Determinants of survival and virulence of Campylobacter

Thesis

DETERMINANTS OF SURVIVAL AND VIRULENCE OF CAMPYLOBACTER

PHILIPPA CAROLINE HARVEY Bsc (HONS)

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

January 2000

Centre for Applied Microbiology and Research

Abstract

The pathogenesis of *Campylobacter* enteritis is not well understood including the mechanisms involved in invasion and translocation across intestinal epithelial cells. The genetic make-up of the pathogen and its responses to different environmental cues are thought to contribute to the organism's ability to survive and cause disease. The extremes of environment which *Campylobacter* can with-stand, and the effect that this has on virulence and invasive ability remains undefined. For the first time, several isolates were compared quantitatively to determine the extent to which intracellular invasion contributes to translocation across epithelial cell monolayers. Translocation ability did not correlate with intracellular invasiveness, suggesting that different "invasion" phenotypes exist among *Campylobacter* isolates. Repeated exposure of *Campylobacter* isolates to Caco-2 cells caused an increase in their ability to invade and survive, which was associated with changes in protein expression. *Campylobacter* was grown in continuous culture under conditions of iron sufficiency, iron limitation, oxidative stress and low pH. Uniquely, growth under oxidative stress and iron replete conditions caused an increase in the invasive ability of *C. jejuni* 81116, which was correlated with the up-regulation of specific proteins. The role of three proteins, HtrB, Tpx and PEB-4, was investigated at the molecular level. Two of the encoding genes, *peb4A* and *htrB*, were found to be essential for viability. Homologous recombination of an inactivated *tpx* gene into the genome of *C. jejuni* caused increased sensitivity to H_2O_2, but did not affect the ability of *C. jejuni* to invade and survive within Caco-2 cell monolayers. This study demonstrated that isolates of *Campylobacter* differ significantly in their virulence potential with respect to their invasive phenotypes. In addition *Campylobacter* grown in well defined continuous culture conditions demonstrated for the first time the importance of iron and oxidative stress as acting as potenital cues for the expression of survival and invasion determinants.

Acknowledgements

I would like to thank and acknowledge the help and support of many colleagues at CAMR. I would like to thank Mark Riches and his Media department for supplying the large amounts of media and agar plates. Also I would like to acknowledge Barry Dowsett for the EM work and Asgar Electricwala for the N-terminal sequencing and the generation of probes.

I would like to acknowledge ECACC for provision of Caco-2 cells for the invasion assays. I would like to thank Thierry Battle for his enthusiasm and encouragement during the investigation of invasion mechanisms and the help from his team including Celine Touchard, John Latigo and Paula Freemantle who sectioned the Transwell units.

I would especially like to thank Brian James, Anne Duguid, Des Purdy and Tom Davies who have all given encouragement, help and support during this study, especially Brian who read the first draft. I am grateful for the time and effort put in by my supervisors, Dr Steve Leach and Dr Ian Henderson for their advice and for their effort in reading numerous versions of manuscripts and chapters. I would also like to thank Dr Andrew Robinson for liasing with the Open University.

Finally I would like to thank my husband Martin and my Mum and Dad for all their love and encouragement during this long period of work.

Dedication

To my Mum and Dad and my husband Martin for all the love and support they have shown me and the sacrifies they have made in allowing me to write this thesis.

Publications

Harvey, P. C., Battle, T. & Leach, S. A. (1999). Different invasion phenotypes of *Campylobacter* isolates in Caco-2 cell monolayers. *J Med Microbiol* **48**, 461-469.

Harvey, P. & Leach, S. (1998). Analysis of coccal cell formation by *Campylobacter jejuni* using continuous culture techniques and the importance of oxidative stress. *J Appl Microbiol* **85**, 387-397.

Leach, S., Harvey, P. & Wait, R. (1997). Changes with growth rate in the membrane lipid composition of *Campylobacter jejuni* and amino acid utilisation by continuous culture. *J Appl Microbiol* **82**, 631-640.

