.* 1979, Wallis 1994, On 2001).

1.2 Taxonomy of *Arcobacter* and *Helicobacter*

Campylobacter cryaerophila (latin; loving cold and air) and *Campylobacter butzleri* were aerotolerant *Campylobacter*-like organisms which were found to have serological and phenotypic differences to other species of *Campylobacter* (Neill *et al.* 1978, Skirrow 1994, Atabay *et al.* 1998, Rice *et al.* 1999). In 1991, these groups of aerotolerant campylobacters were designated to the new genus *Arcobacter* (latin; arc-shaped bacteria) (Kielbauch *et al.* 1991, Vandamme *et al.* 1991, Wesley 1997, Higgins *et al.* 1999, Yan *et al.* 2000). The cells of *Arcobacter* are morphologically similar to those of *Campylobacter*, and the genus was proposed on the basis of differences in nucleic acid hybridisations and molecular studies of aerotolerant campylobacters (Rice *et al.* 1999). There are four species of *Arcobacter* which were formerly included in *Campylobacter*. Three of these have been isolated from humans and animals; *A. butzleri*, *A. skirrowii* and *A. cryaerophilus*. The type species, *A. nitrofigilis* has only been reported from the roots of a salt marsh plant, Spartina (Skirrow 1994, Wesley 1997, Atabay *et al.* 1998, Manke *et al.* 1998, Mansfield and Forsythe 2000, Wesley *et al.* 2000, Yan *et al.* 2000).

Soon after the recognition of the new genus *Campylobacter* as a common human pathogen, a report came from Warren and Marshall in 1983 from Perth, Australia which showed that the stomach was commonly colonised by a new bacterium, first known as *Campylobacter pyloridis*, then named *C. pylori*. This was later to become *Helicobacter pylori* and was the beginning of the new genus *Helicobacter* which currently consists of 10 species (Skirrow 1994, Kim *et al.* 2000). *H. pylori* is the species of the most clinical importance as a human pathogen.

1.3 The Biology, Transmission and Pathogenicity of *Arcobacter* and *Helicobacter*

1.3.1 *Arcobacter* spp.

There are two major phenotypic differences which are used to distinguish between campylobacters and arcobacters; arcobacter is aerotolerant and grows at lower temperatures (15-25°C) than campylobacters (Vandamme *et al.* 1991, Skirrow 1994, Wesley 1997, Mansfield and Forsythe 2000). The most frequent reports of isolations of arcobacters are from cattle and pigs suffering abortion and enteritis (Kielbauch *et al.* 1991, Skirrow 1994, Wesley 1997, Wesley *et al.* 2000, Yan *et al.* 2000). *A. butzleri* is the species most frequently isolated from animals and from humans with diarrhoea and/or abdominal cramp (Kielbauch *et al.* 1991, Mansfield and Forsythe 2000, Yan *et al.* 2000, Corry and Atabay 2001). *A. cryaerophilus* has been isolated from aborted as well as healthy livestock and from human diarrhoeal stool samples (Fernandez *et al.* 1995, Dediste *et al.* 1998). There have also been reports of bacteraemia and septicaemia in humans caused by *Arcobacter* spp. (On *et al.* 1994, Hseuh *et al.* 1997, Wesley 1997, Yan *et al.* 2000).

Transmission of arcobacter has been largely related to contaminated water (Kielbauch *et al.* 1991, Musmanno *et al.* 1997, Wesley 1997, Rice *et al.* 1999). Arcobacters are more adapted for survival in the environment than campylobacters due to their tolerance of oxygen and low temperatures (Wesley *et al.* 2000). They are also less sensitive to anaerobic digestion than faecal bacteria, so the land application of digested sludges may contaminate water sources and cause higher risks of infection, particularly for livestock (Stampi *et al.* 1999). A high prevalence of *Arcobacter* has been found in chicken carcasses from both supermarkets and abattoirs in the UK, Canada and the US (Lammerding *et al.* 1996, Atabay and Corry 1997, Atabay *et al.* 1998, Manke *et al.* 1998, Corry and Atabay 2001) suggesting that poultry is a major reservoir for *Arcobacter* spp. Wesley and Baetz (1999), however, reported infrequent detection of *Arcobacter* spp. in poultry caecal samples and suggested that birds

are not a natural reservoir for arcobacters and the prevalence in poultry must be due to extensive post-slaughter contamination.

There is very little information on the pathogenicity of arcobacters, but a few groups have made attempts to characterise some of the organism's pathogenic factors. Some cases of invasive *A. butzleri* infection have been reported such as those causing bacteraemia (Yan *et al.* 2000). A study of 18 *A. butzleri* isolates from river water samples were each examined for their virulence characteristics (Musmanno *et al.* 1997). Toxicity was assessed based on cytotonic, cytotoxic and cytolethal distending factors upon Vero and CHO cells and adhesivity and invasivity assays were performed using HeLa and INT 407 cells. All strains but one induced cytotoxic effects on cells in culture but no invasiveness was observed. The non-cytotoxic strain did however exhibit cytotonic-like effects causing elongation of CHO cells and was the only strain which adhered to the cells *in vitro* (Musmanno *et al.* 1997). *A. cryaerophilus* has been shown to cause invasive infection in a human (Hsueh *et al.* 1997) and has also been assessed for its toxigenic and invasive abilities. Two strains were tested, both of which caused distension of ileal loops with fluid accumulation and enhanced electrolyte concentrations in a rat ileal loop test (cytotonic toxicity). Both strains were also found to be invasive upon HEp-2 cells (Fernandez *et al.* 1995). These are the only reports of pathogenic properties which have been found in arcobacter apart from a report of alpha-haemolysis (Atabay *et al.* 1998) which may be involved in iron acquisition and/or hampering of the immune response (Rowe and Welch 1994, Wassenaar 1997).

1.3.2 *Helicobacter* spp.

Members of the genus *Helicobacter* are spirally curved with polar flagella which are usually multiple and nearly always sheathed, except *H. pullorum*, the sheath being continuous with the outer membrane (Skirrow 1994). The sheath, and very powerful urease activity, are thought to allow the bacteria to survive in the stomach lumen (Montecucco *et al.* 1999). Infection with *H. pylori* is strongly associated with gastritis, peptic ulceration and gastric cancer although the latter association is still not fully characterised (Skirrow 1994, Kim *et al.* 2000). *H. pylori* colonises humans and animals also causing enteritis and rarely, animal

abortion. It is present in 95% of duodenal and 70-80% of gastric ulcer patients as well as in healthy individuals (Wesley 1997).

Routes of transmission of *H. pylori* have been proposed which include contact between humans (oral to oral or faecal to oral transmission), contact between humans and animals and the ingestion of contaminated foods or water. *H. pylori* has been detected in water using PCR and it has been proposed that a 'viable but non culturable' state (see section 1.4) may be responsible for the long-term survival of *H. pylori* in water (Wesley 1997).

The pathogenesis of *H. pylori* is complex. At the epithelial cell level *H. pylori* enzymes generate toxic molecules such as ammonia (urease), lysolecithin (phospholipases) and acetaldehyde (alcohol dehydrogenase). A vacuolating cytotoxin (VacA) is expressed in 50-60% of strains although the gene is present in all strains. This toxin is associated with ulcer development. A haemolysin is also present which is associated with phospholipase A activity (Megraud 1994, Grant *et al.* 1997, Dorrell *et al.* 1999). The *CagA* gene (cytotoxin associated gene A) encodes a 128 kDa immunodominant surface antigenic protein which is always associated with more severe forms of disease. The exact function of the CagA protein is unknown, but the *cagA* gene resides within a large pathogenicity island. This is a cluster of virulence genes which has been acquired by the horizontal transfer of DNA, as the GC content of this is different to the rest of the *H. pylori* genome. Many pathogens contain these islands of about 10-200kb, which are absent from non-pathogenic memebers of their species and carry a variety of virulence determinants. The origin of the pathogenicity island is, however, unknown (Montecucco *et al.* 1999).

1.4 Clinical Symptoms and Transmission of *Campylobacter* spp.

C. jejuni is the biggest reported bacterial cause of enteric infection in the human population with infection rates considerably higher than those of *Salmonella* (Tauxe 1992, Wallis 1994, Atabay and Corry 1997, Ketley 1997, Leach 1997, Bacon *et al.* 1999, Frost 2001). Reports of *C. jejuni* enteritis have risen dramatically since its recognition in the late 1970's and by 1989 there were over 30 000 reported cases in England and Wales (Griffiths and Park 1990, Ketley 1995). Ten years later in 1999, the figures in England and Wales had

risen significantly to approximately 60 000 reported cases (CDSC 2001). Although there was a slight drop to 54 000 reported cases in 2000, campylobacter is still the most common cause of bacterial gastrointestinal infection and is thus a major public health and economic burden. The number of cases reported to the CDSC is, however, an under-representation of the actual numbers of *Campylobacter* cases as many people with milder symptoms choose not to seek medical advice (Griffiths and Park 1990, Tauxe 1992, Skirrow 1994, Ketley 1995 and 1997, Leach 1997, Wheeler *et al.* 1999). It has been estimated that the ratio of campylobacter cases in the community to cases reaching national surveillance is 7.6:1 (Wheeler *et al.* 1999), thus indicating that the actual figure for campylobacter cases in 2000 was closer to 400 000.

There is a seasonal variation in *Campylobacter* infections in the UK with the peak number of cases occurring in late spring and autumn. There are also differences in patterns of disease and clinical symptoms between developed and developing countries. In developed countries a more severe inflammatory, dysentery-like diarrhoea containing blood and leukocytes usually occurs whereas in developing countries the diarrhoea is usually mild, watery and non-inflammatory (Klipstein *et al.* 1985, Calva *et al.* 1989, Florin and Antillon 1992, Ketley 1997, Leach 1997, Fields and Swerdlow 1999). The symptoms described in the UK and other industrialised countries begin with abdominal pain, fever, headache, malaise and mental confusion following an incubation period of 1-7 days. Profuse diarrhoea rapidly proceeds which lasts 2-3 days, is accompanied by abdominal pain, and blood and leukocytes are present in the faeces. The symptoms are very similar to those of other enteropathogens such as *Salmonella*, except that the abdominal pain is much more severe in *Campylobacter* cases which has led to the misdiagnosis of appendicitis in some cases (Griffiths and Park 1990, Skirrow 1994, Wooldridge and Ketley 1997). Infection can result in a severe illness lasting a week or longer, and relapses sometimes occur, but the disease is usually self-limiting. Complications of the disease are rare but some are serious such as neurological disorders which will be discussed in section 1.12. The peak incidence rate of *Campylobacter* infection in developed countries is in young adults and young children. A different pattern of immunity is seen in developing countries whereby a higher exposure in early life leads to a peak in children, few cases in adults and a high rate of asymptomatic carriage. This

suggests that immunity is acquired at a young age in these countries. This early immunity and asymptomatic carriage does not occur in industrialised countries (Calva *et al.* 1989, Ketley 1997, Fields and Swerdlow 1999).

C. jejuni is largely considered as a foodborne pathogen, but is unable to grow on food surfaces due to its oxygen sensitivity and high temperature requirement (Ketley 1995, Leach 1997). Common vehicles of transmission are undercooked meat, in particular poultry, inadequately pasteurised milk and inadequately treated or untreated water supplies (Walker *et al.* 1986, Fricker and Park 1989, Wallis 1994, Ketley 1997, Leach 1997). Poultry is considered to be the major reservoir and source of campylobacter infections (Harris *et al.* 1986, Fricker and Park 1989, Florin and Antillon 1992, McFarland and Neill 1992, Ketley 1995, Prasad *et al.* 1996). Harris *et al.* (1986) suggested from the results of an American study that the consumption of chicken contributed to approximately half the cases of *C. jejuni/coli* infection. *Campylobacter* resides as a commensal in the intestines of many animals and birds, and of particular importance, in chickens. Here, the bacteria do not cause infection but faecal contamination of the chicken carcasses from their intestines occurs during slaughter, thus making them vehicles of infection which enter the human food chain (Harris *et al.* 1986, Ketley 1997, Fields and Swerdlow 1999). It has been reported that approximately 80% of raw chickens sold in the UK are contaminated with campylobacters (Corry and Atabay 2001). The successful transmission of this pathogen is largely due to its ability to survive in environmental reservoirs such as water (Rollins and Colwell 1986) and the low infective dose required to induce illness. As few as 800 organisms are required for the onset of infectious diarrhoea in human hosts (Black *et al.* 1988). The survival of campylobacter in water sources is considered an important virulence factor whereby the organism enters a dormant-like protective state known as the viable but non-culturable (VNC) state. This is when the organism transforms into a coccoid shape which is resistant to environmental stresses. It has been reported that once in the VNC state, campylobacters cannot be cultured on standard laboratory media, but can cause infection in a live host (Lai-King *et al.* 1985, Rollins and Colwell 1986, Moran and Upton 1987, Jones *et al.* 1991). The organism may thus be able to survive on foods and various food preparation surfaces and not be detectable on laboratory media, yet still capable of causing

disease. Cross-contamination from raw to cooked foods is therefore a major problem (Harris *et al.* 1986, Atabay and Corry 1997, Leach 1997).

1.5 *Campylobacter* Pathogenicity and Virulence Factors

Following ingestion of campylobacter, the organism enters the intestine, initially infecting the jejunum and the upper ileum. The organism then spreads to the rest of the ileum and the colon where it damages epithelial cells and disrupts the absorptive capacity of the intestine. Inflammatory changes take place in the mucosa, often with abscess formation similar to those seen in *Salmonella* and *Shigella* infections (Skirrow 1994). Bacteraemia has only been recorded in 0.15% of cases but this may be more common due to the ability of *C. jejuni* to translocate across intestinal cells (Ketley 1997, Harvey *et al.* 1998).

C. jejuni possesses many pathogenic mechanisms which enable it to infect the host. Motility, adherence, invasion and toxin production are the four major virulence properties (Wallis 1994, Prasad *et al.* 1996, Leach 1997, Wassenaar 1997) and the first three of these are involved in the ability to penetrate the host mucosal surface. This is the first and most important pathogenic activity of campylobacters. The spiral shape and powerful flagella allow the organism to move in a corkscrew-like fashion through viscous matrices such as the intestinal mucus (Skirrow 1994, Wooldridge and Ketley 1997, van Vliet and Ketley 2001). Various outer membrane proteins, glycoproteins and carbohydrate moieties are thought to be involved in the adherence to, and invasion of epithelial cells (Skirrow 1994, Ketley 1997).

Flagella and LPS have been shown to be involved in the adherence of *C. jejuni* to INT 407 and mucosal cells (McSweegan and Walker 1986). Invasion of HEp-2 (Prasad *et al.* 1986) and INT 407 (Konkel *et al.* 1992a) cells has also been demonstrated. The latter study also demonstrated the ability of *C. jejuni* to survive within epithelial cells and elicit a cytotoxic effect. The importance of flagella in bacterium-cell interactions has been demonstrated by the production of a *pflA* mutant (paralysed but full-length flagellum) which showed slightly reduced adhesion and greatly reduced invasion of enterocyte-like cells (Yao *et al.* 1994). The exact mechanisms by which the flagella interact with host cells are

not fully understood, but the general consensus is that it is the motility provided by the flagella which is needed for invasion and adhesion.

Invasion is thought to be a primary mechanism of damage to the colonic mucosa leading to inflammation and diarrhoea. Several new bacterial proteins are synthesised during interaction with epithelial cells in culture, of these *de novo* synthesised proteins, at least one is required for invasion (Wooldridge and Ketley 1997, Harvey *et al.* 1998, Konkel *et al.* 1999). Adhesion of bacteria to mucosal surfaces is a critical step for colonisation. Chemotaxis is also a very important factor in colonisation, this is used to direct the motility of the cells causing net movement either up or down chemical concentration gradients (Wallis 1994, Ketley 1997, Leach 1997, van Vliet and Ketley 2001). Many components of the bile and mucin are chemo-attractants including sugars, in particular L-fucose, and amino acids including L-aspartate, L-cysteine, L-glutamate and L-serine. This is likely to be a significant factor in the affinity of *C. jejuni* to the gastrointestinal tract (Walker *et al.* 1986, Wallis 1994). Studies of the *cheY* gene in *C. jejuni* showed a significant interaction between this gene and the flagellar motor. A *CheY* mutant was produced which was nonchemotactic and straight swimming. Tumbling swimming to move rapidly away from chemical repellents is via the flagella-CheY response (Marchant *et al.* 1998).

Most of the cellular components which interact with host cells are outer membrane components. The outer membrane consists of numerous proteins and lipopolysaccharides many of which have pathogenic properties including toxins, binding proteins and iron regulatory proteins. The Gram negative outer membrane has some unique and complex mechanisms which contribute to the virulence of this group of bacteria. Section 1.6 gives a more detailed account of this important attribute of many pathogens.

1.5.1 Phase Variation

The genetic and phenotypic variability of *C. jejuni* is a very important pathogenic mechanism which is becoming increasingly understood following the completion of the genome sequencing of *C. jejuni* NCTC 11168. The genomic sequence is an invaluable resource for studies of the pathogenic potential of *C. jejuni* which has already identified a lack of classical operon structure and

repetitive DNA as well as hypervariable sequences. The *C. jejuni* sequence has been reported to show hypervariable sequences which give rise to rapid phase variation (gene switching on/off) in *C. jejuni*, some of which are coincident with clusters of genes responsible for LOS biosynthesis and flagellar modification (Parkhill *et al.* 2000, Wren *et al.* 2001). This consolidates theories of other investigators to be discussed in sections 1.6.6 and 1.6.7 which describe phase variation in the flagella and the LPS of *C. jejuni* respectively. Many other reports of phenotypic variation and gene 'switching' *in vivo* have arisen over the past decade (Taylor 1992), but there was no genetic evidence to support them until now. Harvey *et al.* (1998) studied ten different *C. jejuni* isolates for their ability to translocate across and to invade Caco-2 cells (enterocyte-like cell line). They found that all the strains were of different invasion phenotypes and there was a vast variation in the genes expressed by all *C. jejuni* strains. Wassenaar and Blaser (1999) also reported genotypic and phenotypic variability and suggested the likelihood of genome plasticity in *C. jejuni*, that is, the existence of a wide number of strains with many different characteristics. The switching of gene expression is controlled by a number of regulatory systems. *C. jejuni* appears to have a broader repertoire of regulatory systems than *H. pylori* which has a similar sized genome. This may be expected due to the fact that *C. jejuni* is found in a more diverse range of ecological niches than *H. pylori* (Parkhill *et al.* 2000). Many regulatory systems have been identified within campylobacters which control the response of the organism to a range of environmental stimuli. For example, an iron response regulator gene, *fur*, has been identified (Wooldridge *et al.* 1994, Ketley 1997, van Vliet *et al.* 1998). There are also several two-component signal transduction systems which have been identified in *Campylobacter* that respond to various stimuli (Ketley 1997). These systems consist of a sensor protein and a response regulator protein. Many proteins involved in the stress/heat-shock response (section 1.6.4) are components of these signal transduction systems. This enables *Campylobacter* to respond to a wide range of environmental stresses by switching on/off the expression of certain functional proteins (Ketley 1997). *Campylobacter* spp. have also been shown to possess genetic exchange mechanisms such as plasmid transfer and other natural transfer mechanisms eg. via conjugation and transposons (Wang and Taylor 1990, Wassenaar *et al.* 1993), which may give rise to the acquisition

of pathogenic properties from other bacteria. An example of this in closely related organisms is the acquisition of the pathogenicity island by *H. pylori* (Montecucco *et al.* 1999).

Bacterial antibiotic resistance genes are commonly carried on plasmids as are virulence properties such as attachment factors, toxin production and invasiveness (Bradbury and Munroe 1985). There have been widespread reports of plasmids in *C. jejuni* (Marri *et al.* 1984, Bradbury and Munroe 1985, Ansary and Radu 1992, Velazquez *et al.* 1995, Ruiz *et al.* 1998, Bacon *et al.* 2000) with the general view that 19-53% of *C. jejuni* strains carry plasmids many of which are R (antibiotic resistance) plasmids that are transmissible among *Campylobacter* spp. but not to *E. coli* (Marri *et al.* 1984, Bradbury and Munroe 1985, Ansary and Radu 1992, Velazquez *et al.* 1995, Ruiz *et al.* 1998, Bacon *et al.* 2000). Until recently it was also generally considered that plasmids in *Campylobacter* did not play a role in pathogenicity, but had a significant role in antibiotic resistance (Bradbury and Munroe 1985, Bacon *et al.* 2000). A recent study on *C. jejuni* 81-176 found that this strain contained two plasmids, each about 35kb in size (Bacon *et al.* 2000). One of these was an R plasmid carrying a *tetO* gene which encodes tetracycline resistance (termed pTet). The other (termed pVir) was found, following partial sequence analysis, to have open reading frames (ORFs) encoding predicted proteins which were very similar to *H. pylori* proteins. One of these *H. pylori* proteins is encoded by the *cag* pathogenicity island, the genes of which encode proteins with homology to type IV secretion systems. It was found that a mutation of two of these genes affected the virulence of *C. jejuni* 81-176. It was therefore concluded that plasmids in *C. jejuni* do play a role in virulence contrary to previous beliefs, and evidence of a plasmid-mediated type IV secretion system was found. Type IV secretion systems are protein secretion systems similar to conjugation systems, whereby a pilus-like structure is used to deliver virulence proteins to the host cell (Christie 2001). The pVir plasmid in *C. jejuni* 81-176 was also reported to be highly stable whereas other *C. jejuni* plasmids are lost rapidly upon subculturing (Bacon *et al.* 2000).

The aforementioned authors hence provide substantial evidence that differences in the virulence and pathogenicity of *C. jejuni* strains may be due to

genetic variability. This could be a major factor in the identification of different pathogenic mechanisms in *C. jejuni*.

1.6 The Gram Negative Outer Membrane

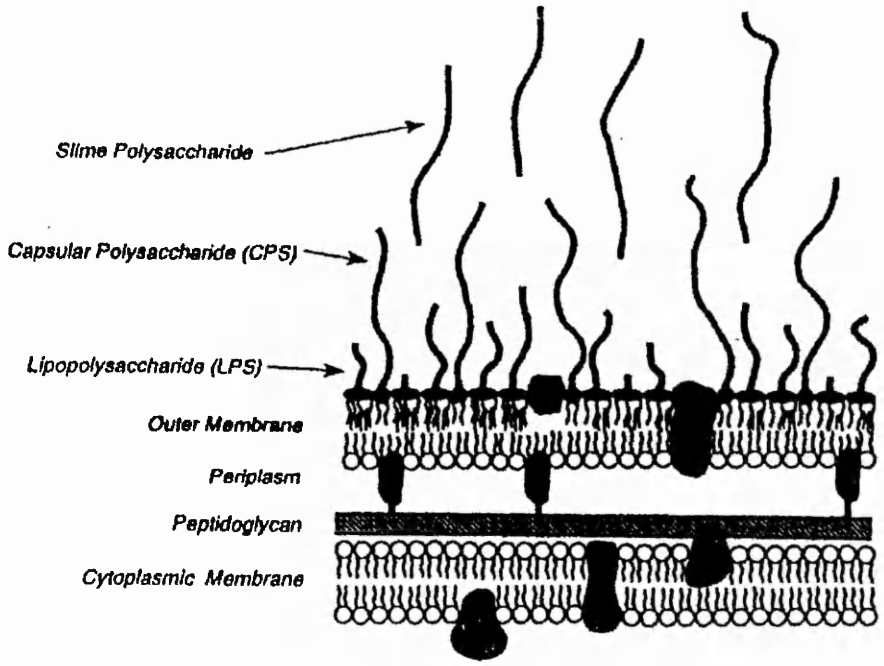
The outer membrane is important in the pathogenesis and virulence of Gram negative bacteria. It is a complex multilayered system which acts as a protective interface between the bacterial cell and its immediate environment (Logan and Trust 1982, Dawes and Sutherland 1992). The inner membrane of the Gram negative bacteria is equivalent to the cytoplasmic membrane of Gram positive bacteria. This is a structure that is permeable to water and some small hydrophobic compounds, but provides rigidity for the maintenance of cell shape as well as protection against lysis. The cytoplasmic membrane also contains the components for electron transport, oxidative phosphorylation, solute transport, biosynthesis of cell-surface components and some enzyme complexes (Whitfield and Valvano 1993). The space between the inner membrane and the outer membrane of Gram negative bacteria is known as the periplasm. Here, a range of molecules are found including enzymes, nutrient-binding proteins and other osmoprotectants (Dawes and Sutherland 1992). Finally, the external protective layer of Gram negative bacteria is the outer membrane (OM). This is absent in Gram positive bacteria which are consequently not as resistant as Gram negative cells to toxic compounds in their environment such as bile salts.

The OM is a complex asymmetrically bilayered structure consisting of lipopolysaccharide (LPS), protein and phospholipid in a ratio of 2:2:1 (Dawes and Sutherland, 1992). Most of the phospholipid is phosphatidylethanolamine (Nikaido and Vaara 1987, Dawes and Sutherland 1992). Fig. 2 shows the structure of the Gram negative cell envelope including the outer membrane.

The lipopolysaccharide (LPS) is unique to the outer membrane in Gram negative bacteria and is not present in Gram positive bacteria at all (Nikaido and Vaara 1987, Dawes and Sutherland 1992). Its presence in the outer leaflet of the OM results in an atypical bilayer which can provide a hydrophilic boundary on the cell surface (Whitfield and Valvano 1993). Surface hydrophilicity is, however, often maintained by a layer of extracellular polysaccharide (EPS) in bacteria from diverese ecological niches. This is also termed the capsule or slime

layer. It is often an adherent, cohesive layer which completely surrounds the cell and is referred to as the CPS (capsular polysaccharide) in Fig. 2. The capsule is highly hydrated (about 99% water) and can protect organisms from desiccation, phage infection, phagocytosis and antibodies. The capsule is hence associated with virulence in pathogenic bacteria (Dawes and Sutherland 1992, Whitfield and Valvano 1993).

Fig. 2: The Gram Negative Cell Envelope



(Whitfield and Valvano 1993)

The OM has specific and non-specific channels for nutrients and ions required for growth. Selected nutrients (eg. sugars, amino acids) are allowed through the OM by passive diffusion into the periplasm through diffusion channels. From the periplasm they are actively transported into the cytoplasm across the cytoplasmic membrane. These diffusion channels are water-filled proteins called 'porins' (Inouye 1979, Nikaido 1992, Moat and Foster 1995). The OM also allows the passage of selected molecules out of the cell, for

example, some toxins are secreted into the external environment. The primary function of the OM is to act as a protective, selective barrier but it is also very important in host-pathogen relationships. It participates in the attachment of the pathogen to the host cell surface (Logan and Trust 1982, Nikaido and Vaara 1987) and the acquisition of iron from the host. The OM also imparts resistance to phagocytosis, bactericidal activities of the serum and various toxic compounds such as bile salts (Inouye 1979, Logan and Trust 1982, Dunn *et al.* 1987).

1.6.1 Outer Membrane Proteins (OMPs)

The OM of Gram negative bacteria has a characteristic protein composition which is entirely different from that of the cytoplasmic membrane (Nikaido and Vaara 1987). It consists of multiple copies of a small number of 'major proteins', some of which have characteristic molecular weights (Dawes and Sutherland 1992) and most of which migrate in the molecular mass range 30-45 kDa (Kervella *et al.* 1992). These major proteins are also known as porins and allow the diffusion of solutes through the OM. There are also a variety of up to 50 less abundant or 'minor proteins'. They play a variety of roles in the physiology of the cell, many are receptors for phages, bacteriocins and some nutrients and specific metabolites (Dawes and Sutherland 1992, Moat and Foster 1995). The most abundant minor protein is the Braun protein, 7.2 kDa (Nikaido and Vaara 1987, Moat and Foster 1995).

The porins exist as a group also commonly known as the 'major outer membrane proteins' and are fairly stable among *Escherichia coli*, *Salmonella* Typhimurium and other related pathogens (Nikaido and Vaara 1987, Dawes and Sutherland 1992). They allow passage of solutes of up to 600Da in *E. coli* and 4000Da in *Pseudomonas aeruginosa*. Porins include OmpA, OmpC, OmpF and LamB and many exist as trimers within the peptidoglycan. LamB in *E. coli* is involved in the passage of maltose and maltodextrin, and OmpA (35.2 kDa in *E. coli*) serves as a receptor for several phages as well as being important in peptide uptake and amino acid transport.

1.6.2 *Campylobacter* Outer Membrane Proteins (OMPs)

A wide range of *Campylobacter* OMPs have been studied with various roles ascribed to them. Many are antigenic factors involved in adhesion and binding to cells, many are structural and there are some which are secreted, invasive and toxic. The most common OMP is the major outer membrane protein (MOMP), but many others have been identified and characterised.

1.6.2.1 *Campylobacter* major outer membrane protein (MOMP)

The MOMP of *C. jejuni* is approximately 43 kDa (often reported as 41-45 kDa) and has many reported roles. These include structural organisation of the OM, pore activity, nutrient uptake, adhesion to cultured cell membranes and cytotoxicity (Bacon *et al.* 1999, De *et al.* 2000). It is a porin which shows no significant homology with other porins, although it has a common antigenic site with *E. coli* OmpC which was discovered following immunocrossreactivity studies (Bolla *et al.* 1995). It does, however, exert the physicochemical and typical pore-forming properties of a porin (Bolla *et al.* 1995, De *et al.* 2000). Early reports stated that the MOMP was a 45 kDa matrix protein which was heat-modifiable and capable of associating in oligomers (Logan and Trust 1982, Huyer *et al.* 1986, Kervella *et al.* 1992). Later, more detailed studies revealed that *C. jejuni* MOMP is a trimeric protein which yields different types of molecule following different solubilisation methods (Bolla *et al.* 1995, Zhuang *et al.* 1997, De *et al.* 2000). After mild solubilisation conditions (0.01% SDS) a high molecular mass trimer (120-140 kDa) was observed. With a higher concentration of SDS (0.1%), a folded monomeric form of 35 kDa was seen, and heating to 96°C resulted in a single band monomer of 45 kDa (Zhuang *et al.* 1997, Bolla *et al.* 1998). The trimeric form is hence generated by the association of three folded monomers and the 35 kDa form is likely to be a transient intermediate of this OMP assembly. The result is the expression of two isomeric forms of this pore-forming protein (Dunn *et al.* 1987, Bolla *et al.* 1995, Bolla *et al.* 1998, De *et al.* 2000). Bolla *et al.* (1998) reported that the channel properties of the two protein structures are different which affects their conductance and selectivity, however, this was later contradicted by De *et al.* (2000) who reported

that the monomer and the trimer essentially have the same pore-forming properties. These studies have also revealed that the MOMP is structurally related to the super-family of trimeric bacterial porins (Kervella *et al.* 1992, Bolla *et al.* 1995, Zhuang *et al.* 1997). Electron microscopy studies have shown that the MOMP is present as a regular, hexagonal lattice which covers the entire cell surface (Amako *et al.* 1996, De *et al.* 2000). MOMP represents 90% of total *C. jejuni* OMP and is probably the major structural protein of the OM, its invariable presence suggesting that it is essential for the organism (Blaser *et al.* 1983, Penn 2001).

Another significant property of the MOMP is its predominance as an antigenic protein. Due to this property the MOMP has been proposed as a potential candidate for a vaccine against *C. jejuni* (Schroder and Moser 1997, Bacon *et al.* 1999). It has been reported several times to have powerful membrane binding properties and consequently plays a crucial role in the adhesion of *C. jejuni* to host cell membranes (Moser and Schroder 1995). Moser *et al.* (1992) described ELISA experiments which demonstrate the probable role of MOMP in the binding of OM preparations to INT 407 tissue culture cell membranes. Further evidence confirming this was reported by Moser *et al.* (1997) and Schroeder and Moser (1997). Additionally, the surface-exposed epitopes of MOMP have been shown to be structural in nature. Zhang *et al.* (2000) aligned the amino acid sequences of MOMP from 22 strains of *C. jejuni* including NCTC 11168. They discovered that there were 7 localised variable regions dispersed amongst highly conserved regions. The surface-exposed epitopes were among the highly conserved regions which is important for MOMP-based diagnostic tools and vaccines (Zhang *et al.* 2000).

The final properties of the MOMP which are significant for its role in pathogenicity are reports of cytotoxicity. Moutinho-Fragaso *et al.* (1998) described a toxic factor (TF) which, after partial purification and N-terminal sequence analysis, was found to most likely resemble a porin. More substantial evidence for this was described by Bacon *et al.* (1999) who identified and characterised a cytotoxic porin-LPS complex produced by *C. jejuni*. After N-terminal sequence analysis of a single cytotoxic protein of 45 kDa it was shown to have 97% sequence homology with *Campylobacter* MOMP. A more detailed account of this can be found in section 1.11.4.3.

To summarise, the work to date on the *C. jejuni* MOMP has demonstrated that it has a vital role as a structural and nutrient-acquiring protein which imparts a certain amount of heat-stability to the outer membrane. Furthermore, it is capable of membrane adhesion and toxicity thus identifying it as an extremely important virulence factor of *C. jejuni*.

1.6.2.2 Other *Campylobacter* OMPs

Many other OMPs with a wide range of activities have been identified in *C. jejuni*. It is widely believed that *Campylobacter* proteins ranging between 25 and 42 kDa play a role in bacterium-cell interactions (Kervella *et al.* 1992). OmpA, OmpC, OmpF and a maltoporin have been identified by sequence comparison of a number of *C. jejuni* OMPs following a crude sarkosyl extraction (Schroeder and Moser 1997). There has, so far, been a demonstration of only one porin-like protein in *C. jejuni* other than the MOMP (Bolla *et al.* 2000), although there are up to 160 predicted membrane proteins in the genome sequence which may include additional porins (Penn 2001). Omp50 is a new porin which has been purified, characterised and sequenced by Bolla *et al.* (2000). This porin was purified by ion exchange chromatography and named Omp50 due to its molecular weight (50kDa) and its location in the outer membrane. No significant sequence similarity was found between Omp50 and other porin protein sequences including that of MOMP. Omp50 has a stable monomeric conformation that can only be denatured by high temperature in the presence of detergent. This, coupled with low single-channel conductance that is typical of numerous other monomeric porins, suggests that Omp50 belongs to the OmpA-like family of monomeric porins which includes the Hop family of porins of *H. pylori* (Bolla *et al.* 2000).

A group of major common antigenic proteins have been identified in *C. jejuni* referred to as PEB1 (28 kDa), PEB2 (29 kDa), PEB3 (30 kDa) and PEB4 (31 kDa) (Pei *et al.* 1991, Pei and Blaser 1996, Guerry 1997, Penn 2001). N-terminal sequence analysis shows that all four proteins are different but the first 35 amino acids of PEB2 and PEB3 are 54% homologous. These proteins all have a strong cationic nature which may aid in anchoring the proteins within the membrane and also in interactions with the host. PEB1 and PEB3 were found to be the common antigens recognised by convalescent sera from both *C. jejuni* or

C. coli-infected patients and are hence likely to be involved in the immunity against these infections that is often acquired in developing countries. They are therefore considered as strong potential candidates for a vaccine against *Campylobacter* infections (Pei *et al.* 1991). PEB 1 is a surface-exposed antigen which is highly conserved in all *C. jejuni* and *C. coli* isolates (Pei and Blaser 1993, Guerry 1997). The PEB1 structural gene (*peb*1A) was cloned and sequenced from *C. jejuni* 81-176. It was found to exhibit significant homology with the *Enterobacteriaceae* binding proteins GlnH, LAO and HisJ. Significant overall homology was also found between this gene and amino acid transport systems in other Gram negative bacteria (Pei and Blaser 1993, Guerry 1997). PEB3 is partially homologous with potential adhesins including class 1 pili from *Neisseria meningitis*, and *Escherichia coli* heat-labile enterotoxin B subunit (Pei and Blaser 1996, Manning *et al.* 1998). PEB3 has also been implicated as having an important role in the colonisation of chickens due to studies where up-regulation of this protein was observed following chicken passage (Manning *et al.* 1998). PEB4 is a major antigen that has been demonstrated to elicit an antibody response in infected patients (Burucoa *et al.* 1995) and shows high sequence similarity to a *Bacillus* export protein (Guerry 1997). Kervella *et al.* (1993) described two immunogenic proteins of 27 and 29 kDa that specifically adhered to HeLa cells and corresponded immunologically to PEB1 and PEB4. These two proteins are targets for the human immune system during infection and have been suggested as candidate antigens for a *C. jejuni* vaccine (Pei and Blaser 1996).

Campylobacters release OM during growth as do other Gram negative bacteria. These released OM fragments are often pathogenic factors of infection eg. some are toxins such as the heat-labile enterotoxin which is released from old cultures of *E. coli* (Williams and Clarke 1998). The CiaB protein (43kDa) is one of at least eight campylobacter proteins ranging from 12.8 to 108 kDa which were shown to be secreted into the culture medium when *C. jejuni* 81116 and several clinical isolates were incubated with INT 407 cells. Confocal microscopy was used to demonstrate the translocation of the CiaB protein into the cytoplasm of the host cell (Konkel *et al.* 1999). It is therefore believed that this protein is required for the entry of *C. jejuni* into host cells. The deduced amino acid sequence of the CiaB protein shares sequence homology with type III

secreted proteins of other bacterial pathogens which are associated with the invasion of host cells. Type III secretion systems are contact-dependent secretion systems (hence are stimulated upon contact with host cells) which are ancestrally related to the flagella apparatus with rod- and hook-like components (Plano *et al.* 2001). This secretion system in many other Gram negative pathogens resides within a pathogenicity island (see section 1.3.2, p6), but it is not clear whether or not *C. jejuni* CiaB is based within such a pathogenicity island (Konkel *et al.* 1999).

Two different proteins which bind fibronectin have been reported within *C. jejuni*. The first is CadF, 37 kDa, which is essential in the colonisation of newly-hatched chicks. Mutants not expressing CadF failed to colonise and suggestion was made for the use of this as a defined-antigen vaccine in chickens (Ziprin *et al.* 1999). The second is a 59 kDa protein which is involved in binding to fibronectin and INT 407 cell membranes. Following N-terminal sequencing the first 14 amino acids were identical to those of CadF. The reason for this sequence homology was unknown (except that they both bind fibronectin) and it was suggested that the 59 kDa protein may be a partially dimeric form of CadF (Moser *et al.* 1997).

Finally, certain OMPs in *C. jejuni* have been reported to have an apoptotic effect upon chicken lymphocytes. In chickens *C. jejuni* is generally considered as a commensal and not a pathogen; however, bacteria usually colonise chickens for the remaining life of the bird so this is therefore a food safety concern. It has thus been suggested that *C. jejuni* may be capable of achieving immune avoidance in chickens due to OMPs which cause apoptosis of chicken lymphocytes *in vivo*. This would hence suggest the presence of membrane-associated toxic proteins in *C. jejuni* which cause cell death. A phospholipase A has also been identified in *C. jejuni* which is implicated in virulence due to its effects on haemolytic activity *in vitro* (Grant *et al.* 1997, Snijder *et al.* 1999, Penn 2001). Further details of this and the role of protein toxins will be discussed more thoroughly in sections 1.8 to 1.11.

1.6.3 Stress and Heat-Shock Proteins

Additional outer membrane proteins are produced when growth is limited by iron or phosphate deficiency, and some are induced by such things as maltose (Watson 1990). A variety of environmental stimuli including extremes of temperature, osmolarity, pH, and exposure to atmospheric oxygen, are known to trigger the synthesis of a set of highly conserved proteins named stress or heat-shock proteins (HSPs) (Thies *et al.* 1999a). Many of these are, however, present in unstressed cells and play essential roles under normal physiological conditions (Watson 1990). A minor protein may therefore become a major protein when its production is fully induced (Inouye 1979). Porins are also markedly affected by extremes of osmotic pressure, temperature, and nutrient starvation (Dawes and Sutherland 1992).

1.6.4 Stress and Heat-Shock Proteins in *C. jejuni*

The production of outer membrane proteins in response to limiting growth conditions, including porins which allow passage of specific nutrients, was mentioned in section 1.6.1. *Campylobacter* spp. are known to possess such proteins, for example, iron-regulated proteins which are induced under iron-limiting growth conditions. Iron acquisition is vital for bacterial survival and the ability to acquire iron under such conditions is considered an important virulence factor. *C. jejuni* has been found to produce three iron-repressed OMPs of 70, 75 and 80 kDa, referred to as ChuA, CfrA and Iro80, respectively. The production of these proteins is induced only under iron-limiting conditions (van Vliet *et al.* 1998). There is also evidence to suggest that the 75 kDa iron-regulated protein (CfrA) is expressed by *C. jejuni* during infection and stimulates the immune response in children infected with *C. jejuni* (Schwartz *et al.* 1990).

The most intensively studied heat shock proteins (Hsps) are those of the 60 kDa (GroEL) and 70 kDa (DnaK) families (Watson 1990, Thies *et al.* 1999a). The GroEL protein works as a pair with a 10 kDa GroES protein which are together encoded by a GroESL bicistronic operon. This operon was cloned and sequenced in *C. jejuni* and was found to show a high level of homology to other bacterial GroES and GroEL proteins. After purification the protein was also

recognised by an anti-Hsp60 monoclonal antibody. The quantity of GroESL protein produced by *C. jejuni* was demonstrated to markedly increase after a temperature upshift (Thies *et al.* 1999 a). The *dnaK* gene of *C. jejuni* was also cloned and sequenced. It was found to encode a protein with a high degree of homology with other bacterial 70 kDa heat shock proteins. Following heat shock, a rapid increase in dnaK mRNA was detectable which reached its maximum after 20-30 minutes (Thies *et al.* 1999b). Wu *et al.* (1994) also detected proteins in the range of 10-120 kDa which were induced and/or released by selective pH or heat treatments.

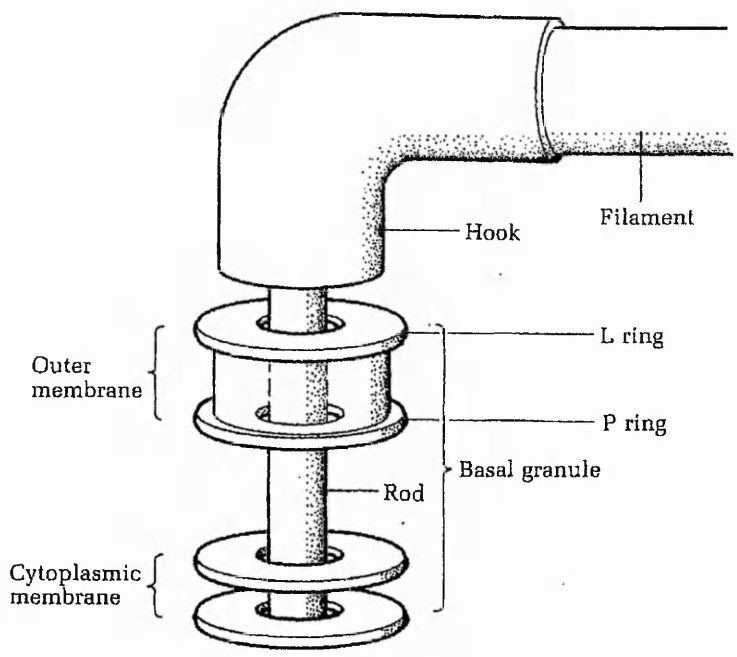
1.6.5 Flagella

Flagella are the organelles that allow motility in bacteria. They are helical structures several times the length of the bacteria composed of subunits of a single protein, flagellin. The filament of each flagellum is attached to the cell membrane at a structure known as the basal granule or disc. A filament is joined by a hook to a rod and a set of rings located in the cell envelope (Fig. 3). Typically in Gram negative bacteria, a set of four rings (L, P, S and M) is associated with LPS, peptidoglycan and the outer and inner face of the cytoplasmic membrane respectively. Many bacteria move end over end, and some bacteria glide (Dawes and Sutherland 1992). *Campylobacter* spp., however have a very distinct, darting, corkscrew-like motility (Holt *et al.* 1994).