Presentations

Poster

Harvey P. & Leach S. (1998). The response of *Campylobacter jejuni* to iron availability-effects on protein expression and invasion. 140th SGM Nottingham.

Harvey, P. C., Battle, T. & Leach, S. A. (1997). *Campylobacter* strain variation in epithelial cell translocation mechanisms and novel protein expression correlated with increased invasion. 9th International workshop on Campylobacters, Helicobacters and related organisms, South Africa.

Oral

Harvey, P. C., Battle, T. & Leach, S. A. (1997). Increased invasiveness of, and novel protein expression occurs in *Campylobacter jejuni* as a result of passage through Caco-2 monolayers. 136th SGM, Reading.

Harvey, P. C. & Leach, S. A. (1996). Invasiveness of *Campylobacter jejuni* and the increased virulence on passaging through Caco-2 cells. 173rd Meeting of the Pathological society of Great Britain and Ireland, Southampton University.

Contents

Chapter 3 Interaction of *Campylobacter* spp. with Caco-2 cell monolayers

Chapter 4 Effects of different environmental conditions on the survival, protein expression and virulence of *Campylobacter*

List of Figures

List of Tables

Abbreviations

Abbreviation	In full
1-D	1-dimensional gel electrophoresis
2-D	2-dimensional gel electrophoresis
ABCD	Aces buffered chemically defined medium
ANOVA	Statistical test, Analysis of variance
ATR	Acid tolerance response
BPB	Bromophenol blue
BSA	Bovine serum albumin
CAM	Chorio-allantoic membrane
CBA	Columbia blood agar plates
CDSs	Predicted coding sequences
CDSC	Communicable Disease Surveillance Centre
Cm^R	Chloramphenicol resistance gene
CPHL	Central Public Health Laboratories
Cpmod4	Complex medium
CTC	Cyanoditoyl tetrazolium chloride
DAPI	4,6-diamidino-2-phenylindole dihydrochloride
DOT	Dissolved oxygen tension
DTT	Dithiothreitol
ECACC	European Collection of Animal Cell Cultures
EDTA	Ethylenediaminetetra-acetic acid
EM	Electron micrographs
FCS	Foetal calf serum
GBS	Guillian Barré Syndrome
h	Hour
IEF	Isoelectric focusing
IPTG	Isopropyl-β-D-thiogalactopyranoside
LPS	Lipopolysaccharide
MEM	Minimal essential media
min	Minute
Mwt	Molecular weight marker
NEAA	Non-essential amino acids
PBS	Phosphate buffered saline
Pefabloc	4,2-amino-ethyl benzene sufonyl fluoride
PHLS	Public Health Laboratory Service
pI	Isoelectric point
s	Second
SDS	Sodium dodecyl sulphate
SE	Standard error
TAE	Tris-acetate buffer
TEER	Trans-epithelial electrical resistance
TEM	Transmission electron micrograph
TCA cycle	Tribcarboxylic acid cycle
Tris	Tris(hydroxymethyl)methylamine
VNC	Viable nonculturable
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Declaration

I declare that the research presented in this thesis is all my own work, except where otherwise indicated, and has not been submitted elsewhere for a research degree

Chapter 1

INTRODUCTION

1.1 Historical Aspect

Campylobacter spp. are one of the most common causes of bacterial enteritis in the developed world. They are often classified as "new pathogens" due to the culture conditions for these micro-organisms not being clearly established until relatively recently. *Campylobacter* was first isolated in 1909, associated with abortion in sheep and cattle, and placed initially in the genus *Vibrio* due to the observed spiral morphology. In 1947, *V. fetus* (later classified as *Camplyobacter fetus*) was cultured from human blood following septic abortion from a pregnant woman and for the first time was implicated in causing human disease (Walker *et al.*, 1986). In the following years *Campylobacter* was more frequently isolated from human body fluids and was suspected to be an opportunistic pathogen (Allos & Blaser, 1995). Its association with enteric infections was proposed by King (1957) following the isolation of strains from the bloodstream of post-diarrhoea patients. However, the presence of the organism could not be confirmed from infected stools until the 1970s, due to its fastidious nature (see section 1.2) and the lack of suitable detection methods. The development of selective media containing antibiotics, for example Skirrow's medium, has improved detection of *Campylobacter* spp. in clinical material (Skirrow, 1977). Recent developments in PCR have also lead to the detection of viable and non-viable *C. jejuni* in foodstuffs using reverse transcription PCR to detect mRNA (Sails *et al.*, 1998)

The new genus *Campylobacter*, based on the Greek word for curved rod, was proposed by Sebald & Véron in 1963 within the family Spirillaceae, reflecting the micro-organisms' differences from the *Vibrio* genus. Two of these differences arose in their inability to use

sugars either oxidatively or fermentatively, and nucleotide base composition (Walker *et al.*, 1986). Another reason for the re-classification was that some *V. fetus* strains were also found to grow best at 42°C, which was not a characteristic of those micro-organisms of the *Vibrio* genus.

The genus *Campylobacter*, contains a variety of species, though the two most closely related are the enteropathogenic species *Campylobacter jejuni* and *Campylobacter coli*, of which there are more than 100 serotypes (Skirrow, 1990). Other species have been identified as enteropathogenic, including *Campylobacter upsaliensis* and *Campylobacter lari* (Ketley, 1997). The distinction between these species was based on DNA–DNA hybridization techniques, which demonstrated less than 35 % nucleotide similarity. *C. upsaliensis* was initially isolated from dogs and tested as a catalase weakly positive or negative strain (Steele *et al.*, 1985). *C. lari* was first isolated from seagulls and differs from *C. jejuni* in that it is resistant to nalidixic acid (Skirrow & Benjamin, 1980). Other species have been isolated from cases of human enteritis including *Campylobacter fennelliae* and *Campylobacter cinaedi*. However, little is known with regards to the epidemiology of these particular species, which were known previously as "*Campylobacter*-like" micro-organisms. Other species, for example, *Campylobacter hyointestinalis* and *Campylobacter fetus* subspp. Fetus, have been associated occasionally with human diarrhoeal disease (Griffiths & Park, 1990).

1.2 Microbiology

Enteric campylobacters are slim, Gram-negative, short spiral rods being 1.5-5.0 μm in length and 0.2–0.5 μm across (Leach, 1997). These spiral curved micro-organisms have tapering ends and possess a polar flagellum at one or both ends of the cell. This structure allows the organism to propel itself through the viscous environment of the mucus layer

surrounding the intestinal epithelium. Work has demonstrated that tumbling *C. jejuni*, when exposed to a viscous environment subsequently swim in straight lines with an increased velocity when compared to those in a non-viscous environment (Szymanski *et al.*, 1995). This was found to enhance the binding of *C. jejuni* and invasion into Caco-2 cells.

In older, or stressed cultures, *Campylobacter* cells may alter in morphology to become spherical or coccoid in appearance (Leach, 1997). This is thought to result from exposure to a number of environmental stresses (see section 1.13). *C. jejuni* and *C. coli* are microaerophilic requiring low levels of oxygen, typically between 3 to15 %, with higher concentrations being toxic to the organism. These are significantly lower concentrations than other aerobic pathogens are able to tolerate. The organism also requires a carbon dioxide concentration of 3 to 5 % for optimal growth. *C. jejuni and C. coli* are thermophilic, with optimum growth at 42°C, probably reflecting the temperature of their normal habitat, the intestines of certain birds, though the organism is able to grow at 37°C. Other species, for example *C. cinaedi*, grow only at 37°C and not at 25°C or 42°C.

1.3 Molecular Aspect

Campylobacter spp. have a genome of 1, 641, 481 bp, which encodes 1, 654 CDSs, and consists of AT rich DNA, with an AT ratio of approximately 70 % (Taylor, 1992, Parkhill *et al.*, 2000). The genome is small in size compared to other enteric pathogens, for example the 4.6 Mbp genome of *Escherichia coli*. This may reflect the micro-organisms' requirement for complex media and their inability to metabolise carbohydrates and other complex substances (Griffiths & Park, 1990).