1.6.6 *Campylobacter* Flagella

The flagellum is the locomotory organelle and motility is absolutely essential for *C. jejuni* to colonise the gastrointestinal tract and cause disease (Yao *et al.* 1994, Guerry 1997, see section 1.5, p9, *pflA* mutant). This has been demonstrated in studies of nonmotile mutants of *C. jejuni* which were shown to completely lose their ability to invade the gastrointestinal tract (Power *et al.* 1992) and INT 407 cells. Adherence to INT 407 cells of nonmotile mutants was also significantly reduced (Yao *et al.* 1994).

Fig. 3 The Bacterial Flagellum



(Dawes and Sutherland 1992)

Flagellin, approximately 62 kDa, is the subunit of the flagellar filament and is recognised as the major immunodominant protein during infection (Wu *et al.* 1991, Nachamkin and Yang 1996, Guerry 1997). It is the flagellin antigen which is the basis of the Lior heat-labile serotyping scheme. The flagellar hook protein is the structure which mediates the rotation of the flagellar filament thus imparting the unusual, rapid, darting motility which is characteristic of the *Campylobacter* genus. On removal of the flagellar filament the hook proteins were found to be 92.5 or 94 kDa. They carried both cross-reactive and specific non-exposed epitopes as well as serospecific epitopes which were over-exposed on the surface of the assembled hook. It was therefore suggested that the *Campylobacter* hook protein is likely to be the antigenically variable domain (Power *et al.* 1992).

Another important aspect of the campylobacter flagellins is that they are glycosylated. This is a posttranslational modification which is surface exposed on the flagellar filament and confers serospecificity. They have also been shown to include a sialic acid residue which suggests the possibility of molecular mimicry between glycosylated flagellin and eukaryotic glycoproteins (Guerry 1997, Szymanski *et al.* 1999). A genetic locus of *C. jejuni* 81-176 was characterised which appeared to be involved in a general glycosylation system of multiple proteins. The genes were named *pgl* (for protein glycosylation). The glycosyl moieties appeared to be immunodominant and loss of the carbohydrate components from the glycoproteins caused a drastic reduction in their reactivity with antisera (Szymanski *et al.* 1999). Flagella are therefore considered as very important virulence factors and this system of general protein glycosylation could have many implications for cell-host interactions including the molecular mimicry of human gangliosides.

The examples above have given some indication of the extensive level of variation of different OMPs expressed, whether they be cell-associated, secreted and/or stress-induced, where each plays its own individual, essential role in the pathogenesis of *C. jejuni.*

1.6.7 Lipopolysaccharide (LPS)

The LPS is a unique constituent of the bacterial OM which is not found anywhere else in the cell except as a biosynthesis intermediate (Nikaido and Vaara 1987). Structurally, LPS is comprised of three genetically, chemically and antigenically distinct regions. The first of these is Lipid A. This is the least variable component which anchors LPS to the bacterial membrane and has a vast number of biological activities. It is also referred to as the 'endotoxin' as it is highly reactive within the host, provoking a range of immunological and biochemical responses to infection such as fever and inflammation. Where present in high amounts it can also induce septic shock, coagulation of the blood and even death (Zahringer *et al.* 1994, Moat and Foster 1995, Moran 1995). Endotoxins are the major components of the outer leaflet of the OM, whereas the inner leaflet is mainly composed of phospholipids (Moran 1995). The second region is the oligosaccharide core which is relatively consistent among Gram

negative bacteria and includes two characteristic sugars; keto-deoxyoctonic acid (KDO) and a heptose. The final region of LPS is the O antigen. This is a long carbohydrate chain comprised of up to 60 repeating units which, in turn, are made up of 2-6 glycose residues. The O-specific chain is responsible for the heat-stable O-antigens of the bacteria and serves to identify multiple serotypes by the binding of serotype-specific antibodies (Zahringer *et al.* 1994). The O antigen shows enormous structural variability and often contains sugars which are not found in other polymers (Dawes and Sutherland 1992). In addition to the endotoxic lipid A, the O antigen is a very important virulence factor which is absent in some enterobacterial pathogens. LPS are immunodominant antigens of most Gram negative bacteria and these antigenic determinants protrude through the bacterial OM (Schnaitman and Klena 1993). The LPS is thus a potent immunostimulator that additionally participates in various physiological membrane functions essential for the growth and survival of the bacterial cell (Zahringer *et al.* 1994).

1.6.8 *Campylobacter* Lipopolysaccharides (LPS)

The LPS of *C. jejuni* has proven to be a mystery to many investigators over the past 15-20 years due to the discovery of an extensive diversity of the LPS expressed in different strains (Penner and Aspinall 1997). Many studies have reported the detection of short-chain (rough type) LPS only, rather than the classical long-chain LPS which is typical of members of the *Enterobacteriaceae*, such as *Salmonella.* Mills *et al.* (1985) proposed two structures of LPS which would account for their serological heterogeneity. The first possibility was that the LPS has a structure termed lipooligosaccharide (LOS) consisting of lipid A to which is attached a short oligosaccharide. The second possibility offered was that LPS consists of lipid A with a core resembling those of the *Enterobacteriaceae* but to which is attached a single repeat unit rather than a long O side chain. Logan and Trust (1984) also studied the LPS of 20 strains of *C. jejuni*, all of which were of low molecular weight and typical of LPS lacking O antigens (LOS). Many other authors supported these findings, for example, Penner and Aspinall (1997) reported the presence of O chains in 16 strains of *C. jejuni* and their absence in 22 strains and Oldfield *et al.* (1998) reported that the

majority of strains produced LOS and not LPS. Moran (1997) also found that approximately one third of *C. jejuni* serostrains had smooth LPS and the others all appeared to express rough LPS. Furthermore, the O-antigen locus was found to be present in isolates of *C. jejuni* with a surprisingly high degree of conservation (Oldfield *et al.* 1998). Many of these studies were performed using SDS-PAGE and a carbohydrate silver stain, and long-chain LPS was usually only identified after immunoblotting with homologous antisera (Preston and Penner 1987). The identification of long-chain LPS only following SDS-PAGE and silver staining of *C. jejuni* strains, without immunoblotting was, however, reported by some groups, for example Blake and Russell (1993).

Evidence was found which indicated that *C. jejuni* was able to shift the antigenic makeup of its cell surface by exposing new determinants or losing others (Mills *et al.* 1992). This demonstrates the high variation in genetic expression of *C. jejuni.* Mills *et al.* (1992) also suggested that this antigenic variation occurred *in vivo* and that the host had an effect on the LPS which caused a 'switch' in antigenic expression. It was thus the general consensus until recently that most *C. jejuni* strains expressed low weight lipooligosaccharide (LOS) which is typical of *Neisseria*, *Haemophilus* and *Bordetella* spp. which are more variable in structure than the highly conserved long-chain LPS of *Salmonella* spp. (Aspinall *et al.* 1993, Fry *et al.* 1998). The relevance of this in the virulence of *C. jejuni* was still debatable but some suggestions were put forward. Aside from the obvious advantage of the apparent ability to 'switch on/off' genes and antigen expression (known as phase variation and antigenic variation, respectively), an observation was made that serum-resistant *C. jejuni* strains all had smooth LPS profiles, but serum-sensitive *C. jejuni* strains all had rough LOS profiles (Perez-Perez and Blaser 1985). It has been reported that it is the core oligosaccharide that contains sialic acid groups and is hence able to mimic the structure of human gangliosides (Aspinall *et al.* 1993, Moran 1997, Mills *et al.* 1998). This is commonly implicated in autoimmune diseases in humans such as Guillain-Barré syndrome. A more detailed account of this is given in section 1.12.1. The lipid A of *C. jejuni* differs structurally from the classical enterobacterial lipid A but is antigenically similar and displays comparable endotoxic activities to those of enterobacterial LPS (Moran 1997). More light has been shed on the virulence properties of *C. jejuni* due to the recent

completion of sequencing of the *C. jejuni* 11168 genome. A study by Fry *et al.* (2000) reported the cloning and sequencing of the *wla* gene cluster which is involved in the sequence of the *C. jejuni* LPS molecule. Sequence alignment showed homology with *galE* genes which are essential for the synthesis of smooth LPS in *Salmonella.* A *C. jejuni galE* mutant was constructed which showed a reduction in its ability to adhere to and invade INT 407 cells and a 20-fold reduction in its ability to take up DNA and integrate it into the genome (Fry *et al.* 2000).

The most important recent discovery is the detection of a capsular polysaccharide in *C. jejuni* for the first time. Suggestion was made by Chart *et al.* (1996) that the heat-stable antigens in *C. jejuni* were probably capsular and not long-chain LPS. No further implications of this have been made until a recent discovery by Karlyshev *et al.* (2000), who searched the *C. jejuni* 11168 genome database and found genes similar to *E. coli* genes involved in the biogenesis of the K1 and K5 capsular polysaccharide. High molecular weight LPS was demonstrated in all the *C. jejuni* strains tested and this LPS was shown to be biochemically and genetically unrelated to LOS with similarities to capsular polysaccharides. *C. jejuni kpsM*, *kpsS* and *kpsC* mutants lost their ability to produce O-antigen, so the previously described O-antigen of *C. jejuni* was demonstrated for the first time to be a capsular polysaccharide. The role of the capsular polysaccharide has therefore been previously underestimated in the virulence of *C. jejuni* and could be more intimately involved in survival mechanisms such as the VNC state.

1.7 Serotyping of Campylobacters

Within the species *C. jejuni* there are many different strains, only some of which have been associated with disease. There is still no distinct correlation between different strains and type and/or severity of illness, or even the vehicle of transmission. The antigenic diversity of these strains allows serotype differentiation. Penner serotyping uses heat-stable lipopolysaccharide (LPS) antigens in a passive haemagglutination assay (Penner and Hennessy 1980), a method which has identified over 60 serotypes. The Lior serotyping scheme is a slide agglutination method based on heat-labile flagella antigens (Lior *et al.*

1982) which has identified more than 100 serotypes (Walker *et al.* 1986, Griffiths and Park 1990, Wassenaar and Blaser 1999). New serotyping schemes and modifications of the Penner and Lior schemes have been described (Griffiths and Park 1990) to aid discrimination between *C. jejuni* strains including many DNA-based schemes such as pulsed-field gel electrophoresis and PCR-RFLP (Fields and Swerdlow 1999).

There is, however, a lack of correlation between serotypes and DNA profiles and no single method can be relied upon for definitive typing thus making comparisons between different laboratories very difficult (Fields and Swerdlow 1999). As a result of this, a project called Campynet has recently been set up. Campynet is a network project financed by the EU for the harmonisation and standardisation of molecular typing methods for campylobacters. The project includes researchers from 23 institutes in 11 different countries in the EU who aim to provide standardised molecular typing methods for *C. jejuni* and *C. coli* in an attempt to facilitate epidemiological studies of these foodborne pathogens (http://www.svs.dk.campynet/).

1.8 Toxins

1.8.1 Bacterial Toxins

The term 'bacterial toxin' refers to a whole host of bacterial products which may be internally anchored or secreted externally and includes enzymes and other proteins with specific activities upon host target cells. A toxin can be defined as 'a bacterial product whose principal common feature is that they are harmful to various sensitive hosts when administered in relatively low doses' (Arbuthnott 1978). Other definitions of toxins are 'a microbial substance able to induce host damage' and 'any microbial product or substance that is harmful or lethal to cells, tissue cultures or organisms' (Williams and Clarke 1998). The human body is an abundant source of nutrients and toxin production is just one of several sophisticated mechanisms that bacteria use to combat the human immune defences to acquire them. The human body is thus an ecological niche for a bacterial pathogen (Williams and Clarke 1998). The amount of toxin secreted into the surrounding medium varies for different toxins but is in the range of 1-

500 mgl^{-1} though it does not usually exceed 100mgl^{-1} (Arbuthnott 1978). Only a small proportion of all bacterial genera (approximately 10%) include species which are pathogenic to humans and animals (Stephen and Pietrowski 1986). Different strains of the same species often vary in their toxin-producing capabilities. Relatively few toxins secreted from bacteria have been shown experimentally to influence the pathogenicity of the producing organism although they produce harmful/lethal effects upon host cells in culture. Additionally, many bacteria produce toxins, known as bacteriocins, that are lethal to other bacterial species but do not affect eukaryotic cells (Williams and Clarke 1998). It is therefore only the toxins which are of clinical importance in the pathogenesis of disease in humans which will be subject to discussion and reference during the current investigation.

1.8.2 Classification of Toxins

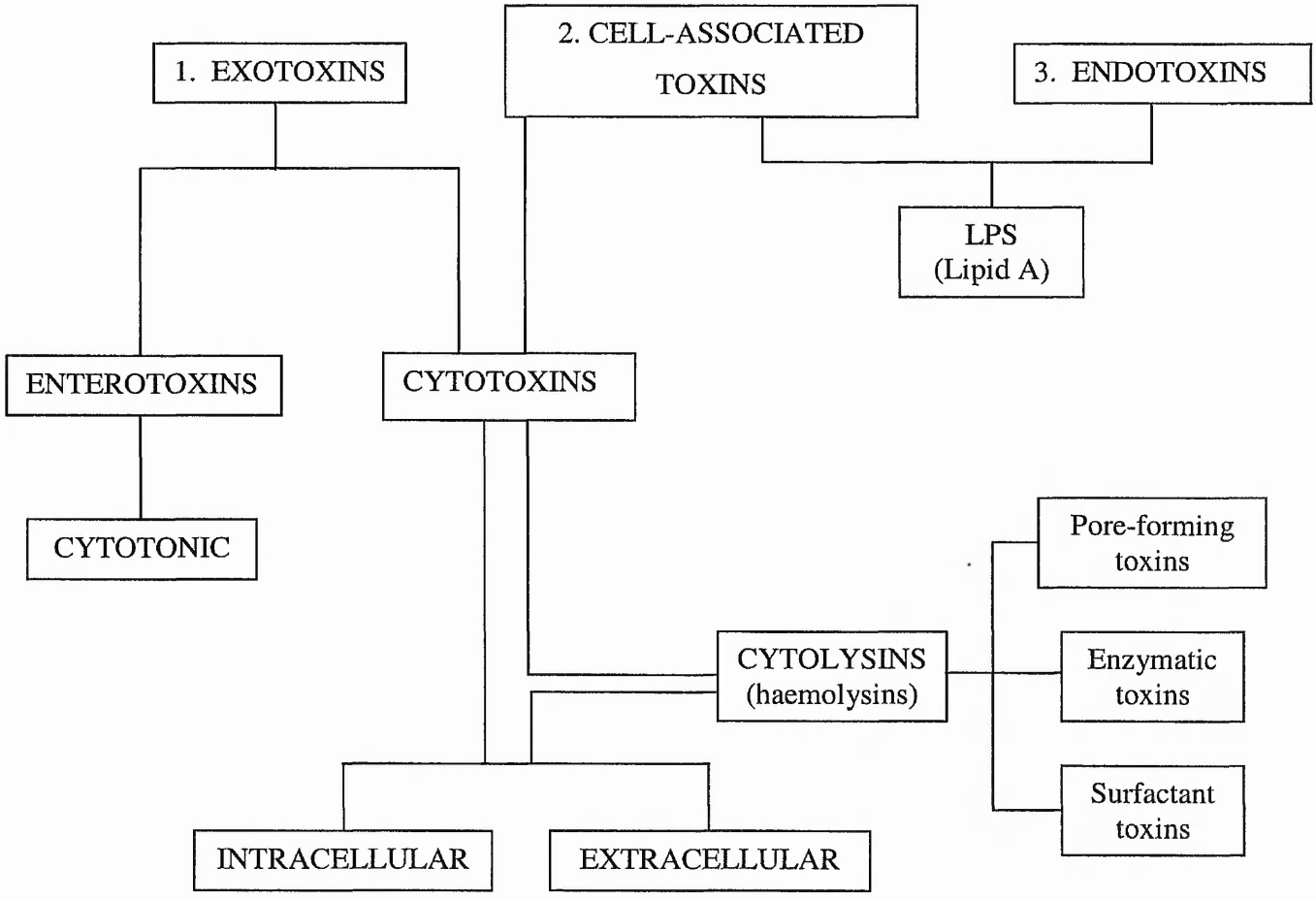
The nomenclature of different types of toxin in the literature varies greatly from author to author and many old classifications of toxins are no longer valid due to recent advances. A useful guide is to remember that most toxins are named according to their mode of action/target cells eg. haemolysins (red blood cells), neurotoxins (nerve cells), enterotoxins (gut cells) (Balfanz *et al.* 1996). The following classification scheme represents many current opinions on toxin classification and will be used throughout the rest of this report. The flow diagram in Fig. 4 shows a schematic representation of general toxin classification. The first division of the bacterial toxins is into three major classes; (1) Exotoxins (2) Cell-Associated Toxins and (3) Endotoxins. These are further categorised as later explained.

(1) Exotoxins, as indicated by their name, are produced intracellularly and exported out of the cell to elicit their effect. They are secreted proteins whose site of action may be some distance from the site of infection. They exert their specific effects at low concentrations, often by enzymatic activities (Arbuthnott 1978, Balfanz *et al.* 1996, Williams and Clarke 1998). The subgroups of exotoxins consist of cytotoxins, enterotoxins and some neurotoxins.

(2) Cell-associated toxins are anchored to the cell and include protein components of the outer membrane which are surface exposed. This group of

toxins represent outer membrane structural entities which have key functions in the organism (Stephen and Pietrowski 1986). The subgroup of cell-associated toxins consist only of cytotoxins. Endotoxins are also sometimes referred to as 'cell-associated toxins'.

Fig. 4: Flow Diagram of General Toxin Classification



(3) Endotoxins are located inside the bacterial cell and are integral components of the lipopolysaccharide in the Gram negative membrane known as lipid A (see section 1.6.7). There is no significant release of toxin into the surrounding environment during normal cell growth and survival, and the host only comes into contact with endotoxin following cell lysis due to such things as phagocytic attack or antimicrobial therapy. This can then be fatal for the host as a massive immune response is initiated which often leads to septic shock (Arbuthnott 1978, Balfanz *et al.* 1996, Williams and Clarke 1998).

Enterotoxins: Bacterial toxins which act directly on gut tissue and cause biochemical and/or structural lesions which lead to diarrhoea. Enterotoxins are exported out of the cell and are not normally cell-associated (ie. they are exotoxins). This definition strictly excludes old classifications such as staphylococcal enterotoxins which are in fact neurotoxins; the vomiting they initiate is mediated via interaction with the vagus nerve (Stephen and Pietrowski 1986).

The enterotoxins themselves can also be split into two categories i) Cytotonic Enterotoxin and ii) Cytotoxic Enterotoxin. Cytotonic enterotoxins induce net fluid secretion by interfering with biochemical regulatory mechanisms and causing ion flux changes. This leads to profuse, watery diarrhoea without causing any histological damage or cell death. Cytotoxic enterotoxins induce actual damage to intestinal cells in order to cause net fluid secretion and subsequent diarrhoea (Stephen and Pietrowski 1986, Agbodaze 1999).

Neurotoxins: A toxin is classified in this group if at least part of the secretory activity of the toxin is attributable to the release of one or more neurotransmitters from the enteric nervous system or if the toxin alters smooth muscle activity (Sears and Kaper 1996).

Cytotoxins: Cytotoxins can be defined as toxins which cause direct cell damage and/or death in order to elicit their effects upon the host. Cytotoxins generally fall into one of three groups dependent upon their mode of action; i) those acting on extracellular targets on the plasma membrane ii) those acting on intracellular targets and iii) those toxins which cause cell lysis (cytolysins).

Cytolysins/Haemolysins: The term 'cytolysins' refers to a whole range of bacterial toxins which lyse cells using different modes of action and are also often termed 'haemolysins', so-called due to their ability to lyse red blood cells. Many are, however, toxic to other cells as well such as leukocytes. Haemolysins are generally considered to be virulence factors although their contribution towards disease varies, activity upon leukocytes may be a survival mechanism of the pathogen against the human immune system. The importance of erythrocyte lysis is unclear but it has been suggested that this is a mechanism of iron uptake in many pathogens. It does, however, serve as a convenient phenotype for screening cytolytic activity *in vitro* (Rowe and Welch 1994). Green zones surrounding colonies on blood agar are referred to as α-haemolysis and clear

zones are referred to as β-haemolysis (Misawa *et al.* 1995). Haemolysins are split into three categories depending on their mechanism of action: i) enzymatic ii) pore-forming iii) surfactant. The enzymatic haemolysins include the *Clostridium perfringens* α-toxin or lecithinase. The RTX toxin family (see section 1.11.2.1) are pore-forming cytolysins and the *Staphylococcus aureus* delta (δ) toxin is an example of a surfactant toxin (Rowe and Welch 1994).

1.8.3 The Origin of Toxins

The genes which control toxin production and expression vary among different strains of the same species and are often located in plasmid DNA. This has been well-established for the *E. coli* enterotoxin and the staphylococcal epidermolytic toxin (Arbuthnott 1978). It is considered likely that *E. coli* LT was acquired from *Vibrio cholerae* CT by plasmid transfer due to their identical subunit structures and modes of action. Suggestion has been made that many other toxins with similar or identical structure and function have been acquired by vertical transfer of the genes i.e. from a common ancestral bacterium before it diverged into two or more groups. Alternatively the genes may have been acquired by horizontal transfer across taxonomic divisions within plasmids or transposons. For example, cholera toxin and pertussis toxin have similar activities but are produced in widely divergent species with different receptors. Both act by ADP ribosylation of adenylate cyclase which causes the elevation of cyclic AMP levels, but pertussis toxin targets the respiratory tract and cholera toxin targets the intestinal tract (Williams and Clarke 1998).

1.9 Toxins Produced by *Campylobacter* spp.

Campylobacter jejuni (and *C. coli*) have been reported to produce at least eight different toxins consisting of one enterotoxin and seven cytotoxins. This may yet prove to be an over- or under- representation of the actual numbers of toxins produced by *Campylobacter* spp. due to various discrepancies of the organism itself, the research groups and techniques involved.

A wide number of strains from a wide range of sources have been tested for their toxic potential, and significant strain to strain variation may account for differences in the reports of toxin production. There is a great deal of contradictory evidence in the literature surrounding many of the cytotoxins and, in particular, the enterotoxin. Additional variation is found between the laboratories, where different culture media, extraction and purification methods, and tissue culture cell lines have been employed for these studies. Many of the authors also have not attempted to correlate their findings with those of other research groups and a lack of standardisation of the methods and cell lines used makes this a very difficult and laborious task.

Attempts have, however, been made to correlate toxicity results with the clinical symptoms induced by the isolates, but again, findings are contradictory and no clear conclusions to this have been made. It has largely been suggested that the type of diarrhoea that the patient presents with is likely to directly correspond with the phenotype of the strain and the types of toxin being expressed (Klipstein *et al.* 1985, Johnson and Lior 1986, Walker *et al.* 1986, Daikoku *et al.* 1989, Fricker and Park 1989, Perez-Perez *et al.* 1989, Mahajan and Rodgers 1990, Florin and Antillon 1992, Prasad *et al.* 1996, Wassenaar 1997, Lee *et al.* 2000). Strains which are invasive and/or produce invasive, cell-destructive cytotoxins are thought to correspond with patients suffering the inflammatory, invasive type of diarrhoea which contains blood and leukocytes. Strains which produce enterotoxin are thought to correspond with patients suffering from profusely watery diarrhoea. This hence, at least theoretically, links the type of toxin being produced with the clinical status of the host. It is also possible that a number of different toxins are being produced but the one which is produced most abundantly is the one which dominates the clinical manifestations of the host. Alternatively, it has also been suggested that other mechanisms (such as invasion, leading to inflammation and fluid secretion in the intestine) are being employed by campylobacters to evoke these symptoms and toxins do not play an important role in the disease process (Perez-Perez *et al.* 1989, Ketley 1997, Leach 1997). This is, however, rather ambiguous considering that the broad definition of a toxin includes any molecule produced by the bacteria which causes damage or disruption to cells within the host.

The first *Campylobacter* toxin which will be discussed is the enterotoxin which is thought to be related to cholera toxin and has been a subject of debate for many researchers for many years.

1.10 Enterotoxins

The first bacterial enterotoxins to be recognised and described were the *Vibrio cholerae* enterotoxin (CT) and the *E. coli* heat-labile toxin (LT) (Wassenaar 1997). Bacterial enterotoxin activity can be demonstrated *in vitro* by the elongation of Chinese hamster ovary (CHO) cells or rounding of mouse adrenal tumour (Y-1) cells. Other effective methods are the measurement of intracellular cyclic AMP (cAMP) levels, or an ELISA using either GM_1 gangliosides or antisera to cholera toxin or *E. coli* heat-labile toxin. *C. jejuni* has been reported to produce an enterotoxin which is very similar to cholera toxin (CT) and is referred to as cholera-like toxin (CLT), *Campylobacter* cytotonic toxin (CCT) or *C. jejuni* enterotoxin (CJT). This will be described further in section 1.10.3

1.10.1 Cholera Toxin (CT)

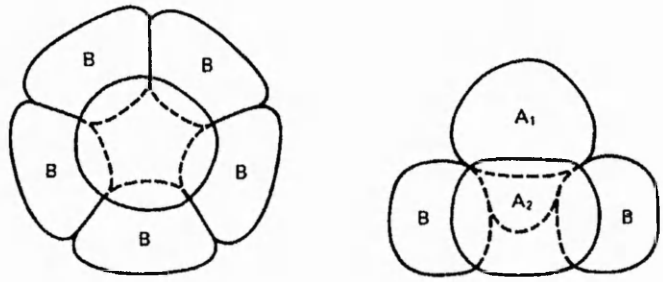
Cholera is a clinical-epidemiological syndrome caused by *Vibrio cholerae* (Kaper *et al.* 1995). It remains an important problem in many developing countries and any area where water supplies can become contaminated (Spangler 1992). Humans usually contract cholera from food or water that has been contaminated by the faeces of a cholera victim. The ingested bacteria multiply in the alimentary canal, adhere to specific receptors in the small intestine, and after 2-5 days cause the sudden onset of nausea, vomiting, diarrhoea and abdominal cramp (Stephen and Pietrowski 1986). Severe dehydration and cramps are the consequence of a rapid, extreme loss of fluid and electrolytes during the course of infection (Ganguly and Kaur 1996). Loss of fluid from the gut in the form of liquid faeces, often referred to as 'rice water stools', may be as great as 20 litres a day in severe cases (Stephen and Pietrowski, 1986). Cholera can be treated with antibiotics and oral rehydration therapy but it is still an extremely debilitating and often fatal disease (Spangler

1992). Hypovolemic shock, acidosis and death can ensue if prompt and appropriate treatment is not initiated (Kaper *et al.* 1995).

The enterotoxin of *V. cholerae*, known as cholera toxin (CT), was first isolated by Robert Koch in 1887 following his proposal that the agent responsible for cholera produced a 'special poison' which acted on the intestinal epithelium (Kaper *et al.* 1995). It was not until 1959 that De and his colleagues demonstrated that injection of lysates or culture filtrates of *V. cholerae* into rabbit ileal loops led to the accumulation and subsequent outpouring of fluid (Stephen and Pietrowski 1986, Kaper *et al.* 1995). Ten years later the toxin was purified by Finkelstein and LoSpulloto and studies on the structure and mode of action of cholera toxin began (Kaper *et al.* 1995).

Cholera toxin or 'choleragen' (Levine *et al.* 1983, Stephen and Pietrowski 1986) is an oligomeric protein of approximately 84 kDa. It consists of two separate moieties, or subunits; subunit A of 28 kDa and subunit B of 56kDa.

Fig. 5 Subunit Structure of Cholera Toxin



(Stephen and Pietrowski 1986)

Further studies revealed that subunit B consists of an aggregate of five smaller proteins, each of 11 kDa, and that subunit A can be proteolytically cleaved into A_1 and A_2, of 23 and 5 kDa, respectively. The A and B subunits are associated by covalent bonds and the A subunit lies on the central axis of a ring of five B subunits. The five B subunits of cholera toxin form a very stable pentamer structure. It is the A_2 subunit which is believed to serve in holding the A_1 and B subunits together in tertiary configuration by powerful internal protein-

protein interactions (Levine *et al.* 1983, Stephen and Pietrowski 1986, Ganguly and Kaur 1996).

Cholera toxin is the prototype of the AB_5 subunit toxins, in which the subunits each have specific functions. The B subunit is responsible for binding of the toxin to its eukaryotic cell receptor and the A subunit possesses specific enzymatic function that acts intracellularly (Ganguly and Kaur 1996, Sears and Kaper 1996). Subunit A is unable to attach itself to cell receptors and subunit B has no enzymatic activity, so the two covalently linked subunits are required for the full, biologically active form of the cholera toxin (Stephen and Pietrowski 1986). Subunit B is therefore immunodominant and antibodies directed against it are much more efficient at neutralising toxin activity than are antibodies directed against the A subunit (Kaper *et al.* 1995). The intestinal receptor for CT B subunit is ganglioside GM_1 and the intracellular target enzyme of subunit A is adenylate cyclase. The B subunit initiates translocation of subunit A across the cell membrane. Binding of CT requires at least two of the B subunits to interact with GM_1 (Ganguly and Kaur 1996). Two modes of entry of cholera toxin into cells were proposed by Gill in 1976 using the following model (Fig. 6):

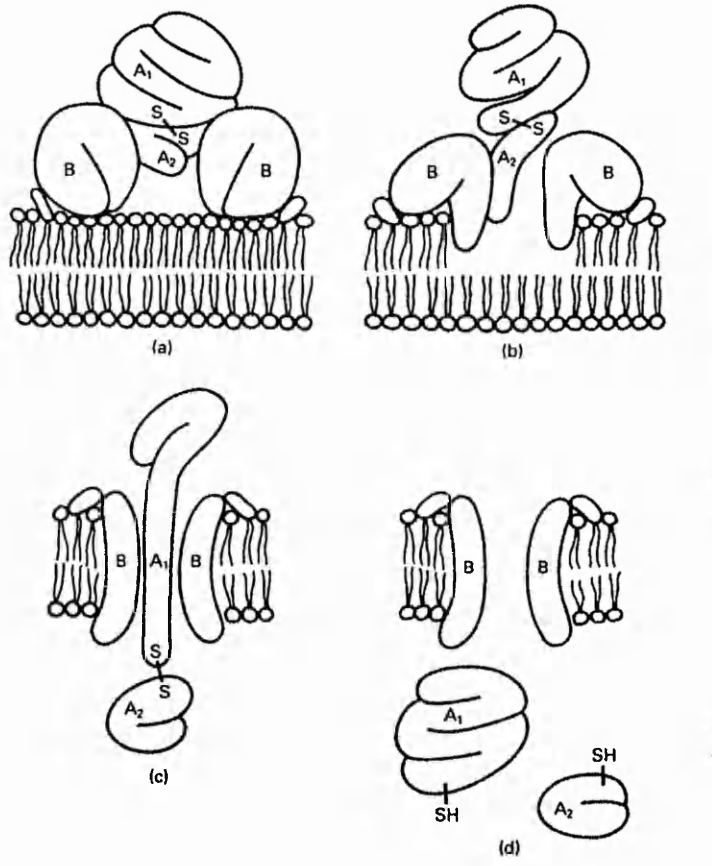
(1) Intimate interaction between all five of the B subunits and the cell membrane may be sufficient to allow A_1 to cross the membrane unaided, the B subunits playing no further role in the process.

(2) Internal hydrophobic protein-protein forces of the B subunits are replaced by lipid-protein (ganglioside) interaction (a), thus causing unfolding of the B subunits to form a hydrophilic tunnel through which A can diffuse (b) and (c). Here, thiol agents reduce the disulphide bridge between fragments A_1 and A_2 (d). The cleaved A_1 is then free to exert its enzymatic action in the cytoplasm (Fig. 6).

The intracellular target of CT is adenylate cyclase which is one of the most important regulatory systems of the eukaryotic cell. Subunit A of CT activates this enzyme by ADP-ribosylation which, in turn, mediates the transformation of ATP to cyclic AMP (cAMP). Adenylate cyclase is normally activated/inactivated in reponse to a variety of stimuli and this is regulated by the G proteins. The G proteins are a large family of eukaryotic proteins that couple receptors to a variety of enzymes and ion channels in eukaryotic cell membranes. After CT has bound to GM_1, there is a lag time of 15 to 60 minutes before the

activation of adenylate cyclase. This lag time is the time it takes for the A_1 peptide to translocate across the membrane and come into contact with the G proteins. The resultant increase in cyclic AMP concentrations leads to increased Cl^- secretion by intestinal crypt cells and decreased NaCl-coupled absorption by villus cells. The net movement of electrolytes into the lumen causes a transepithelial osmotic gradient which causes water flow into the lumen. This soon creates a massive volume of water which overwhelms the adsorptive capacity of the intestine (Kaper *et al.* 1995). The final result is voluminous, watery diarrhoea which is rich in electrolytes, including Na^+, K^+, Cl^- and HCO_3^- (Levine *et al.* 1983). Massive dehydration and shock occur following these events.

Fig. 6 Mechanism of Entry of Cholera toxin



(Stephen and Pietrowski 1986)

Cholera toxin is encoded by chromosomal genes in *V. cholerae* and is the prototype of a number of bacterial toxins which have an AB_5 structure and a similar mode of action. The most remarkably similar enterotoxin, both structurally and functionally, is the *E. coli* heat-labile toxin (LT).

1.10.2 *E. coli* Heat-Labile Enterotoxin (LT)

E. coli LT is a large, immunogenic, heat-labile protein with a molecular weight of 90-96 kDa. It resembles CT structurally, functionally, biologically and immunogenically and shares 80% amino acid sequence homology with CT. It is a hetero-hexameric compound of one A and five B subunits, with the pentameric B subunit responsible for binding the toxin, and the A subunit responsible for the irreversible activation of adenylate cyclase. There are, however, some differences between LT and CT. CT is secreted into the culture supernatant during growth, but LT remains cell-associated within the periplasm and is not secreted (Spangler 1992). It can only apparently reach its site of action following bacterial death and lysis (Williams and Clarke 1998). Holmgren (1985) postulated that in the human or animal intestine *in vivo*, gut fluid factors may be responsible for releasing LT from the periplasm and activating it by proteolytic nicking. The receptor molecule for CT is ganglioside GM_1, but LT will bind to GM_1 and galactose glycoprotein. Additionally, besides activating adenylate cyclase, LT has been reported to activate calcium-dependent protein kinases and elevate intracellular calcium levels causing phosphorylation of membrane proteins. The ultimate effects are still the accumulation of water and electrolytes in the intestinal lumen.

The remarkable structural and functional similarities between LT and CT suggest that they may be evolutionarily related. The gene encoding LT is plasmid encoded and shows 80% homology with CT. It has thus been postulated that the LT gene is a foreign gene acquired by *E. coli* and transmitted by species-to-species (horizontal) transfer from the *V. cholerae* CT gene ancestor (Spangler 1992). LT is responsible for a much milder disease in adult humans (Rhodigiero *et al.* 1999) than the severe, dehydrating, cramping diarrhoea of CT, and is also known as traveller's diarrhoea (Spangler 1992).

1.10.3 *C. jejuni* Enterotoxin

The production of an enterotoxin by *C. jejuni* was first reported in 1983 by Ruiz-Palacios *et al.* who reported elongation of CHO cells and increased intracellular cAMP levels in vitro. They also demonstrated that culture supernatants of *C. jejuni* strain INN-1-79 caused intraluminal fluid secretion in the rat ileal loop test (RILT) model. This was followed by a considerable number of other reports on enterotoxin detection in *C. jejuni.* Klipstein and Engert (1984a) reported that many strains produce a heat-labile enterotoxin which they refer to as 'CJT'. They demonstrated cytotonic effects by the rounding of Y-1 cells and elongation of CHO cells and also by fluid secretion in ileal loops. The immunological cross-reactivity between the campylobacter enterotoxin and CT and LT was first demonstrated by Ruiz-Palacios *et al.* (1983) and many others have since reported neutralisation of the toxin with antisera against CT and/or LT (Klipstein *et al.* 1984a and 1984b, Mathan *et al.* 1984, McCardell *et al.* 1984, Goossens *et al.* 1985a). This led to the development of an ELISA based on binding to CT antibody which was used to screen supernatants (McCardell *et al.* 1984). Enterotoxic activity in the supernatant was confirmed by Mathan *et al.* (1984), Klipstein and Engert (1984a and b), Johnson and Lior (1984 and 1986), Goossens *et al.* (1985a), Klipstein *et al.* (1985), Kawaguchi *et al.* (1989), Lindblom *et al.* (1989), Daikoku *et al.* (1989 and 1990), Collins *et al.* (1992), Florin and Antillon (1992) and Suzuki *et al.* (1994).

The common tests for enterotoxin activity were established as rounding of Y-1 cells, elongation of CHO cells and neutralisation with anti-CT and/or anti-LT. The RILT (rabbit or rat intestinal loop test) model was also used but was found in most cases to only show a positive result with rats, and not rabbits (Ruiz-Palacios *et al.* 1983, Wadstrom *et al.* 1983, Klipstein and Engert 1984a and 1984b, Saha and Sanyal 1990, Wassenaar 1997). Neutralisation by ganglioside GM_1 was also reported in a number of studies (Klipstein and Engert 1984a and b, Klipstein *et al.* 1985, Calva *et al.* 1989) and a GM_1-based ELISA has frequently been used for screening enterotoxin in *Campylobacter* spp. (Mathan *et al.* 1984, McCardell *et al.* 1984 and 1986a, Klipstein *et al.* 1985, Lindblom *et al.* 1989, Perez-Perez *et al.* 1989, Collins *et al.* 1992). The *C. jejuni* enterotoxin is often referred to as heat-labile cytotonic toxin. It is called

cytotonic due to its reversible effects upon Y-1 and CHO cells with no resultant long-term damage (McCardell *et al.* 1984, Johnson and Lior 1988a, Florin and Antillon 1992). Enterotoxin production has also been detected in some strains of *C. coli* (Johnson and Lior 1984 and 1986, Belbouri and Megraud 1988, Lindblom *et al.* 1989 and 1990) and in *C. lari* (Johnson and Lior 1986). Some contradiction is apparent in the reports of heat-lability. Johnson and Lior (1984 and 1986) reported heat-stability of the toxin, showing that no toxicity was lost after heating to 70°C for 30 minutes. Heat-lability, on the other hand, was reported by numerous investigators, firstly by Ruiz-Palacios *et al.* (1983) who demonstrated almost complete inactivation at 56°C for 1 hour and at 96°C for 10 minutes. The latter heat/time combination was confirmed to inactivate the toxin by Klipstein and Engert (1984b), and Florin and Antillon (1992) later supported this by demonstrating the inactivation of enterotoxin at 100°C for 10 minutes. McCardell *et al.* (1984) reported the inactivation of both culture supernatant and purified enterotoxin after heating to 90°C for 15 minutes. Goossens *et al.* (1985a) failed to report on heat-sensitivity.

Purification of the enterotoxin has been attempted by some groups. Klipstein and Engert (1984a) purified the enterotoxin, or CJT as they referred to it, from *C. jejuni* strain INN-73-83 by the application of a method used for LT based on affinity to the ganglioside GM_1. They reported that purification resulted in a nearly 23 000-fold increase in the specific activity of CJT, which gave a positive response at concentrations as low as 40pg. Unfortunately, no attempt was made to determine the molecular weight of the toxin. McCardell *et al.* (1984) purified the toxin from the supernatant of strain CH5 using an affinity column with conjugated anti-CT. The rounding of Y-1 cells by affinity-purified material confirmed that the campylobacter cytotonic toxin, or CCT as this group called it, had been successfully purified. SDS-PAGE revealed one band at 70 kDa with an isoelectric point of 9.0, whereas the pI of CT on the same gel was 6.6. Daikoku *et al.* (1990) were also successful in partial purification of the enterotoxin by gel filtration from the culture supernatant of a strain isolated from the stools of a child in Hakodate Chuou Hospital. The purified fraction revealed three bands of 68, 54 and 43 kDa on SDS-PAGE and enhanced the adenylate cyclase activity of HeLa cell membranes by 1.5-fold over that of the control.

Affinity column chromatography was carried out with anti-cholera toxin immunoglobulin and ganglioside GM_1. The anti-CT affinity column fraction exhibited a band at 68 kDa on SDS- and native- PAGE. The eluent from the GM_1 column exhibited two bands at 68 and 54 kDa on SDS-PAGE. The authors hence suggested that the 68 kDa polypeptide should have an immunological relationship with cholera toxin and the 68 and 54 kDa polypeptides might be responsible for the recognition of ganglioside. The purified toxin exhibited three bands on SDS-PAGE and one band on native PAGE (68 kDa) and the hypothesis given by the authors was that the holotoxins of the enterotoxin would aggregate each other and form oligomers as the native form. No sugars or glycoproteins were detectable in these fractions by a periodic acid-Schiff (PAS) stain.

It has additionally been reported by a number of research groups that the culture media used for growth of *C. jejuni* and the treatment of cells prior to testing has a marked influence on the demonstration of enterotoxin. Ruiz-Palacios *et al.* (1983) demonstrated a 16-fold increase in enterotoxin production from *C. jejuni* INN-1-79 grown in Brucella broth supplemented with polymyxin B or 0.25% L-asparagine, 0.25% L-serine and 0.25% L-glutamic acid. Polymyxin B treatment of the culture filtrates has also been recommended to enhance the recovery of enterotoxin by other investigators (Johnson and Lior 1984, Klipstein and Engert 1984a and 1984b, Kawaguchi *et al.* 1989). Goossens *et al.* (1985a) proposed a new medium modified from that of Ruiz-Palacios *et al.* (1983) that consisted of Brucella broth supplemented with 0.25% L-asparagine, 0.25% L-serine, 0.25% L-glutamic acid, 0.05% L-cystine, 0.5% yeast extract and 0.48% dextrose (pH 7.1). This was designated HG broth and was recommended by the authors for high titres of enterotoxin, based on experiments with 25 clinical isolates of *C. jejuni*. Klipstein and Engert (1984b) then reported that maximal *C. jejuni* INN-73-83 enterotoxin production was achieved by growth at 42°C for 24h under agitation in GC medium supplemented with 1% IsoVitaleX.

Maximal enterotoxin activity was also reportedly found in cultures approaching the stationary phase (Klipstein and Engert 1984b, Daikoku *et al.* 1989). The addition of iron compounds to the growth medium was also recommended by McCardell *et al.* (1986a). They concluded that the yields of CCT from a wide range of clinical, animal and environmental *C. jejuni* and *C.*

coli isolates were vastly enhanced by the iron concentration. The oxidative state of the iron (ferrous or ferric) also affected toxin production in *Campylobacter* spp., with ferric producing the greater yields. Additional evidence for this was given by Suzuki *et al.* (1997) who found that treatment with an organic iron compound, haemin, activated enterotoxin production in strain AK11. It has been recommended by the above two groups as well as by Saha and Sanyal (1990) that Casamino acid-yeast broth (CYE) with additional iron supplements should be used for the production of enterotoxin.