The availability of antibiotic resistance genes that are functional in *Campylobacter*, for example the chloramphenicol resistance gene *cat*, and are able to function in other species,

for example *E. coli*, have allowed some genetic manipulation of campylobacter. Shuttle-vectors have been constructed to contain these antibiotic resistance genes with *E. coli* and *C. coli* origins of replication (Yao *et al.*, 1993), which can function in both micro-organisms. These vectors allow plasmids to be manipulated by conventional recombinant DNA methods, for example site-directed mutation before being returned to *Campylobacter* for study. As an alternative approach, suicide vectors can be used which contain an origin of replication, an antibiotic resistance gene and a polylinker region to allow for the insertion of the gene. Following insertion into the vector, an unique restriction site within the gene can be used to clone a second antibiotic resistance gene. The presence of this second antibiotics resistance marker can be used to select *E. coli* containing the mutated plasmid, and the resulting plasmid is used to transform *Campylobacter* (see section 5.1.2). On return to the *Campylobacter* genome, if sufficient homology exists between the plasmid and the gene, homologous recombination occurs. The suicide vector, which lacks a replication site, is unable to replicate in the bacteria and is removed from the bacteria. This allows the gene in the plasmid to replace the original gene in the chromosome. To detect such an event *Campylobacter* must be selected for on media containing the same antibiotic as used during the gene insertion.

However, identification of genes has been hindered through the difficulty of genetic exchange mechanisms, which can be inefficient (see section 5.1.2). Natural gene transfer systems have been designed in *Campylobacter*, with plasmid transfer reported in *Campylobacter* with a frequency range of $1x10^{5}$-$1x10^{3}$ transconjugants per recipient cell in a 24 h mating period (Labigne-Roussel *et al.*, 1987). Some strains of *Campylobacter* are naturally competent for DNA uptake without special treatment such as $CaCl_2$ or heat shock, with the process found almost to be independent of growth phase, with early log phase bacteria slightly more competent than late log-phase cells. Interestingly, transformation of

Campylobacter with plasmid DNA is less efficient than with chromosomal DNA. Alternatively, plasmid DNA can be introduced through electroporation where high voltage reversibly permeabilizes the biomembranes allowing uptake of DNA. Miller *et al.*, (1988) was able to electroporate plasmid DNA into *Campylobacter* at frequencies of 1.2×10^6 transformants per μg of DNA. Work by Wassenaar *et al.*, (1993) identified two strains which were competent for uptake of chromosomal DNA by electroporation and other strains which were naturally competent. The frequency of recombination was found to be increased if long homologous regions were included with the returning foreign DNA.

Developments in molecular techniques have lead to the sequencing of the genome of *C. jejuni* 11168 by the Sanger Centre, which was completed in September 1998 and published recently (Parkhill *et al.*, 2000). The shotgun technique was employed whereby the genome was sheared by ultrasonic disintegration into small segments (1.8-2.3 kb) which were ligated into the vector pUC18 and sequenced by PCR (Karlyshev *et al.*, 1999). Each insert was sequenced from each end, until the complete sequence could be aligned. Interestingly, no prophages, plasmids insertion elements or pathogenecity islands were present in the genome. The data has been released on the Internet (http://www.sanger.ac.uk/Projects/C_jejuni/) and has allowed genes to be identified by homology to known gene sequences in other micro-organisms. These advances has created a wealth of information on *Campylobacter*, but conventional genetic approaches are still required to determine gene function, for example site directed mutations can be created in a precise manner to define the role of specific sequences.

1.4 Clinical presentation

Infection with *Campylobacter* causes a wide spectrum of enteric disease, ranging from mild secretory diarrhoea to severe inflammatory diarrhoea (Ketley, 1997). An infective dose as

low as 500-800 micro-organisms has been found to result in symptoms of infection (Black *et al.*, 1988). The incubation period varies between 1-7 days, before symptoms develop which include acute abdominal pain, often with fever and general malaise. The profuse diarrhoea lasts between 2 to 3 days, which in the developed world is often accompanied by frank blood, mucus and inflammatory exudate containing leukocytes. Biopsy specimens of colonic tissue were found to be characterised by oedema, with infiltration of the lamina propria by neutrophils and mononuclear cells (Allos & Blaser, 1995). Degeneration of glandular tissues arises, accompanied by the loss of crypts and the development of crypt absceses (Walker *et al.*, 1986), which has lead to confusion with acute ulcerative colitis. Other mis-diagnoses arise from persistent abdominal pain and discomfort after diarrhoea has ceased. Abdominal complications are rare in *C. jejuni* infections, however, persistent pain can continue after the cessation of diarrhoea and has been mistaken for inflammatory bowel disease or acute appendicitis. Additional intestinal complications of *C. jejuni* infections include toxic megacolon, pseudomembranous colitis and cholecystitis (Allos & Blaser, 1995). Further complications can arise such as gastrointestinal haemorrhage following local mucosal invasion. Systemic infection may occur, though most strains of *C. jejuni* can be killed by normal human serum. However, *C. fetus* has been found to be resistant to killing by serum and is more likely to be isolated from extra-intestinal sites. In developing countries, and occasionally in industrialised nations, non-inflammatory watery diarrhoea is presented, which does not contain blood, mucus or leukocytes (Ketley, 1997). The disease is usually self-limiting and complications are uncommon (Skirrow & Blaser, 1992). The cost of infections in the UK, in health care and in lost productivity was estimated in 1986 to be £233 per case. Using 1986 estimates of cost, and the present numbers of infections, the economic cost could be over £13.7 million.

Patients can occasionally develop post-infection complications with about 1 % developing reactive arthritis (Leach, 1997). The most notable complication is the Guillain-Barré Syndrome (GBS). This peripheral neuropathy, causing weakness in at least two limbs, usually affects the motor, sensory and autonomic nerves supplying the limbs and may also involve respiratory muscles (Hughes & Rees, 1997). The disease is characterised by segmental demyelination of peripheral nerves with mononuclear infiltrates and oedema. *C. jejuni* appears to be the most important trigger of GBS, with 30 % of GBS cases are preceded by *C. jejuni* infection, which develops 1 to 3 weeks following infection. The myelin destruction was thought to be mediated by either a direct toxic effect or by an immunopathogenic mechanism (Allos, 1997). However, no relationship has been established between severity of *C. jejuni* infections and the likelihood of developing GBS, though the Penner serotype O:19 has been pre-dominantly associated with the initiation of GBS. Sera from *C. jejuni* patients with GBS recognise a peripheral nerve myelin specific protein, PO, which suggests that *C. jejuni* has antigens which stimulate production of antibodies that cross-react with peripheral nerve myelin and cause GBS (Mishu & Blaser, 1997). The economic burden of GBS to the US in 1995 was calculated to be $0.2-$1.8 billion, adding further to the other costs of campylobacteriosis.

1.5 Treatment of the disease

Although not usually treated with antimicrobials, *Campylobacter* infections can be treated with a number of antibiotics including macrolides, fluoroquinolones, tetracycline and chloramphenicol. Initiation of treatment within four days from the onset of symptoms has a clinical benefit in reducing diarrhoea. The preferred choice, in industrialised countries, are macrolide drugs, including erthromycin and clarithromycin due to the low level of macrolide resistant strains. Usually a combination of erythromycin followed by ciprofloxacin is prescribed.

There has been a noticeable increase in antibiotic resistance to quinolone compounds in *Campylobacter*, for example, 30 % of strains isolated in a study in Spain were found to be resistant (Acar & Goldstein, 1997). The high level of resistance to fluoroquinolones was thought to be due to their high use in veterinary medicine, where they are used as therapeutic agents or at low levels in animal feed as growth promoters (Acar & Goldstein, 1997).