In contrast to all of the evidence given so far, there are a number of studies which have failed to detect any enterotoxin at all. Coote and Arain (1996) failed to detect enterotoxin in polymyxin-B filtrates using reverse passive latex agglutination with anti-cholera toxin. *C. jejuni* NCTC 11168 was used aswell as three human faecal isolates, 51680, 01110 and 45457. This method has, however, not been used by others so is somewhat non-comparable. McFarland and Neill (1992) were also unable to detect any enterotoxin activity in 71 strains of *C. jejuni* and 4 strains of *C. coli*, isolated primarily from poultry, using a CHO cell distension assay. Perez-Perez *et al.* (1989) used an ELISA with anti-CT and anti-LT as well as a CHO cell assay to screen for enterotoxin production by *Campylobacter* spp. Twenty-two campylobacter isolates from confirmed human cases of *C. jejuni* enteritis were tested, of which 17 were *C. jejuni* and 5 were *C. coli*. *Campylobacter* cultures were grown in CYE broth supplemented with 1μg/ml of ferric chloride. All 22 isolates were negative for enterotoxin and only 10 were found to produce low levels of cytotoxin. The humoral immune response to these toxins was also assessed in 64 patients with inflammatory diarrhoea. A GM_1-based ELISA for detecting serum IgG to cholera-like enterotoxin identified that only 1 of 64 adults showed seroconversion to the enterotoxin. Serum from the patients was also tested for its potential to neutralise cytotoxin, this test also showed absolutely negative results. The authors therefore presented this as major evidence against the roles of either enterotoxin or cytotoxin in the pathogenesis of campylobacter infections in the USA. Other reports, however, have demonstrated serum antibodies against CJT in Mexico, Japan and Africa (Ruiz-Palacios *et al.* 1983 and 1985, Honda *et al.* 1986, Martin *et al.* 1988).

Wadstrom *et al.* (1983), Konkel *et al.* (1992b), and Coote and Arain (1996) were also totally unable to detect or demonstrate any level of enterotoxic activity. Konkel *et al.* (1992b) grew multiple *C. jejuni* isolates, including strain 81-176, for 24-48h in GC medium supplemented with 1% IsoVitaleX enrichment and 1μg of ferric chloride per ml with the addition of 2mg/ml of polymyxin B sulphate 10 minutes prior to harvesting. These media and conditions meet all the suggestions by authors for optimal enterotoxin production. Enterotoxicity was screened using a CHO-K1 tissue culture assay, a GM_1-based ELISA and immunoblotting with both anti-CTA and anti-LTA. Culture filtrates were also examined for the presence of specific ADP-ribosyltransferase activity which is characteristic of LT and CT. The *C. jejuni* filtrates tested showed consistent negative results for all of the above tests. The lack of reproducibility of these tests between different laboratories has raised some serious doubts as to whether or not a *C. jejuni* enterotoxin actually exists. A suggestion by Konkel *et al.* (1992b) was that their failure to detect enterotoxin may have been due to the fastidious nature of *C. jejuni* which may render it difficult to find appropriate culture conditions. This would indicate that enterotoxin production by *C. jejuni* is highly variable and unlike that associated with either *V. cholerae* or *E. coli* which produce enterotoxins under a variety of culture conditions. There have been additional reports that concentrating the supernatant 5-50 fold is necessary to detect these low level toxic effects (Klipstein *et al.* 1985) and that freshly seeded tissue culture cells are often required for the supernatants to induce an effect (Wassenaar 1997). The loss of toxin following storage (Daikoku *et al.* 1989, Florin and Antillon 1992) and subculture (Suzuki *et al.* 1994) suggests that production of enterotoxin is unstable. A study by Fernandez *et al.* (1999) also indicated this, as they demonstrated that non-toxigenic *C. coli* and *C. jejuni* strains can be induced to produce enterotoxin by intraperitoneal passage in mice following freeze-thaw injury.

Significant strain to strain differences in the production and expression of toxins are likely to exist and where different laboratories are isolating different strains, this may account for the differences. This does still raise the question of the significance of these toxins in the disease process. Again, contrasting evidence is given for this as some groups (Klipstein *et al.* 1985, Florin and

Antillon 1992) show strong links between the types of illness presented in the patient and the corresponding toxicity detected in the lab. Florin and Antillon detected an enterotoxic effect of rounding of Y-1 cells in supernates from 62% of strains from children with watery diarrhoea, in 28% of strains from children with bloody, inflammatory diarrhoea and in 34% of strains from chickens on sale at a local store in a village in Costa Rica. Klipstein *et al.* (1985) clearly demonstrated a correlation between 6 *C. jejuni* strains isolated from persons with watery, secretory-type diarrhoea and production of enterotoxin. The broth filtrates of all six strains were also shown to cause fluid secretion in ligated ileal loops. No correlation was found between enterotoxin activity and prevalent Lior or Penner serotypes (Lindblom *et al.* 1989) or certain biotypes (Everest *et al.* 1992).

The real doubt over the existence of the enterotoxin has been caused by the lack of homology at the DNA level between *Campylobacter spp.* genes and CT and LT genes (Baig *et al.* 1986, Calva *et al.* 1989, Konkel *et al.* 1992b). Baig *et al.* (1986) did succeed in demonstrating nucleotide sequence homology between the LT-B probe (*E. coli*) and DNA from five *C. jejuni* strains, despite previously reporting that DNA from enterotoxigenic *C. jejuni* strains INN-1-79 and INN-83 (described and provided by Ruiz-Palacios *et al.* 1983) did not hybridise with *E. coli* LT DNA. Additionally, Calva *et al.* (1989) produced a mixed oligo probe of the B subunits of CT and LT which they called 'CT-LT/Btrp88'. This was shown to hybridise with the DNA of 13 *C. jejuni* clinical isolates, although the LT AB gene probe did not. The authors suggested that this shows similarities between CJT and LT-B or CT-B which may be confined to small regions. Konkel *et al.* (1992b), in contrast, found no hybridisation with this probe or any combination of others of the conserved LT and CT A subunits. This supports the previous statements of Baig *et al.* (1986) and Calva *et al.* (1989) that *C. jejuni* DNA does not directly hybridise with that of CT or LT and very small regions only may show homology at the DNA level. Significant homology at the serological level was demonstrated by all these groups except Konkel *et al.* (1992b). Additional findings have also been made in the sequencing of the *C. jejuni* NCTC 11168 genome which confirm the reported lack of DNA homology. No genes were found to bear any homology with CT or LT genes within the *C. jejuni* genome (Parkhill *et al.* 2000).

It seems therefore that the controversy about enterotoxin is based on a minority of reports of lack of enterotoxin detection. Most reports give evidence of enterotoxin production and there are only a few which cannot demonstrate the presence of enterotoxin at all. Wong *et al.* (1983), though they only referred to cytotoxicity, stated that 'it is hazardous to deduce that an enterotoxin is absent merely on the failure to demonstrate its activity in an *in vitro* system'. The results of the DNA homology, coupled with the lack of homology between enterotoxin genes in the *Campylobacter* genome, provide the only real evidence that causes serious doubts. The possibility that an enterotoxin is present which does not bear any genetic resemblance to cholera toxin or *E. coli* LT remains to be clarified. One group who reported a lack of enterotoxin also reported a lack of cytotoxin from clinical samples (Perez-Perez *et al.* 1989 and 1992). When compared to the abundance of reports on different cytotoxins in the following section, it can only be concluded that there was some fundamental difference in their experimental technique and/or strains than the others.

1.11 Cytotoxins

Many cytotoxins exist within groups which are common to a wide range of bacteria, such as Shiga toxins and haemolysins. There are reports of various cytotoxins from *C. jejuni* including (i) shiga-like cytotoxin (ii) haemolytic cytotoxin (iii) cytolethal distending toxin (CLDT) (iv) vero positive cytotoxin (v) vero negative cytotoxin (vi) porin cytotoxin (vii) hepatotoxin and (viii) other unclassified cytotoxins (see following sections). Many research groups have only been able to detect one or two of these toxins and their detection seems to rely on the use of various different techniques, culture media and tissue culture cell lines. Due to the lack of comparison between the authors and different methods used, a comparison and correlation of the results is not easy. The following is an attempt to broadly categorise the toxins reported to date with respect to the different cell lines and methods used. *Campylobacter* cytotoxins which exist within groups will be discussed first, beginning with Shiga toxins.

1.11.1 Shiga Toxin and Shiga-like Toxins

There has, to date, been only one report of a Shiga-like toxin (SLT) detected in *Campylobacter* spp. by Moore *et al.* (1988). The term Shiga-like toxin is used to describe a whole family of molecules based on structural and functional similarities/identities with the family prototype, Shiga toxin (Donohue-Rolfe *et al.* 1991).

1.11.1.1 Shiga Toxin

Shiga toxin is a potent exotoxin produced by *Shigella dysenteriae* type 1 strains which acts biochemically by the inhibition of protein synthesis (Olsnes *et al.* 1981, Reisbig *et al.* 1981, Donohue- Rolfe *et al.* 1991, Agbodaze 1999, Mainil 1999). It has three biological activities: cytotoxicity, enterotoxicity and neurotoxicity and was initially termed a neurotoxin due to the paralysis and death of rabbits following injection of crude cell-free extracts of *Shigella dysenteriae*. It was later discovered that the toxin was involved in multiple activities including the major clinical manifestations of shigellosis: diarrhoea and dysentery (Donohue-Rolfe *et al.* 1991).

Shiga toxin shares the mechanism of inhibition of protein synthesis with diphtheria toxin, but also causes the enterotoxic effect of fluid accumulation in the intestine, a mechanism more closely related to that of cholera toxin. The fluid accumulation caused by shiga toxin is, however, not due to the elevation of intracellular cyclic AMP levels which is the mechanism used by cholera toxin. It has been demonstrated in a rabbit model to be due to selective targeting and inhibition of intestinal adsorptive cells (Donohue-Rolfe *et al.* 1991). Shiga toxin is, however, structurally similar to cholera toxin. It too has an AB_5 subunit structure whereby subunit A is responsible for the biochemical effect of the toxin and subunit B mediates the binding of the toxin to cell surface receptors; the two held together by a disulphide linkage (Olsnes *et al.* 1981, Reisbig *et al.* 1981, Donohue-Rolfe *et al.* 1991). The complete toxin molecule is approximately 65 kDa with an A subunit of 32 kDa and five B subunits each of 7.7 kDa (Olsnes *et al.* 1981, Donohue-Rolfe *et al.* 1991). In contrast, the binding specificities and biochemical modes of action of shiga toxin and cholera toxin are totally different. Shiga toxin subunit B binds to Galα1$\rightarrow$4Gal-containing glycolipids

and the biochemical effect of shiga toxin subunit A is the inhibition of protein synthesis by cleavage of the N-glycosidic bond of adenine at nucleotide position 4324 in the 28S rRNA of the 60S ribosomal unit (Donohue-Rolfe *et al.* 1991).

1.11.1.2 *E. coli* Shiga-like toxins (SLTs)

Some strains of *E. coli* produce protein toxins that are biologically, structurally and antigenically related to shiga toxin, hence termed shiga-like toxins/SLTs (Agbodaze 1999). The first report of this was in 1977 when an *E. coli* isolate was found to be cytotoxic to Vero cells and distinct from the well-known heat-labile (LT) and heat-stable (ST) toxins of *E. coli*. For this reason, the term *E. coli* verotoxin is often used and VTEC refers to verotoxigenic *E. coli*. After discovery that the cytotoxin of *E. coli* O157:H7 associated with hemorrhagic colitis was neutralised by antiserum to purified shiga toxin, the toxin was then called shiga-like toxin (SLT) (Donohue-Rolfe *et al.* 1991). Further studies indicated that two plasmids were present in *E. coli* O157:H7 which carried the structural genes for one cytotoxin which was neutralised and one cytotoxin which was not neutralised by antiserum to shiga toxin. They were termed shiga-like toxin I (SLTI) and shiga-like toxin II (SLTII) respectively. These toxins are also known as VT1 (Verotoxin 1) or Stx1 and VT2 (Verotoxin 2) or Stx2 (Agbodaze 1999, Mainil 1999). Gene sequencing has revealed that SLTI is virtually identical to shiga toxin and SLTII shows 56% homology to shiga toxin based on predicted amino acid sequences (Donohue-Rolfe *et al.* 1991).

1.11.1.3 *Campylobacter* spp. SLT

Of 36 human enteritis isolates of *C. jejuni* and *C. coli*, 11 (10 *C. jejuni* and 1 *C. coli*) produced low levels of cell-associated SLT. This was determined by a cytotoxicity assay of cell lysates and supernatants upon HeLa cells as well as neutralisation studies with a monoclonal antibody to the B subunit of *E. coli* SLTI and rabbit anti-shiga toxin. Twenty-seven per cent of *C. jejuni* infected patients also had shiga toxin neutralising activity in their sera (Moore *et al.* 1988). The results of this study did not convincingly implicate *C. jejuni* SLT in the pathogenesis of disease as it is produced in very low amounts and some of the *C. jejuni* isolates which produced SLT were from asymptomatic patients.

Additionally, 8 of the 15 *C. jejuni* isolates that did not produce SLT were clearly associated with inflammatory diarrhoea (Moore *et al.* 1988). To date, there have been no further reports of SLT production in campylobacters, thus, the only conclusion that can be reached is that SLT is not involved in the pathogenesis of disease in *Campylobacter* spp. Suggestions were made that the *Campylobacter* Vero positive cytotoxin was related to the SLT, but as no neutralisation of Vero toxin with anti-shiga toxin serum was proven, it will be assumed that they are separate toxins.

1.11.2 Haemolysins (Cytolysins)

C. jejuni was originally reported to be a non-haemolytic organism (Arimi *et al.* 1990, Tay *et al.* 1995, Akan *et al.* 1998). There have, however, been numerous reports on the haemolysis of *C. jejuni* and some attempts made at establishing their role within the host during infection. *Helicobacter pylori* is also known to produce a haemolysin, the genes of which were cloned and characterised (Drazek *et al.* 1995). Most *Arcobacter* spp. are considered to be non-haemolytic with the possibility that *A. skirrowii* is alpha-haemolytic (Nachamkin 1995). There has, however, been one report to date of alpha-haemolysis in *A. butzleri* (Atabay *et al.* 1998). A haemagglutinating antigen in *Arcobacter* was also reported by Tsang *et al.* (1996). Recent studies on *V. cholerae* haemolysin have shown it to have additional vacuolating activity on mammalian cells (Coelho *et al.* 2000). This may indicate similarities with the vacuolating cytotoxin in *H. pylori.* Haemolysins are produced by a very diverse range of organisms with very diverse modes of action, functions in pathogenesis (where determined) and target cell range. The exact role in pathogenesis of many of them remains to be established, but some of the better characterised ones will be briefly reviewed.

1.11.2.1 The RTX Toxin Family

The RTX family represents the most broadly occurring family of bacterial exotoxins to be found among Gram negative bacteria. The RTX (repeats in toxin) family name has been proposed on the common presence of tandem copies of a nine-amino acid repeat in each of the toxin proteins which is responsible for

calcium-binding (Welch *et al.* 1992). This identical number of genes in an operon encodes each separate member of the RTX family, all of which show high sequence homology (Braun *et al.* 1991). They also share the property of being secreted by a leader-independent pathway (Welch *et al.* 1992). The individual toxins within this family often exhibit different cell and host specifications (Welch 1991).

The prototype of the RTX family is *E. coli* haemolysin (HlyA) which exhibits cytolysis on a broad range of cells. There are many other members of the family including the leukotoxins of *Pasteurella haemolytica* (LktA), *Actinobacillus pleuropneumoniae* (AppA and AppA1), *Actinobacillus actinomycetemcomitans* (AaltA), *Bordetella pertussis* haemolysin (CyaA) and *Proteus vulgaris*. *E. coli* carries the RTX gene on plasmids, but others are usually encoded on the chromosome (Braun *et al.* 1991). It is therefore speculated that multiple horizontal transfer has led to the divergently evolved group of RTX toxins (Welch *et al.* 1992).

The RTX toxins produce pores in target cell membranes which disrupt the membrane permeability and lead to lysis of the cells. When subjected to sublytic amounts of the *E. coli* HlyA *in vitro*, cells undergo rapid and profound metabolic changes such as loss of membrane receptor function and prolonged depression of ATP levels. They represent important virulence factors for pathogens that share extra-intestinal niches; these include mammalian environments such as the skin, mouth and respiratory tract as well as aquatic and terrestrial environments such as soil, marshes, ponds, rivers and streams. For example, many organisms that commonly reside in the gastrointestinal tract of humans and animals are abundant in human sewage as well as soil and watercourses following excretion from animals. These represent extra-intestinal niches for large numbers of bacteria including *E. coli*, *Clostridia*, *Bacillus*, *Proteus*, *Vibrio* and *Campylobacter*, of which many, but not all, are pathogenic. The periodontal cavity of mammals is a niche in which certain strains of *Campylobacter* and *Actinobacillus* are pathogens, and the respiratory tract is also a niche for bacterial pathogens such as *Streptococcus* and *Bordetella*. A limited number of ancestrally related pore-forming toxin families have been formed. These toxins probably serve multiple purposes for the pathogen in the complicated interaction between pathogen and host, for example, destroying

phagocytic cells to resist the host immune defences. Full characterisation of the precise roles of these toxins has yet to be elucidated (Welch 1991, Welch *et al.* 1992).

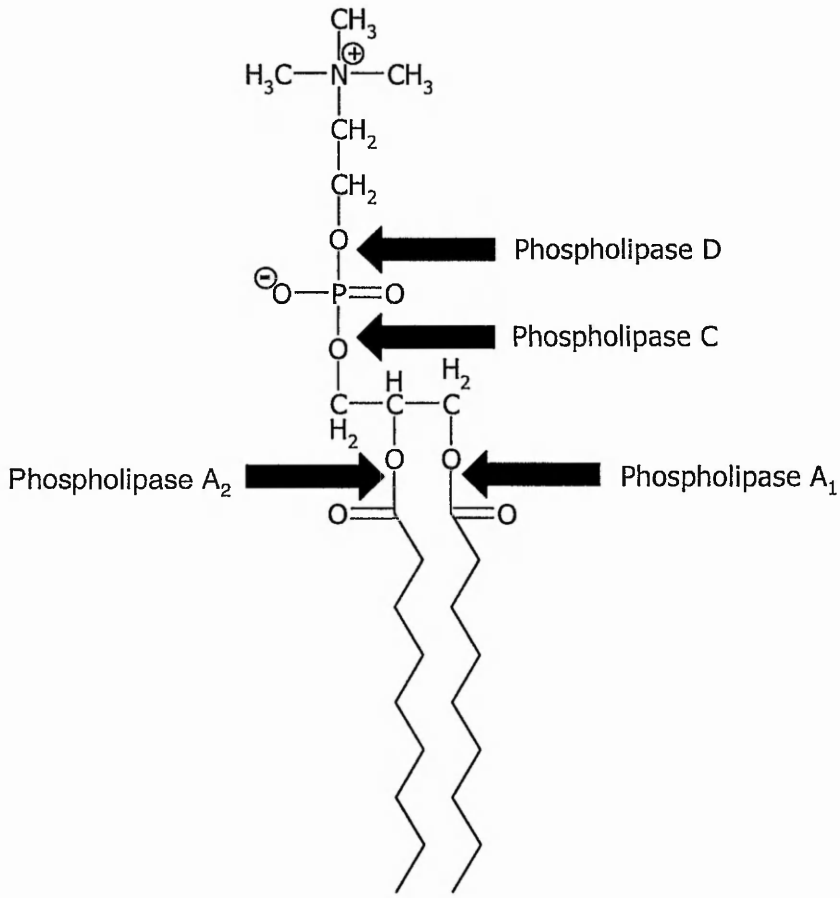
1.11.2.2 The Thiol-Activated Cytolysins

The thiol-activated cytolysin (TACY) family is a prominent group of bacterial toxins, of which Streptolysin O (SLO) is the prototype. These toxins have several unique characteristics that are not found in other cytolytic toxins which classify them as a group (Tweten 1997, Billington *et al.* 2000). The group is named due to the sensitivity of their cytolytic activity to oxygen and their activation by reducing compounds. They are hence also referred to as 'oxygen-labile' toxins although many of them do not necessarily express either of these phenotypes, and it has been concluded that thiol-activation is not an important property of these toxins. The name has however stuck despite its apparent irrelevance to many toxins within the group (Tweten 1997, Billington *et al.* 2000). More importantly, they are synthesised as water-soluble, single-chain polypeptides with molecular masses ranging from 47-60 kDa and are lytic for eukaryotic cells by the formation of pores in the cell membrane. Cholesterol is the major target cell receptor for these toxins and a small amount of free cholesterol can inhibit their lytic activity (Billington *et al.* 2000). Members of the group show 30-60% similarity in primary amino acid sequence which suggests that these toxins have developed from a single primordial gene (Tweten 1997). They contain an almost invariant undecapeptide sequence (ECTGLAWEWWR) located near the C-terminus of the protein. TACYs were originally described as haemolysins due to their lytic activity against erythrocytes from a range of animal species. Their pathogenic effects are, however, more subtle than simple lysis of host cells and include interference with host immune cell functions and cytokine induction. Proteins of the TACY family bind cholesterol-containing membranes and oligomerise to form transmembrane pores up to 30nm in diameter. Members of this group are found in more than 20 species of Gram positive bacteria and are intimately involved in the pathogenesis of infection by species such as *Arcanobacterium pyogenes*, *Clostridium perfringens*, *Listeria monocytogenes* and *Streptococcus pneumoniae* (Tweten 1997, Billington *et al.* 2000).

1.11.2.3 Bacterial Phospholipases

A large group of enzymatic haemolysins which have been studied and characterised are the phospholipases. Phospholipids are key components of all eukaryotic cell membranes, serving important roles such as scaffolding for membrane proteins, separating the intracellular and extracellular environments and intracellular signalling molecules. It is therefore not surprising that bacteria have evolved systems to hydrolyse phospholipids. Bacterial phospholipases consist of four main groups: C, D, A_1 and A_2 which are categorised according to their site of cleavage of phosphatidylcholine (Fig. 7). The role of phospholipases in the pathogenesis of infection is diverse (Titball 1998). They may be directly toxic and their interaction with cell membranes can take several forms (Songer 1997). The bacterial phospholipase C enzymes are the best characterised group.

Fig. 7 Site of cleavage of phosphatidylcholine by phospholipase A_1, A_2, C and D



(Titball 1998)

Phospholipase C enzymes are produced by a wide variety of pathogenic and non-pathogenic bacteria and are divided into three groups based on their amino acid sequence and substrate specificities. The first is phosphatidylcholine-preferring enzymes which have a wide substrate specificity. This group can again be subdivided according to their Gram negative and Gram positive origins. Phospholipase C enzymes play an important role in the pathogenesis of disease including roles in invasion, establishment of a host infection and evasion of the host immune system. Their renowned ability to cause cell lysis as a result of damage to the cell membrane has led to the testing of their haemolytic capacity upon erythrocytes as a measure of this effect. Some phospholipase C enzymes are membrane active and others act by becoming inserted into the membrane and causing phospholipid hydrolysis (Titball 1998).

The *C. perfringens* α-toxin (also known as phospholipase C or lecithinase) is the best studied of the clostridial phospholipases and probably the most well-documented bacterial phospholipase C enzyme. It was the first toxin to be classified as an enzyme (Jolivet-Reynaud *et al.* 1988) and is the most potent phospholipase C characterised to date (Songer 1997). It hydrolyses lecithin which has been demonstrated in laboratories using egg yolk lecithin (MacFarlane and Knight 1941, Miles and Miles 1947, Jolivet-Reynaud *et al.* 1988). Phosphatidylcholine is the main substrate for this enzymatic toxin, but sphingomyelinase is also hydrolysed to a lesser extent. The toxin has lethal, necrotising and cytolytic activities and has been proven to play a key role in the pathogenesis of gas gangrene (Jolivet-Reynaud *et al.* 1988, Titball 1997). It elicits a variety of subtle effects on cells which leads to tissue dysfunction, shock and death. It is able to cause mistrafficking of neutrophils so they do not enter infected tissues and it causes vasoconstriction and platelet aggregation which can reduce blood supply to infected tissues, thus creating an anoxic environment in the host tissues for the bacteria to replicate. This is how the onset of gangrene occurs (Titball 1998, Titball *et al.* 1999).

All phospholipases, including phospholipases A and E, contain a certain amount of phospholipase C activity (Titball 1997) and this has the most important effects in terms of pathogenicity. Even in cases where the role of

phospholipases has been unequivocally demonstrated in virulence, the specific molecular functions of the enzyme remains to be defined (Songer 1997).

A phospholipase A (PldA) has been demonstrated in association with haemolytic activity in both *Helicobacter pylori* and *Campylobacter coli* (Grant *et al.* 1997, Dorrell *et al.* 1999). In *C. coli*, the *pldA* gene encodes a 35kDa outer membrane protein which is homologous to the *E. coli* PldA protein. A *pldA* mutant of *C. coli* was deficient in phospholipase A activity and was also demonstrated to have reduced haemolytic activity (Grant *et al.* 1997). The PldA of *H. pylori* was shown to have reduced haemolytic activity and it was additionally implicated in colonization of the gastric mucosa (Dorrel *et al.* 1999). A homologue of this protein has also been detected in *C. jejuni* (Brok *et al.* 1998, Penn 2001). The phospholipase A outer membrane proteins of both *C. jejuni* and *H. pylori* are implicated in virulence and show homology to the larger family of conserved outer membrane phospholipases A which includes *E. coli* PldA (Brok *et al.* 1998, Snijder *et al.* 1999, Penn 2001). Sequence comparison of phospholipase A proteins from eleven different bacteria including *C. jejuni*, *C. coli* and *H. pylori* revealed that 30 amino acids were completely conserved and similar residues were found in 48 positions (Brok *et al.* 1998). *H. pylori* also has a phospholipase C which has been shown to act independently of the *cagA* gene, thus the role of this enzyme in pathogenesis remains unknown (Bode *et al.* 2001).

1.11.2.4 *Campylobacter* Haemolysins

There are numerous reports on the haemolytic activity of campylobacters, despite the fact that they are supposedly non-haemolytic organisms (Arimi *et al.* 1990, Tay *et al.* 1995, Akan *et al.* 1998). The lack of an efficient method for the demonstration of haemolytic activity may explain why they were originally classed as non-haemolytic. There are still many discrepancies among the methods and results, which is a possible indication that a number of different haemolysins are being produced, each of which is expressed under a different set of conditions. An early report of haemolysins present in campylobacters was that of Arimi *et al.* (1990). Eighty-three *C. jejuni* clinical isolates, nineteen sewage isolates and NCTC strains 11168 and 11322 were screened. They showed that haemolysis on conventional sheep or horse blood agar plates (blood

agar base) was poor, but reported a high level of haemolysis (92.3% of *C. jejuni* and 21.7% of *C. coli* strains) upon blood plates made with HI (Heart Infusion) or BHI (Brain Heart Infusion). They reported that this was due to campylobacters having complex growth requirements for the stimulation of haemolysis in culture. Basal media containing peptones and a high content of animal tissue extracts is required for the stimulation of haemolysis by *V. cholerae* or *E. coli*. They additionally reported that this haemolysin was a heat-stable protein which was produced in ageing cultures (incubation for 4d was required) and may be released on cell death and lysis (Arimi *et al.* 1990).

Many other reports followed this of haemolysins which were expressed poorly and inconsistently on conventional blood agar plates (Pickett *et al.* 1992, Tay *et al.* 1995, Istivan *et al.* 1998) so other approaches were adopted for detection of haemolytic activity. Tay *et al.* (1995) reported the successful use of blood agarose plates (2% erythrocytes added to 1% agarose), but showed even more success with the use of a microplate assay (94 of 100 *Campylobacter* clinical isolates were haemolytic). The microplate assay was adapted from Dominguez-Rodriguez *et al.* (1986), and many other groups tried this method with varying successes. A contact haemolysis assay has also been recommended by some authors who state that the bacteria and blood need to be in close contact in order to observe the haemolytic effect (Pickett *et al.* 1992, Istivan *et al.* 1998). Tay *et al.* (1995) also reported that incubation conditions were important, recommending that an aerobic incubation at 42°C for 18-24h was the most successful. Akan *et al.* (1998) also recommended aerobic incubation conditions. This, however, contradicts many other investigators who show that microaerobic conditions yield the most haemolytic activity (Arimi *et al.* 1990, Misawa *et al.* 1995, Istivan *et al.* 1998). Many groups have confirmed each other's findings in that prolonged incubation periods are required for the detection of haemolysin due to its production in ageing cultures (Arimi *et al.* 1990, Hossain *et al.* 1993, Misawa *et al.* 1995, Istivan *et al.* 1998). It has also been suggested that the haemolysins are cell-associated and are not detected in cell-free supernatant (Istivan *et al.* 1998). There are some contrasting reports on heat-sensitivity. Istivan *et al.* (1998) and Tay *et al.* (1995) demonstrated heat-lability (100°C for 30 minutes) of the haemolysins. Hossain *et al.* (1993), however, reported the

presence of at least two haemolysins in 15 *C. jejuni* isolates screened, one of which was heat-stable (100°C) and one of which was heat-labile (80°C). The differences in results could therefore be clarified by this discovery that *C. jejuni* produces two or more different haemolysins.

The growth media which have been found to be effective for the production of haemolysins vary. Brucella broth has been widely used and reported as the most suitable medium by many groups (Hossain *et al.* 1993, Misawa *et al.* 1995, Istivan *et al.* 1998) but there are mixed reports on the suitability of conventional blood agar (i.e. made with Blood Agar Base). A broad range of erythrocyte species have also been used for testing for haemolytic activity including rabbit, horse, sheep, chicken, ox and human. Misawa *et al.* (1995) and Tay *et al.* (1995) reported that haemolytic activity was not influenced by the species of blood, however, Hossain *et al.* (1993) reported that the most haemolysis occurred on rabbit erythrocytes and virtually no haemolysis was detected on chicken erythrocytes. The authors suggested that this may have important implications in the near commensal relationship between *C. jejuni* and the adult chicken. Istivan *et al.* (1998) also demonstrated haemolytic activity which was minimal upon chicken erythrocytes and maximal upon human and rabbit erythrocytes. Additionally, Akan *et al.* (1998) reported that rabbit erythrocytes were the most sensitive to the haemolytic activity of 20 *C. jejuni* and *C. coli* strains isolated from chickens. No difference was apparent between washed and whole blood cells, indicating a lack of inhibitory molecules such as antibodies to haemolysin in the plasma (Arimi *et al.* 1990). It has also been reported that haemolysin production does not correlate with biotype (Tay *et al.* 1995). Other influential parameters investigated were pH and gaseous concentration (Misawa *et al.* 1995) and iron concentration (Pickett *et al.* 1992, Istivan *et al.* 1998). Misawa *et al.* (1995) reported that α- and β-haemolytic zones were apparent on conventional blood agar, pH 6 with a high concentration of CO_2 in the gas mixture, following the application of 12 *C. jejuni* strains, some of which were human isolates, grown in Brucella broth. When the pH was adjusted to 6.5 by changing the CO_2 concentration, the α-haemolytic zones immediately disappeared. β-haemolytic zones disappeared after prolonged

incubation. They additionally reported that a microaerobic gas mixture containing hydrogen is better than that containing nitrogen.

Pickett *et al.* (1992) investigated the effects of iron present in the media upon the haemolytic activity of numerous clinical isolates of *C. jejuni* including 81-176. The ability of pathogenic bacteria to acquire iron in the host has been shown to be of critical importance in establishing infection. Within humans, bacteria must utilise iron-containing compounds and many organisms can use haemoglobin as an iron source including *V. cholerae*, *Y. pestis*, *H. influenzae* and some *Neisseria* spp. Heme is also utilised by many pathogens such as *N. gonorrhoeae* and *H. influenzae*. It has thus been speculated that haemolytic activity may be an important method of iron acquisition employed by bacteria within the human host (Rowe and Welch 1990, Pickett *et al.* 1992, Wassenaar 1997). No iron regulation was apparent in the results of this study and the authors suggested that 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). A study by Istivan *et al.* (1998), however, reported that the haemolysin was iron-regulated and the haemolytic titre decreased upon the addition of surplus iron to the media. *C. concisus* was used in this study, whereas Pickett *et al.* (1992) used *C. jejuni*. This may account for the difference, however, the discrepancies in this and many of the other results does suggest that much more work is needed before any firm conclusions can be made about the nature and role of these haemolysins.

Hossain *et al.* (1993) were the only group who attempted to characterise the the mechanism of action of *C. jejuni* haemolysins. They tested the effect of egg-yolk lecithin and cholesterol on haemolytic activities to see if there was an interaction between the haemolysin and a specific phospholipid in the erythrocyte membrane (ie. testing for phospholipase C activity). This was shown to have no effect on haemolytic activity, hence no phospholipase activity was detected. However, Grant *et al.* (1997) identified a 35kDa PldA phospholipase A outer membrane protein in *C. coli* which was homologous to the PldA protein of *E. coli*. They additionally demonstrated its role in haemolysis by the reduced haemolytic activity of a *pldA* mutant and thus suggested that this protein plays some role in *Campylobacter* virulence. The phospholipase A protein has since been identified in the outer membrane of *C. jejuni* following complete

sequencing of the *C. jejuni* NCTC 11168 genome (Penn 2001), but further characterisation of the enzyme's activities in *C. jejuni* has not yet been reported.

Park and Richardson (1995) cloned a genetic determinant from *C. jejuni* NCTC 11351 which conferred a haemolytic phenotype when expressed in *E. coli*. They identified a 10Kb insert which conferred haemolytic activity but did not show significant homology to previously characterised haemolysins. The protein did, however, reveal homology to a group of periplasmic binding proteins which are components of transport systems for ferric siderophores. Siderophores are highly specific iron chelators which are produced by many pathogenic bacteria for iron acquisition during infection. The protein (36.2kDa) conveyed a haemolytic phenotype when cells were grown on plates but no haemolytic activity was detected in whole cell lysates or supernatants. It was therefore concluded that this was a false haemolytic phenotype and the haemolysis observed was due to lysis of the *E. coli* host by the overexpression of a protein which may have a toxic nature. This protein was hence a component of a periplasmic-binding-protein-dependent system for ferric siderophores and not a true haemolysin at all (Park and Richardson 1995). Two closely related genes *csxA* and *csxB* were recently cloned from *C. rectus* which is a periodontal pathogen in humans and were found to show characteristics of the RTX toxin family. These genes were, however, absent in all other *Campylobacter* and *Helicobacter* species examined (Braun *et al.* 1999). Thus, no progress has yet been made on the characterisation of *C. jejuni* haemolysins. McCardell *et al.* (1986b) detected a toxin in about 45% of 60 *C. jejuni* and *C. coli* strains which was haemolytic for rabbit blood cells, trypsin-sensitive, heat-labile and neutralised by an antibody to the cytolysin produced by *V. cholerae* non-01. No further characterisation of this toxin was carried out and this is the only report of neutralisation with antiserum to other cytolysins. Until more facts are established, the role of haemolysins in pathogenesis will remain a mystery. Tay *et al.* (1995) did, however, propose a potential role of *C. jejuni* haemolysin as a virulence factor similar to the haemolysin found in *Shigella* spp. Haemolytic activity is used by these organisms to lyse phagocytic vacuoles for the release of virulent shigella into the cytoplasm (Tay *et al.* 1995). This suggestion for a potential role needs further clarification and it may be that a similar role is revealed for the haemolysin(s) of *C. jejuni*.

1.11.3 Cytolethal Distending Toxin (CLDT)

Johnson and Lior (1998a) were the first to describe a novel heat-labile toxin cytolethal to CHO, Vero, HeLa and HEp-2 cells and inactive upon Y-1 cells from the culture filtrates of *Campylobacter* spp. They termed this new toxin Cytolethal Distending Toxin (CLDT) due to its effect of progressive distension and eventual death upon tissue culture cells. Of 718 strains of *C. jejuni*, *C. coli*, *C. fetus* subsp. *fetus* and *C. laridis*, 295 (41%) were found to produce CLDT. Responses to CLDT were remarkably similar in several tissue culture cell lines.

CLDT was reported to be distinct from previously reported cytotoxins and the cholera-like enterotoxin although effects on CHO cells between CLDT and enterotoxin could not be distinguished after 24h. Both caused elongation of CHO cells after this period, and effects on Y-1 cells were the only way to discriminate between the two in these early stages. After 2-4 days the CLDT-treated cells showed extensive distension and death in contrast to the enterotoxin-treated cells which only became moderately elongated and were not killed. Incubation for up to 96h was therefore mandatory to differentiate between cytotonic and cytotoxic activity. For this reason, and the fact that 41% of 500 isolates tested were positive for CLDT production, Johnson and Lior (1988a) suggested that CLDT may have been referred to as a cytotonic toxin/enterotoxin until now. They also suggested that there is a high potential for previous, present and future misinterpretation of enterotoxic and CLDT effects. Strains producing CLDT were shown not to be geographically related nor related by serotype or biotype. *Campylobacter* strains were found to be very stable in their expression of CLDT and continued to excrete toxin even after continuous subculture. The toxin could only be neutralised by homologous rabbit anti-toxin, was trypsin-sensitive, non-dialysable and over 30 kDa in molecular weight. This description of a CLDT by Johnson and Lior, coupled with work on CLDTs in *E. coli* and *Shigella*, triggered the designation of a new toxin family (Johnson and Lior 1988b).

The CLDT family are produced by a small but diverse group of bacterial pathogens and affect a wide variety of mammalian cells (Pickett and Whitehouse 1999). The CLDT of *E. coli* was characterised before much progress was made with *Campylobacter* spp. CLDT (Pickett *et al.* 1996, Pickett and Whitehouse

1999). It was not until 1996 that the structural genes encoding the CLDT of *C. jejuni* 81-176 were cloned, sequenced and characterised (Pickett *et al.* 1996). Degenerate primers were constructed from regions of the DNA sequence of CLDT from *E. coli*. These were used to amplify a fragment from the *C. jejuni* genome which encoded CLDT. This fragment was then used as a probe to clone the complete locus of CLDT from *C. jejuni* 81-176 (Pickett *et al.* 1996, Wassenaar 1997). The nucleotide sequence revealed 3 genes: *cdtA*, *cdtB* and *cdtC* encoding proteins with predicted sizes of 30116, 28989 and 21157 Da respectively. *E. coli* CLDT also has 3 structural genes, *cdtA*, *cdtB* and *cdtC* and the toxins of both *C. jejuni* and *E. coli* are strongly hydrophobic suggesting that one of the proteins is a lipoprotein (Wassenaar 1997).

Studies on the mechanism of action of the CLDTs were first reported in 1997 by Aragon *et al.* and Peres *et al.* who both described a cell cycle blockage caused by CLDT. Peres *et al.* (1997) noted that a 4h interaction between *E. coli* CLDT and HeLa cells induced the formation of giant mononucleated cells blocked in G_2/M phase. Aragon *et al.* (1997) reported that CLDT-treated cells accumulated actin stress fibre-like structures which were accompanied by an apparent blockage of cell division. Toxin treated cells failed to divide yet cell viability remained high for the first 4 days. Whitehouse *et al.* (1998) also demonstrated that CLDT from *C. jejuni* 81-176 caused a rapid and specific cell cycle arrest in HeLa and Caco-2 cells. They subsequently determined this to be a G_2 phase block as immunofluorescent studies indicated that arrested cells had not entered M phase. This is a novel mechanism of action unlike that of any other known bacterial toxin (Aragon *et al.* 1997, Whitehouse *et al.* 1998, Eyigor *et al.* 1999, De Rycke *et al.* 2000). *C. jejuni* 81-176 and 11168 CLDTs have been inactivated by insertional mutation of the *cdtB* toxin subunit. *CdtB* mutant strains were shown to be unaffected in enteric colonisation abilities in immunodeficient mice but impaired invasiveness in blood, spleen or liver tissues was demonstrated (Purdy *et al.* 2000). This therefore suggests the possible role of CLDT in *C. jejuni* invasion of mammalian cells.

More recently, the CdtB protein was identified as being responsible for the cytotoxic activity of CLDT within the host cell by means of DNase activity. Amino acid sequence comparisons revealed similarities between this protein and deoxyribonuclease (DNase) 1-like proteins. This protein was subsequently

confirmed as a DNase (Lara-Tejero and Galan 2000). This group also went on to demonstrate that CdtB is the active subunit of a tripartite toxin in which CdtA and CdtC form the heterodimeric subunit required for the delivery of CdtB, hence all three proteins are necessary for the activity of CLDT (Lara-Tejero and Galan 2001). A CLDT receptor has not yet been identified (Pickett and Whitehouse 1999), but Bag *et al.* (1993) reported the presence of specific proteins on HeLa and CHO cells that bind the CLDT of *C. jejuni* strain CJ1. This group also developed a receptor-based ELISA for detection of CLDT activity which was reported to be faster than a cytotoxic assay and insensitive to enterotoxin (Bag *et al.* 1993).

CLDTs have been detected in many other bacteria including some species of *Shigella* (Aragon *et al.* 1997, Pickett and Whitehouse 1999), *Haemophilus ducreyi* (Gelfanova *et al.* 1999, Pickett and Whitehouse 1999) and, more recently, some species of *Helicobacter* (Chien *et al.* 2000, Young *et al.* 2000). Genes encoding CLDT in *Helicobacter* were identified as a cluster of three genes: *cdtA, cdtB* and *cdtC*. *CdtB* genes from *H. hepaticus, H. bilis, H. canis* and from two novel *Helicobacter* spp. were cloned and sequenced and all of them were found to show 50-65% homology to the *cdtB* group of genes of *C. jejuni* and *H. ducreyi* (Chien *et al.* 2000, Young *et al.* 2000).

In conclusion, CLDT is produced by a number of *Campylobacter* spp. as well as by many other pathogenic organisms. It is a DNase which causes a cell cycle block in the G_2 phase. This does, at present, seem likely to play a significant role in the disease process caused by *C. jejuni.*

1.11.4 Other *Campylobacter* Cytotoxins

1.11.4.1 Vero Positive Cytotoxin

There have been numerous reports of a campylobacter toxin that is active on Vero cells which has been referred to by Florin and Antillon (1992), Wassenaar (1997) and Schulze *et al.* (1998) as Vero positive cytotoxin, and will be referred to here also as Vero positive cytotoxin. The first reports of this were in 1985 by Klipstein *et al.* and in 1986 by Johnson and Lior. Klipstein *et al.* (1985) assessed the cytotoxicity of 20 clinically isolated strains of *C. jejuni* using Vero and HeLa cell lines. They also screened for enterotoxins and correlated the

clinical status of the patient with toxicity for each strain. Invasiveness and cytotoxicity were shown to be compatible with the occurrence of bloody, inflammatory diarrhoea. All six strains isolated from bloody, invasive-type diarrhoea demonstrated cytotoxicity and high invasive properties upon Vero and HeLa cell lines. Johnson and Lior (1986) were the next to demonstrate a cytotoxic effect from culture supernatant on CHO, HeLa and Vero cells by a trypan blue exclusion method. They discovered cytotoxic effects by a study of morphological changes of the cells and attempted neutralisation experiments with *E.coli* VT (SLT) antitoxin and *Clostridium difficile* antitoxin, neither of which were successful.

Florin and Antillon (1992) also found supernatants from 79 *C. jejuni* strains isolated from chickens, and from children with diarrhoea, to elicit a cytotoxic effect on HeLa and Vero cells. This result was additionally demonstrated upon MRC-5 cells. They reported a similar morphological response in all three cell types; rounded cells with a wrinkled, shrunken appearance after 24h, then degeneration and death of the cells after a few days. The cytotoxin was heat-labile (100°C for 10 minutes) and preliminary purification experiments revealed that the toxin had a major band of 100 kDa on SDS-PAGE. The authors suggested that this toxin might be the campylobacter analogue of the shiga-like toxin (or VT) produced by *E. coli* which is active on Vero cells. Neutralisation studies, however, showed that *E. coli* VT antitoxin was ineffective.

Coote and Arain (1996) demonstrated the cytotoxicity of *C. jejuni* NCTC 11168 on HeLa and Vero cells using an MTT cytotoxicity assay. A description of the MTT assay can be found in the Results chapter, section 3.4. A low level (not quantified) of cytotoxicity compared to that of verotoxin-producing *E. coli* was detected in cell extracts only, not in the supernatant. This different result may reflect the differences in the method used here than in the previous reports. Previous researchers found cytotoxicity in the cell supernatant (cell extract not tested) using studies of morphological changes. It is possible that the lower result in the MTT assay is due to the fact this assay only detects cell viability, whereas a morphological study will show early signs of cytotoxic effects before cell viability is lost. The MTT assay of Coote and Arain used an incubation time

of bacteria with cell lines for 28h, while the results of Florin and Antillon (1992) suggest that only morphological changes are apparent after 24h and cell degeneration and death does not occur until a few days later. It is therefore likely that the same toxin was found in both instances but a lower level of cytotoxin was present in the supernatant which was not detected by the method of Coote and Arain. McFarland and Neill (1992) also found a toxin active on Vero cells, as well as CHO-K1, foetal calf lung and chicken embryo fibroblast cells.

A cytotoxin from *C. jejuni* 81116 which was recently reported by Lee *et al.* (1998 and 2000) falls into this category. They reported a cytotoxin which was consistently lethal to numerous cell lines and initially acted by causing rounding and detachment of cells. The toxin is active upon CHO, INT 407, HeLa, J774 and Vero cells. During the first 12h, the cytotoxicity was located within the bacterial cell and after 24h the level of cytotoxicity in the supernatant increased with incubation until 72h, when 100% cell rounding was achieved. The LPS in the assay was measured and found to be negligible and thus unlikely to have induced a cytotoxic effect. The crude fraction was very resistant to storage and retained its cytotoxicity after storage at –20 and -70°C for over 24 months. The cytotoxicity was lost, however, after 4 weeks at 4°C, but heat-stable at 100°C for 30 minutes. The authors reported this to be a novel toxin due to its resistance to freezing and heat-stability. Heat stability, however, as stated by Wassenaar (1997), is an unreliable parameter for the comparison of different toxins due to the different levels of purity of the toxins imparting different heat sensitivities. There are no other reports of the stability of the Vero positive toxin, thus, it cannot be distinguished from the Vero toxin and will remain in this category.

Previous detection of a heat-stable toxin released into the supernatant using a ^{51}Cr-release assay was reported by Lam (1993) which also indicates that the toxin reported by Lee *et al.* (1998 and 2000) was by no means 'novel'. Another toxin which seems to correspond with the results of Lee *et al.* was reported by a group of Japanese researchers in the late 1980's and early 1990's (Daikoku *et al.* 1989 and 1990, Kawaguchi *et al.* 1989 and Mizuno *et al.* 1994). They described a toxin which was active upon HeLa and CHO cells and was heat-labile and trypsin sensitive but no neutralisation studies or Vero cell cytotoxicity assays were performed. Solubilisation studies indicated that the

toxin was produced intracellularly in the early log phase, was detected as a membrane-associated form in stationary phase and was released after the stationary phase of growth (Kawaguchi *et al.* 1989, Daikoku *et al.* 1990, Mizuno *et al.* 1994). From the evidence so far, it is feasible that this toxin is the same as the Vero positive toxin described by Lee *et al.* (1998 and 2000) due to its presence within the bacterial cell in early growth phases and its presence in the supernatant after stationary phase. Lee *et al.* (1998 and 2000) also reported cytotoxic activity upon CHO and HeLa cells, as well as upon INT 407 and Vero cells. Daikoku *et al.* (1989) reported that the toxin was lost upon subculture which also corresponds with the work by Lee *et al.* (1998 and 2000) who stated that although toxicity was retained after storage at –70 and -20°C, it was rapidly lost after 2-4 weeks at 4°C. Both groups hence reported instability of the toxin. Some cytotoxic activity was detected in the LPS fraction by Kawaguchi *et al.* (1989) but Lee *et al.* (2000) maintained that the LPS fraction was not responsible for the cytotoxic effect due to its presence at a very low level (1μg). The only real contrast between these two reports is the heat-stability reported by Lee *et al.* (2000) compared to the heat lability reported by the Japanese researchers. As previously discussed, heat stability is a fairly unreliable means of classifying and comparing toxins but the vast difference in results may be of significance in this case. The Japanese group report that the toxin was heat-labile at 60°C (method of Guerrant *et al.* 1987), but Lee *et al.* (2000) reported heat-stability at 100°C for 30 minutes. In both cases, however, the toxin was sensitive to trypsin confirming that it was proteinaceous.

1.11.4.2 Vero Negative Cytotoxin

A Vero negative cytotoxin was also reviewed by Wassenaar (1997) and briefly by Florin and Antillon (1992) and Schulze *et al.* (1998). Wassenaar (1997) summarises this to be a cytotoxin of approximately 70 kDa which is heat-labile and trypsin-sensitive, active against HeLa, CHO, HEp-2, and INT 407 cells, inactive against Vero cells and not neutralised by anti-Shiga or anti-*Clostridium difficile* toxins. This molecular weight of 70kDa is, however, contradictory between reports.

The first report of this toxin was in 1983 by Wong *et al.* with their description of a cytotoxic effect on HeLa, HEp-2, MRC-5 and CHO cells from culture filtrates of 11 *C. jejuni* isolates from humans with gastroenteritis. The cytotoxin showed a negative reaction against Vero cells and was sensitive to trypsin treatment and heating to 100°C. The same group later followed this work up by using a ^{51}Cr-release assay to demonstrate the toxic effects in more detail. Release of 54-68% of the label from HeLa cells in the presence of toxin and virtually no release in the absence of toxin indicated that the toxin disrupted membrane integrity which, in turn, led to cell death. The purified toxin had a molecular weight of 30 kDa, and the toxic activity was lost upon subculture (Pang *et al.* 1987). Pang *et al.* (1987) also correlated the toxicity with clinical symptoms in the host. Toxin activity was detected in faecal filtrates from patients with *C. jejuni* as the only pathogen isolated from stools, but to further implicate the toxin in the disease mechanism, it was also demonstrated that the inoculation of rabbits with concentrated toxin alone consistently produced diarrhoea.

Guerrant *et al.* (1987) described a cytotoxin active upon HeLa and CHO, but not Vero, cell lines. This toxin was not neutralised by anti-Shiga or anti-*Clostridium difficile* toxins and the molecular weight was estimated to be 50-70 kDa based on the weight range of cytotoxic fractions following column chromatography. Heat lability at 60°C was also reported as well as partial lability to trypsin. Two further reports which fit into this toxin group were Fricker and Park (1989) and Mahajan and Rodgers (1990). Fricker and Park also attempted to correlate cytotoxicity with clinical manifestations of the patients. They demonstrated that all 10 strains isolated from patients with invasive diarrhoea were cytotoxic to HeLa, HEp-2 and MRC-5 cells, but were not toxic to Vero cells. Mahajan and Rodgers (1990) isolated a 68 kDa protein from the supernatant of *C. jejuni* which was heat-labile and trypsin-sensitive. Cytotoxin-like properties were demonstrated by the induction of rounding of CHO cells, and enterotoxin properties were eliminated by its failure to bind in a GM_1-based ELISA. The toxin was also found to be lethal for fertile chicken eggs, chicken embryo fibroblasts, CHO and INT 407 cells. The binding capacity of the toxin was also assayed using an ELISA. The cytotoxin was shown to bind to INT 407

cells with indication that a protein or glycoprotein-like receptor on the cells mediated this binding. Toxicity was lost upon freeze-thawing and the authors suggested the likelihood that this cytotoxin was the same as the one reported by Guerrant *et al.* (1987).

McCardell *et al.* (1986b) reported a cytotoxin which may possibly fit in this group except that they observed heat and trypsin stability. The cytotoxin was 70 kDa and active on CHO cells. No tests on Vero cells were carried out. Wassenaar (1997) included this toxin in the Vero negative group, but also referred to the Vero negative toxin as the 70 kDa toxin. There are reports of a cytotoxin with a molecular weight approximately in this range (Guerrant *et al.* 1987, Mahajan and Rodgers 1990), but as another report revealed the purified toxin to be 30 kDa (Pang *et al.* 1987), it seems a little too presumptive to name the toxin a '70 kDa Cytotoxin'. The name 'Vero negative cytotoxin' hence seems to be a more accurate name for this toxin.

1.11.4.3 Porin (MOMP) Cytotoxin

The major outer membrane porin (MOMP) of *C. jejuni* which has been previously discussed in section 1.6.2.1 has also been found to demonstrate cytotoxic activity. The first reported incidence of this was in 1996 by Moutinho-Fragaso *et al.* from a study of 63 *C. jejuni* and 43 *C. coli* clinical isolates. At the time, they reported this toxin as a new toxic factor 'TF' which caused elongation of Vero and HEp-2 cells and a rounding of CHO cells, inducing a high percentage of cell death in the latter two cell types after 72h. The toxin was demonstrated to be lethal for several tissue culture cell lines and was differentiated from enterotoxin (causes elongation of CHO and rounding of Y-1 cells) and from CLDT (causes progressive distension and death in all tissue culture cell lines). The toxin was reported to be a protein due to its partial heat-lability and trypsin-sensitivity. Further characterisation of the toxin was not carried out until 1998 when the same group isolated it from *C. coli* strain P26, isolated from a human gastroenteritis patient. The most pronounced effects were noted in HEp-2 cells after 48h exposure and purification experiments revealed two cytotoxic proteins which were 45 and 50 kDa on SDS-PAGE. N-terminal sequence analysis was performed on these two proteins and a BLAST search used to identify homologous proteins. BLAST (Basic Local Alignment Search

Tool) is an internet-based tool which searches relevant protein, nucleotide and gene databases for homologous matches. This revealed that the protein shared homology with OMPs from *Wolinella succinogenes* and the MOMP from C. *jejuni* (Moutinho-Fragaso *et al.* 1998).

Studies by another group showed similar results with *C. jejuni* strain 2483. Bacon *et al.* (1998) identified and characterised a cytotoxic porin-LPS complex from *C. jejuni* 2483 which was active upon a number of cells. They reported the highest activity upon HEp-2 cells which correlates with the findings of Moutinho-Fragaso *et al.* (1998). Following sequence analysis, they discovered that the protein contained the amino terminus of the cytotoxic porin protein from *C. jejuni*. This group also concluded that MOMP (or PorA as they referred to it) is highly conserved in *C. jejuni* and *C. coli* but not among other *Campylobacter* spp. They continued this work and following purification experiments, a single protein band of 45 kDa with a high molecular weight carbohydrate moiety was revealed. The cytotoxicity was heat-labile at 70°C and resistant to inactivation with glycosidases. This therefore demonstrated that it was not the carbohydrate portion of the LPS within the complex that imparted its cytotoxicity. They also found that the LPS was not an integral component of the cytotoxicity and probably served as a protective role, providing heat and trypsin resistance. Following N-terminal sequencing the protein revealed 97% homology with the *C. jejuni* MOMP. The MOMP is invariably present in *C. jejuni* and has been found to elicit an immune reponse in both animals and humans (Bacon *et al.* 1998 and 1999, Penn 2001). The authors hence suggested the PorA (MOMP) protein to be a suitable candidate molecule for detection of *C. jejuni* and for vaccine development against *C. jejuni*.

Another toxin which has been allocated a place in this group of toxins is the Cytolethal Rounding Toxin (CLRT) reported by Hanel *et al.* (1998) and Schulze *et al.* (1998). It seems likely that the effects described by Schulze *et al.* (1998) on tissue culture cells correspond with those observed by Moutinho-Fragaso *et al.* (1996). Both groups reported a rounding of CHO cells and whereas Moutinho-Fragaso *et al.* reported the effects on Vero cells to be 'elongation', Schulze *et al.* described the Vero cells as forming 'spindle-like structures' following incubation with the toxin. It is therefore feasible that the

two groups were describing the same toxic effect, but the only way to verify this would be a comparison of the morphological changes discovered by both groups. Moutinho-Fragaso *et al.* also reported elongation of HEp-2 cells and toxicity to other cell lines but no further morphological descriptions. Schulze *et al.* reported the rounding of HeLa cells, but again no further morphological descriptions were given. Toxicity to Vero cells was also described which suggests this toxin may belong to the Vero positive toxin group, but morphological differences were observed. Florin and Antillon (1992) described rounding and shrinking of all cell lines exposed to the Vero positive toxin, whereas Schulze *et al.* (1998) and Hanel *et al.* (1998) reported the rounding but no change in size of CHO and HeLa cells. It is therefore concluded that the toxin described by Schulze *et al.* (1998) as CLRT corresponds more closely with the MOMP toxin described by Moutinho-Fragaso *et al.* (1996 and 1998) and Bacon *et al.* (1998 and 1999) than with the Vero positive toxin, and is hence the MOMP. This also presents the possibility that many of the Vero negative and uncategorised toxins reported by others could also fall into this group due to the predominance and abundance of MOMP in *C. jejuni* strains.

1.11.4.4 Hepatotoxic Cytotoxin

The only reports of a hepatotoxin produced by *Campylobacter* spp. are by Kita *et al.* (1990 and 1992). They managed to isolate and purify hepatotoxic factor(s) from whole-cell lysates of *C. jejuni* GIFU8734. The hepatic lesions were reported to be very similar to those evoked by *C. jejuni* infection, and a single intravenous injection of 10μg of the purified toxin consistently resulted in hepatitis in mice. This was determined by histology and liver function tests. Tissue culture studies with mouse hepatocytes demonstrated that low concentrations of the hepatotoxic factor caused the release of hepatic enzymes without cytolysis but high concentrations of the factor induced cytolysis. The hepatotoxic factors were neutralised using antiserum to the hepatotoxic factor, but neutralisation studies using antisera to *C. jejuni* LPS and *E. coli* LT failed to have any effect. The question is whether or not this hepatotoxic factor has any consequence or role in the infection of humans by *C. jejuni*. The answer appears to be probably not without further confirmation. Hepatotoxic strains were

isolated from asymptomatic patients which alone is not a basis for dismissal due to the differences in immunity of humans. The hepatotoxic factor additionally did not induce morphological changes in CHO and Y-1 cells. Of all the toxins discussed in the previous sections there is not one which has not been shown to cause toxicity/morphological changes upon CHO cells. It therefore seems that the toxin described here is either a very different toxin to all the others reported, yet does have clinical manifestations in human *C. jejuni* infection, or alternatively does not play a role in *C. jejuni* disease in humans. Until further reports of its role in humans are described, the latter case is assumed to be correct.

1.11.4.5 Unclassified *Campylobacter* Cytotoxins

Many other cytotoxins have been reported which have not been investigated sufficiently to be allocated to one of the aforementioned categories.

Yeen *et al.* (1983) reported a cytotoxin from 8 out of 11 *C. jejuni* strains that was heat-labile, trypsin-sensitive and induced characteristic rounding of HEp-2, HeLa and MRC-5 cells. This toxin is definitely not the CLDT or MOMP but could possibly be enterotoxin, as no studies of CHO or Y-1 cells have been reported. Alternatively, it could be either the Vero positive or Vero negative cytotoxin which both produce rounding of a number of cell lines, but are distinguished from each other by their toxicity or lack of toxicity on Vero cell lines, respectively.

A cytotoxin effective by rounding and death of CHO cells, but not tested on Vero cells, which could not be neutralised by anti-Shiga toxin was reported in 33 out of 39 *C. jejuni* strains by Goossens *et al.* (1985b), and again by Everest *et al.* (1992). This again could be either enterotoxin, Vero negative cytotoxin or Vero positive cytotoxin. Perez-Perez *et al.* (1989) reported a cytotoxic effect characterised by rounding of CHO cells by 3 polymyxin B-treated *C. jejuni* strains PEN1, PEN2 and PEN3. This again could be one of many toxins, but they ruled out the presence of enterotoxin using a ganglioside GM_1-based ELISA and CHO cell culture assay. This group were very doubtful of the clinical significance of toxin production by *Campylobacter* spp. due to the low levels of cytotoxin, lack of enterotoxin and the lack of a host immune response against these toxins.

Prasad *et al.* (1996) detected a cytotoxin in all three *C. jejuni* strains isolated from patients with invasive diarrhoea, they also found cytotoxicity in one isolate from a patient with watery diarrhoea. Supernatants from the *C. jejuni* isolates were tested and elicited a toxic effect upon HeLa and HEp-2 cells which was more pronounced in the former cell line. No toxicity tests using Vero cells were performed so once again, this toxin is very difficult to categorise.

1.12 Extraintestinal Complications of *Campylobacter* Enteritis

The single most important complication reported is the development of Guillain-Barré Syndrome. Other extraintestinal complications of campylobacter include reactive arthritis (Bremell *et al.* 1991, Peterson 1994), Bickerstaff's brainstem encephalitis or BBE (Yuki *et al.* 2000), pancreatitis and endocarditis (Janvier *et al.* 2000, Yuki *et al.* 2000).

1.12.1 Guillain-Barré Syndrome

Guillain-Barré Syndrome (GBS) is the most common cause of neuromuscular paralysis in the developed world since the eradication of polio (Allos 1997, Nachamkin *et al.* 1998a, Hartung and Keiseier 1999, Nachamkin *et al.* 1999, Prendergast *et al.* 1999, Hadden and Gregson 2001). It is an autoimmune disorder of the nervous system that involves loss of reflexes, paralysis and weakness of the respiratory muscles (Jacobs *et al.* 1997, Allos 1998, Nachamkin *et al.* 1998a, Prendergast *et al.* 1998, Ho and Griffin 1999). Miller Fisher Syndrome (MFS) is another form of GBS which affects the eyes (causes ophthalmoplegia).

GBS is self-limiting and reaches its worst within approximately 2-3 weeks, although 15-20% of patients are left with severe neurologic defects, and in some cases it is fatal (Nachamkin *et al.* 1998a). GBS is commonly preceded by a bacterial or viral infection (Gregson *et al.* 1998, Prendergast *et al.* 1999), of which *C. jejuni* is now the most common in up to 66% of cases (Allos 1997, Moran and Prendergast 1998, Nachamkin *et al.* 1998, Ho and Griffin 1999, Hughes *et al.* 1999, Prendergast *et al.* 1999, Hadden and Gregson 2001). Neurologic symptoms are more likely to be severe and/or irreversible when GBS

is preceded by *C. jejuni* (Allos 1997). Certain serotypes of *C. jejuni* are over-represented in patients with GBS preceded by *C. jejuni* infection, in particular serotypes O:19 in Japan and O:41 in South Africa (Gregson *et al.* 1998, Prendergast *et al.* 1998, Sheikh *et al.* 1998, Nachamkin *et al.* 1999, Prendergast *et al.* 1999, Endtz *et al.* 2000, Janvier *et al.* 2000, Wassenaar *et al.* 2000, Hadden and Gregson 2001).

The mechanism by which *C. jejuni* is assumed to be responsible for the onset of these illnesses is molecular mimicry. *C. jejuni* has many epitopes on its lipo-oligosaccharide which mimic human gangliosides (Jacobs *et al.* 1997, Allos 1998, Nachamkin *et al.* 1998b, Ho and Griffin 1999, Prendergast *et al.* 1999, Toyka 1999, Janvier *et al.* 2000, Hadden and Gregson 2001). Host antibodies against bacterial antigens are still present post-infection and thus begin to attack similar host epitopes. *C. jejuni* epitopes which mimic ganglioside GM_1 are the most abundant ones associated with GBS patients (Allos 1998, Hughes *et al.* 1999, Nachamkin *et al.* 1999). The likelihood of developing GBS after a *C. jejuni* infection has, however, been estimated as fairly low. Allos (1998) estimated the prevalence to be 1 case of GBS following 1000 cases of *C. jejuni*.

1.13 Conclusion

The pathogenic mechanisms of *Campylobacter* spp. are diverse and complicated. Studies of these mechanisms, in particular the toxins, have not yielded much success over the last two decades. A significant problem which hinders research on the mechanisms of campylobacter infection is the lack of an appropriate animal model. Many of the models used so far are unsuccessful for reproducing the human symptoms of *C. jejuni* (Walker *et al.* 1986, Leach 1997, Wassenaar and Blaser 1999, Fields and Swerdlow 1999, Newell 2001). The sequencing of the *C. jejuni* genome is now complete and is a valuable tool for the identification and subsequent analysis of many pathogenic factors. The genome has been sequenced from *C. jejuni* NCTC 11168 and shows hypervariable sequences. There is therefore potential for the contradictory findings between different laboratories to continue due to probable differences between individual strains and significant phase variation within these strains. It may yet take a long time before all the pathogenic factors in all the strains, whether expressed or not, are fully sequenced and identified. The identification of a capsular polysaccharide, following mixed reports and much confusion over LPS structures, is an example of the potential success in this field. The proposal for toxin involvement in the pathogenesis of disease has been partially validated by the sequencing and characterisation of the cytolethal distending toxin (CLDT), as well as identification of the *pldA* gene and its proposed role in haemolysis. The story of the other cytotoxins and the enterotoxin, however, remains to be established at the genetic level, although evidence at the serological and cell culture level is already quite substantial.

Arcobacter is a newly emerging pathogen which is closely related to *Campylobacter*. Very little is known about the potential pathogenic mechanisms of *Arcobacter* spp., thus it seems that much can be learnt from methodologies and research findings in *Campylobacter* spp. A logical path of study would therefore be to mirror investigations on campylobacter toxicity and pathogenicity with arcobacter to try and gain an insight into similar pathogenic mechanisms in these two organisms.

1.14 Aims

The aims of this investigation were to:

1. Determine the cytotoxic effects of *C. jejuni* and *Arcobacter* spp. using mammalian cell culture experiments. *C. jejuni* grown in a range of media and at different ages will be tested to determine the optimum conditions and the most suitable strain for consistent cytotoxin expression.
2. Extract OMPs from *C. jejuni* and *Arcobacter* spp. grown under a range of culture conditions and examine and compare profiles using SDS-PAGE.
3. Determine whether a range of *C. jejuni* and *Arcobacter* spp. strains express haemolytic activity and under what conditions.
4. Characterise any haemolytic activity by determining specific enzyme activity.
5. Determine the crossreactivity of the OMPs from the most cytotoxic strain of *C. jejuni* and various *Arcobacter* strains with antibodies against shiga toxin and cholera toxin.
6. Use oligonucleotide probes to determine the presence of genes encoding the cytolethal distending toxin (CLDT) in strains of *C. jejuni* and *Arcobacter* spp.
7. Purify cholera-like toxin from *C. jejuni* using ion exchange chromatography for initial separation followed by gel electroelution.
8. Obtain N-terminal sequence data of the purified protein toxin band and screen the *C. jejuni* NCTC 11168 genome database for homologous sequences.

Chapter 2 - Materials and Methods

2.1 Strains and Media

Campylobacter strains:

Three different strains of *C. jejuni* were used: NCTC 11351, NCTC 11322 and NCTC 11168. NCTC is the National Collection of Type Cultures (Central Public Health Laboratory, London) and the strain numbers are the ones allocated in this collection. *C. jejuni* NCTC 11351 was the laboratory strain and the other two strains were ordered because they were both clinical isolates from humans with diarrhoea.

Arcobacter strains:

Eleven *Arcobacter* strains were used, the first was *Arcobacter butzleri* NCTC 12481. This was the laboratory strain which was originally isolated from a human with diarrhoea (http://www.phls.co.uk/services/nctc/searcher.html). Three further *Arcobacter butzleri* human isolates were obtained from Dr. S. On, Danish Veterinary Laboratory, Denmark: Rigs 1714, Rigs 15342 and Rigs 16799. Six strains were donated by Dr. Janet Corry, Bristol University. The first two were *Arcobacter butzleri* CA 102 and *Arcobacter cryaerophilus* C3 isolated from ducks. The final four *Arcobacter* spp. were isolated from chickens and identified as strains 1A, 2A, 3A and 5A and have not been speciated. These strains were selected from human, and also from avian sources, to investigate if the same levels and types of toxicity are found from isolates from all sources. This could help to link human illness from *Arcobacter* spp. with consumption of poultry, as it is with *C. jejuni*. A comparison of the toxic capabilities of the two strains *A. butzleri* and *A. cryaerophilus* was also considered to be important.

Media used:

Three different kinds of broth media were used for culturing. These were Brain Heart Infusion (BHI), Brain Heart Infusion supplemented with 1% Yeast Extract (BHI-YE) and Brucella broth supplemented with FBP (B-FBP). FBP supplement consists of 0.9mM $FeSO_4$, 1.3mM NaS_2O_5 and 2.3mM sodium pyruvate.

Three types of solid media were used. Blood agar was most commonly used for culturing purposes. B-FBP agar and washed blood agar (WBA) were also used.

A microaerobic atmosphere was used for culturing *C. jejuni*, and was also often used to culture *Arcobacter spp*. Details of this are given below.

Preparation of Blood Agar (BA)

To 350ml distilled water, 14g blood agar base No.2 (Oxoid) was added and autoclaved. The molten agar was cooled to approximately 50°C before the addition of 25ml sterile, defibrinated horse blood (Oxoid). This was mixed gently before pouring.

Preparation of Washed Blood Agar (WBA)

Blood Agar was prepared with 7% washed horse blood. To wash the blood, 25ml of sterile, defibrinated blood (Oxoid) was centrifuged at 3000xg for 10 minutes. Following this, the supernatant was removed and PBS (see below) was added to make the volume back up to 25ml. This was repeated twice and after the last addition of PBS, the blood was mixed before being added to molten blood agar base (as above) and poured.

Preparation of B-FBP Agar

To 400ml distilled water, 11.2g of Brucella base (Difco) and 6g Agar No. 1 (Oxoid) were added and autoclaved. FBP supplement was used at 10x the concentration in agar than in broth, so 0.25g each of ferrous sulphate, sodium metabisulphite and sodium pyruvate were dissolved into 5ml distilled water and filter sterilised. To each 400ml of molten agar, 5ml of this FBP supplement was added before mixing and pouring.

Preparation of BHI and BHI-YE

To make BHI; brain heart infusion (Oxoid) was prepared and autoclaved according to the manufacturer's instructions. To make BHI-YE; 1% yeast extract (Oxoid) was added to BHI before autoclaving.

Preparation of B-FBP Broth:

Brucella broth (Difco) was prepared and autoclaved as per manufacturer's instructions. To make the FBP supplement, 0.025g each of ferrous sulphate, sodium metabisulphite and sodium pyruvate was dissolved in 5ml distilled water. This was filter sterilised using a 0.2μm pore size Acrodisc filter (Gelman Sciences). To each millilitre of Brucella broth, 30μl of FBP supplement was aseptically added. For example 0.3ml of FBP supplement was added to each 10ml Brucella broth.

Phosphate Buffered Saline (PBS):

PBS is frequently referred to throughout the methodology. In all cases, the following was used: Phosphate Buffered Saline, Sigma; 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH7.4.

Microaerobic Atmosphere:

This was achieved in one of two ways throughout the project.

1. Oxoid AnaeroJars (2.5 litre volume) were used with the addition of a catalyst and a Campylobacter Gas Generating Kit (Oxoid). This was the method used during most of the projects: used in the following sections 2.2, 2.5, 2.6, 2.7, 2.8 and 2.9.
2. A Variable Atmosphere Incubator (VAIN, Donn Whitley) was used with a gas cylinder attached containing a gas mix of 5% O_2, 10% CO_2 and 85% nitrogen (BOC gases). This method was only used in the heamolysis work in sections 2.3 and 2.4.

Chemicals and Reagents:

All chemicals, reagents and enzymes unless otherwise stated were obtained from Sigma Chemicals (Poole, UK).

2.2 Growth Curve Procedures

2.2.1 *C. jejuni* Growth Curve

B-FBP broth and B-FBP agar were the media mainly used during these experiments, although experiments were repeated later with BHI broth. All three *C. jejuni* strains were used in this experiment.

The growth curve procedure was as follows:

1. Cultures were initially grown microaerobically on blood agar for 48h. A loopful of colonies from a BA plate was used to inoculate a 10ml B-FBP broth in a half ounce (approx. 12ml) bottle with a rubber-lined screw cap.
2. Caps were screwed on tight and the broths incubated at 42°C in an aerobic environment for 24h, this was used as the starter culture.
3. Two further 10ml B-FBP broths in half ounce bottles were inoculated with 10µl of starter culture. One of these was used for absorbance, the other one used for plate count readings. Six of these were set up for each experiment

(three for absorbance readings, three for plate count readings) so that the volume of each bottle was not affected too greatly by the number of readings taken. These were then incubated with the caps on tight at 37°C.

4. Plate count readings were taken every hour up to 8 h, then again at 24, 48 and 60h where possible. To do this, 50µl was removed aseptically from the test broth and added to a 450µl B-FBP broth. A tenfold dilution series was made in B-FBP broth and plated out onto B-FBP agar plates. These were then incubated microaerobically at 37°C for 48h before the colonies were counted and the number of colony-forming units (cfu) per ml was calculated.
5. Absorbance readings at 650nm were measured at approximately every 2 h for the first 8 h, then again after 24h, 48h and 60h against a B-FBP blank.

The doubling time of *C. jejuni* was calculated using the calculation

$$G = \frac{T}{N}$$

where G=generation time (doubling time), T=time and N=number of generations.

2.2.2 *A. butzleri* Growth Curve

The following experiment was designed and performed by Claire Hilton (The Nottingham Trent University) using *A. butzleri* NCTC 12481:

1. A loopful of *A. butzleri* culture from a BA plate was used to inoculate each of two 10ml BHI starter broths in flasks.
2. These were incubated overnight on a shaker at 100rpm, 37°C in an aerobic environment.
3. Two millilitres (2% inoculum) was transferred into a 100ml BHI broth in a flask, this was then incubated shaking at 200rpm, 37°C, aerobically.
4. Absorbance readings at 650nm and plate counts (BA) were measured every hour for 5 h, then at 8h, 10h and 30h against a BHI blank.

The doubling time of *A. butzleri* was calculated using the calculation in section 2.2.1 above.

2.3 Detection of Haemolysis in *C. jejuni* and *Arcobacter* spp.

Haemolytic activity in bacteria is thought to be representative of pathogenesis. The following was a series of basic tests to screen all the *Campylobacter* and *Arcobacter* strains for haemolytic activity. This was used as an indication of the possible pathogenic potential of all of the strains.

2.3.1 Screening of BA Cultures for Zones of Haemolysis

For this experiment all three strains of *C. jejuni* and all eleven strains of *Arcobacter* spp. (2.1) were used.

a) Each strain was streaked onto BA. *C. jejuni* strains were streaked onto plates in triplicate and incubated microaerobically at 30°C and 37°C for 48h.

   Each *Arcobacter* strain was streaked onto 3 BA plates for each of 4 conditions i.e. 12 plates in total of each strain. Arcobacter haemolysis was hence tested under the following four conditions in triplicate:

i) Microaerobic incubation at 30°C for 48h

ii) Microaerobic incubation at 37°C for 48h

iii) Aerobic incubation at 30°C for 48h

iv) Aerobic incubation at 37°C for 48h

b) Each strain was streaked onto WBA (washed blood agar) as above.

After 48h, each plate was examined for zones of haemolysis. α-haemolysis was visualised by green zones surrounding the colonies, β-haemolysis was visualised by clear zones surrounding the colonies (Misawa *et al.* 1995).

2.3.2 Microplate Haemolysis Assay

This was a liquid assay which was a slightly modified version of the microplate technique used by Dominguez Rodriguez *et al.* (1986).

Procedure as follows:

1. Two loopfuls (about 10^{10} cells, this was checked using plate counts) from a BA plate were put into 0.5ml PBS.
2. Into the top well of each column, 100µl of suspension from each different strain was added (Microtitre 96U-well plates, Scientific Laboratory Supplies,

Loughborough, UK) and 50µl of PBS was put into the rest of the wells in the plate.

3. Six two-fold dilutions were made from the original cell suspension down to the penultimate well. The final well was left as a negative control.
4. To each well, 100µl of 1% washed horse or sheep blood (see below) was added.
5. The plates were set up with all strains and both horse and sheep blood. They were left to incubate for 6-8h at 37°C. After observations of positive or negative haemolysis were made, the plates were reincubated overnight. Haemolysis was positive where the liquid in the well had turned red indicating lysis of the blood cells. Where the blood cells were still intact, a small red pellet was visible with clear liquid covering it.

Where broth cultures were used, 10ml BHI broths of each strain were centrifuged at 5000xg for 20 min and the pellets resuspended in 0.5ml PBS. Steps 2-5 were then carried out. The assay was also carried out with broth supernatants.

The assay results were assessed visually, and were to be scored according to the lowest dilution which caused haemolysis for each strain.

2.3.3 Liquid Haemolysis Test in Tubes

This test was almost exactly the same as the microplate test except on a larger scale. The results could also be analysed quantitatively, not just visually as with the microplate test. The method is slightly modified from Rowe and Welch (1994).

Procedure:

1. Four loopfuls of each strain from BA (approximately 10^{10} cells according to plate counts) were put into 1ml PBS.
2. Two fold dilutions were made into a further 7 tubes containing 0.5ml PBS.
3. One millilitre of 1% washed horse or sheep blood (see below) was added to each tube.
4. Negative controls were made using PBS and blood only, positive controls were made using 1% Sarkosyl in PBS and blood.
5. The tubes were incubated overnight at 37°C.

6. After incubation, the contents of each tube was vortex mixed and transferred to an Eppendorf tube. These were centrifuged in an MSE MicroCentaur (Sussex, UK) at 11000xg for 10 min.
7. The absorbance of the supernatants of each sample was measured against the negative control at 540nm using a spectrophotometer (PU8675Vis, Phillips Analytical, Cambridge).

These experiments were performed aerobically initially, but repetitions were later carried out under microaerobic conditions in a Variable Atmosphere Incubator.

Where broth cultures were used they were centrifuged as in section 2.3.2 and resuspended in 1ml PBS.

The assay results were assessed visually, and were to be scored according to the lowest dilution which caused haemolysis for each strain.

The percentage haemolysis was calculated using the positive control absorbance reading as 100% and calculating the sample absorbance readings against this.

Preparation of 1% washed horse/sheep blood:

1. Bottles of 25ml sterile, defibrinated horse and sheep blood (Oxoid) were centrifuged at 3000xg for 10 min, 4°C.
2. The supernatant was pipetted off and replaced with sterile PBS.
3. The cells were washed twice or until the supernatant of the blood was almost clear.
4. When the blood supernatant was nearly clear, all but the last centimetre was removed. This was mixed back in with the packed cells.
5. A plain glass capillary tube (Hawksley and Sons, Sussex, UK) was put into the blood which was drawn up the tube by capillary action. The end of the tube was sealed using a Hawksley Cristaseal (Hawksley and Sons, Sussex, UK). This was carried out for a bottle of horse blood and a bottle of sheep blood.
6. The sealed centrifuge tubes were placed opposite each other in a Micro-Haematocrit Centrifuge (Hawksley and Sons, Sussex, UK). The blood was centrifuged for 2 min.
7. Following centrifugation, a Hawksley Reader for the Micro-Haematocrit Centrifuge (Hawksley and Sons, Sussex, UK) was used to determine the concentration of the blood cells (as a percentage). This was then used to

make 1% blood, eg. where the concentration of the blood cells was 85%, 1ml of the blood was added to 84ml of sterile PBS.

2.3.4 Contact Haemolysis Assay

This method was developed from Istivan *et al* (1998). The most important modifications from the liquid test (section 2.3.3) that were made were centrifuging the bacteria and blood cells so that they were in close contact with each other. Secondly the test was incubated under microaerobic conditions. *C. jejuni* strains NCTC 11351 and NCTC 11168 and *Arcobacter* strains NCTC 12481, CA 102, C3, 1A, 2A, 3A, 5A, 16799, 15342 and 1714 were used for this experiment.

Procedure:

1. Broth (BHI) and plate (BA) cultures of each strain grown for 48h at 37°C were used for this test. Viable counts were used to ensure that cell numbers were consistent at 10^{10} per ml of inoculum per test.
2. Cells were resuspended in 0.5ml PBS (cell pellets following centrifugation at 5000xg for 20 min where broths were used; cells harvested from agar surface where plates were used) before the addition of 1ml 1% washed horse or sheep blood.
3. Negative controls were prepared using blood and PBS only (i.e. no bacteria) and positive controls were prepared using 1% Sarkosyl in PBS and blood.
4. A second centrifugation took place after the bacterial cells were mixed with blood at 3000xg for 20 min, 4°C.
5. Supernatants (where clear) were poured off all but about 100µl. If the supernatant was red it was not poured away (the cells had already lysed).
6. The tubes were then incubated microaerobically at 37°C overnight (approximately 16h).
7. Following incubation, 1ml PBS was added to each tube (not where supernatant was left behind). The tubes were vortex mixed and the contents transferred to 1.5ml eppendorf tubes. These were centrifuged in a MSE MicroCentaur (Sussex, UK) at 11000 xg for 10 min.
8. The supernatant was poured off following centrifugation. The absorbance at 540nm of each supernatant was measured against the negative control.

The percentage haemolysis was calculated using the positive control absorbance reading as 100% and calculating the sample absorbance readings against this.

Further modification (for phospholipase tests):

A further modification of this method was made. The blood and the bacteria were centrifuged together for 5 min (3000xg), but the supernatant was left in the tube during the incubation period. This way, the cells were in close contact for the test, but only needed to be vortexed and transferred to eppendorfs to read the results. The test was also performed under aerobic conditions to test whether close contact of the cells only was required for consistent haemolysis.

2.4 Characterisation Studies of Haemolysins

C. jejuni strains NCTC 11351 and NCTC 11168 and *Arcobacter* strains NCTC 12481, 1A, 2A, 3A, 5A, Rigs 16799, Rigs 15342 and Rigs 1714 were used for this set of experiments.

2.4.1 Screening for Phospholipase A Activity

Two phospholipase A inhibitors were used for this experiment, dibucaine and quinocrine. Purified phospholipase A_2 enzyme from *Streptomyces violaceoruber* was used as a positive control to test the efficiency of the inhibitors.

Procedure:

1. A 2mM solution of each of the inhibitors was prepared in sterile PBS.
2. Contact haemolysis was carried out with the further modification in stage 2 of section 2.3.4 (above) as follows; the pellets were resuspended in 0.2ml PBS and the following inhibitors were added: dibucaine (10μM) and quinocrine (10μM). Each of these was added to triplicate contact haemolysis test bottles. Horse blood was the only blood used.
3. Controls without inhibitor were set up for each strain containing bacteria, horse blood and PBS only.
4. Controls without bacterial cells were set up with inhibitors at the above concentration, PBS and blood only.
5. The inhibitors were incubated with bacteria or PBS only for approximately 30 minutes at room temperature before the addition of 1ml 1% horse blood.

6. The tubes were centrifuged at 3000xg for 5 min before being incubated microaerobically at 37°C for approximately 16h (supernatant left in tube).
7. After incubation, the tubes were vortex mixed, then transferred to 1.5ml eppendorf tubes. These were centrifuged in a MSE MicroCentaur (Sussex, UK) at 11000xg for 10 min.
8. The absorbances were measured at 540nm against the negative controls.

The results were expressed as OD540nm readings and were not calculated against a positive control. The enzyme for the positive control proved to be very unstable, and a suitable titer for effective haemolysis was not established.

Preparation of Dibucaine and Quinocrine:

Dibucaine and quinocrine (Sigma) were used at the final concentration of 10μM. Two millimolar solutions of each were made in PBS. The final volume of the test was 1.2ml. To make 10μM from this, 6μl was added into 1.2ml final volume.

Preparation of Phospholipase A_2:

Phospholipase A_2 from *Streptomyces violaceoruber* (Sigma) was used for this experiment. To 50 units of enzyme, 0.5ml of PBS was added so there was 1 unit per 10μl. For each test sample 2 units were required, so 20μl was added to each.

2.4.2 Screening for Phospholipase C Activity

Compound 48/80 (Sigma) is a known phospholipase C inhibitor, and was the inhibitor used for this experiment. Phospholipase C Type 1 from *Clostridium welchii,* also known as *C. perfringens,* (Sigma) was the enzyme used as the positive control.

Procedure:

This experiment was set up in almost exactly the same way as the previous phospholipase A experiment (section 2.8.1) except that 1μg 48/80 and 0.0005 units of Phospholipase C enzyme were used.

Preparation of 48/80:

A stock solution of 1mg/ml 48/80 was prepared in PBS. One microlitre (1μg) of 48/80 was added to the sample.

Preparation of Phospholipase C:

Phospholipase C Type 1 (129 units) from *Clostridium welchii* was used. To this, 1ml PBS was added, so 1 unit equalled 7.75μl. This was diluted 1 in 1000,

hence 3.75µl of this equalled 0.0005 units (the required amount for one test sample).

2.4.2.1 BLAST Search of Phospholipase C Gene Sequence

A BLAST (Basic Local Alignment Search Tool) search was performed using the NCBI database to check for any sequence homology in the *C. jejuni* genome with known phospholipase C alpha toxins (http://www.ncbi.nlm.nih.gov/BLAST?). BLAST is an internet-based tool provided by the NCBI (National Center for Biotechnology Information) database which performs searches for specific protein or nucleotide sequences across a broad range of species. In this case protein sequences of known phospholipase C toxins were searched for within the *C. jejuni* genome.

2.5 Outer Membrane Protein (OMP) Extraction and Analysis from *C. jejuni* and *Arcobacter* spp.

OMPs were extracted and analysed from all three *C. jejuni* strains, *A. butzleri* NCTC 12481, *A. butzleri* CA 102 and A. *cryaerophilus* C3.

2.5.1 Isolation of OMP using Sodium N-Lauroyl Sarcosinate (Sarkosyl)

1. Bacteria were cultured on blood agar under microaerobic conditions for a range of time periods (see below).
2. All the bacteria from one agar plate was harvested with a wire loop into 5ml 10mM Tris (hydroxymethyl) aminoethane, pH 8.0.
3. Sodium N-lauroyl sarcosinate was added to a final concentration of 2% (in this case, 0.5ml of a 20% solution). This was mixed and incubated at room temperature for 30 min.
4. A 100mM solution of phenylmethylsulfonylfluoride (PMSF) was prepared by dissolving 0.174g PMSF into 10ml isopropanol (propan-2-ol, BDH). A 10mg/ml solution of benzamidine was prepared in distilled water. Following part 3 (above), 50µl of each of these solutions was added to the preparation.
5. The outer membrane-peptidoglycan complex was recovered by centrifugation at 40000xg for 60 min at 4°C. The pellet (usually gelatinous and transparent)

was washed once in 2ml 10mM Tris, pH 8.0 with the addition of 20µl each of the above PMSF and benzamidine solutions.

6. The pellets were resuspended in 400µl of 2x sample buffer (see below) and boiled ina boiling water bath in pierced eppendorfs for 5 minutes.
7. Samples were cooled, divided into 20µl volumes and stored at -20°C until required.

To isolate OMP from broth cultures, 10ml BHI broths were incubated microaerobically for various time periods then centrifuged at 5000xg for 20 min. The pellets were resuspended in 5ml 10mM Tris pH 8.0 before proceeding through steps 3-5 as above. In step 6 the pellets were finally resuspended in a smaller volume (250µl) of 2x sample buffer before being boiled, divided into 20µl volumes and stored at -20°C. Pellets from broth cultures were resuspended in less sample buffer than pellets from agar cultures due to the lower cell density obtained from broth cultures.

A series of 2d to 14d old cultures were prepared from all three *C. jejuni* strains, *A. butzleri* NCTC 12481 and CA 102 and *A. cryaerophilus* C3. The *C. jejuni* strains were cultured in BHI, BHI-YE and B-FBP broths and also on Blood Agar plates. *Arcobacter* strains were cultured on Blood Agar only.

Preparation of 2x Sample Buffer (Hancock and Poxton 1988)

0.125M Tris (Tris (hydroxymethyl) aminoethane) pH 6.8

4% SDS (sodium dodecyl sulphate)

20% glycerol

2% 2-mercaptoethanol

0.002% bromophenol blue

To 50ml distilled water, 1.51g of Tris base was added and the pH was adjusted to 6.8 with 1M HCl before the addition of 4g SDS. To this, 20ml of glycerol, 2ml 2-mercaptoethanol and 4ml of a 0.05% aqueous solution of bromophenol blue were added. The solution was then made up to 100ml with distilled water and stored at 4°C (brought to room temperature before use).

2.5.2 BCA Protein Assay

The bicinchoninic acid (BCA) protein assay (Brown *et al.* 1989) was used to determine the protein concentration of each of the OMP samples.

Preparation of BCA Reagents

BCA Reagent A:

1% bicinchoninic acid

2% sodium carbonate

0.16% sodium tartrate

0.4% sodium hydroxide

0.95% sodium hydrogen carbonate, pH 11.25.

Bicinchoninic acid (5g), 10g sodium carbonate, 0.8g sodium tartrate, 2g sodium hydroxide and 4.8g of sodium hydrogen carbonate were added to 450ml distilled water. The pH was adjusted to 11.25 using 50% (12.5M) sodium hydroxide before making the volume up to 500ml with distilled water.

BCA Reagent B:

4% copper sulphate; 4g of $CuSO_4.5H_2O$ was added to 100ml distilled water.

BCA Standard Working Reagent:

This was prepared by the addition of 50 parts Reagent A to 1 part Reagent B.

BCA Assay Procedure

1. Standard protein solutions in the range of 0-1mg/ml were prepared in sterile distilled water using a 1mg/ml BSA stock solution (bovine serum albumin).
2. Each protein to be assayed was diluted by adding 20µl to 1ml distilled water in an Eppendorf tube.
3. Sodium deoxycholate (150µl of 0.1%) was added to each tube.
4. After 5 min at room temperature protein was precipitated by the addition of 72µl TCA (trichloroacetic acid, 50% w/v) and each tube was thoroughly mixed with a vortex mixer (Fisons model WM/250/SC/P).
5. Tubes were then centrifuged at 11000xg using an MSE MicroCentaur (Sussex, UK) and the supernatant was removed by vacuum aspiration.
6. The pellets were washed by the addition of 1ml distilled water, 150µl of 0.1% sodium deoxycholate and 72µl TCA, then mixed with a vortex mixer.

7. Protein was re-dissolved in 50µl of 0.1% sodium hydroxide containing 5% SDS.
8. After the addition of 1ml BCA standard working reagent, each tube was thoroughly mixed with a vortex mixer and incubated at 60°C for 30 min. The absorbance of the samples was measured at 562nm against the 0mg/ml standard as the blank.

The results of the standards were used to construct a calibration graph which was then used to calculate the protein concentrations of each sample assayed.

Using these results the amount of protein loaded onto each SDS-PAGE lane was standardised. On a mini gel, 10µg was loaded and on a large gel, 30µg was loaded. Samples were boiled for 5 min in a boiling waterbath, then cooled before loading.

2.5.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using BioRad Minigel System

OMP samples were loaded onto each lane in the gels in 10µg concentrations. For example, where the protein concentration was 0.6mg/ml, a volume of 16µl was loaded which contained 10µg protein. The gels comprised of a 4% acrylamide stacking gel and a 10% acrylamide separation gel. The gels were electrophoresed with electrode buffer (see below) at 50mA using a BioRad minigel apparatus and a Powerpac 300 (BioRad Laboratories, UK). The gels were run for approximately 1h, or until the tracking dye ran to within 1cm of the bottom of the gel.

Molecular weight markers (Sigma, Cat. No. MW-SDS-200) were also loaded on to the first lane of each gel in 10µl volumes for analysis purposes. The markers were prepared in sample buffer and retained at -20°C until required. The marker mix consisted of:

Carbonic anhydrase	29kDa
Egg albumin	45kDa
Bovine albumin	66kDa
Phosphorylase-b	97.4kDa
β-Galactosidase	116kDa

Preparation of the Acrylamide Gels

The following recipes are slightly modified from Hancock and Poxton (1988).

10% Separating Gel/Lower Gel:

To 2ml distilled water, 8.75ml double-strength separating buffer (see below) and 5.8ml of a 30% acrylamide stock solution were added and mixed in a flask. Following this, 25µl Temed and 875µl of 15mg/ml ammonium sulphate were added and mixed gently. The gel mix was used immediately.

4% Stacking Gel/Upper Gel:

To 3ml distilled water, 5ml double-strength stacking buffer (see below) and 1.5ml of a 30% acrylamide stock solution were added and mixed in a flask. Following this, 20µl of Temed and 500µl of 15mg/ml ammonium sulphate were added. The gel was mixed and used immediately.

Preparation of Double-Strength Separating Buffer

0.75M Tris, pH8.8

0.2% SDS

To 700ml distilled water, 90.86g Tris (hydroxymethyl) aminoethane was added and the pH was adjusted to 8.8 with 1M HCl. Following the addition of 2g SDS the buffer was made up to 1 litre with distilled water.

Preparation of Double-Strength Stacking Buffer

0.25M Tris, pH 6.8

0.02% SDS

To 300ml distilled water, 15.14g Tris was added. The pH was adjusted to 6.8 before the addition of 1g SDS. The buffer was made up to 1 litre with distilled water.

Preparation of Electrode Buffer, pH 8.3

0.025M Tris

0.192M glycine

0.1% SDS

To 1.5L distilled water, 6.06g of Tris and 28.3g of glycine were added. The pH was adjusted to 8.3 before the addition of 2g SDS. The buffer was made up to 2 litres with distilled water.

2.5.4 SDS-PAGE using the Protean II X1 Gel System (BioRad)

SDS-PAGE of the isolated OMP samples was also carried out using gels of size 20cm x 20cm to increase the separation of protein bands on the gel. This required a greater protein loading in each lane (30μg). An increased loading of 30μl of the molecular weight marker mix was loaded. The gels were run at 50mA using a Powerpac 3000 (BioRad) for approximately 5h 30min.

2.5.5 Staining of OMP Gels

Initially, a Coomassie Blue stain (see below) was used for the minigels. Later, when greater separation was required using the larger gel system a Silver Staining Kit was obtained from Sigma Chemicals (Poole). This was approximately 200 times more sensitive than the Coomassie Blue stain, thus was used to identify a greater range of bands following the increased band separation achieved using the Protean 11X1 System.

Once staining was completed the gels were analysed using the QuantiScan software package (Biosoft, Cambridge, UK).

Coomassie Blue Staining Procedure:

1. When gels were left overnight before staining, they were immersed in fixer until ready for staining. Gels not being left overnight were put straight into stain.
2. When the gels were ready they were transferred to the stain and held on a shaker for 90 min at room temperature.
3. The stain was poured off and the gels were immersed in destain until the background was clear. This would normally take about 4 h, with at least two changes of destain.

Fixer: 25% ethanol and 10% acetic acid by volume in distilled water.

Stain: 0.02% Coomassie blue in 25% ethanol, 10% acetic acid as above. (Same as fixer with Coomassie stain added)

Destain: 10% methanol and 10% acetic acid by volume in water.

Silver Staining:

Silver stain kit was obtained from Sigma chemicals and used according to the manufacturer's instructions.

2.6 General Cytotoxicity Screening

2.6.1Preparation of Samples for Cytotoxicity Screening

Blood Agar (BA) Cultures

All *C.jejuni* strains were grown on BA at 37°C microaerobically for 1d, 2d, 3d and 7d before harvesting. Arcobacter strains 12481, CA 102, C3, 1A, 2A, 3A and 5A were only harvested from 2d old BA cultures grown microaerobically at 37°C.

a) Harvesting Procedure:

1. Bacteria from one BA plate was harvested with a wire loop into 1ml PBS per sample.
2. The Eppendorf tubes were vortex mixed after 20 min at room temperature to encourage resuspension of all the bacteria into the PBS.
3. The contents of the tubes were allowed to settle again at room temperature for 20 min. If any lumps of agar or other undissolved material were visible, the bacterial suspension was pipetted into a fresh Eppendorf leaving the solid matter behind.

These samples were then ready for part b) Cell Sonication.

Broth Cultures

All *C. jejuni* strains were grown at 37°C in BHI, BHI-YE and B-FBP microaerobically for 3d. No *Arcobacter* broth cultures were prepared for cytotoxicity screening.

The broths were prepared as follows:

1. Three 10ml broths in 20ml Universal bottles were inoculated using a loopful of culture from a 2d old BA plate. Incubation was carried out at 37°C in a microaerobic jar (Oxoid) with loosened caps.
2. After 3 days, these three broths were used to inoculate (10% v/v) a 300ml broth. This was incubated under the same conditions for 3 days.
3. The broth was centrifuged at 14500xg for 20 min. The supernatant was poured off and retained in the freezer. This was ready for immediate use in the cytotoxicity assay with no further treatment required.
4. The pellet was washed in 20ml PBS and centrifuged at 3000xg for 20 min. The final pellet was resuspended in 5ml PBS.

This sample and the BA sample were then treated further in a sonication step.

b) Cell Sonication

1. One ml quantities of the cell suspensions were distributed into Eppendorf tubes and bath sonicated (QH Kerry Ultrasonics Ltd.) on ice for 15 min.
2. Suspensions were then centrifuged at 8000xg in a MSE MicroCentaur (Sussex, UK) for 30 min.
3. The supernatants and pellets were stored at -20°C until needed.

2.6.2 MTT Cytotoxicity Assay

The cytotoxicity of these cell pellets and supernatants was determined using an MTT (methyl tetrazolium thiazolyl blue) assay. Two cell lines were used for initial testing. The first was N2a, a mouse neuroblastoma cell line (European Tissue Culture Collection (ETCC)), the second was ECV 304, a human endothelial cell line (ETCC). A Chinese hamster ovary (CHO) cell line (ETCC) was also used with strain 11351.

The following procedure was carried out:-

1. 24-well plates were seeded with the following cell numbers:

   N2a cells = 25 000 cells/well

   ECV 304 cells= 12 500 cells/well

   These were incubated in growth medium at 37°C for 24 h.
2. The growth medium was changed and treatment added (ie. bacterial supernatants/cell sonicates). A dilution range of 5% to 0.625% of the total volume of the treatment was added.

   These were incubated at 37°C for 48 h.
3. MTT Assay:

   MTT (50μl) was added and the cells were incubated at 37°C for 45 min. The growth medium (see below) was removed, and 1ml undiluted DMSO (dimethylsulphoxide) was added. The absorbance reading was measured at 570nm. This was then plotted against treatment concentration. Cytotoxicity was plotted as a percentage of the absorbance of the control (PBS or broth medium) and a T-test ($P<0.05$) was used to determine positive cytotoxicity from multiple samples.

Heat and Trypsin Sensitivity

Some of the samples which were cytotoxic were also tested for heat and trypsin sensitivity, by first incubating the samples in trypsin at 37°C for 30 min. After this, they were boiled in a boiling water bath at 100°C for 10 min to denature the trypsin. The same boiling procedure was also used to test the samples for heat sensitivity.

Preparation of growth medium:

500ml DMEM (Sigma): dulbecco's modified eagles' medium with bicarbonate and high glucose

50ml FBS (Sigma): foetal bovine serum, 10%

5ml glutamine (2mM), penicillin G (100 units/ml) and streptomycin (10µg/ml) (Sigma).

The above procedure (2.6.2) was performed by Kevin Spears (The Nottingham Trent University). When this assay was repeated throughout the rest of the experimental work it was performed by Kevin Spears.

2.7 Screening for Specific Toxins

C. jejuni NCTC 11351, *A. butzleri* NCTC 12481, *A. butzleri* CA 102 and *A. cryaerophilus* C3 were used for this set of experiments.

2.7.1 Screening of OMP Samples for Cholera-like Toxin

OMP samples were applied to SDS-PAGE following a BCA protein assay as in 2.5.2 and then a Western blot was performed with each gel. The first step of a Western blot was electroblotting.

a) Electroblotting Procedure

1. SDS-PAGE was carried out as in 2.5.3 with the following amendments; 12.5µg of protein was loaded onto each lane; 5µl of a prestained molecular weight marker (Sigma, Cat. No. MW-SDS-BLUE, 26-180kDa) was used in lane 1; 25µg of a cholera toxin control sample (see below) was loaded in lane 2.
2. Following SDS-PAGE, the gel was removed and immersed in electroblotting buffer for 20 min.

3. During this time 18 sheets of filter paper (Whatman No. 1) per gel were cut to size (10x7cm) and immersed in electroblotting buffer.
4. A sheet of nitrocellulose (Gelman Sciences, 0.45µm membrane pore size) was cut as above and soaked in electroblotting buffer.
5. The graphite electrodes of the transfer unit (BioRad Trans-Blot SD Semi-Dry Transfer Cell) were dampened with absorbent paper soaked with distilled water.
6. 9 sheets of the wet filter paper (above) were placed onto the anode electrode and any air bubbles smoothed out between each sheet.
7. The nitrocellulose was placed on top of the filter paper and the gel placed on top of the nitrocellulose. Air bubbles were excluded at each step.
8. The remaining 9 sheets of wet filter paper were then placed on top of the gel and the cathode electrode was fixed firmly to the top to seal the transfer unit before fitting the lid.
9. A constant current of 150mA was set for 1h (BioRad model 200/2.0 power supply) to electroblot one minigel. If two minigels were electroblotting in the same unit a constant current of 200mA was applied.

Preparation of Cholera Toxin Control:

Cholera toxin B subunit (0.5mg) isolated from *Vibrio cholerae* was obtained from Sigma chemicals. The powder was resuspended into 250µl sterile, distilled water to give a concentration of 2mg/ml. This was stored at 4°C until needed. Just before use, the toxin was diluted 1:2 with 2x sample buffer to give a concentration of 1mg/ml. For example, to load two gels; 25µl of cholera toxin was added to 25µl 2x sample buffer and boiled for 5 min in a boiling water bath to ensure proteins are denatured and dissolved. Twenty-five microlitres was loaded in lane 2 of each gel, which is the equivalent of 25µg per gel. This was more than the sample loadings which were only 12.5µg.

Preparation of Electroblotting Buffer, pH 8.0:

7.24g Tris (hydroxymethyl) aminoethane

33.5g Glycine

600ml Methanol (BDH)

To 2 litres of distilled water, 7.24g of Tris and 33.5g glycine were added. The pH was adjusted to 8.0 before the additon of 600ml methanol. The buffer was then brought to 3 litres with distilled water.

b) Procedure for Probing Blots with Cholera Toxin B Subunit Monoclonal Antibody (Western blotting)

1. The nitrocellulose sheet was removed from the transfer unit. Transfer of the protein onto the nitrocellulose was visible by the transfer of the prestained molecular weight markers.
2. The nitrocellulose sheet was placed into 20ml blocker solution (see below) on a shaker (IKA Labortechnik, model KS 250 basic) at room temperature for 1h.
3. Primary antibody treatment (see below) was added and incubated overnight at 4°C on a shaker (as above).
4. The nitrocellulose was washed for 20 min shaking at room temperature in TBS/Tween (see below). After 20 min, the TBS/Tween was poured away and replaced with fresh TBS/Tween. This procedure was repeated twice.
5. The secondary antibody treatment (see below) was added and incubated shaking at room temperature for 2h.
6. The nitrocellulose was again washed for 3x20 min in TBS/Tween (as above). Following this a brief wash in distilled water and a brief wash in substrate buffer (see below) were carried out.
7. The nitrocellulose was developed by adding 20ml substrate buffer with the addition of 44µl NBT and 33µl BCIP (see below).
8. The reaction was stopped by transferring the nitrocellulose to distilled water.

Preparation of blocker solution:

20ml 1x TBS (tris buffered saline) with 3% BSA (bovine serum albumin).

BSA (0.6g) was dissolved in 20ml TBS.

Preparation of Tris Buffered Saline/TBS (10x):

50mM Tris (hydroxymethyl) aminoethane

200mM NaCl (sodium chloride)

1 litre of 10x TBS:

To 700ml distilled water, 60.55g Tris was added and the pH adjusted to 7.4 with 1M HCl. Following the addition of 116.88g NaCl the buffer was made up to 1 litre with distilled water.

Preparation of TBS/Tween:

To every litre of 1x TBS, 1ml of Tween 20 (Sigma) was added.

Preparation of primary antibody treatment:

Monoclonal antibody against cholera toxin B subunit raised in mouse (BioDesign International) was used at a final concentration of 1 in 5000 following optimisation. The antibody treatment consisted of 20ml blocker with the addition of 4µl of primary antibody.

Preparation of secondary antibody treatment:

Alkaline phosphatase-linked anti-mouse IgG raised in rabbit (DAKO, Denmark) was used at a final concentration of 1 in 1000. The antibody treatment consisted of 20ml blocker with the addition of 20µl of secondary antibody.

Preparation of substrate buffer:

0.75M Tris pH 9.5

30.4g Tris was added to 800ml distilled water and the pH adjusted to 9.5 with 1M HCl before making the volume up to 1 litre with distilled water.

Preparation of NBT:

NBT is nitro blue tetrazolium. This was dissolved in 70% dimethyl formamide (DMF) to a concentration of 75mg/ml.

To 100mg NBT (Sigma), 1.3ml of 70% DMF (BDH) was added.

Preparation of BCIP:

BCIP (5-bromo-4-chloro-3-indolylphosphate) is the substrate for alkaline phosphatase which is turned into a blue colour on development of Western blots.

To 50mg of BCIP (Sigma), 1ml of 100% DMF was added.

2.7.2 Screening of OMP Samples for Shiga-like Toxin

The procedures described in section 2.7.1 were repeated with a monoclonal antibody against shiga toxin and a shiga toxin control (Toxin Technologies, Florida, USA). The shiga toxin control was prepared in the same manner as the cholera toxin control and was loaded onto the gels at the same concentration.

The monoclonal antibody against shiga toxin was raised in mouse so the same secondary antibody was used with the same antibody concentrations and conditions as in section 2.7.1 b).

2.7.3 Screening of *Campylobacter* and *Arcobacter* for the Cytolethal Distending Toxin (CLDT) Genes

Degenerate primers (VAT1 and WM11) for the cytolethal distending toxin genes were constructed as given by Pickett *et al.* (1996) and were obtained from Life Technologies. VAT1 was based on the amino acid sequence VATWNLQG from *E. coli* CdtB. WM11 was based on the amino acid sequence WMILGDFN from *E. coli* CdtB. *C. jejuni* 11168 was used as a positive control for this experiment since the genome of *C. jejuni* had been fully sequenced and the CLDT genes are known to be present. The first step was to extract the DNA and check its purity before carrying out the polymerase chain reaction (PCR).

2.7.3.1 DNA Extraction

This procedure was a modification of Silhavy *et al.* (1984), Cold Spring Harbor Laboratory.

1. A loopful of organisms from BA were used to inoculate 10ml BHI broths in Universal bottles with *C. jejuni* and *Arcobacter*. *C. jejuni* were incubated at 42°C whereas *Arcobacter* were incubated at 30°C, both aerobically with caps screwed on tight.
2. After 48h these broths were subcultured using a 1% inoculum i.e. 0.1ml into 10ml. *C. jejuni* were grown in 10x10ml volumes in half ounce (approx.12ml) bottles with caps on tight, aerobically, at 37°C. *Arcobacter* spp. were grown shaking (100rpm) in 100ml volumes in 500ml conical flasks aerobically at 30°C.
3. After another 48h the cells were collected by centrifugation in 50ml volumes at 13000xg for 20 min.
4. The cell pellets were resuspended in 5ml of 50mM Tris HCl, pH 8.0, 50mM EDTA and then frozen at -20°C.
5. A fresh lysozyme (Sigma) solution 10mg/ml was made in 0.25M Tris HCl, pH 8.0. Half a millilitre of this was added to the frozen cells before

they were thawed with gentle mixing at room temperature. When just thawed the mixture was put on ice for 45 min.

6. 1ml of STEP solution (see below) was added and mixed well before heating at 50°C for 60 min in a gently shaking (80rpm) waterbath (Grant Instruments, Cambridge).
7. 6ml of phenol : chloroform : isoamyl alcohol (25:24:1, supplied as a mixture by Sigma chemicals) was added and gently mixed to emulsify.
8. This was centrifuged at 5000xg for 15 min to separate the layers. The top aqueous layer was transferred to a clean tube by careful pipetting before 0.1 volume (usually approx. 700µl) of 3M sodium acetate, pH 5.5 was added to the aqueous layer and gently mixed.
9. Two volumes (usually approx. 15.4ml) of 100% ethanol (-20°C) were added and gently mixed. The solution was left overnight at -20°C for maximum precipitation.
10. The tubes were centrifuged at 6000xg for 30 min and the pellets washed in 1ml 70% ethanol (-20°C).
11. After the second centrifugation at 8000xg in a MSE Microcentaur the supernatant was poured off and the pellets were left to dry in a fume hood for 30-60 min.
12. The final pellets were resuspended in TE buffer (see below) with 200µg/ml RNase A.

Preparation of STEP solution:

50mM Tris-HCl (Tris (hydroxymethyl) aminoethane hydrochloride), pH 7.5

0.4M EDTA

0.5% SDS

Proteinase K (1mg/ml)

To 150ml distilled water, 0.156g Tris-HCl was added and the pH adjusted to 7.5 with 1M NaOH. To this, 30.4g EDTA and 0.1g SDS were added before making the volume up to 200ml with distilled water. This solution was stored at room temperature until use. Immediately prior to use 1mg/ml Proteinase K was added. This was usually done by dissolving 5mg of Proteinase K powder (Sigma) into 5ml of STEP solution. Five to ten DNA extractions were carried out simultaneously.

Preparation of TE buffer:

50mM Tris-HCl

1mM EDTA

To 75ml distilled water, 0.78g of Tris-HCl was added and the pH was adjusted to 7.5 using 1M NaOH. To this, 0.38g of EDTA was added before making the volume up to 100ml with distilled water.

A 10mg/ml stock of RNase A (Sigma) was prepared and boiled in a boiling water bath for 10 minutes to denature any possible DNases present before being stored frozen until needed. Just before use 200µg/ml was added to TE buffer ie. 20µl was added to each 1ml sample.

2.7.3.2 Determination of DNA Concentration and Purity (Ausubel *et al.* 1990)

The absorbance of the samples at 260nm was measured to calculate the DNA concentration of the samples. Dual absorbance readings at 260nm and 280nm were required to determine the purity of the DNA.

Procedure:

In a silica cuvette, 10µl of each DNA sample was added to 990µl distilled water. The absorbances were measured at 260nm and 280nm on a DU70 Spectrophotometer (Beckman). Distilled water was used to zero the machine.

To calculate DNA content:

50 x Absorbance 260nm x Dilution Factor (100) = DNA content in µg/ml

For example, if the absorbance of a sample at 260nm was 0.062:

50 x 0.062 x 100 = 310µg/ml or 0.310µg/µl.

To calculate DNA purity:

$$\frac{\text{Absorbance 260nm}}{\text{Absorbance 280nm}} = \text{A value of 1.2 or below was preferable for pure DNA.}$$

2.7.3.3 Agarose Gel Electrophoresis

All DNA samples were electrophoresed on agarose gels to make sure they were suitably pure for PCR. The calculation in the previous section was a good indication of purity, but the quality of samples can be visually assessed by means

of gel electrophoresis. BioRad Mini-Sub Cell GT electrophoresis equipment was used.

Preparation of Agarose Gels:

1% agarose gels were used:

To 50ml of 1x TAE buffer, 12.5µl of a 10mg/ml solution of ethidium bromide solution was added. To this, 0.5g of molecular grade agarose (Promega) was added and heated on full power (2-3 min) in a microwave oven with regular mixing until completely dissolved. This mixture was allowed to cool to approximately 50°C before pouring. The gel holder was placed on the bench (not in the gel tank) with combs fixed in place and tape sealing the ends for the gel to be poured into (about 25ml into each gel holder). This took about 20 min to set, after which time distilled water was poured into the gel holder as the combs were removed to fill the wells with water.

Preparation of TAE buffer:

1x working solution:

0.04M Tris-acetate

0.001M EDTA

50x stock solution:

To 700ml distilled water, 242g Tris was added and dissolved. To this, 57.1ml of glacial acetic acid was added followed by the addition of 100ml of a 0.5M EDTA solution, pH 8.0. The buffer was finally made up to 1 litre with distilled water. Before use 1µl of a 10mg/ml solution of ethidium bromide was added to each 20ml of TAE buffer.

Procedure for gel electrophoresis:

The set gels in holders were placed into the gel tanks (BioRad Mini-Sub Cell GT). TAE buffer (1x) with ethidium bromide was added to the tanks until almost full (needed to cover surface of gels by about 0.5cm). A DNA sample (5µl) was loaded into each well. Before loading each sample was mixed with 2µl 5x loading buffer (Promega) and 3µl distilled water giving a final volume of 10µl. A negative control was loaded into one lane of each gel. This consisted of 8µl distilled water and 2µl loading buffer (no DNA). Once loading was finished, the lids were placed onto the gel tanks and they were plugged into a BioRad 300

Powerpac. The gels were electrophoresed at 50 Volts for 30 minutes then visualised using a Transilluminator (San Gabriel, model no. CA91778). Grab IT software (Synoptics, Cambridge, UK) was used to photograph any images.

2.7.3.4 Polymerase Chain Reaction (PCR)

Preparation of Primers:

The final amount of primers required in the PCR mix was 100pmoles. The PCR mix total was 50µl and 1µl of each primer was added. Therefore the final concentration of the primers needed to be 100µM (100pmoles/µl).

Primer VAT1 (Life Technologies) = 63 nmol

If 1ml was added = 63 nmol/ml

= 63 000 nM
= 63µM

Needed 100µM, so:

63/100 = 0.63ml or 630µl.

Therefore, to Primer VAT1 630µl TE buffer was added giving a final concentration of 100µM (thus 1µl = 100pmoles).

Primer WM11 (Life Technologies) = 51.1 nmol

If 1ml was added = 51.1 nmol/ml

= 51 100 nM

= 51.1µM

Needed 100µM, so:

51.1/100 = 0.511ml or 511µl.

Therefore, to Primer WM11 511µl of TE buffer was added giving a final concentration of 100µM (thus 1µl = 100pmoles).

Preparation of dNTPs (deoxynucleotide triphosphates):

dTTP, dCTP, dGTP and dATP (100mM each) were obtained from Sigma chemicals. From each of these, 10µl was pipetted into an eppendorf with 60µl distilled water. The stock dNTP mixture therefore contained 2.5mM of each dNTP.

Preparation of samples:

Approximately 0.5µg of DNA sample was put into each PCR mixture in a 5µl volume. The concentration of each DNA sample was calculated as in section

2.7.3.2. Using this, the samples were diluted so that the correct amount could be loaded.

Sample calculation of loading volume:

Sample DNA content = 0.212µg/µl. So in 5µl of this there was 1.06µg. This could then be diluted 1 in 2 to give a final concentration of 0.53µg in 50µl.

Preparation of PCR master mix:

For each sample to be tested the following mix was required:

1µl Primer 1 (VAT 1)

1µl Primer 2 (WM 11)

1µl dNTP stock

5µl DNA sample

5µl 10x PCR buffer

2.5µl Taq polymerase

34.5µl distilled water

When many samples were being tested at once a large 'master mix' was made. For example, if 7 samples were to be tested plus a negative and positive control, 9x the above mixture would be made (without samples and Taq) and aliquoted into 9x 42.5µl. The samples can then be added and the Taq polymerase added at a later stage (see below). The negative control was distilled water, the positive control was *C. jejuni* NCTC 11168.

PCR Amplification Cycles:

The PCR machine (Hybaid Thermocycler) was first loaded with samples for a 5 min incubation at 95°C, after which 2.5µl of Taq polymerase was added to each sample. Following this, the machine was set to perform 30 amplification cycles of 95°C for 5 min, 42°C for 2 min and 72°C for 3 min.

The PCR products were then loaded onto a 1% agarose gel as in section 2.7.3.3. A molecular weight marker (DNA 100bp ladder, Promega) was loaded into the first lane. This was to determine the molecular weight of the final PCR products. The gels were visualised using Grab-IT software (Synoptics, Cambridge, UK).

2.8 Effects of *C. jejuni* Toxins upon CHO Cell Morphology

This procedure was performed by Kevin Spears (TNTU) as a follow-up to the MTT cytotoxicity assay. Cultured CHO cells were exposed for 48h to extracts from *C. jejuni* culture grown in BHI broth as in the MTT assay (section 2.6.2, parts 1 and 2). The cells were then fixed and stained with Coomassie brilliant blue dye before inverted light microscope photography.

2.9 Purification of Enterotoxin (cholera-like toxin) from *C. jejuni* NCTC 11351 using Ion Exchange Chromatography

2.9.1 Preparation for Ion Exchange Chromatography

Ion exchange chromatography of a whole protein extraction from *C. jejuni* NCTC 11351 was the first step towards toxin purification.

2.9.1.1 Bacterial Cell Harvesting

C. jejuni NCTC 11351 was the only strain used for this experiment. The cells were harvested from both BHI and from BA. The BHI broths (300ml) were grown and harvested in exactly the same way as in section 2.6.1 for general cytotoxicity screening. The supernatants from the BHI cultures were used for ion exchange chromatography. BA plate cultures (5-10) were incubated microaerobically for 48 hours at 37°C, then the growth on each of these plates was harvested into 5ml PBS. Using this suspension 100-150 BA spread plates were prepared (100µl per spread plate). These were incubated microaerobically for 3 days at 37°C before their growth was harvested into 1ml PBS per plate and then pooled together. The sample was split into 10 x 15ml tubes for bath sonication (QH Kerry Ultrasonics Ltd.). This took place for 20 min on ice before samples were pooled into 3 x 50ml tubes and centrifuged at 13000xg for 30 min at 4°C. The pellet in each tube was resuspended in 5ml PBS, the three suspensions were pooled into 15ml and stored at -20°C. The cell-free extracts (10 x 15ml volumes) were pooled and used for the next step.

2.9.1.2 Whole Protein Extraction

Proteins were precipitated from the BA cell-free extract (as above, 150ml) and the BHI supernatant (300ml) using ammonium sulphate precipitation.

Procedure for ammonium sulphate precipitation:

1. To 150 ml BA cell-free extract, 84.41g of NH_4SO_4 was added.
   To 300ml BHI supernatant, 168.82g of NH_4SO_4 was added.
   This was done in large conical flasks placed in the bottom of ice buckets stirred with a magnetic flea. The ammonium sulphate was added slowly over a period of 30 min – 1h.
2. The flasks were left on magnetic stirrers overnight at 4°C.
3. The samples were split into 50ml volumes and centrifuged for 20 min at 13000xg, 4°C.
4. The pellets were resuspended in Buffer A without benzamidine (see below). The BA pellets were resuspended in 15ml Buffer A, the BHI pellets were resuspended in 30 ml Buffer A.
5. The samples were put into dialysis tubing (0.45µM pore size, 5cm width) and dialysed in 2 litres of Buffer A with benzamidine for 1 hour at 4°C. This buffer was then replaced with 4 litres of fresh Buffer A and left to dialyse for 3 days at 4°C.
6. The samples were transferred from dialysis tubing into centrifuge tubes and centrifuged at 5000xg for 10 min. The pellet was resuspended in 5ml PBS and stored frozen at -20°C in the event that it should be needed again. The supernatant was then ready for use. This was to remove any debris for the ion exchange column.

Preparation of Buffer A (with/without benzamidine):

50mM Tris-HCl, pH 7.8

1mM EDTA

1mM PMSF

(1mM benzamidine)

10mM 2-mercaptoethanol

2.9.2 Ion Exchange Chromatography (IEC)

The ion exchange column used was a BioRad Econo-Pac High Q Cartridge, 5ml. The system used for running the column was a BioRad BioLogic HR system. The low salt buffer used was Buffer A with benzamidine. The high salt buffer was Buffer A with benzamidine and 0.5M NaCl (initial experiments were carried out using 1M NaCl).

Washing the cartridge:

The cartridge was pre-washed to prepare it for use before each run of the column system. This was done by first washing the cartridge with low salt buffer for 2 min with the pump flow rate set to 2ml/min. The cartridge was next washed with high salt buffer for 10 min with the pump flow rate set to 6ml/min.

Program run on the BioLogic HR System:

1. The cartridge was equilibrated with an equal volume (5ml) of low salt buffer at pump flow rate 2ml/min.
2. The sample was loaded onto the column at flow rate 2ml/min.
3. The cartridge was washed with 20ml low salt buffer at 2ml/min to remove any unbound material from the sample.
4. A linear gradient was run from 0-0.5M NaCl for 100ml at 2ml/min (1h 30 min).
5. The cartridge was washed with 5ml high salt buffer at 2ml/min to wash off any remaining bound proteins.
6. The cartridge was washed with 2.5ml low salt buffer at 2ml/min to bring the gradient back down.

The cartridge was finally washed with 20ml low salt buffer at 2ml/min

Regeneration of the cartridge:

1. The cartridge was washed with 20ml high salt buffer at 6ml/min.
2. A final wash of the cartridge at 6ml/min with 20ml low salt buffer was carried out. The cartridge was then ready for another sample programme to begin.
3. If the column was stored for any period of time between uses, a further wash with 50ml 20% ethanol at 6ml/min was carried out. The cartridge was then stored in 20% ethanol until the next use.

The cartridge was regenerated after each run.

2.9.3 Analysis of Ion Exchange Samples

2.9.3.1 BioRad DC Protein Assay

Each sample was analysed for protein content using the BioRad DC protein assay kit according to the manufacturer's instructions.

2.9.3.2 Estimation of Protein Content Using a Spectrophotometer (Absorbance 280nm)

Each sample was analysed for protein content by measuring the absorbance 280nm against a buffer A blank using a DU70 spectrophotometer (Beckman).

2.9.3.3 Reactivity with Cholera Toxin Antibody

All samples were tested for reactivity with cholera toxin antibodies using a dotblot.

Procedure for DotBlot:

A Schleicher and Schull SRC 96D Minifold 1 Dot Blotting unit was used.

1. One sheet each of filter paper (Whatman No. 1) and nitrocellulose (Gelman Sciences, 0.45µm membrane pore size) was cut to the appropriate size to fit the unit and soaked in Tris Buffered Saline (section 2.7.1).
2. The filter paper was placed onto the unit, then the nitrocellulose placed on top of this. The unit was clamped together, then attached to a vacuum pump.
3. 250µl of TBS was pipetted into all of the holes on the unit. This was repeated to make sure that sample was being drawn through all the holes.
4. The samples were loaded into the holes (50µg of sample in total volume of 250µl).
5. The unit was washed through again with 250µl TBS, then left to dry under vacuum for approximately 30 min.
6. The membrane was then removed from the blotting unit and treated as in the Western blotting procedure, steps 2-8, section 2.7.1 b).

2.9.3.4 Pooling and MTT Assay of IEC Samples

The samples were pooled where high reactivity with the cholera toxin antibody and a high protein content was observed and the pools were dialysed overnight at

4°C in PBS. This was to remove NaCl and any other ingredients from the buffer which may interfere with the cytotoxicity assay. The cytotoxicity of each pool was analysed using an MTT assay. The procedure for this is described in 2.6.2.

2.9.4 Gel Elution of IEC Pools

1. The proteins within each pool were concentrated using a centrifuge filter unit (Millipore Ultrafree-15, Sigma) at 5000xg for 30 min.
2. The pools were then resuspended in sample buffer and applied onto SDS-PAGE in high concentrations. The Protean X11 20x20cm gel system (BioRad) was used for maximum sample loading and separation.
3. Molecular weight markers (prestained, 20µl) were loaded in lanes 2 and 8. Pool 1 was loaded into lanes 3 and 9, pool 2 was loaded into lanes 4 and 10 and pool 3 was loaded into lanes 5 and 11. The gel was run at 50mA for approximately 5h 30min.
4. When the gel was removed from the SDS-PAGE equipment it was cut in half using a scalpel so there were two smaller gels each containing a marker lane, pool 1, pool 2 and pool 3.
5. One half of the gel was immersed in Copper Stain (Copper staining and destaining kit, BioRad) and the other half was Western blotted (see section 2.7.1).
6. The band which was reactive on the Western blot was then identified on the copper stained gel. This band was excised from the gel (x3 for each pool) and destained (Copper staining and destaining kit, BioRad).
7. Each excised band was chopped up into tiny pieces before being applied to an electro-elution unit (Mini Protean II, BioRad). The elution unit was filled with CAPS buffer (see below). Each pool to be eluted was put into a separate corner tube of the elution unit. The lid was closed and the elution was run for 4h at 20mA using a BioRad 300 Powerpac.
8. On removal from the gel elution tubes, a small sample was taken from each pool for MTT testing. A protein assay (BioRad DC Assay) was again carried out to make sure that enough protein was eluted from the gel pieces.
9. These samples were again concentrated, resuspended in sample buffer and the SDS-PAGE procedure repeated as in steps 2-4 above.

10. Both halves of the gel were electroblotted (Trans-Blot Cell, BioRad) in CAPS buffer instead of electroblotting buffer. One half of the gel was blotted onto nitrocellulose and the other half was blotted onto Immobilon membrane (Immobilon-Psq PVDF transfer membrane, pore size 0.1μm, Sigma). Two sheets of filter paper were put either side of each membrane for the wet blotting procedure.
11. The electroblotting was carried out for 16h (overnight) at 20 volts using a BioRad 300 Powerpac.
12. The nitrocellulose was probed with monoclonal anti-cholera toxin B subunit (BioDesign International) and developed. The band which was reactive against the antibody was matched to a band on the Amido Black stained Immobilon sheet (see 13). This was the band which was chosen for sequencing.
13. The Immobilon was stained using Amido Black stain for approximately 30 min. This was then destained using Amido Black destain to remove the background stain. This was then ready for sequencing.

Preparation of CAPS buffer:

10mM CAPS (3-(cyclohexylamino)-1-propanesulphonic acid)

10% methanol, pH 11.0

To 700ml distilled water, 2.21g of CAPS (Sigma) was added. To this, 100ml of methanol was added and the pH was adjusted to 11.0. The volume was made up to 1 litre using distilled water.

Preparation of Amido Black Stain:

Amido Black (Sigma) 0.1% (w/v) in 45% Methanol, 10% acetic acid and 45% distilled water.

Preparation of Amido Black Destain:

90% methanol

10% distilled water

2.9.5 Sequencing of Cholera Toxin Reactive Band

The sequencing was carried out by Kevin Bailey at the University Hospital, Nottingham. The protein was sequenced by Edman degradation on an Applied Biosystems Model 473A automated protein sequencer. The sequence obtained was submitted into a BLAST search (Basic Local Alignment Search Tool) in the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/BLAST?) to identify genes in the *C. jejuni* genome with the closest sequence homology. Sequence alignment was then performed with the protein identified in the BLAST search and cholera toxin A and B subunits using GCG (Genetics Computer Group) software.

Chapter 3 – Results

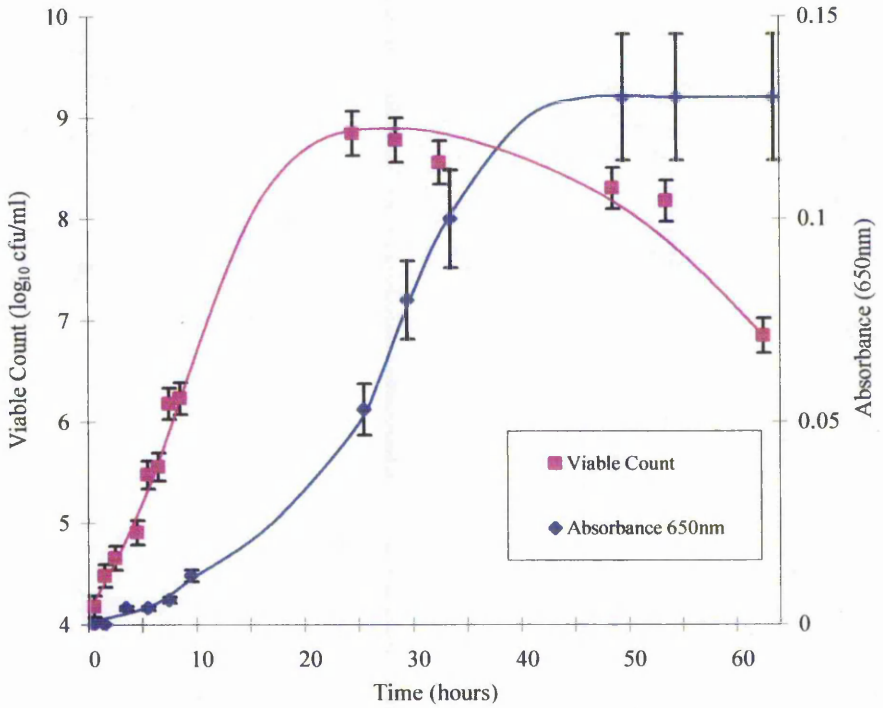
3.1 Growth Curve Results

Growth curves were performed to establish the patterns of growth of *C. jejuni* in liquid media. Many toxins are detected during stationary phase and in ageing cultures so it was important to establish at what time points the different phases of growth took place. This provided useful information towards the establishment of suitable culture conditions used for cytotoxicity testing.

3.1.1 *Campylobacter jejuni* Growth Curve

Figures 8 and 9 show the growth curves of *C. jejuni* NCTC 11351 and NCTC 11168, respectively. The growth medium used for both organisms was B-FBP.

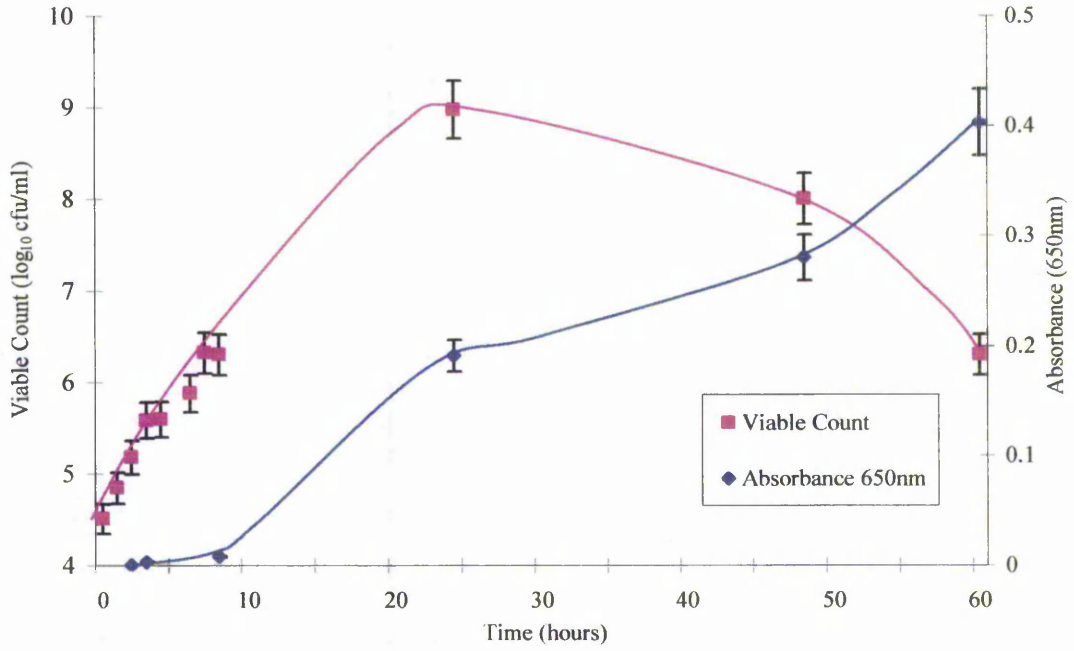
Fig. 8: Growth Curve of *C. jejuni* NCTC 11351



Error bars: indicate standard error in 5 sets of viable count and absorbance data.
Experiment was performed at 37°C.

In Fig. 8 the viable count of *C. jejuni* NCTC 11351 shows an exponential phase up to about 24h. From 28h onwards, the cell number began to decrease steadily until 60h, suggesting that the stationary phase occurred between approximately 20 and 30 h. The viable count then declined steadily over the next 48 h. The absorbance readings show a distinct lack of correlation with the cell numbers. The absorbance increased steadily with the cell numbers up to 24h, then continued to increase as the viable count declined. The absorbance readings were also very low when the viable count was at its highest thus indicating that absorbance is not a very reliable representation of the growth of *C. jejuni*.

Fig. 9: Growth Curve of *C. jejuni* NCTC 11168



Error bars: indicate standard error in 5 sets of viable count and absorbance data.
Experiment was performed at 37°C.

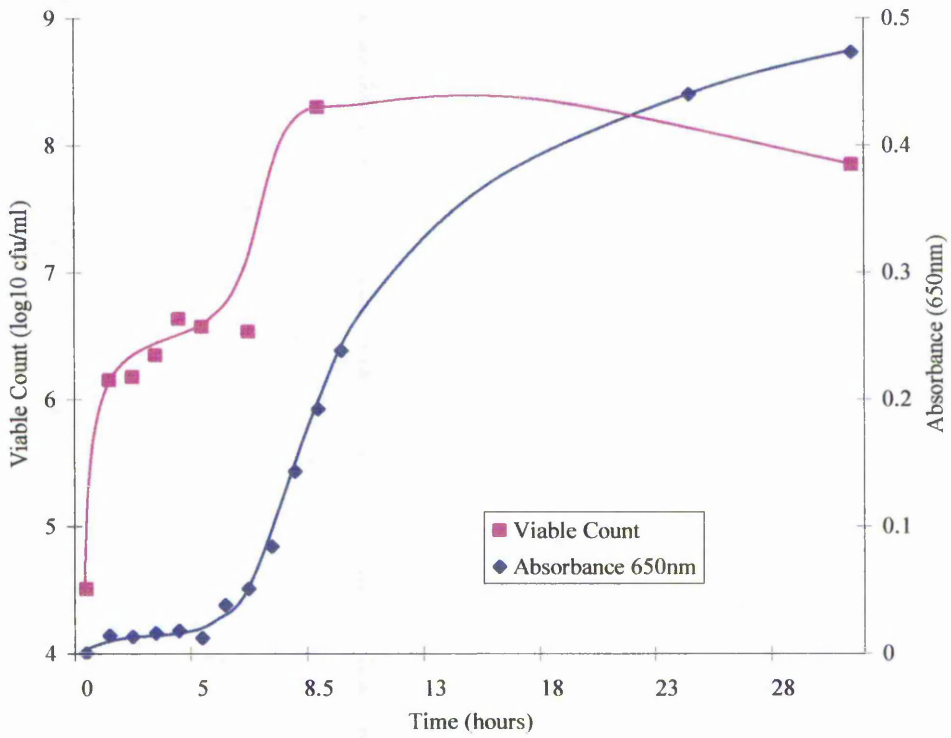
The growth curve of *C. jejuni* NCTC 11168 (Fig. 9) shows a very similar pattern to strain 11351 (Fig. 8). Little difference was found in maximum cell number between the two strains, but the absorbance readings were variable. Stationary phase again seemed to occur between approximately 20 and 30h in *C. jejuni* 11168 (Fig. 9), with a steeper decline from 48h onwards. Also in Fig. 9, as in Fig. 8, the absorbance values gradually increased during the rapid increase in

viable cell numbers (exponential phase), then continued to increase rapidly as the viable cell numbers declined.

There was also a significant difference between the absorbance readings in Figs. 8 and 9. Many of the readings during exponential phase on Fig. 8 were below 0.1 and the maximum reading was approximately 0.13. The absorbance range on Fig. 9 was much wider, but low absorbance readings were invariably obtained for both strains. The absorbance readings were thus an unreliable parameter for determination number of cells and growth phase. The doubling time of both *C. jejuni* NCTC 11351 and NCTC 11168 was 4h as determined by the calculation given in section 2.2. This calculation was from measurements taken between 0 and 8h.

3.1.2 *Arcobacter butzleri* Growth Curve

Fig. 10 Growth Curve of *A. butzleri* NCTC 12481



The growth curve data in Fig. 10 were supplied by Claire Hilton (The Nottingham Trent University) as a comparative study. *A. butzleri* NCTC 12481

was grown in BHI for this experiment. The growth curve of *A. butzleri* was similar to that of *C. jejuni* except it had a shorter doubling time of 2.6h (taken between 0 and 5h). This resulted in an earlier stationary phase that began at approximately 10 hours. The exponential phase was between 0-9 hours. The absorbance reading was found to be more reliable in *A. butzleri* growth curves than in *C. jejuni* for determining approximate cell numbers in mid-late exponential and early stationary phase (e.g. between 5 and 10 h). The absorbance readings were very reproducible at these time periods although they do continue to rise during stationary phase, as in *C. jejuni*. In *C. jejuni*, however, there was often variation between absorbance readings in different experiments at specific time points where the viable count was the same. Hence, absorbance readings were not reliable for determining approximate cell numbers and growth phase at any time point in the *C. jejuni* growth curve (Figs. 8 and 9).

3.2 Detection of Haemolysis in *C. jejuni* and *Arcobacter* spp.

Haemolysis is a convenient laboratory phenotype used to establish whether or not bacteria have cytolytic activity. Thus, simple tests can be performed to check for lysis of red blood cells which can supply valuable information about the potential toxicity and pathogenicity of the organisms. Some very potent bacterial toxins are haemolysins including the *Clostridium perfringens* α-toxin, a phospholipase (Songer 1997), and *E. coli* HlyA, a member of the RTX family of toxins (Welch *et al.* 1992). There are some reports of haemolytic activity in *Campylobacter* and *Helicobacter* and also a report of haemolysis mediated by *Arcobacter* spp (see section 1.11.2). The aim of the following section was to detect and characterise haemolytic activity in *C. jejuni* and *Arcobacter* spp.

3.2.1 Screening of BA Cultures for Zones of Haemolysis

Table 1 shows the results for the inoculated unwashed blood plates which were incubated under a range of conditions and screened for haemolytic zones. From the data shown, it is clear that no haemolysis occurred under aerobic conditions. Various amounts of haemolysis occurred with most *Arcobacter*

strains under microaerobic conditions. No haemolysis was detected with either of the three *C. jejuni* strains or *A. butzleri* NCTC 12481. All the haemolysis observed was identified as α-haemolysis (green zones on BA).

Table 1: Haemolytic Reaction of *Campylobacter* and *Arcobacter* with Unwashed and Washed Horse BA

	Incubation Conditions				
	30°C O_2[1]	30°C mO_2[2]	37°C O_2	37°C mO_2	37°C mO_2
C. jejuni	Unwashed	Unwashed	Unwashed	Unwashed	Washed
strain	BA	BA	BA	BA	BA
11351	NG[3]	0[4]	NG	0	2
11322	NG	0	NG	0	2
11168	NG	0	NG	0	2
Arcobacter	Unwashed	Unwashed	Unwashed	Unwashed	Washed
strain	BA	BA	BA	BA	BA
12481	0	0	0	0	2
CA 102	0	3	0	2	3
C3	0	3	0	2	3
1714	0	3	0	2	3
16799	0	3	0	3	3
15342	0	3	0	2	2
1A	0	3	0	2	2
2A	0	2	0	2	2
3A	0	2	0	3	3
5A	0	3	0	3	3

[1] O_2 = aerobic
[2] mO_2 = microaerobic
[3] NG = No Growth; haemolysis could only be measured where the bacteria had grown
[4] A score of 0-3 was used for haemolysis. This was based on three separate tests, where each positive result scored 1.

The results of haemolysis with washed blood agar can also be seen in Table 1. All of these plates were incubated microaerobically at 37°C for 48h. Differences were apparent by comparison with the assay using unwashed blood; firstly, *A. butzleri* NCTC 12481 showed almost consistent haemolysis with washed agar, whereas with unwashed agar, this strain was consistently non-

haemolytic. The rest of the *Arcobacter* strains were haemolytic most of the time, but there was still some inconsistency. There was also evidence of *C. jejuni* strains being haemolytic when grown on washed blood agar which had been non-haemolytic when grown on unwashed blood agar. These results with washed BA were, however, found to be more consistently positive than those with unwashed agar after repeating the experiment 3 times for both *Arcobacter* spp.and *C. jejuni.*

3.2.2. Microplate Haemolysis Assay

The microplate haemolysis assay was performed as a fast, convenient method of testing numerous samples for their haemolytic potential against two species of blood. A similar method was used by Tay *et al.* (1995) and Akan *et al.* (1998) and was originally described by Dominguez Rodriguez *et al.* (1986) to demonstrate the haemolytic activity of *Listeria* spp. Tables 2 and 3 show the results obtained from the microplate haemolysis assay method. The assay results were assessed visually, and were to be scored according to the lowest dilution which caused haemolysis for each strain. However, tests were scored positive where all seven of the dilutions in the microtitre well had caused haemolysis of the blood cells; no results were obtained in which haemolysis only occurred down to a particular dilution number. Results were taken after 8h, and then after overnight incubation; however, no change was seen in any of the tests between these times, so the result given is representative of both incubation periods.

Table 2: *C. jejuni* Microplate Haemolytic Reaction

C. jejuni strain	Sheep[1] blood + BHP[2]	Sheep blood + BHS[3]	Sheep blood + BA[4]	Horse blood + BHP	Horse blood + BHS	Horse blood + BA
11351	2[5]	0	3	2	0	3
11322	2	0	3	2	0	3
11168	2	0	3	2	0	3

[1] All blood used was washed
[2] Cell pellets harvested from BHI cultures
[3] Cell-free supernatant from BHI cultures
[4]Cells harvested from Blood Agar cultures
[5]A score of 0-3 was used for haemolysis. This was based on three separate tests, where each positive result scored 1.

These results were also rather variable and inconsistent. It can be concluded immediately that no haemolysis was detected using the BHI supernatants from each strain. Secondly, *Arcobacter* spp. were more haemolytic on horse blood than on sheep blood (Table 3), yet less haemolytic overall than *C. jejuni*. *C. jejuni* whole cells were almost consistently haemolytic upon horse and sheep blood (Table 2). Many of the *Arcobacter* BA-grown cultures were consistently non-haemolytic upon sheep blood. A high amount of variation was recorded for the other results on Table 3 (*Arcobacter* spp.).

Table 3: *Arcobacter* spp. Microplate Haemolytic Reaction

Arcobacter strain	Sheep[1] blood + BHP[2]	Sheep blood + BHS[3]	Sheep blood + BA[4]	Horse blood + BHP	Horse blood + BHS	Horse blood + BA
12481	2[5]	0	3	3	0	3
CA 102	1	0	0	1	0	2
C3	2	0	1	3	0	1
1714	1	0	0	2	0	2
15342	1	0	0	1	0	1
16799	0	0	0	2	0	2
1A	1	0	0	1	0	2
2A	1	0	1	1	0	1
3A	2	0	0	3	0	1
5A	1	0	0	2	0	2

[1]All blood used was washed
[2]Cell pellets harvested from BHI cultures
[3]Cell-free supernatant from BHI cultures
[4]Cells harvested from Blood Agar cultures
[5]A score of 0-3 was used for haemolysis. This was based on three separate tests, where each positive result scored 1.

3.2.3 Liquid Haemolysis Test

This test was successfully used by Hossain *et al.* (1993) to demonstrate the haemolytic activity of *C. jejuni*, and was reported by Rowe and Welch (1994) as a standard test for the demonstration of haemolytic activity. The liquid haemolysis test results were as variable as those in the previous experiments.

Some strains repeatedly showed a very haemolytic response in one test, then in the next test were totally non-haemolytic. For example, the first test for *A. butzleri* NCTC 12481 gave a haemolytic titre of over 80% and in the second test was less than 10%. Similar sets of results were produced for all strains of *C. jejuni* and *Arcobacter* spp. in both aerobic and microaerobic conditions. The results are not presented as this experiment was not continued due to the development of the contact haemolysis test below.

3.2.4 Contact Haemolysis Test

The contact haemolysis test was recommended by some authors who claimed that the bacteria and blood needed to be in close contact in order to observe the haemolytic activity of *C. jejuni* (Pickett *et al.* 1992, Istivan *et al.* 1998). This test was performed in an attempt to improve the detection of haemolytic activity, and was subsequently found to be the most successful test for consistent detection of haemolysis. Microaerobic conditions were also still required, as performing this test under aerobic conditions yielded variable, and often negative, results (data not shown). Fig. 11 shows the results of this assay for *C. jejuni* strains grown on BA and BHI with both sheep and horse blood. The data were still slightly variable with regard to the extent of haemolysis.

Fig. 11 Contact Haemolysis Assay of BA and BHI Grown *C. jejuni*

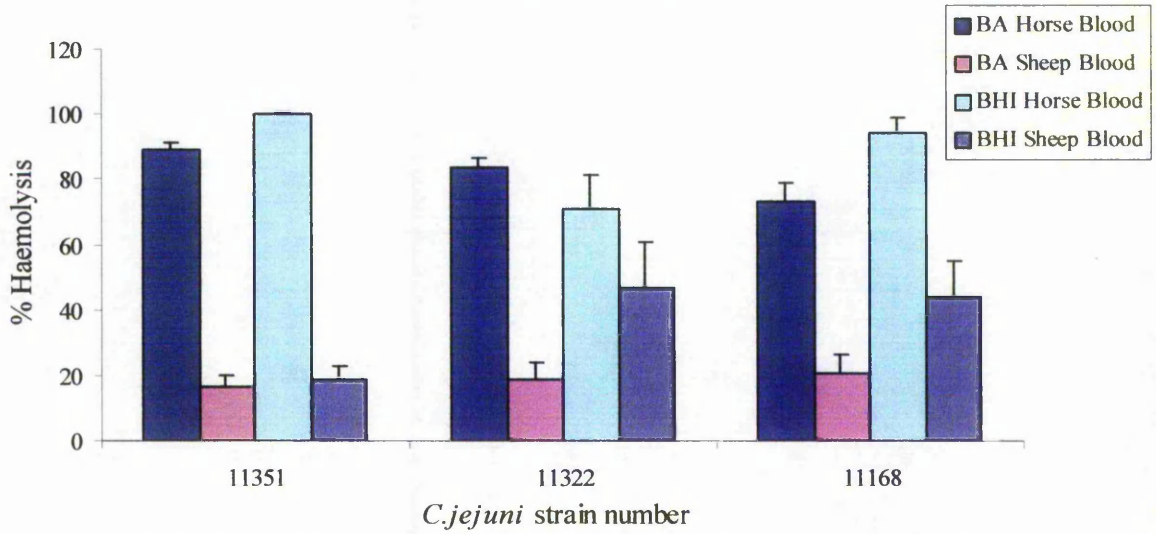


Fig. 11: Absorbance at 540nm was measured of the supernatants following centrifugation of test samples. Percentage haemolysis was calculated using negative controls of washed sheep and horse blood with the addition PBS only and a positive control of 1% Sarkosyl and washed sheep

or horse blood. The absorbance reading of the positive control was 100% haemolysis, and the percentage haemolysis of the samples was calculated using their absorbance readings against this. Legend indicates the media in which the bacteria were cultured (BA or BHI) and the type of blood used in each test (Horse/Sheep). Error bars indicate standard error of 5 sets of data.

Fig. 12 Contact Haemolysis Assay of *Arcobacter* spp. Grown on BA

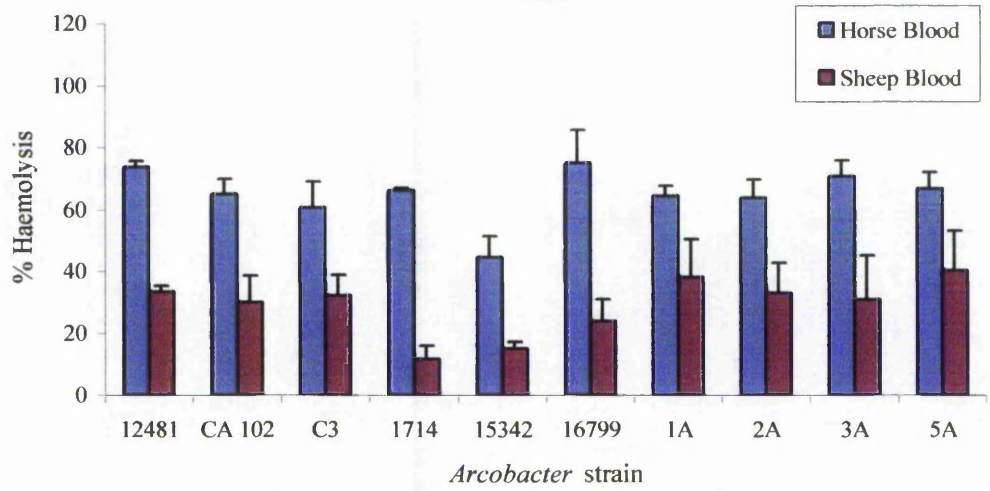


Fig. 12: Absorbance at 540nm was measured of the supernatants following centrifugation of test samples. Percentage haemolysis was calculated using controls of washed sheep and horse blood with the addition of PBS only. Error bars indicate standard error of 5 sets of data.

Fig. 13 Contact Haemolysis Assay of *Arcobacter* spp. Grown in BHI

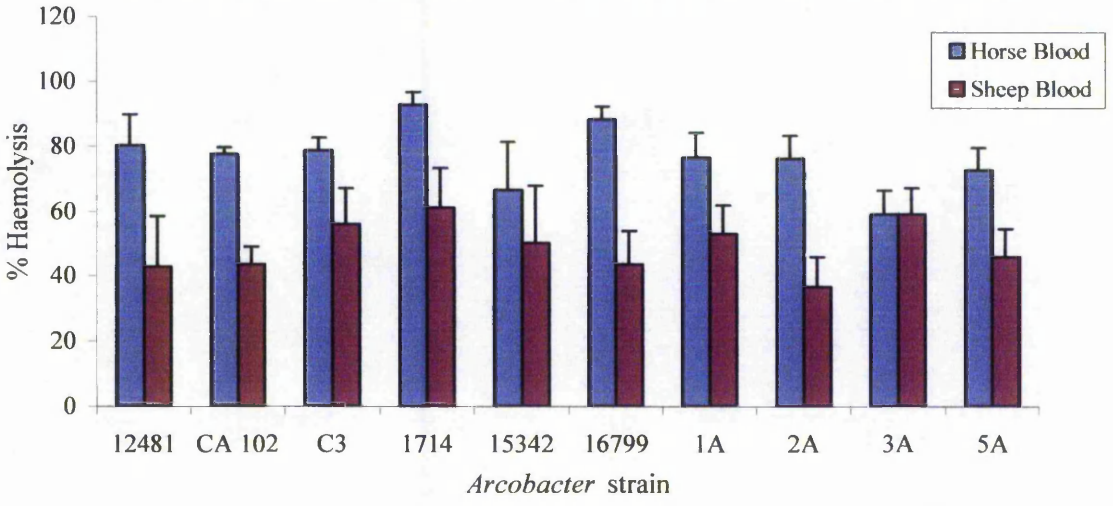


Fig. 13: Absorbance at 540nm was measured of the supernatants following centrifugation of test samples. Percentage haemolysis was calculated using controls of washed sheep and horse blood with the addition PBS only. Error bars indicate standard error of 5 sets of data.

The general trend seen in the results of this test, in both *Arcobacter* spp. (Figs. 12 and 13) and *C. jejuni* (Fig. 11), was that the haemolytic effect was stronger in horse blood than in sheep blood. In most of the results with horse blood the bacterial cells caused at least 50% lysis of the blood cells. In contrast, the percentage haemolysis of the sheep blood samples was much lower, often in the range of 20-40%. This difference was not quite so prominent in the *Arcobacter* spp. grown in BHI (Fig. 13), which showed a high percentage of haemolysis overall, ranging from 40-99%.

All strains grown on BA, except *Arcobacter* 1A and 3A, were significantly more haemolytic on horse blood than on sheep blood, as shown by the paired t-Test ($P<0.05$). Of the BHI-grown samples, all *C. jejuni* strains were significantly more haemolytic on horse blood than sheep blood, but only *A. butzleri* NCTC 12481, 1714 and 16799 were significantly more haemolytic upon horse blood in a paired t-Test. None of the other *Arcobacter* strains showed any significant difference between haemolysis in horse and sheep blood. Overall, a higher percentage of lysis on both blood species was seen with the *C. jejuni* samples than the *Arcobacter* samples.

3.2.5 Screening Haemolytic Strains for Phospholipase Activity

Phospholipase activity was of interest as many haemolysins of other bacteria are known to be phospholipases (see section 1.11.2.3). The best-characterised of these is the phospholipase C (alpha toxin) of *Clostridium perfringens*. It has also been established that there is a phospholipase A present in the outer membrane of *C. jejuni* NCTC 11168 (Penn 2001) and also in *Helicobacter pylori*, both of which are possible virulence factors (Snijder *et al.* 1999). Phospholipases A in *Campylobacter coli* and *Helicobacter pylori* have been demonstrated to contribute to haemolytic activity (Grant *et al.* 1997, Dorrell *et al.* 1999). A phospholipase C has also been reported in *H. pylori*, but its role in pathogenesis is unknown (Bode *et al.* 2001). These experiments were therefore carried out to investigate the presence of phospholipases (A and C) in *C. jejuni* and *Arcobacter* spp. and to establish if these enzymes were responsible for the haemolysis detected in previous experiments.

3.2.5.1 Phospholipase A Activity

Inhibitors of phospholipase A did not have any significant effects on the haemolytic properties of *A. butzleri* and *C. jejuni*. However, as the phospholipase A_2 enzyme control gave a very weak haemolytic response, it was difficult to measure the amount of inhibitor required to prevent haemolysis by phospholipase A. A change of buffer for resuspension of phospholipase A_2 to Tris-buffered saline, pH 8.0, instead of phosphate buffered saline, pH 7.4, did not activate the enzyme further. This was done because the optimum pH of the enzyme was indicated to be 8.0 on the product specification sheet. The highest concentrations of inhibitor that did not lyse the red blood cells in the absence of bacteria were used. Neither phospholipase A, dibucaine nor quinocrine had any significant effect on the haemolytic response of *Campylobacter* and *Arcobacter*, as determined by statistical analysis using the Mann-Whitney U-Test (data not shown).

3.2.5.2 Phospholipase C Activity

Inhibitors of phospholipase C also seemed to have little effect upon the haemolytic response of *C. jejuni* and *A. butzleri*. In this case, the exogenously added phospholipase C enzyme was extremely potent and only a small amount was required to lyse blood cells. The inhibitor 48/80 had little effect upon the pure enzyme (results not shown) but was used at the highest concentration possible without causing blood cell lysis in the absence of bacteria. Due to these problems with the control, the results were recorded as absorbance 540nm and not as a percentage of the control as per Figs. 11, 12 and 13.

Fig. 14 shows the results of the test with phospholipase C and its inhibitor. A Mann-Whitney U-Test ($P<0.05$) was performed to determine the significance of these results. The addition of 1μg of 48/80 significantly reduced the haemolytic activity of *C. jejuni* NCTC 11168 ($P=0.0304$) and *A. butzleri* Rigs 16799 ($P=0.0304$). The statistical analysis did not reveal significant differences in the data for the other strains due to the variation in the results as indicated by the standard error bars. However, it can be seen that there is a trend towards a reduction in haemolysis with the presence of the inhibitor 48/80 in all of the strains except *A. butzleri* NCTC 12481. This inhibitory effect is particularly

prominent, yet not statistically proven, in *C. jejuni* NCTC 11351 and *A. butzleri* 1714. It seems therefore that the phospholipase C inhibitor reduces the activity in all of the *C. jejuni* and *A. butzleri* strains except *A. butzleri* NCTC 12481, thus implicating the involvement of a phospholipase C enzyme in the haemolysis caused by *C. jejuni* and *A. butzleri.* A BLAST search was performed using known phospholipase C enzyme whole sequences in FASTA format to check for homology in the *C. jejuni* NCTC 11168 genome. No sequence homology was found between the *C. jejuni* NCTC 11168 genome and phospholipases C (alpha toxins) from *Clostridium perfringens*, *Clostridium bifermentans* and *Bacillus cereus*.

Fig.14 Haemolysis Test with Phospholipase C Inhibitor (48/80)

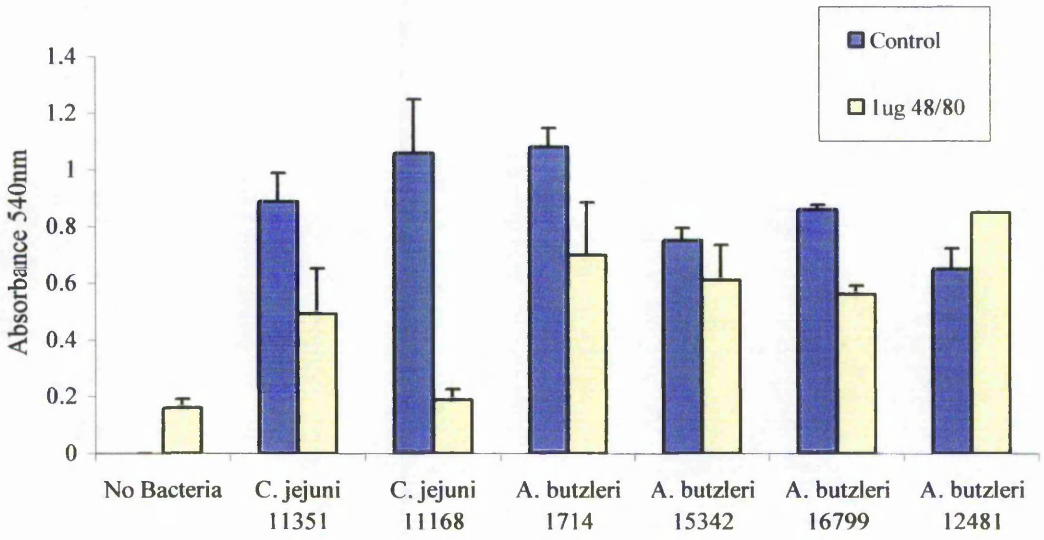


Fig. 14: Controls indicate contact haemolysis assay conditions with no inhibitor (ie. washed horse blood and bacteria resuspended in PBS). Bacteria were cultured on BA for this experiment. A 'no bacteria' sample was also tested to examine the effects of the inhibitor alone upon the horse blood cells washed in PBS. Absorbance at 540nm was measured of the supernatants following centrifugation of samples. Error bars indicate standard error of 5 sets of data.

3.3. SDS-PAGE Analysis of OMP Extracts

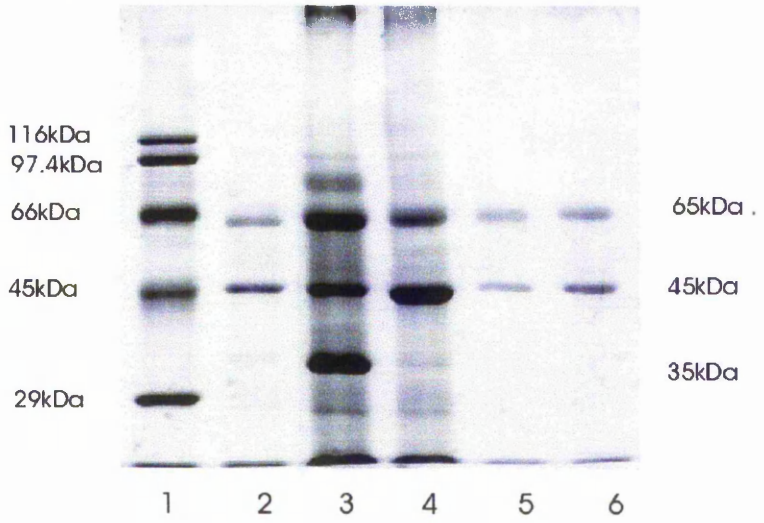
The aim of this section was to study physiological adaptations of *C. jejuni* and *Arcobacter* spp. grown under different conditions. The outer membrane proteins were extracted and analysed using SDS-PAGE. Bacterial toxins are often either cell-associated outer membrane proteins or proteins which are

transported to the outer membrane before being released from the cell. Culture conditions and the age of the culture also affect toxin production, thus changes in protein profiles under different conditions may be indicative of changes in toxin expression.

3.3.1 *Campylobacter* Results

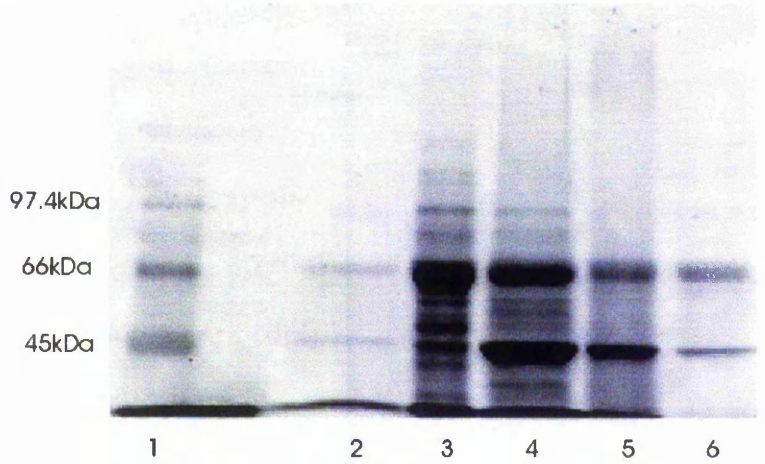
Figs. 15, 16 and 17 show the SDS-PAGE profiles of OMPs from *C. jejuni* NCTC 11351 and 11168 on minigel systems stained with Coomassie Blue. Figs. 15 and 17 contain OMPs extracted from BA grown *C. jejuni* NCTC 11351 and 11168 respectively. Fig. 16 contains OMPs from BHI broth grown *C. jejuni* NCTC 11351.

Fig. 15 OMP Profile of BA Grown *C. jejuni* NCTC 11351



OMP samples (10ug protein per lane) were separated on a 10% denaturing polyacrylamide minigel and stained with Coomassie blue.
From left to right:
Lane 1: Molecular weight marker 29-116kDa
Lane 2: 2 day old *C. jejuni*
Lane 3: 3 day old *C. jejuni*
Lane 4: 5 day old *C. jejuni*
Lane 5: 7 day old *C. jejuni*
Lane 6: 10 day old *C. jejuni*

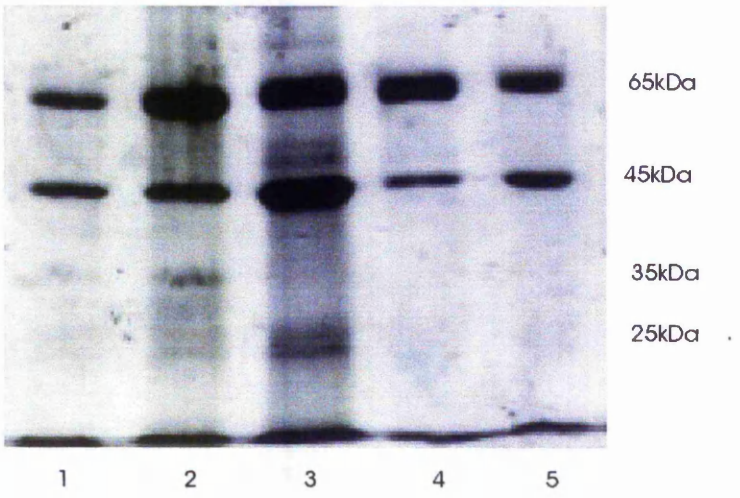
Fig. 16 OMP Profile of *C. jejuni* NCTC 11351 Grown in BHI Broth



OMP samples (10ug protein per lane) were separated on a 10% denaturing polyacrylamide minigel and stained with Coomassie blue.
From left to right:
Lane 1: Molecular weight markers 45-97.4kDa
Lane 2: 1 day old *C. jejuni*
Lane 3: 2 day old *C. jejuni*
Lane 4: 4 day old *C. jejuni*
Lane 5: 5 day old *C. jejuni*
Lane 6: 7 day old *C. jejuni*

Two major bands which can be seen on all the gels (Figs. 15-18) were at 65 kDa and 45 kDa. These were also the two main bands present on *C. jejuni* NCTC 11322 OMP samples (results not presented). Many samples did not show any other bands. Coomassie blue stain detects the major bands from the outer membrane protein extractions, but is not sensitive enough to detect all the bands. Additional bands can be seen on the 3-5d old BA grown samples on minigels of both strains (Figs. 15 and 17), and on the 2 and 4d old BHI grown samples on the minigel (Fig. 16). There was a particularly dominant additional band at 35 kDa on the 3d old BA grown *C. jejuni* NCTC 11351 sample (Fig. 15). Bands at 35 and 25 kDa were also visible on 3 and 5d old BA-grown *C. jejuni* NCTC 11168 samples respectively (Fig. 17). The OMP samples from BHI-cultured *C. jejuni* NCTC 11351 (Fig. 16) showed a greater diversity of bands in the range of 20-60 kDa than from those from cells cultured on BA (Fig. 15).

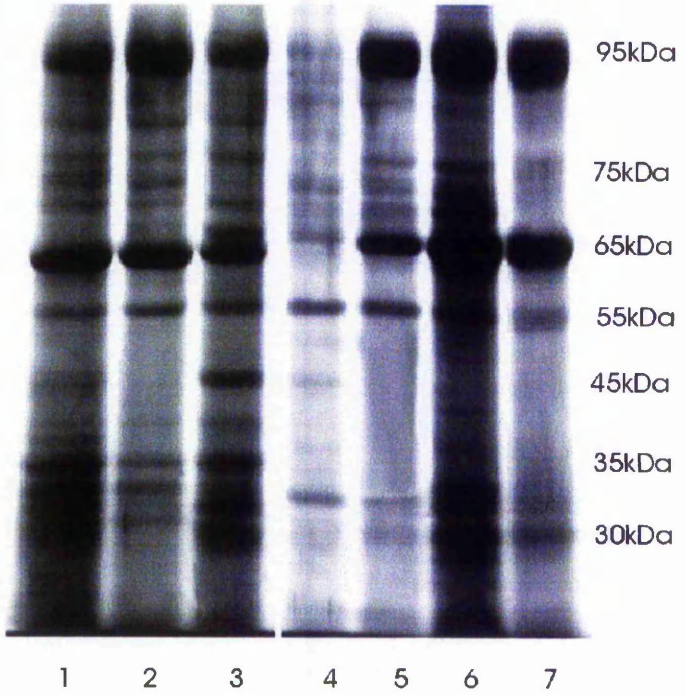
Fig. 17 OMP Profile of BA Grown *C. jejuni* NCTC 11168



OMP sample (10ug protein per lane) were separated on a 10% denaturing polyacrylamide minigel and stained with Coomassie blue
From left to right:
Lane 1: 2 day old *C. jejuni*
Lane 2: 3 day old *C. jejuni*
Lane 3: 5 day old *C. jejuni*
Lane 4: 7 day old *C. jejuni*
Lane 5: 10 day old *C. jejuni*

Silver stain is up to 200 times more sensitive than Coomassie blue stain (Hames and Rickwood 1990), subsequently, when minigels were stained with Silver there were too many poorly resolved bands. Silver stain was therefore used only for the 20x20cm gels, where greater separation of the bands was achieved. Fig. 18 is a Silver stained 20x20cm gel of *C. jejuni* NCTC 11351, *C. jejuni* NCTC 11322 and *C. jejuni* NCTC 11168 outer membrane proteins extracted from broth cultures. The two main bands on this gel were at 95 kDa and 65 kDa. Many other bands were present on this gel including a prominant 55 kDa band, but the 45 kDa band that was prominant on the Coomassie stained gels was not prominant on the Silver stained gels. The 45 kDa band can, however, be seen in lanes 1 and 3 (Fig. 18) which contain OMPs from *C. jejuni* NCTC 11351 cultured in B-FBP and BHI-YE respectively. It can also be seen in BHI-grown *C. jejuni* NCTC 11322 (Fig. 18, lane 4).

Fig. 18 Silver Stained OMP Samples from 3 day old Broth Cultures of *C. jejuni* NCTC 11351, NCTC 11322 and NCTC 11168

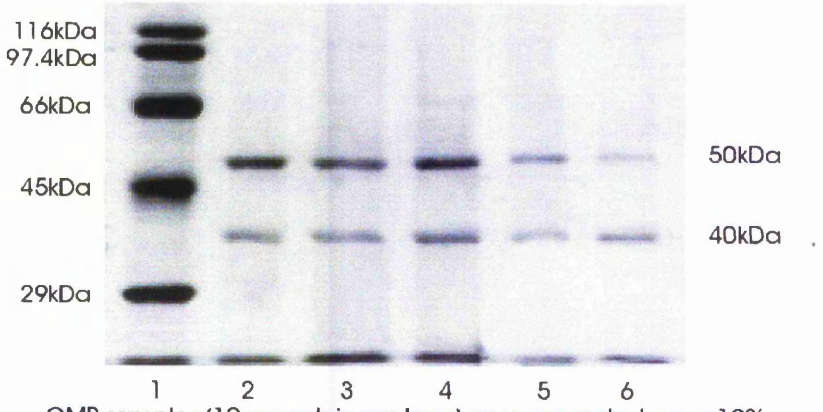


OMP samples (30ug protein per lane) from 3 day old broth cultures of *C. jejuni* were separated on a large 10% denaturing polyacrylamide gel and stained with silver.
From left to right:
Lane 1: *C. jejuni* NCTC 11351 cultured in B-FBP
Lane 2: " " " cultured in BHI
Lane 3: " " " cultured in BHI-YE
Lane 4: *C. jejuni* NCTC 11322 cultured in BHI
Lane 5: " " " cultured in BHI-YE
Lane 6: *C. jejuni* NCTC 11168 cultured in BHI
Lane 7: " " " cultured in BHI-YE

3.3.2 *Arcobacter* Results

Fig. 19 shows a Coomassie blue stained minigel of OMPs from *A. butzleri* NCTC 12481. Two major bands were visible at 40 and 50 kDa, which are similar in relative position to the 45 and 65 kDa bands on the *C. jejuni* minigels (Figs. 15-17).

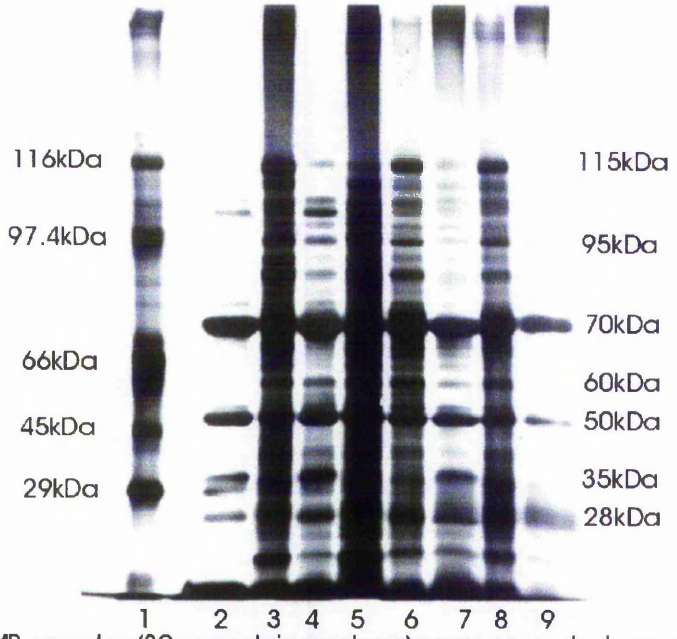
Fig. 19 Coomassie Stained OMP Extracts from *A. butzleri* NCTC 12481 Grown on BA in a Microaerobic Atmosphere



OMP samples (10ug protein per lane) were separated on a 10% denaturing polyacrylamide minigel and stained with Coomassie blue
From left to right:
Lane 1: Molecular weight marker 29-116kDa
Lane 2: 2 day old *A. butzleri*
Lane 3: 3 day old *A. butzleri*
Lane 4: 5 day old *A. butzleri*
Lane 5: 7 day old *A. butzleri*
Lane 6: 10 day old *A. butzleri*

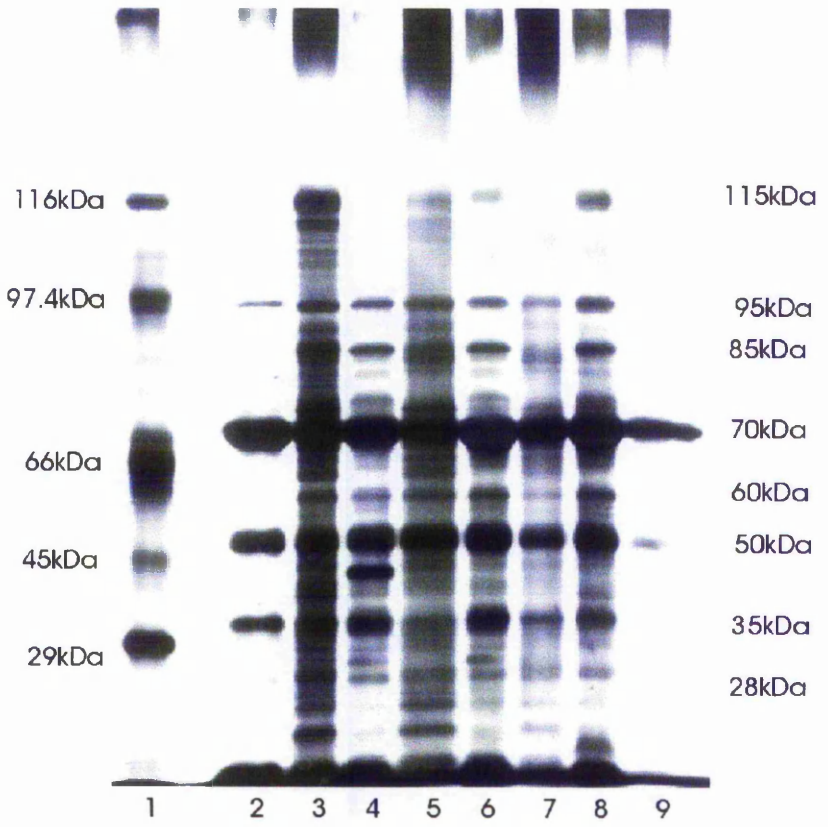
Fig. 20 shows a large silver stained gel of OMPs from *A. butzleri* NCTC 12481, grown for various periods under aerobic and microaerobic conditions. The major OMP bands expressed under all conditions were at 50, 70, 95 and 115 kDa. *A. butzleri* NCTC 12481 showed a higher diversity of OMP bands under aerobic conditions rather than microaerobic conditions in cultures aged 2d and 4d. This pattern was reversed in older cultures of 7d and 10d in which the microaerobic cultures showed a higher diversity of bands than the aerobic cultures. The same pattern was observed in *A. butzleri* CA 102, as shown in Fig. 21.

Fig. 20 Silver Stained Gel of *A. butzleri* NCTC 12481 OMP Extracts from Aerobic and Microaerobic BA Cultures



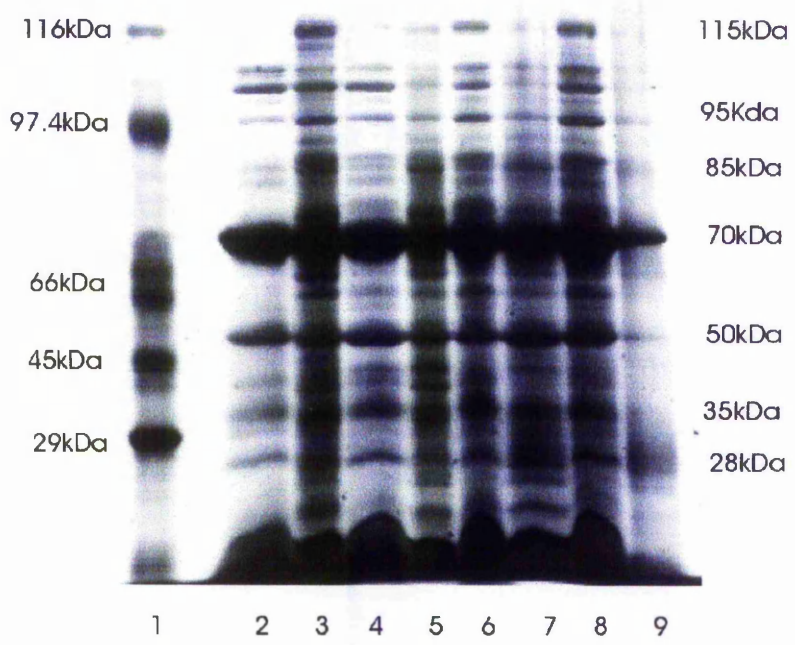
OMP samples (30ug protein per lane) were separated on a large 10% denaturing polyacrylamide gel and stained with silver.
From left to right:
Lane 1: Molecular weight markers 29-116kDa
Lane 2: 2 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 3: " " " " " " an aerobic atmosphere
Lane 4: 4 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 5: " " " " " " an aerobic atmosphere
Lane 6: 7 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 7: " " " " " " an aerobic atmosphere
Lane 8: 10 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 9: " " " " " " an aerobic atmosphere

Fig. 21 Silver Stained Gel of *A. butzleri* CA 102 OMP Extracts from Aerobic and Microaerobic BA Cultures



OMP samples (30ug protein per lane) were separated on a large 10% denaturing polyacrylamide gel and stained with silver.
From left to right:
Lane 1: Molecular weight markers 29-116kDa
Lane 2: 2 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 3: " " " " " " an aerobic atmosphere
Lane 4: 4 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 5: " " " " " " an aerobic atmosphere
Lane 6: 7 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 7: " " " " " " an aerobic atmosphere
Lane 8: 10 day old *A. butzleri* grown in a microaerobic atmosphere
Lane 9: " " " " " " an aerobic atmosphere

Fig. 22 Silver Stained Gel of *A. cryaerophilus* C3 OMP Extracts from Aerobic and Microaerobic BA Cultures



OMP samples (30 ug protein per lane) were separated on a large 10% denaturing polyacrylamide gel and stained with silver.
From left to right:
Lane 1: Molecular weight markers 29-116kDa
Lane 2: 2 day old *A. cryaerophilus* grown in a microaerobic atmosphere
Lane 3: " " " " " " an aerobic atmosphere
Lane 4: 4 day old *A. cryaerophilus* grown in a microaerobic atmosphere
Lane 5: " " " " " " an aerobic atmosphere
Lane 6: 7 day old *A. cryaerophilus* grown in a microaerobic atmosphere
Lane 7: " " " " " " an aerobic atmosphere
Lane 8: 10 day old *A. cryaerophilus* grown in a microaerobic atmosphere
Lane 9: " " " " " " an aerobic atmosphere

A. cryaerophilus C3 showed a similar pattern of results according to temperature, age and oxygen concentration as the two *A. butzleri* strains (Figs. 20 and 21).

Arcobacters and campylobacters appear to have a similar outer membrane protein profile with slight variations in the molecular weights. The *Campylobacter* major OMPs include proteins of 95, 65 and 45 kDa whereas

Arcobacter spp. have major OMPs at 95, 70, 50 and 40 kDa. Both the *Campylobacter* 45 kDa protein and the *Arcobacter* 40 kDa protein appear to be preferentially detected by the Coomassie blue, and not the Silver stain procedure.

3.4 MTT Cytotoxicity Assay

The MTT assay was used to screen for the presence of cytotoxin in extracts of *C. jejuni* and *Arcobacter* spp. Initial testing was performed to establish which strain was the most consistently toxic and also to establish which culture medium consistently yielded bacterial extracts with the highest toxicity. From this, a defined set of conditions was established to begin the purification of a toxin.

The MTT assay is a spectrophotometric assay which assesses the proliferation of cells through their metabolic activity. It is a dye reduction assay using methyl tetrazolium thiazolyl blue dye (MTT), which is reduced from a yellow colour to blue by the metabolic activity of live cells. A colour reaction is therefore obtained which can be measured spectrophotometrically. Lower absorbance readings compared with the controls indicate reduced mammalian cell growth or viability. A paired t-test was used to assess which samples are significantly toxic using 95% confidence limits.

3.4.1 Cytotoxicity of *C. jejuni* Extracts

Table 4 shows an overview of the *C. jejuni* samples extracted from various culture media that were tested for toxicity against different mammalian cell lines. Fig. 23 shows the cytotoxicity of three BA-grown extracts of *C. jejuni* NCTC 11351 towards N2a cells.

The N2a cell line was a mouse neuroblastoma cell line that had been established in the laboratory as a good general cytotoxicity model for various other toxicity studies. This was used for initial screening with bacterial extracts from cultures grown in BHI broth, BHI-YE broth and on BA. B-FBP broth was also used in initial experiments but this was abandoned due to the cytotoxicity of the broth control against N2a cells. The cytotoxicity of bacterial extracts from 3d old cultures at dilutions of 2.5%, 5% and 10% are presented in Table 4. As shown in Fig. 23, maximum cytotoxicity was produced in the bacterial extract

dilutions of 5% and above, and maximum cytotoxicity was detected in extracts from 2 and 3d old cultures. One day-old cultures exhibited negligible cytotoxicity.

Table 4: Cytotoxicity of Culture Extracts from *C. jejuni* NCTC 11351, NCTC 11168 and NCTC 11322

Strain & Media (3d growth)	Cell Lines and Dilutions of Bacterial Extracts Applied					
	N2a			ECV304		
C. jejuni 11351:	2.5%	5%	10%	2.5%	5%	10%
BA	38.1*[3]	20	18	91	83	/[4]
BHI S/N[1]	85*	70.3	69	95	96	98
BHI-YE S/N	98.6*	101.6	102.3	85.3	92.6	89.6
BHI C/S[2]	89.6	96.3	100	88.3	85.3	84
BHI-YE C/S	110	98.5	99	119	113.6	113.6
C. jejuni 11168:						
BA	85.6	78.3	89.6	NT[5]	NT	NT
BHI S/N	87	91.5	84.7	NT	NT	NT
BHI C/S	94	96	98	NT	NT	NT
C. jejuni 11322:						
BA	90.3	79.3	77.3	NT	NT	NT
BHI S/N	98.6	69.3	67.3	NT	NT	NT
BHI C/S	88.3	85.3	84	NT	NT	NT

Table 4: Results presented as mean percentage of control cell viability. 5-8 sets of data were used for each result. Bacterial extract dilutions of 2.5%, 5% and 10% only are presented with the cell lines N2a and ECV 304. Results of toxicity towards ECV 304 cells are only presented from extracts of *C. jejuni* NCTC 11351, as this was the strain chosen for more detailed studies.
[1]S/N = Bacterial cell-free supernatant
[2]C/S = Cell pellet sonicate
[3]* = Heat- and trypsin-sensitive
[4]/ = Insufficient data to present (less than 5 repeats of experiment)
[5]NT = Not Tested

From preliminary experiments, it was deduced that extracts from *C. jejuni* NCTC 11351 grown on BA and in BHI were cytotoxic towards N2a cells (Table 4). However, it was established at an early stage that cytotoxicity was not detectable in extracts from BHI-YE cultures. Therefore, extracts from BHI-YE were eliminated from the cytotoxicity screening using N2a cells during

preliminary studies. Hence, the only results presented from BHI-YE extracts were those of *C. jejuni* NCTC 11351 (Table 4).

Fig. 23 Cytotoxicity Assay of *C. jejuni* NCTC 11351 using N2a Cells

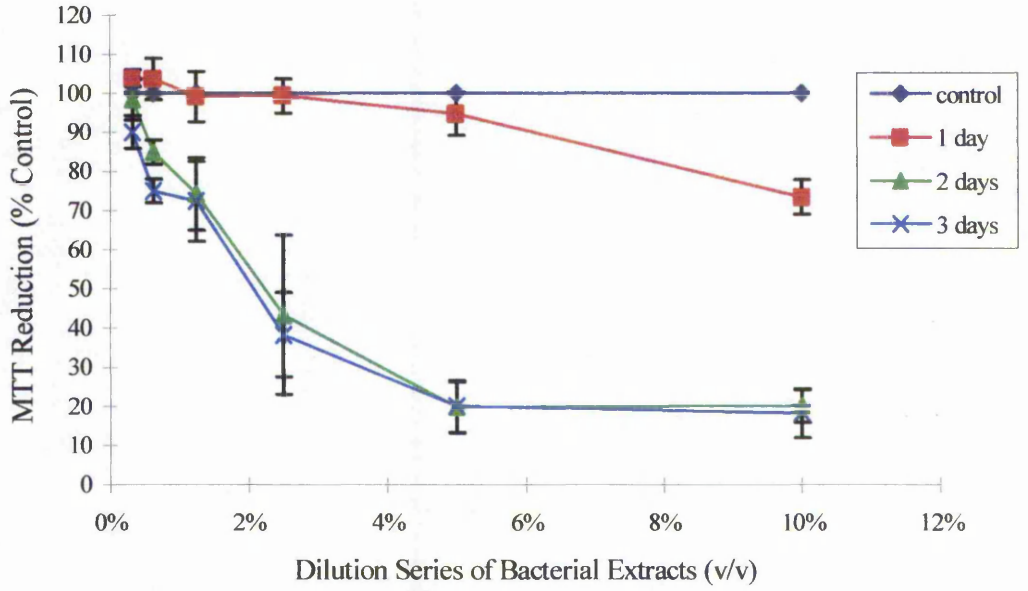


Fig. 23: Results are presented as mean percentage of control cell viability. The error bars represent standard deviations from 5-8 sets of data. The bacterial extracts are from BA cultures grown for 1d, 2d and 3d as indicated in the legend.

A comparison of the three different strains screened for cytotoxicity indicated that extracts from *C. jejuni* NCTC 11351 were showing higher levels of cytotoxicity than the other two strains, NCTC 11168 and 11322. This was particularly evident in extracts of *C. jejuni* NCTC 11351 from BA-grown cultures which resulted in only 20% remaining viability (of the control) of N2a cells at a 5% dilution of the bacterial extract (see Table 4 and Fig. 23). Extracts of *C. jejuni* NCTC 11351 from BHI-grown culture supernatant were also shown to be more cytotoxic than extracts from the sonicated cell pellet of BHI-grown cultures. *C. jejuni* NCTC 11351 was found to be the most cytotoxic strain in extracts from BA-grown cultures and BHI-grown culture supernatants. Therefore, extracts from this strain grown in these media were subsequently chosen for toxin purification experiments.

The latter stages of the cytotoxicity screening experiments were to test the *C. jejuni* NCTC 11351 extracts using alternative cell lines. The first of these

was a human endothelial cell line, ECV 304. Table 4 shows the cytotoxicity of extracts from *C. jejuni* NCTC 11351 cultures towards this cell line. It can be seen that the cytotoxic effects of the extracts are not as great towards this cell line as their effects towards the N2a cell line. The greatest effects were again seen in extracts from BA-grown cultures. In contrast with the results with the N2a cell line, the extracts from BHI-grown culture supernatants were less cytotoxic than extracts from cell pellets of BHI-grown cultures towards ECV 304 cells. Thus, N2a cells were used in preference to ECV 304 cells as they were more sensitive to the cytotoxicity of the *C. jejuni* extracts. The final cell line to be tested was the CHO (chinese hamster ovary) cell line which has been widely used in *C. jejuni* toxicity experiments (Wong *et al.* 1983, Klipstein and Engert 1984a, McCardell *et al.* 1984, Johnson and Lior 1988a, Florin and Antillon 1992, Bag *et al.* 1993, Lee *et al.* 1998 and 2000). Extracts from *C. jejuni* NCTC 11351 BA-grown cultures and BHI-grown culture supernatants were shown to be significantly cytotoxic towards the CHO cell line (data not presented). The cytotoxicity of these extracts was additionally found to be heat- and trypsin-sensitive, suggesting that the toxin was a protein.

3.4.2 *Arcobacter* Cytotoxicity Results

No significant cytotoxic activity was detected in extracts from any of the *Arcobacter* strains tested. All of the samples were from 3d old BA cultures and were tested on the N2a cell line.

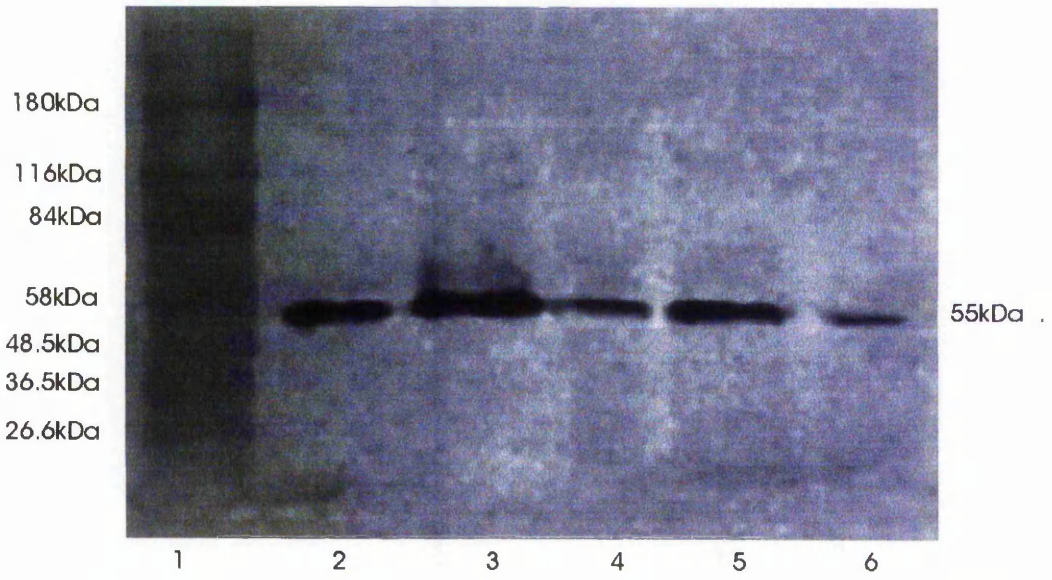
3.5 Screening for Specific Toxins

The following experiments were performed to screen *C. jejuni* and *Arcobacter* spp. strains for toxins that have previously been reported in *C. jejuni*. The OMP extraction and SDS-PAGE methods used for the analysis of protein profiles were used again here. Proteins were electroblotted and probed with anitbodies against Shiga toxin and Cholera toxin. DNA extractions were also performed to test for the presence of the CLDT genes using PCR.

3.5.1 Screening of OMP Samples for Cholera-like Toxin

As shown in Fig. 24, Western blots of all the *C. jejuni* NCTC 11351 samples revealed bands that cross-reacted with the cholera toxin B subunit monoclonal antibody. The reaction was strongest with extracts from the 2d old sample, with a weaker reaction evident from the 3d and 7d old samples. The reactive band was at approximately 55 kDa. OMP extracts from *Arcobacter butzleri* Rigs 1714, 16799, 15342 and NCTC 12481 did not demonstrate any cross-reactivity. Hence, no evidence of cholera-like toxin was found with these samples.

Fig. 24 Cross-reactivity of *C. jejuni* NCTC 11351 OMP Extract with Antibodies to Cholera Toxin B-subunit



OMP samples from BA-grown C. *jejuni* were electrophoresed (12.5ug protein per lane) and western blotted then probed with a cholera toxin B subunit monoclonal antibody. From left to right:
Lane 1: Prestained molecular weight marker 26.6-180kDa
Lane 2: Cholera toxin B subunit
Lane 3: 2 day old C. *jejuni* NCTC 11351
Lane 4: 3day old C. *jejuni* NCTC 11351
Lane 5: 4 day old C. *jejuni* NCTC 11351
Lane 6: 7 day old C. *jejuni* NCTC 11351

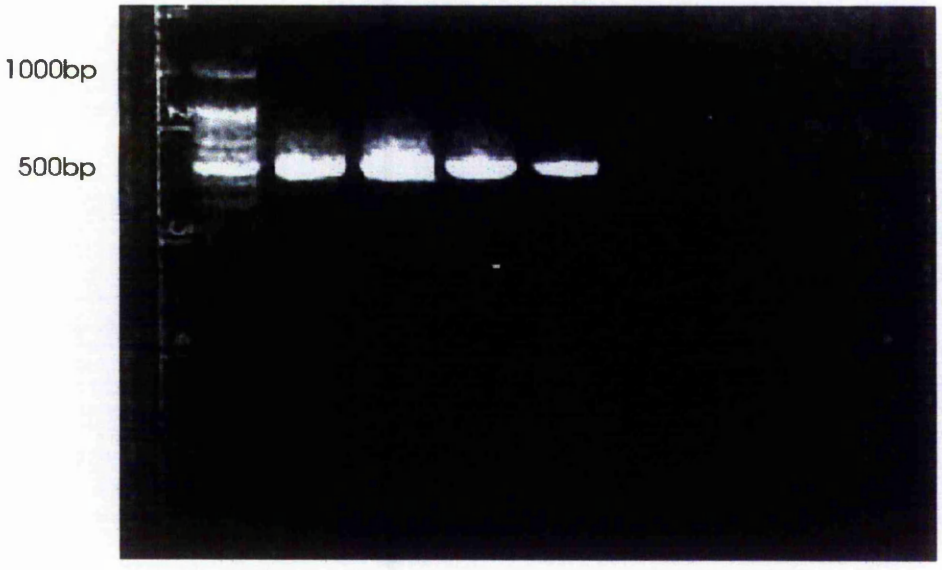
3.5.2 Screening of OMP Samples for Shiga-like Toxin

A picture of these results has not been included due to the lack of success of the experiment. The antibody was non-specific and on some occasions bound to all the proteins on the blot and on other occasions did not bind to anything. Variations were made in protein loadings applied to the blots. At the lower concentrations no binding occurred and at the higher concentrations the antibody bound to all the protein on the blot. A range of antibody concentrations was also tried with protein loadings fixed at 12.5µg. This was also unsuccessful. Another problem was the lack of reaction from the Shiga toxin positive control. Even with higher concentrations of antibody, no response was found with the toxin control. The same results were found with both *A. butzleri* and *C. jejuni* outer membrane protein extracts.

3.5.3 Screening of *Campylobacter* and *Arcobacter* DNA for the Cytolethal Distending Toxin (CLDT) Genes

Fig. 25 shows the PCR products of DNA samples from both *C. jejuni* NCTC 11351 and NCTC 11168. *C. jejuni* NCTC 11351 showed a positive result for the presence of cytolethal distending toxin genes. *C. jejuni* NCTC 11168 was run as a positive control in this experiment, but the presence of the CLDT genes in *C. jejuni* NCTC 11351 adds to the toxicity data for this strain. The bands all ran almost parallel to the 500bp marker, indicating that the molecular weight of the PCR products are approximately in this range. As the expected molecular weight of the CLDT genes is 494bp (Pickett *et al.* 1996), this confirms that the PCR products are within the expected range. DNA extracts from *A. butzleri* strains CA 102, C3, 1714, 15342, 16799 and NCTC 12481 were also screened with the same probes, but none of them showed any detectable presence of the CLDT genes.

Fig. 25 PCR Products of *C. jejuni* NCTC 11351 and NCTC 11168 DNA using CLDT Probes



PCR products of DNA extracted from *C. jejuni* probed with primers VAT1 and WM11 for the CLDT genes.
From left to right:
Lane 1: Molecular weight marker 100-1000bp
Lane 2: BHI broth grown *C. jejuni* NCTC 11168
Lane 3: B-FBP broth grown *C. jejuni* NCTC 11168
Lane 4: BHI broth grown *C. jejuni* NCTC 11351
Lane 5: B-FBP broth grown *C. jejuni* NCTC 11351
Lane 6: Negative control:- PCR mix and sterile distilled water

3.6 Effects of *C. jejuni* 11351 Toxins upon CHO Cell Morphology

The morphology of CHO cells following exposure to bacterial extracts can indicate what sort of toxin is present. Expression of CLDT can be assessed using this method. Cell-free supernatants from BHI broth-grown *C. jejuni* NCTC 11351 were previously demonstrated to be cytotoxic towards mammalian cells using the MTT assay. These cytotoxic supernatants were further studied for their morphological effects on CHO cells. This resulted in a reduction in cell number as well as distension of CHO cells. Fig. 26 shows Coomassie blue-stained CHO cells which have been treated with BHI broth alone for 48 hours (control). Fig. 27 shows Coomassie blue-stained CHO cells following exposure

to a cytotoxic level (determined by previous MTT assays) of culture extract (5% of total volume of N2a cell culture medium) for 48 hours.

Fig. 26 Control CHO Cells

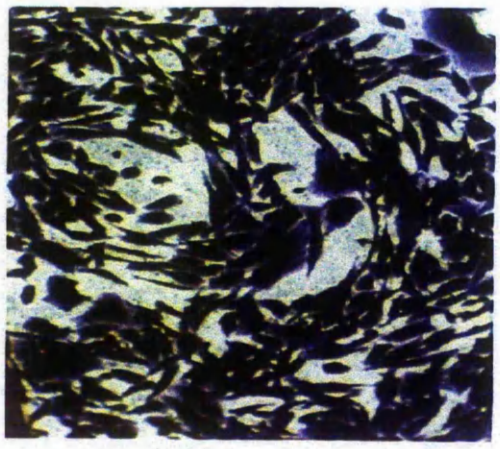
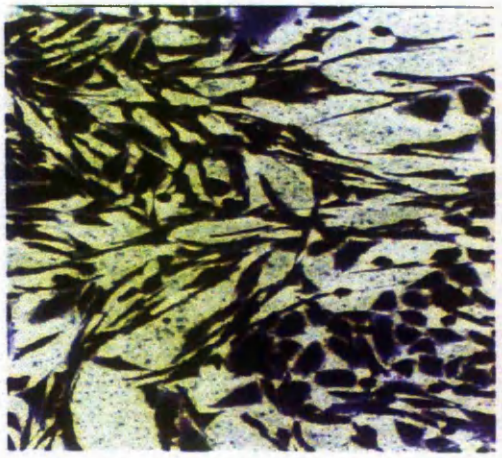


Fig. 27 Toxin-treated CHO Cells



3.7 Purification and Sequencing of Cholera-like Toxin

At this stage, it was established that *C. jejuni* NCTC 11351 contained a protein that was cytototoxic to mammalian cell lines and that OMP preparations contained a protein which was cross-reactive with cholera toxin antibodies. The hypothesis that a cholera-like toxin was present in the OMP of *C. jejuni* was therefore pursued, and the next step was to purify this toxin. It was taken into consideration that this toxin may share a mode of action as well as its mammalian cell binding site with cholera toxin or, alternatively, it may share mammalian cell binding site homology but have a very different mode of action. For this reason, both cross-reactivity with anti-cholera toxin antibody and the MTT assay were used to screen samples throughout purification. Following the toxicity data of previous experiments, it was decided to use BHI broth supernatants of *C. jejuni* NCTC 11351 cultures as well as BA-grown culture sonicates, from cells grown for 3 days microaerobically, for the purification of cholera-like toxin.

3.7.1 Ion Exchange Chromatography

Preliminary ion exchange experiments were performed with both BA extracts and BHI supernatants of *C. jejuni*. A 0-1M NaCl gradient was used initially to separate protein fractions, which was later optimised to 0-0.5M NaCl. Fig. 28 shows a preliminary purification experiment.

Fig. 28: Absorbance at 280nm of Ion Exchange Fractions from BHI Supernatant on a 0-1M NaCl Concentration Gradient

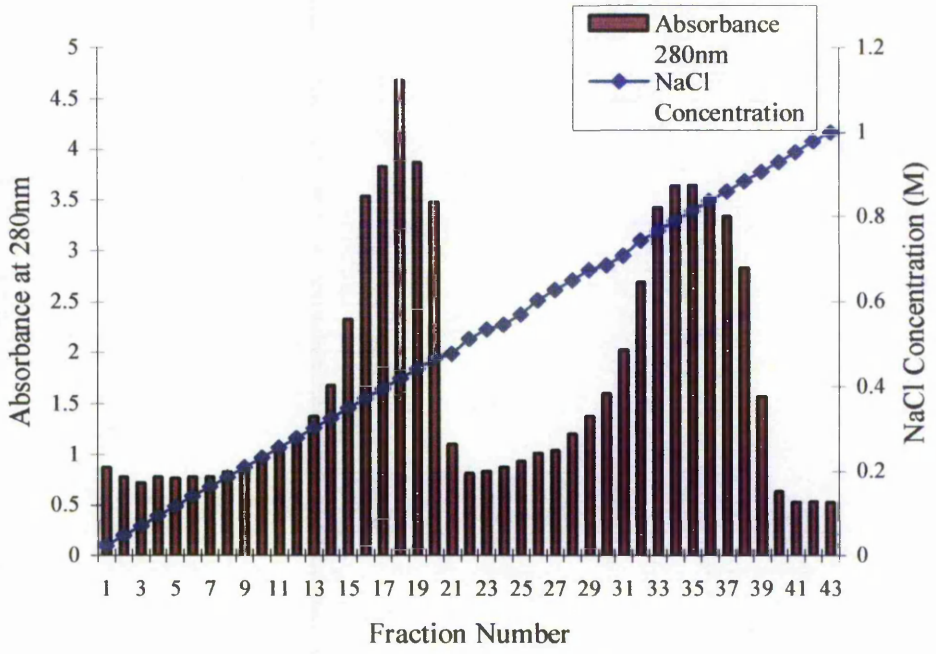


Fig. 28: Preliminary purification experiment: proteins were precipitated from an extract of *C. jejuni* NCTC 11351 cultured on BA, then applied to an ion exchange chromatography column. Forty-three fractions were obtained each of which were tested for cross-reactivity with anti-cholera toxin antibodies and absorbance at 280nm of each was measured.

The first large eluted peak on Fig. 28 yielded the samples that were reactive with the cholera toxin B subunit antibody. It can be seen from the salt gradient that all these fractions were within 0-0.5M NaCl, thus the salt gradient was adjusted to 0-0.5M in subsequent experiments. Preliminary purification experiments revealed that the BHI supernatant was not an ideal starting material due to its low density of proteins as compared to *C. jejuni* cells harvested from BA cultures. Thus, large volumes of protein extracts from cells grown on BA,

which yielded a high density were used with a 0-0.5M NaCl gradient ion exchange column to obtain the following results (Fig. 29).

Fig. 29 shows the results of a separation run on the BioRad BioLogic HR system. Protein was analysed by protein assay, absorbance at 280nm and reactivity to cholera toxin antibody. The reactivity to the cholera toxin antibody was the most important measurement as the intention was to purify a cholera-like toxin. A high protein content in the pooled samples was also essential for gel elution and sequencing purposes. Insufficient levels of protein for N-terminal sequencing was a problem which had been encountered during many early attempts. This was also the main reason for using BA-grown cultures which yielded a much higher cell density than broth supernatants. The fractions were pooled according to antibody reactivity as indicated in Fig. 29 overleaf.

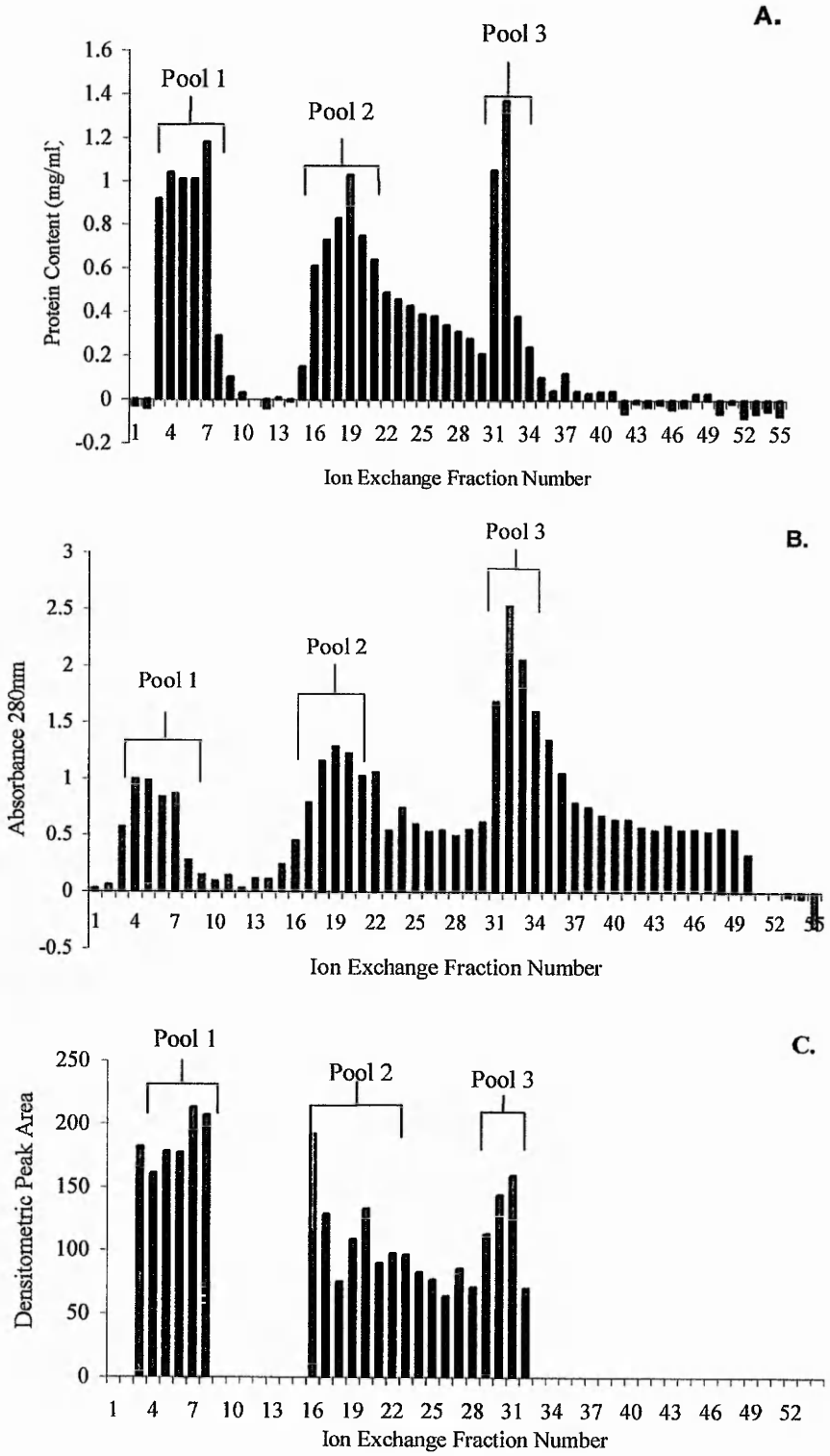
Pool 1: Fractions 3-8 (unbound peak)

Pool 2: Fractions 16-21 (peak 1, NaCl-eluted)

Pool 3: Fractions 30-33 (peak 2, NaCl-eluted)

Fig. 29 Analysis of Ion Exchange Fractions

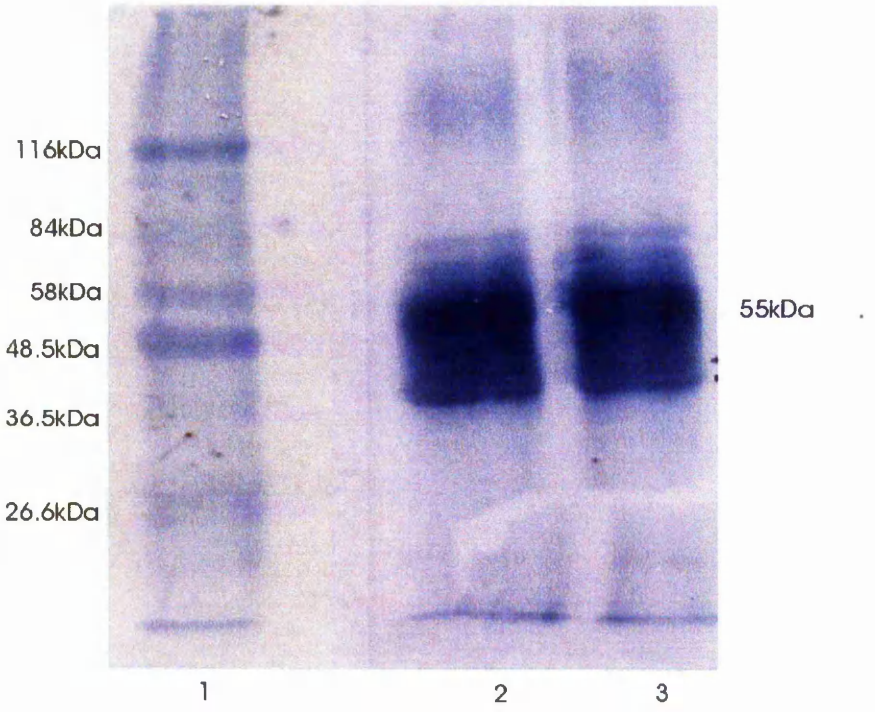
A. Protein content (mg/ml) of ion exchange fractions
B. Absorbance at 280nm of ion exchange fractions
C. Reactivity of ion exchange fractions with anti-cholera toxin antibody



3.7.2 Gel Elution Step

An MTT assay was used to assess cytotoxicity within the pools before further purification and analysis (Kevin Spears, TNTU). The pools were all still cytotoxic, and the correspondence of this with the persistent cross-reactivity with anti-cholera toxin supported the suggestion that a cholera-like toxin may be present that demonstrates serological homology to cholera toxin, but possibly with a different mode of action. This would additionally explain the lack of sequence homology between *Campylobacter* DNA (from multiple clinical isolates) and cholera toxin that has previously been reported (Calva *et al.* 1989, Konkel *et al.* 1992, section 1.10.3). The pooled samples were subsequently run in duplicate on a large (20x20cm) gel. Following Western blotting of one half of the duplicate gel, the 55 kDa band in pool 2 was the only band that continued to show cross-reactivity with the anti-cholera toxin B subunit antibody (results not shown). Its corresponding band at 55 kDa on the other half of the gel was excised and the proteins eluted from it. These proteins were subsequently applied to another gel which was electroblotted half onto Immobilon and half onto nitrocellulose (Western blot). The Amido Black-stained Immobilon membrane had a stained band at 55 kDa which was excised for N-terminal sequence analysis. This band corresponded with a 55kDa band reactive with cholera toxin on the Western blot. This was, however, only partially purified as there were still other bands present on the blot. Fig. 30 shows two lanes of protein pool 2 transferred onto Immobilon membrane and stained with Amido Black. The 55 kDa band was the band which was N-terminally sequenced.

Fig. 30 Protein Pool 2 Following IEC and Gel Elution Blotted onto Immobilon Membrane and Stained with Amido Black



Pool 2 from the ion exchange column was electroeluted from SDS-PAGE, blotted onto Immobilon membrane and stained with Amido Black..
From left to right:
Lane 1: Molecular weight markers 26.6-116kDa
Lanes 2&3: Pool 2 from the ion exchange column

3.7.3 N-Terminal Sequence Analysis

N-Terminal sequencing of the 55kDa band was performed by Mr. Kevin Bailey at the Protein Sequencing Unit, Queens Medical Centre, Nottingham. The protein was sequenced by Edman degradation on an automated protein sequencer. A mixed signal was obtained which yielded two different amino acids for nine cycles:

1. T+A
2. E+P
3. L+V
4. K+E
5. E+I
6. A+G
7. I+V
8. K+V
9. D+L

The amino acids from this mixed signal were put into BLAST searches in various combinations. The sequence which produced a positive 'hit' is indicated where the amino acid letter is underlined. The sequence was as follows:

T (threonine)

P (proline)

L (leucine)

E (glutamic acid)

E (glutamic acid)

A (alanine)

I (isoleucine)

K (lysine)

D (aspartic acid)

3.7.4 BLAST Search Sequence Homology

This N-terminal sequence showed significant homology with the MOMP of *Campylobacter jejuni*. The expected N-terminal sequence for residues 1-9 of MOMP is TPLEEAIKD, which matches exactly the N-terminal sequence shown above. The approximate molecular weight of this protein is 43 kDa (based on amino acid content) whereas the protein that has been N-terminally sequenced above was approximately 55 kDa on SDS-PAGE. Moutinho-Fragaso *et al.* (1996 and 1998) and Bacon *et al.* (1998 and 1999) reported a cytotoxic porin protein with sequence homology to the MOMP and the latter group managed to purify it. A comparison of the sequence matches of these groups, the results obtained in the current report and those of the *C. jejuni* MOMP (Bolla *et al.* 1995) are presented in Table 5 overleaf. The partially purified toxin from this work shows N-terminal sequence alignment with the other proteins but has a higher apparent molecular weight.

Zhang *et al.* (2000) aligned the amino acid sequences of MOMP from 25 strains of *C. jejuni* including NCTC 11168 and discovered that there were 7 variable regions dispersed among highly conserved sequences. The above sequence result, TPLEEAIKD, is amongst the highly conserved sequences reported.

Table 5: Sequence Comparisons of the Partially Purified Toxin from *C. jejuni* NCTC 11351with MOMP Purified by other Research Groups

Toxin	**Amino acid sequence**	**Molecular weight (Method)**	**Campylobacter Species & strain**
Partially purified cholera-like toxin (This study)	TPLEEAIKD	55 kDa (SDS-PAGE) (45 kDa: sequence analysis)	*C. jejuni* NCTC 11351
Toxin 1 (Mouthino-Fragaso *et al.* 1998)	TPLEEAIKDI(F)DL(V)N(S)GV[1]	45 kDa (SDS-PAGE and sequence analysis)	*C. coli* P26
Toxin 2 (Mouthino-Fragaso *et al.* 1998)	TPLEEAIKDIDVSGV	50 kDa (SDS-PAGE and sequence analysis)	*C. coli* P26
Porin toxin (Bacon *et al.* 1999)	TPLEEAIKDVDVSGVLRYRYDTGNFDRNFVN	45 kDa (SDS-PAGE and sequence analysis)	*C. jejuni* 2483
MOMP (Bolla *et al.* 1995)	TPLEEAIKDVDVSGVLRYRYDTGNFDKNF*N[2]	43 kDa (SDS-PAGE and sequence analysis)	*C. jejuni* 85H

[1]Brackets ( ) indicate that either one of two amino acids were present eg. I (F) indicates that one or the other was present.

[2]Asterix * indicates that an amino acid was missing.

3.7.5 Sequence Alignment of MOMP and Cholera Toxin Subunits A and B

Analysis of the sequences of the *C. jejuni* MOMP and the *V. cholerae* enterotoxin A and B subunits showed little sequence alignment. The alignment of MOMP with the cholera toxin A subunit (Fig. 31) showed 42% similarity and 33% identity at the N-terminus of the cholera toxin A subunit (amino acid numbers 1-70.). This does not appear to be significant due to the random occurrence of amino acid identical matches. There were many other 'similar' amino acids, but these are not representative of homology. There was no sequence of any length which is homologous between the two, however, it is

possible that this may be enough to indicate slight functional homology between the two proteins. There is currently no evidence to prove this.

Fig. 31 Sequence alignment of MOMP and Cholera toxin A subunit

```
MOMP:    3 LVKLSLVAALAAGAFSAANATPLEEAIK....DVDVSGVLRYRYDTGNFD 48
           :||:  |  :   .|| ||   |  |       ::  || |  |  .  ||
  CT:    1 MVKIIFVFFIFLSSFSYANDDKLYRADSRPPDEIKQSGGLMPRGQSEYFD 50

MOMP:   49 KNFVNNSNLNNSKQDHKYRAQVNF 72
             :    | ||     ||    |  |
  CT:   51 RGTQMNINL....YDHARGTQTGF 70
```

Match display thresholds for the alignments:

| = Identity

: = 2

. = 1

This is a similarity score of the amino acids to one another on the basis of their nucleic acid composition and chemical properties, with 2 indicating similarity and 1 indicating slight similarity.

The alignment of MOMP with cholera toxin B subunit (Fig. 32) showed 33% similarity and 33% identity. Again, there was no homologous sequence of significant length and the similarities were towards the C-terminus of the cholera toxin B subunit only (amino acid numbers 104-124). However, this slight homology at the C-terminus may be sufficient to explain cross-reactivity between *C. jejuni* MOMP and anti-cholera toxin B subunit antibodies.

Fig. 32 Sequence alignment of MOMP and Cholera toxin B subunit

```
MOMP:     1 ...MKLVKLSLVAALAAGAFSAANATPLEEAIKDVDVSGVLRYRYDTGNF 47
                ||   .     |  | | ||
 CT:    101 AKVEKLCVWNNKTPHAIAAISMAN..........................  124
```

Match display thresholds for the alignments:

| = Identity

: = 2

. = 1

Chapter 4 – Discussion

4.1 Growth Curves

The growth of *C. jejuni* was demonstrated to be fairly slow with a doubling time of 4 hours (Figs. 8 and 9). A similar growth pattern was obtained for *A. butzleri* (Fig. 10) but with a faster doubling time of 2hr 36 minutes. It was discovered that absorbance readings remained very low and varied during the *C. jejuni* growth curve, therefore viable counts were used as the more reliable method to determine cell number and growth phase.

An interesting pattern observed in the growth curves was the continued increase in absorbance readings when the cell numbers were in decline. This can be seen after 24h on the *C. jejuni* growth curves (Figs. 8 and 9), and after 8.5h on the *A. butzleri* growth curve (Fig. 10). This is likely to be due to morphological changes of the bacteria as they enter stationary phase. *C. jejuni* are rod-shaped cells which are known to change into a coccoid morphology when entering stationary phase (Lai-King *et al.* 1985, Moran and Upton 1987, Jones *et al.* 1991). A study by Thomas *et al.* (1999) showed that the organisms first began to elongate and swell in early stationary phase before budding into coccoid-shaped cells. *Arcobacter* morphology closely resembles that of *Campylobacter*, so it is likely that this morphological transition would be made by *A. butzleri* cells aswell.

Turbidity measurements are the most convenient and simplest way to determine biomass in bacterial cultures (Koch 1984, Nester 1978). The method is based on the fact that bacteria scatter light which is passed through the cell suspension. The more light scattered, the less is measured by the detector, corresponding to a higher optical density (OD). However, this technique is subject to certain errors and limitations (Koch 1984, Nester 1978). When bacterial cells are relatively uniform in size distribution (and consequently mass), the light-scattering is proportional to cell mass. Larger cells will scatter more light than small cells (Nester 1978), therefore, changes in the morphology of *A. butzleri* and *C. jejuni* cells during stationary phase results in an increase in the size (and consequently mass) of the bacterial cells, which would scatter more light resulting in a higher absorbance reading. Although the cell number

remained the same, the absorbance readings continued to rise as more and more of the bacteria underwent this morphological transition from smaller rod-shaped cells, to swollen and elongated, and finally larger coccoid cells, scattering a larger proportion of the light.

The growth curve data provided valuable information about the behaviour of *C. jejuni* and *A. butzleri in vitro*, and also indicated the time period after which the organisms began to age. This was important for later experiments to detect toxins, many of which are produced in ageing organisms. It was not considered necessary to carry out a full study of the growth curves of all strains used in the project. This experiment was used as guidance for later work, and similar behaviour was expected and often observed from the other strains of *C. jejuni* and *Arcobacter* spp.

4.2 Haemolytic Activity

This set of experiments has demonstrated that all *C. jejuni* and *Arcobacter* strains tested were capable of α-haemolytic activity, but certain conditions were often required for the expression of this activity. All cultures were grown for 48h so that they should be well into stationary phase and approaching death phase as previous investigators stated that haemolysis was only detected in ageing cultures. The first condition which was shown to induce haemolysis was a microaerobic atmosphere (Table 1). *C. jejuni* strains cannot grow aerobically and consistently failed to express haemolytic activity on conventional blood agar plates. *A. butzleri* NCTC 12481 did not express haemolytic activity on conventional BA either, but the rest of the *Arcobacter* strains did. It can be seen from the results in Table 1, however, that the haemolysis was inconsistent and was only detectable in microaerobic conditions. This suggests that the organisms are expressing different proteins in survival conditions (aerobic) and virulence conditions (microaerobic). Under aerobic conditions the organisms are surviving and growing in a sub-optimal environment. The fact that no haemolysis occurred under these conditions suggests that the haemolysin(s) is only expressed when the pathogen comes into contact with the host or conditions mimicking those of the host. Haemolysis on conventional BA was still not consistent in a

microaerobic atmosphere and no significant difference could be seen between 30°C and 37°C incubation (Table 1). It is possible that the animal blood used in the agar may contain inhibitory agents such as antibodies against the bacteria (Arimi *et al.* 1990). This was another factor which may possibly affect haemolysis and could explain the lack of consistency of the results with *Arcobacter* spp. and the total lack of haemolysis in the *C. jejuni* results. To eliminate this potential inhibitory factor, the experiment was repeated using washed BA. This did seem to yield more consistent results with the *Arcobacter* strains. A positive haemolytic reaction was also detected in some of the *C. jejuni* experiments using washed BA (Table 1).

The microplate assay again yielded highly variable results (Tables 2 and 3). It was concluded that this effect was probably due to the presence of oxygen during the test. Exposure to oxygen has already been demonstrated to have an inhibitory effect on haemolysis and this is the most plausible explanation for inconsistent results from both the microplate and the liquid tests. The liquid test also showed a high degree of variability in a microaerobic atmosphere (results not presented), suggesting that another factor was involved in the induction or inhibition of haemolysis caused by *C. jejuni* and *Arcobacter* spp. It could also be seen that the haemolysin was cell-associated, as only tests using broth pellets or agar cultures produced haemolytic reactions whilst spent medium had no effect (Tables 2 and 3).

This possibility was supported by the contact test, which finally gave consistent detection of haemolysis (Figs. 11, 12 and 13). Aerobic incubation of this test yielded many negative and variable results (data not shown), thus it was concluded that a microaerobic atmosphere was required as well as close contact between the bacterial cells and the red blood cells. Slightly variable levels of haemolysis were seen in each test for each strain and differences were observed in the level of haemolysis in the different species of blood (horse and sheep). This confirmed the work of Istivan *et al.* (1998), Pickett *et al.* (1992) and many other authors who found a different level of haemolysis with different blood species (Hossain *et al.* 1993, Akan *et al.* 1998, Istivan *et al.* 1998). In this investigation *C. jejuni* cultures grown on BA and in BHI were found to cause more haemolysis in horse blood than sheep blood (Fig. 11). *Arcobacter* spp.

cultures from BA were more haemolytic towards horse blood, but many BHI-grown cultures were also highly haemolytic towards sheep blood (Figs. 12 and 13). The use of media containing animal products such as BHI was recommended by Arimi *et al.* (1990) to induce a fully haemolytic effect. This may explain the enhanced haemolysis of sheep blood cells by BHI-grown *Arcobacter* spp. (Fig. 13), although no such effect was observed in BHI-grown *C. jejuni* (Fig. 11).

Phospholipase A activity was not found to be associated with haemolytic activity in *C. jejuni* or *Arcobacter* spp. (data not presented). The lack of haemolytic activity of the exogenously added phospholipase A_2 enzyme itself at high concentrations, and the lytic activity of the inhibitors upon blood cells at relatively low concentrations, made it impossible to assess the effective concentration of the inhibitor needed to block its specific enzyme. It is therefore arguable whether or not the phospholipase A inhibitors would have been efficient in counteracting any phospholipase A activity in *C. jejuni* and *Arcobacter* spp. The only conclusion that can be drawn is, at the concentrations used, the two phospholipase A_2 inhibitors used were unable to prevent haemolysis. It is known that there is a phospholipase A enzyme present in the *C. jejuni* outer membrane (Penn 2001). Additionally, it is postulated that this phospholipase is responsible for haemolytic activity due to studies on *C. coli* (Grant *et al.* 1997, van Vliet and Ketley 2001), and also due to sequence homology between this phospholipase A protein and others known to demonstrate haemolytic activity (Brok *et al.* 1998). Unfortunately, in this case it was not possible to conclude whether or not phospholipase A activity was present in *C. jejuni* and *Arcobacter* spp. under these conditions, or if it was involved in haemolysis.

The phospholipase C inhibitor, 48/80, successfully reduced the haemolytic effect caused by many strains of *C. jejuni* and *A. butzleri* (Fig. 14). The phospholipase C inhibitor was, however, not particularly effective against the activity of the phospholipase C enzyme of *Clostridium perfringens*. Slight reductions in the haemolytic activity of the exogenously added phospholipase C enzyme were seen after addition of the inhibitor, but it was negligible in many cases and did not effectively prevent haemolysis (data not shown). It must be considered, however, that the phospholipase C (or alpha toxin) of *Clostridia* is the most potent type of phospholipase C (Songer 1997). It is therefore very

unlikely that if *C. jejuni* and *Arcobacter* spp. were producing a phospholipase C enzyme that it would be as potent as that of *Clostridia.* Given that the inhibitor has a small effect on *C. perfringens* phospholipase C, it is feasible that the inhibitor would have a rather more significant effect on a less potent phospholipase C enzyme. The haemolytic activities of *C. jejuni* NCTC 11351 and 11168 and *A. butzleri* 1714, 15342 and 16799 were reduced following treatment with 48/80 (Fig. 14), but statistical analysis using a Mann-Whitney U-Test ($P<0.05$) only revealed significant reductions in haemolysis by *C. jejuni* NCTC 11168 and *A. butzleri* 16799. *A. butzleri* NCTC 12481 was the only strain tested in which haemolytic activity was not reduced following addition of the inhibitor 48/80 (Fig. 14). It can thus be concluded that a phospholipase C enzyme is likely to be either responsible for, or associated with, haemolytic activity in many strains of *C. jejuni* and *A. butzleri.* This does, however, need further clarification.

Hossain *et al.* (1993) tested for phospholipase C activity and its effect on the haemolytic activity of *C. jejuni* using an egg yolk lecithin test. They concluded that phospholipase C (lecithinase) was not the enzyme responsible for haemolysis in *C. jejuni.* The results of the current investigation using an alternative method are inconsistent with this finding and suggest that phospholipase C may be involved in the haemolytic activity of *C. jejuni* and *A. butzleri.*

In summary, this set of experiments demonstrated that an α-haemolysin(s) was produced by both *Arcobacter* spp. and *C. jejuni* which was cell-associated, oxygen-sensitive, produced in stationary/ageing cultures and was only detected when the bacteria were in close association with red blood cells. The presence of the haemolytic activity by exponential cultures was not tested in this study but has previously been investigated in *C. jejuni* (Arimi *et al.* 1990, Hossain *et al.* 1993, Misawa *et al.* 1995, Istivan *et al.* 1998), indicating that haemolysin production is an ageing response. In ageing cultures one of the main stresses for the organisms is the lack of nutrients. Production of haemolysins to gain extra nutrients under stress conditions supports the theory that haemolysins are produced by bacteria as a mechanism of iron acquisition (Rowe and Welch 1994). Uptake of iron from mammalian cells by this mechanism may therefore

only take place in ageing or stressed bacteria. Organisms entering the mammalian gut from food or water sources are, however, already likely to be in a stressed state. Iron acquisition via haemolysis may be an immediate effect following entry into the host and may also play a role in invasion.

4.3 SDS-PAGE Analysis of OMP Extracts

The OMP gels of *C. jejuni* indicate few major differences. Using a minigel system and a Coomassie blue stain the major OMP bands were observed at 45 and 65 kDa (Figs. 15-17). These are likely to be the *C. jejuni* MOMP and flagellin, respectively, based on approximate molecular weights. These are both immunodominant proteins which are present in abundance in the OM of *C. jejuni*. The MOMP is found over the entire cell surface (Amako *et al.* 1996, De *et al.* 2000) and representing up to 90% of the total OMP (Blaser *et al.* 1991).

A higher diversity of OMPs was observed in the BHI broth grown samples (Fig. 16) than on the BA grown samples from *C. jejuni* NCTC 11351 (Fig. 15). This may be due to the BHI being a more complex medium containing animal products and complex peptides. This could be optimal for the maximal expression of many campylobacter virulence proteins, as was discovered by Arimi *et al.* (1990) for the production of haemolysins. The optimum environment for campylobacters is in the intestines of mammals and birds, where they are thought to produce many pathogenic factors that have adverse effects on the host. The abundance of proteins in the BHI grown cell extracts may hence represent the production of these pathogenic factors. It must also be noted that this was observed in the 2d sample in particular, and also in the 4d sample from cells grown in BHI (Fig. 16). This may have been due to the ageing of the bacteria as they are thought to produce certain proteins and toxins in the stationary phase and in ageing cultures. Maximal enterotoxin production (Klipstein and Engert 1984b, Daikoku *et al.* 1989) has been reported in the stationary phase. Haemolysins are also cell-associated proteins which are expressed maximally in ageing cultures (Hossain *et al.* 1993, Arimi *et al.* 1990, Istivan *et al.* 1998, Misawa *et al.* 1995). It is therefore a possibility that the increased diversity of OMPs in ageing broth cultures is related to the virulence and pathogenic mechanisms of *C. jejuni*. There are also extra proteins in the BA-

grown samples aged 3d in *C. jejuni* NCTC 11351 and 4d in *C. jejuni* NCTC 11168 (Figs. 15 and 17 respectively). These bands may also be due to the release of certain proteins during ageing, for example due to blebbing. The proteins will initially be transported to the OM before being released which is why they are found in these OMP preparations. Blaser *et al.* (1983) and Vandamme and Goossens (1992) stated that the electrophoretic profile of outer membrane proteins from *Campylobacter* is stable and not affected by different culture conditions. However, differences are apparent in the samples in the current investigation. This may be due to methodological variations between laboratories, for example in the extraction procedure, culture conditions, strains used and their behaviour in culture media. It is also likely that the bacteria grown on BA age and become nutrient-stressed faster than those in broths due to the higher density of growth and lack of mobility on agar plates. Hence, the BA-grown cultures may be approaching death and no longer producing certain proteins, such as those required for substrate metabolism, resulting in fewer protein bands on SDS-PAGE.

The large gel photograph of all 3 *C. jejuni* strains (Fig. 18) is a silver stained gel of broth samples. Silver stain is up to 200 times more sensitive than Coomassie blue (Hames and Rickwood 1990) and the larger gel system increases band separation, so a much higher number of bands would be expected in this gel. The broths are all complex nutrient sources which are further supplemented in the case of B-FBP and BHI-YE for the enrichment of *C. jejuni* growth and a high yield of OMPs is therefore expected. As many OMPs serve as diffusion channels or receptors for specific metabolites (Dawes and Sutherland 1992), when cultured in a very complex medium, the bacteria would be expected to express more of these specific functional proteins. It has also been shown that certain virulence factors are induced in nutrient-rich media including some toxins (Ruiz-Palacios *et al.* 1983, Goossens *et al.* 1985a, Arimi *et al.* 1990).

The major difference between Fig. 18 (silver stained) and Figs. 15-17 (Coomassie blue stained), is that the Coomassie stained gels exhibit two major OMP bands at 45 and 65 kDa. The two major bands on the silver stained gel are, however, at 65 and 95 kDa. These may represent the flagella components, flagellin and the hook protein which are reported to be dominant proteins of 62 and 92.5 or 94 kDa respectively (Power *et al.* 1992, Guerry 1997). No

significant band at 45 kDa can be seen. The reason for this was probably due to the sensitivities of different proteins to silver stain. Some proteins stain either poorly or not at all and it has been suggested that the absence or low content of cysteine residues in proteins may contribute to poor silver staining (Hames and Rickwood 1990). The amino acid composition of the *C. jejuni* MOMP was studied by Schroeder and Moser (1997) and Zhang *et al.* (2000), and both groups reported a lack of cysteine residues, thus giving a possible explanation for its detection by the Coomassie blue stain, but not by silver stain. The exact mechanisms of how silver stain works and which proteins are sensitive or insensitive are unknown (Hames and Rickwood 1990). This gives an explanation for the results presented in this chapter which show a predominant band at 45 kDa with the Coomassie blue stain which is not distinguishable in most cases with the silver stain. It is apparent that the silver stain does not easily detect the 45 kDa band, which is presumably the MOMP, and may only be detecting the 45 kDa band when it is present in very high concentrations. The extraction procedure itself may cause variations in the amount of particular proteins which are isolated. The large silver stained gels exhibit a full range of OMP bands up to 95 kDa which are likely to include a diverse range of proteins including porins, enzymes, structural, adhesive and binding proteins.

To fully study and analyse the exact contents of each OMP sample it would be necessary to use further separation techniques such as 2D gel electrophoresis and subsequent protein characterisation. This would require a significant amount of further work and the time restraints and aims of this project meant that it was not feasible. A full analysis of the OMP content of *C. jejuni* would also require the application of different extraction techniques. The Sarkosyl method is only one of a number of techniques for this, each of which has its advantages and disadvantages and can include or exclude different groups of proteins.

The most interesting observation of the *Arcobacter* spp. gels, particularly of *A. butzleri*, is the differences between aerobically and microaerobically grown cells (Figs. 20-22). *A. butzleri* cultures of 2-4d are well beyond stationary phase (which starts at approximately 10h) so are likely to be ageing, stressed cultures. At this stage they express a wider diversity of OMP bands when cultured aerobically rather than microaerobically. In cultures which are older still (eg. 10

days) and likely to be significantly losing viability, a wider diversity of OMPs is expressed in microaerobic rather than aerobic cultures (Figs. 20-22). The reasons for this are unclear. A possibility is that the high numbers of OMPs are directly related to a stress response. A microaerobic environment, such as that found in certain sites of mammalian intestines, is likely to be the optimum for *Arcobacter* spp., as they appear to be adapted as pathogens to mammalian hosts (Kielbauch *et al.* 1991, Skirrow 1994, Wesley 1997, Mansfield and Forsythe 2000, Yan *et al.* 2000, Wesley *et al.* 2000). Aerobic cultures of 2-4d growth are probably producing stress proteins and employing survival mechanisms for this sub-optimal environment. After 7 and 10d, however, *Arcobacter* spp. cultured aerobically are probably rapidly losing viability and death and lysis will be occurring imminently. This would explain the decreased number of OMPs and the weak bands at 50 and 70 kDa which are the major bands in other samples. If this explanation is correct, the microaerobic cultures would be closer to their optimal conditions after 2-4d growth and probably surviving easily, hence expressing dominant, major bands at 50 and 70 kDa. After this, they would become stressed and employ survival mechanisms including various stress proteins, which explains the increase in OMP diversity in the older (7-10d) microaerobic cultures (Figs. 20-22). Additionally, some of these extra bands could be degradation products. This is only a potential hypothesis of the events that could cause these differences in OMP profiles and much more work is required to clarify any of the above scenarios.

Differences between silver stain and Coomassie blue stain were again evident in gels of *Arcobacter* OMPs and very similar to the situation in *C. jejuni*. The Coomassie stain of *Arcobacter* spp. OMPs (Fig. 19) shows the expression of two major bands at 40 and 50 kDa. The silver stain, however, detects two major bands at 50 and 70 kDa but not the 40 kDa band (Figs. 20-22). This re-iterates the different sensitivities of proteins to these two stains and suggests that *Arcobacter* spp. may contain a 40 kDa OMP similar to the 45 kDa band of *C. jejuni*, which is probably the MOMP.

4.4 MTT Cytotoxicity Assays

This assay demonstrated the presence of cytotoxic activity in extracts from all strains of *C. jejuni*. However, *C. jejuni* NCTC 11351 was the most cytotoxic of the three strains, particularly in extracts from cultures grown on BA and in BHI-grown culture supernatants (Table 4). This was subsequently the strain chosen for further toxin characterisation and purification due to its consistently high levels of cytotoxicity (Table 4 and Fig. 23) towards different mammalian cell lines (Table 4). The N2a cell line was the most sensitive to the cytotoxicity of extracts from *C. jejuni* NCTC 11351 (Table 4 and Fig. 23), but CHO cells were also found to be sensitive (data not presented). ECV 304 cells were not very sensitive to the cytotoxicity of these extracts (Table 4). The cytotoxicity of extracts from BA-grown cultures and BHI-grown culture supernatants of *C. jejuni* NCTC 11351 was also heat- and trypsin-sensitive, which suggested that it was mediated by a protein.

As toxicity towards mammalian cells was demonstrated in extracts from older bacterial cultures (after 2-3d), it may be due to the ageing processes of the bacteria; for example, it could be a toxic factor released due to cell lysis or blebbing of the cell contents. Methods for the detection of cytotonic toxicity (eg. RILT assay, elongation of CHO cells and rounding of Y-1 cells) were not applied so the presence of this type of toxin could not be elucidated from the results at this stage.

The extracts from *Arcobacter* spp. tested did not demonstrate any cytotoxic activity although some of the strains were isolated from humans with diarrhoeal illness (*A. butzleri* NCTC 12481, *A. butzleri* Rigs 1714, 15342 and 16799). It may be that this method of cytotoxicity screening and/or the particular cell lines used were not sensitive to toxins detected in *Arcobacter* spp. Additionally, as was suggested in section 1.11.4.1 on the Vero Positive Cytotoxin, it may be that the MTT assay was a less sensitive method for the detection of certain toxins than studying morphological changes to tissue culture cells. The MTT assay only detects changes in cell viability, therefore the presence of cytotonic toxins or toxins that cause progressive damage for a few days before lethality to the cell may not be detected by this method.

4.5 Screening of OMP Extracts for Cholera-like and Shiga-like Toxins

The strong cross-reactivity of a 55kDa band with the anti-cholera toxin B subunit antibody suggested the presence of a cholera-like toxin in *C. jejuni* NCTC 11351 extracts (Fig. 24). Whether the mode of action of this toxin would be similar to that of cholera toxin cannot be elucidated from these results. It does, however, show that there is an OMP present in *C. jejuni* which is serologically homologous to cholera toxin. As the binding site of cholera toxin in mammalian cells is gangloside GM_1 (Aspinall *et al.* 1993, Moran 1997, Allos 1998, Mills *et al.* 1998, Toyka 1999, Hughes *et al.* 1999) it may be that *C. jejuni* contains an OMP that binds GM_1. However, it has been established from studies of Guillain-Barré Syndrome that *C. jejuni* LPS is also capable of binding GM_1 (section 1.12.1). It is therefore possible that LPS present in the OMP fractions could cause this reaction. Another consideration is that a cytotonic toxin with a similar mode of action to the cholera toxin would not necessarily reduce cell viability as such, as was previously detected by the MTT assay. Therefore, the results presented so far lead to the possibility of two conclusions:

1. *C. jejuni* NCTC 11351 may be producing a range of toxins including both a cytotonic cholera-like toxin and cytotoxic factors.
2. *C. jejuni* NCTC 11351 produces an outer membrane protein which is serologically similar to cholera toxin and may be a cytotoxin with a different mode of action to that of cholera toxin.

The fact that *A. butzleri* and *A. cryaerophilus* OMP extracts did not cross-react with the anti-cholera toxin antibody suggests that any toxins or other virulence factors produced by *Arcobacter* spp. do not share any serological homology with cholera toxin.

The experiments on serological homology between *C. jejuni* and *Arcobacter* spp. OMPs and Shiga toxin were inconclusive. The lack of specificity of the antibody, coupled with the lack of reaction from the Shiga toxin control, invalidated any results obtained from this experiment.

4.6 CLDT Gene Screening

The CLDT genes were shown to be present in *C. jejuni* NCTC 11351, as well as the positive control *C. jejuni* NCTC 11168 (Fig. 25). This does not, however, provide any evidence of whether or not the CLDT is being expressed in either strain. If expressed, the CLDT may account for some of the toxicity observed in the MTT assay, however, an MTT assay is not specific for CLDT detection. CLDT causes extensive cell distension for the first 2-3 days with very little reduction in cell viability (Johnson and Lior 1988a). The MTT assay used in this investigation measures cell viability over a period of 48h and therefore may not detect the cytotoxic effects of a CLDT in this short space of time.

The CLDT genes were not detected in *Arcobacter* spp. CLDT genes are stable and present in a range of bacterial species. As degenerate primers were used which were constructed from *E. coli* genes, it seems very unlikely that *Arcobacter* spp. would contain a similar set of genes which were not detected by these primers. This, coupled with the lack of toxicity observed in MTT assays and the absence of cross-reactivity of OMP extracts with anti-CT antibodies suggests either low levels or absence of toxin production in the *Arcobacter* spp. tested. However, toxins may be produced which do not include CLDT and do not share serological similarities with cholera toxin. Other tests would be required to investigate this possibility.

4.7 Effects of *C. jejuni* Extracts on CHO Cell Morphology

The distending effects of the cell-free extracts from supernatants of *C. jejuni* NCTC 11351 cultures upon CHO cells (Fig. 26) suggests the presence of a CLDT-like toxin in the culture supernatant. Distension of CHO cells is, however, also induced by the *Campylobacter* cytotonic or cholera-like toxin (Johnson and Lior 1988a). The morphological effects of these two toxins upon CHO cells is indistinguishable after 24h (or 48h, as in this experiment), and an incubation period of 96h is required for the differentiation of the two (Johnson and Lior 1988a). After this period, CLDT causes extensive distension and death to CHO cells, whereas cytotonic toxin causes moderate, reversible distension and is not lethal to the cells. However, the fact that the presence of CLDT genes in

C. jejuni NCTC 11351 was demonstrated in the previous section (3.5.3) is consistent with the possibility that these morphological effects were caused, at least in part, by CLDT. The presence of a cholera toxin-like protein had also been previously demonstrated by the cross-reactivity of *C. jejuni* NCTC 11351 OMPs with cholera toxin B-subunit antibody (section 3.5.1). It may therefore be that both a CLDT and a cholera-like toxin are present in the *C. jejuni* NCTC 11351 samples. In order to determine whether one toxin may be having a more dominant effect than the other, the CHO cells would need to be treated for 96h before morphological observation to allow the different toxic effects to be distinguished. The reduction in cell number in this experiment and reduced cell viability in the MTT assay (section 3.4), however, do not necessarily correlate with the presence of a cytolethal distending toxin or a cholera-like cytotonic toxin. CLDT activity generally causes cell distension for only the first 24-48h of incubation, and cell death does not occur until after 2-4 days. The cholera-like toxin is cytotonic and does not cause cell death at any stage. The fact that a cytotoxic effect was obtained after 48h in the MTT assay raises the possibility that CLDT and cholera-like toxin may not be the only toxins present in the *C. jejuni* cultures or that they may be acting differently and more quickly in our cell culture conditions. It is likely that CLDT and/or cholera-like toxins are dominant in culture supernatants due to the morphological effects observed upon CHO cells. If a different cytotoxin were dominant, it is likely that a different morphological effect would be observed, for example, cell rounding caused by the MOMP cytotoxin (Moutinho-Fragaso *et al.* 1996 and 1998).

Thus far, it seems highly likely that both a cholera-like toxin and a CLDT are present in these cultures. The results of the MTT assay and the inability to distinguish the morphological effects between cytotonic and cytolethal distending toxins, suggest that there is possibly a third toxin present. This toxin could be causing the cytotoxic effects in the MTT assay, as the CLDT cytotoxicity may not be detected and a cholera-like toxin is unlikely to be detected in an MTT cell viability assay. Due to the serological evidence of a cholera-like toxin, the results again lead to two possible conclusions. Firstly, there may be a range of cytotoxic and cytotonic factors present in *C. jejuni* culture extracts which elicit a range of effects upon the CHO cells in culture, including both a cholera-like toxin and a CLDT. Secondly, there may be a

CLDT present which caused the morphological effects upon CHO cells but was not responsible for the decreased viability detected by the MTT assay. In addition, a cholera-like toxin may be present which has a similar binding domain in mammalian cells to the cholera toxin, but a different mode of action. Hence, it was cytotoxic in the MTT assay but did not cause the morphological effects observed upon CHO cells.

4.8 Toxin Purification and Sequencing

The sequence data obtained from the partially purified toxin of *C. jejuni* NCTC 11351 suggests a close match with the MOMP of *C. jejuni* (Table 5). The molecular weights of these two proteins, however, do not match. Additionally, the molecular weight does not match those of two previous attempts to purify the cholera-like toxin in *C. jejuni* which were 70 kDa from strain CH5 (McCardell *et al.* 1984) and 68 kDa from a clinical isolate (Daikoku *et al.* 1990). The results appear, therefore, somewhat ambiguous. If the molecular weight of the toxin partially purified from *C. jejuni* NCTC 11351 was in the range of that of the MOMP (40-45 kDa) then a plausible conclusion may be that a covalently bound carbohydrate moiety binds the cholera toxin antibody. Bacon *et al.* (1999) reported that the cytotoxic MOMP is a porin-LPS complex and the reactivity of LPS from *C. jejuni* with GM_1 gangliosides has been well-documented in investigations into the cause of Guillain-Barré Syndrome (see section 1.12.1). GM_1 is the binding site for cholera toxin, therefore it is feasible that LPS in *C. jejuni* that mimicks GM_1 could be responsible for this reaction with the cholera toxin antibody. This would have therefore given a false positive reaction of the MOMP cytotoxin with anti-cholera toxin antibodies, suggesting that this could be the cholera-like toxin that has been detected in previous reports. The fact that very little sequence alignment is present between cholera toxin B subunit and MOMP supports the possibility that the cholera antibody reaction is an artefact. This would be a definitive conclusion if it were not for the fact that the cholera-like toxin partially purified from *C. jejuni* NCTC 11351 was 55 kDa, and that purified by two previous research groups, was of molecular weight 68-70 kDa. These are rather significant differences in molecular weight from each other, and from the 40-45 kDa of MOMP. It is feasible that the protein sequenced in this

report is not the same protein that was previously reported to be cholera-like. Two questions therefore remain to be answered. Firstly, how a different size protein (55 kDa from *C. jejuni* 11351) to those previously reported (68-70 kDa from CH5 and a clinical isolate) inferred anti-cholera toxin reactivity. Secondly, how a protein with a published molecular weight of 45 kDa was sequenced from a band on Immobilon at 55 kDa.

The most feasible explanation for the aforementioned differences is that the MOMP is glycosylated, or has covalently bound LPS, and this can be a significant posttranslational modification in which the carbohydrate and/or LPS groups could cause the protein to migrate at the higher molecular weight of 55 kDa. It must be considered that molecular weights are being compared from two different methods; the sequenced protein has an estimated molecular weight of 45kDa based on amino acid sequence, but the 55kDa band was determined using electrophoresis. Mobility on SDS-PAGE can vary due to a number of factors, one of which is protein glycosylation. An excess ratio of SDS to polypeptide of at least 3:1 is required during protein dissociation on SDS-PAGE, but many glycoproteins are known to behave anomalously even when SDS is in excess. SDS is bound only to the protein part of the molecule and this reduced SDS binding causes a reduction in the net charge. This in turn lowers the polypeptide mobility during electrophoresis causing the proteins to migrate at artificially high molecular weights (Hames and Rickwood 1990).

The carbohydrate groups on glycosylated proteins commonly contain sialic acid groups (Szymanski *et al.* 1999). This may mimick GM_1 and cause cross-reaction with anti-cholera toxin antibody. The MOMP would have elicited a cytotoxic effect in the MTT assay as this protein has been demonstrated to be cytotoxic (Moutinho-Fragaso *et al.* 1998, Bacon *et al.* 1998 and 1999). This protein has also been demonstrated to have an associated LPS moiety which serves a protective role and does not contribute to the cytotoxicity (Bacon *et al.* 1999). The explanation that the protein purified and sequenced in this investigation which was cytotoxic, cross-reactive with anti-CT and of the higher molecular weight of 55 kDa was actually a glycosylated MOMP is hence the most plausible answer. Immunoblotting of the partially purified fraction with an antibody to the MOMP could be done to confirm this.

Other groups who identified and/or purified the cholera-like toxin from *C. jejuni* reported it to be a cytotonic toxin (Klipstein and Engert 1984a and 1984b, McCardell *et al.* 1984 and 1986b, Collins *et al.* 1992, Suzuki *et al.* 1994). Its presence was detected by cross-reactivity with antisera, RILT (rat or rabbit ileal loop test) assays and morphological changes in CHO and Y-1 cells. The *C. jejuni* enterotoxin has not been reported to be a cytotoxin that kills tissue cultured cells; it is a cytotonic toxin with the reversible effects of fluid accumulation and cell distension. Thus, the MTT assay was probably not the most suitable assay to detect the enterotoxin, as it is a measure of cell viability. It did, however, indicate the possibility that a protein was present which was cytotoxic and also cross-reacted with anti-cholera toxin antibody. Cell distension was also observed in CHO cells, which is a common assessment of the presence of cytotonic toxin; however, these effects are indistinguishable from those of the CLDT for the first 2-3 days (Johnson and Lior 1988a). However, the presence of CLDT genes in *C. jejuni* NCTC 11351 suggested strongly that the CLDT elicited these effects on CHO cells. These studies would also have benefited from further purification of the toxin so that any other proteins were completely removed from the eluted fraction and the only remaining protein was the 55 kDa cholera-like protein. It would be of further interest to determine the carbohydrate/LPS content of the purified fraction. This would help to establish whether the carbohydrate moiety of a glycoprotein inferred the reaction with anti-cholera toxin antibody, and would ensure that the cytoxicity in the MTT assay was caused by the protein rather than the LPS fraction. Unfortunately, the time limitations of the project meant that many of these useful ideas were never put into practice.

Chapter 5 – Conclusions

C. jejuni is a well-known human pathogen that was recognised in association with foodborne disease over 20 years ago. *Arcobacter* is a newly emerging organism that is closely related to *Campylobacter* and shares many similar characteristics. Much research into *C. jejuni* pathogenicity, toxicity and OMP profiles has already been carried out, but very little progress has been made with investigations into *Arcobacter* disease mechanisms, or in fact, whether it is responsible for disease in humans. SDS-PAGE profiles of *A. butzleri* and *A. cryaerophilus* were compared to those of *C. jejuni* and it was apparent that a major outer membrane protein was present in *Arcobacter* spp. which behaved similarly to the MOMP of *C. jejuni*. The two proteins were found to respond in the same way to different staining procedures, except that the *C. jejuni* MOMP is 45 kDa (Figs. 15-17) and the corresponding *Arcobacter* protein is 40 kDa (Fig. 19). In the MTT assay, however, *Arcobacter* spp. did not demonstrate any cytotoxicity. If this protein is the *Arcobacter* equivalent of the *Campylobacter* MOMP it certainly does not share all of the same properties. *Campylobacter* MOMP is, after all, known to be cytotoxic (Moutinho-Fragaso *et al.* 1996, Bacon *et al.* 1998 and 1999). *A. butzleri* and *A. cryaerophilus* also showed changes in protein profile according to oxygen concentration (Figs. 20-22). A more diverse OMP range apparently corresponded with a stress response and the cells seemed the least stressed in a microaerobic, instead of aerobic atmosphere. This change in OMP profile was probably due to the switching on or off of pathogenic or survival factors. In an aerobic environment survival proteins would probably be the most predominant but in a microaerobic environment different proteins for colonisation and interaction with the host may become more abundant. The most dominant bands of the *C. jejuni* OMPs were likely to be the flagellin (65 kDa) and the MOMP (45 kDa) and these may be the corresponding dominant proteins in *Arcobacter* spp. It must also be considered that many *C. jejuni* proteins, including flagellin, are glycosylated (Szymanski *et al.* 1999) which can affect mobility on SDS-PAGE (Hames and Rickwood 1990). These molecular weights may therefore be inaccurate, but without clarification of this it will be assumed that the proteins have migrated correctly.

C. jejuni NCTC 11351, NCTC 11322 and NCTC 11168 were all shown to be cytotoxic to N2a cells (Table 4). *C. jejuni* NCTC 11351 demonstrated the highest cytotoxicity of the three strains from BA-grown culture extracts and BHI-grown culture supernatant extracts towards N2a cells, and was also toxic to CHO cells, thus was the strain chosen for toxin purification. OMP preparations from *C. jejuni* NCTC 11351 showed strong positive reactivity at 55 kDa with a monoclonal antibody against cholera toxin (Fig. 24). This therefore strongly indicated that a cholera-like toxin was present in *C. jejuni* NCTC 11351 which may also be responsible for the cytotoxicity. The CLDT genes were detected in *C. jejuni* NCTC 11351 and NCTC 11168 (Fig. 25), but were not proven to be expressed in the samples tested. Distension of CHO cells was observed following a 48h incubation (Fig. 27), an effect which suggests the presence of either the CLDT and/or cytotonic activity, but cannot be differentiated without prolonged incubation. It is therefore a possibility that the cytotoxicity was induced by the CLDT, however, an MTT assay is not specific for CLDT detection. The MTT assay is also not a reliable method for detection of cholera-like toxin as it measures cell viability. Cholera toxin and the cholera-like toxin reported from *C. jejuni* do not cause lethality to cells, but are cytotonic toxins and cause cells to undergo certain reversible, morphological changes due to excess fluid accumulation (Johnson and Lior 1988a, Florin and Antillon 1992, McCardell *et al.* 1984). During purification, however, it was discovered that fractions which cross-reacted with cholera toxin antibody also elicited a cytotoxic effect in the MTT assay. It was thus assumed during purification that there was a cholera-like toxin present which had a similar binding domain, but slightly different mode of action, than that of cholera toxin itself.

The cholera-like toxin was partially purified from BA-grown cultures of *C. jejuni* NCTC 11351 using cholera toxin B subunit antibody reactivity, protein assays and the MTT assay to confirm its presence at each stage during the purification procedure (Fig. 29). The end product which was N-terminally sequenced was, however, the MOMP (Table 5). This protein is 45 kDa which did not match the cholera-like toxin detected in immunoblots at 55 kDa, but could explain the cytotoxicity. MOMP has been reported to be a cytotoxic porin-LPS complex (Bacon *et al.* 1999). The most likely explanation is that CHO groups of the LPS complex were binding the cholera toxin antibody. *C. jejuni*

LPS is known to contain sialic acid groups which can mimick ganglioside GM_1, which is also the binding site of cholera toxin, and this is the postulated mechanism for the onset of Guillain-Barré syndrome. Many other proteins are glycosylated in *C. jejuni* and commonly contain sialic acid within the glycosylated region (Szymanski *et al.* 1999).

The MOMP is predicted to be 45 kDa based on its amino acid sequence, but glycosylation is a common posttranslational modification of *Campylobacter* proteins (Szymanski *et al.* 1999) which can alter their mobility and resultant molecular weight on SDS-PAGE (Hames and Rickwood 1990). The high titre in the protein assays during purification would indicate the presence of the MOMP due to its abundance in the OM. MOMP represents about 90% of the total OMPs of *C. jejuni* (Blaser *et al.* 1983), so a high protein content would correlate with a high MOMP content in the fractions. The MTT assay would also have indicated the presence of MOMP due to the evidence that it is a cytotoxic porin (Moutinho-Fragaso *et al.* 1998, Bacon *et al.* 1998 and 1999). The MOMP has been demonstrated to be a porin-LPS complex with a very dense carbohydrate to protein ratio of 4:1 (Bacon *et al.* 1999). This would almost certainly contain sialic acid groups which would be highly likely to bind to cholera toxin (CT) B subunit antibody.

The molecular weight of the purified MOMP in these experiments was 55 kDa which did not match the 68 kDa (Daikoku *et al.* 1990) and 70 kDa (McCardell *et al.* 1984) cholera-like enterotoxins detected and partially purified by other groups. This suggests that the toxin partially purified at 55 kDa was a very different protein to those previously reported as cholera-like toxins. It is, however, a possibility that these previous researchers also detected the MOMP or other glycosylated proteins which migrated at a higher molecular weight and cross-reacted with cholera toxin antibodies. This may be true in some reports which did not perform cytotonic assays, but the detection of cytotonic activity by many groups suggests the definite presence of a cholera toxin-like protein with a cytotonic, and not cytotoxic, mode of action. If these groups had, in fact, purified the MOMP, they would not detect reversible, cytotonic activities and the effects upon CHO cells would be very different. The toxic effects of MOMP were differentiated from those of enterotoxin by Moutinho-Fragaso *et al.* (1996). Enterotoxin (cholera-like toxin) was shown by other groups to cause elongation

of CHO cells and rounding of Y-1 cells (Ruiz-Palacios *et al.* 1983, Wadstrom *et al.* 1983, Johnson and Lior 1988a, Saha and Sanyal 1990), yet MOMP was demonstrated to cause rounding of CHO cells (Moutinho-Fragaso *et al.* 1996).

The main conclusion from the purification of cholera-like enterotoxin was that the methods used in this investigation and the subsequent results led to the firm indication that a glycosylated MOMP was the purified toxin. This led to the additional conclusion that the morphological effects observed upon CHO cells were, in fact, a result of CLDT activity and not cytotonic activity. Hence, CLDT genes were not only detected in *C. jejuni* NCTC 11351, but were also shown to be expressed due to the effects noted upon CHO cells. It can thus be assumed that the following two toxins were detected in cultures of *C. jejuni* NCTC 11351:

1. CLDT was detected using PCR to identify the presence of these genes and its effects were observed in culture by the distension of CHO cells. It may also have been partially responsible for the cytotoxicity in the MTT assay.
2. MOMP was detected which was cytotoxic in an MTT assay, cross-reacted with cholera toxin B subunit antibodies, and was finally partially purified and sequenced.

There may have been other toxins present, but the methods and results presented in this investigation only provide evidence for the presence of these two toxins.

Arcobacter spp. did not demonstrate any cytotoxicity, nor did it cross-react with antibodies against shiga toxin or cholera toxin. Additionally, no CLDT genes were detected in *A. butzleri* which strongly indicates either that this organism does not produce toxins, or that it produces toxins which are very different to those produced by *C. jejuni*. The strains tested included human clinical isolates of *A. butzleri* and hence were possible pathogenic strains.

Haemolytic toxins were detected in *C. jejuni* and *Arcobacter* spp. In all strains of both organisms cell-associated alpha-haemolytic activity was detected in ageing cultures, the production of which was oxygen-sensitive, more lytic upon horse than sheep blood and only detectable when the bacteria were in close contact with the blood cells. This haemolytic activity was possibly involved in iron uptake as indicated by Istivan *et al.* (1998), but there was a possibility that it may play other roles in pathogenicity such as invasion or evasion of the host

immune responses (for example, lysis of neutrophils). Possible involvement of phospholipase C activity in haemolysis was detected in many of the *C. jejuni* and *A. butzleri* strains. Reduced haemolysis by *C. jejuni* NCTC 11168 and *A. butzleri* following the addition of a phospholipase C inhibitor was shown to be of statistical significance. Other strains also had a visible reduction in their haemolytic activity following the addition of a phospholipase C inhibitor, suggesting the potential role of a phospholipase C enzyme in the haemolytic activity of both *C. jejuni* and *A. butzleri*. However, no sequence homology was found between *C. jejuni* NCTC 11168 genes and the phospholipase C alpha toxins of *Clostridium perfringens*, *Clostridium bifermentans* and *Bacillus cereus*.

This report gives an overview of the screening and detection of different toxins in *C. jejuni* and *Arcobacter* spp. No toxicity apart from haemolytic activity was demonstrated by *Arcobacter* spp. It can, however, be seen by the wide range of methods and conditions involved in toxin expression and detection that the production of toxins by *Arcobacter* may still be detected but it will depend upon finding the correct assay conditions. A thorough account of all the screening methods, cell lines and assays used for investigations into *C. jejuni* pathogenic factors was given in Chapter 1. This brings to attention the need for standardising methods for the detection and screening of different toxins and much can be learnt from this by investigators of *Arcobacter* pathogenicity.

There are still, however, few defined mechanisms and absolute answers to the questions raised about *C. jejuni* pathogenicity. Despite the fact that two decades have passed since initial investigations into *C. jejuni* foodborne disease and the genome of *C. jejuni* NCTC 11168 has recently been published, little progress in defining pathogenic mechanisms has been made. No genes encoding toxins other than the CLDT and a haemolysin were identified, and no pathogenicity islands were identified within the *C. jejuni* 11168 genome (Parkhill *et al.* 2000). The variation between strains regarding genetic and antigenic diversity and expression has indicated that sequencing the genome of one strain of *C. jejuni* is insufficient. There are still many genes within the *C. jejuni* genome with unknown functions. The possibility that one of these genes of unknown function encodes a cholera-like (cytotonic) enterotoxin or that other toxin genes are present still remains. It is also feasible that the genes which encode the cholera-like toxin are highly unstable and not present within a number

of strains, including *C. jejuni* NCTC 11168. This would account for the varying reports of the ability to detect enterotoxin activity. No enterotoxin activity was detected in *C. jejuni* 11168 *in vitro*, although it has been demonstrated to exhibit cytotoxicity upon HeLa and Vero cell lines in an MTT assay. The cytotoxicity was obtained from a cell extract and not supernatant, and was more active from 48h cultures rather than 24h (Coote and Arain 1996). Groups studying enterotoxin (Daikoku *et al.* 1989, Florin and Antillon 1992, Suzuki *et al.* 1994, Fernandez *et al.* 1999) and other toxins (Daikoku *et al.* 1989, Mahajan and Rodgers 1990, Lee *et al.* 1998 and 2000) reported that expression of the toxins was unstable and toxicity was frequently lost upon subculturing or following refrigerator or freeze-thaw storage. This supports the theory that the genes encoding this and other toxins are very unstable and leads to the suggestion that they may even be plasmid-mediated. *E. coli* LT is known to be plasmid-mediated and the recent revelation that *Campylobacter* plasmids (Bacon *et al.* 2000) are involved in virulence further validates this suggestion.

This study was an attempt to define the toxic capabilities of *C. jejuni* and *Arcobacter* spp. and to purify a toxin from *C. jejuni* for sequence analysis. This proved to be an extremely difficult task, but significant progress was made and this was the first time that purification of a toxin cross-reactive with cholera toxin reached the stage of N-terminal sequence analysis. The sequence data indicated, however, that the MOMP of *C. jejuni* was the protein present in the purified and sequenced fraction. This caused confusion as the MOMP is 45 kDa and the cholera-like band which was sequenced was 55 kDa and suggested reasons for this have previously been discussed. It has been concluded that the results of this investigation strongly suggest that the MOMP was the toxin which was purified. Further investigation into the possible presence of a cholera-like enterotoxin is still required with a more specific ion exchange system and the use of a cytotonic toxin assay as well as the MTT assay. It needs to be established if the MOMP is capable of causing cytotonic as well as cytotoxic effects such as the RILT test and rounding of Y-1 cells. If so, the evidence would strongly suggest that previous detection of a cholera-like toxin was actually detection of the MOMP. This would account for some of the differences between the abilities of different research groups to detect the presence of a cholera-like toxin. If the MOMP does not demonstrate any cytotonic activities then the mystery of the

'cholera-like toxin' will continue. There is still a strong possibility that other glycosylated proteins may cross-react with cholera toxin antibody. Additionally, there is still a possibility that a highly unstable, potentially plasmid-mediated cholera-like toxin is present in some *C. jejuni* strains. Further investigation is needed before this issue is properly resolved. Further investigation is also needed into the pathogenic potential of *Arcobacter* spp., as this organism does not appear to share similar toxic and immunologically cross-reactive properties to those of *Campylobacter* spp., except a cell-associated, oxygen-sensitive α-haemolysin. This organism may yet produce one or more toxins which were not sensitive to the methods used in this investigation.

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