Resistance to erythromycin and quinolone compounds was proposed to be chromosomally mediated, with quinolone resistance caused by a mutation in *gyrA* which encodes the A subunit of DNA gyrase (Piddock, 1995). Alternatively resistance may arise through mutation in regulatory genes which alter the permeability or the efflux capacity of the bacteria (Acar & Goldstein, 1997). Resistance to other antibiotics including tetracycline, kanamycin and chloramphenicol has been found to be encoded on plasmids (see section 1.3) and can be transferred by bacterial conjugation. In response to the general concern of antibiotic resistance in food-borne pathogens and other clinical micro-organisms, committees have been established in the UK to determine its distribution.

1.6 Epidemiology

The incidence of *Campylobacter* enteritis has risen steadily in the UK partially due to improved detection methods and reporting procedures to CDSC (Skirrow, 1977). In 1998, 58 059 laboratory-confirmed *C. jejuni* faecal isolates were reported to CDSC, a 15.7 % increase on the number reported in 1997 (PHLS web site). This figure is probably an underestimate due to failure of patients in reporting diarrhoea to doctors. Infection with *Campylobacter* appears to be seasonal in the UK and USA, with a consistent rise in May peaking in July, which may reflect the warmer temperatures and changes in eating habits,

for example, more buffets and barbecues. Cases arise most commonly in infancy and early adulthood with males more susceptible up until the age of 40 (Allos & Blaser, 1995).

Campylobacter enteritis is considered a food-borne disease, (Griffiths & Park, 1990). Unlike cases of *Salmonella*, infection is not thought to result from person to person contact, but arises through consumption of contaminated foodstuffs. The main source of infection in humans has been identified as animals used for food (Adak *et al.*, 1995). Other sources have included unpasteurised milk, water and sewage contamination and domestic pets. Food-borne infections are usually sporadic in nature, whereas water or milk-borne infections occur generally as outbreaks. Twenty-one outbreaks were reported to CDSC between 1992–1994, resulting in illness in 706 people. Eight outbreaks were caused by consumption of contaminated food involving mainly chicken meat or cross-contamination from turkey meat. Consumption of unpasteurised milk was indicated in a further eight outbreaks. In a school in Gloucestershire an outbreak was linked to drinking pasteurised milk from bottles whose tops had been pecked by birds, a route which has been previously reported to be involved in other outbreaks of *Campylobacter* (Stuart *et al.*, 1997). Five outbreaks resulted from consumption of contaminated water (Peabody *et al.*, 1997). Water-borne outbreaks of *Campylobacter* arose through contaminated water sources which were under-chlorinated or poorly maintained. Public supplies of water in the UK are normally chlorinated to a sufficient level to prevent the survival of *Campylobacter*.

Chickens and other birds are regarded as the natural hosts of *Campylobacter*, reflected in the bacterium's adaptation for optimum growth between 42°C and 43°C, the temperature of the avian gastrointestinal tract. The large scale mechanised processing of poultry can lead to the deposition of faeces on the carcasses, causing previously uninfected poultry to become cross-contaminated. This may account for the high rate of isolation of

Campylobacter from poultry sold in many of the major outlets. In the US, 69 % of broiler carcasses bought from a local supermarket were contaminated with *C. jejuni* (Willis & Murray, 1997). Similarly in Germany, *C. jejuni* was detected on fresh chicken breast meat and isolated from 33 % of the samples (Geilhausen *et al.*, 1996). A study in the UK isolated *Campylobacter* from 37 % of the chickens bought from major outlets (Which Magazine, October, 1996). Human infection from poultry arises through several routes including consumption of raw, or undercooked products, and cross-contamination of other products through handling of the raw product.

1.7 Host Response

The humoral response is important in the control of *C. jejuni* infections, and acts as a primary defence mechanism. After the onset of illness, symptoms generally diminish as anti-*C. jejuni* antibodies appear. In a study by Black *et al.*, (1988), those volunteers which were infected and developed symptoms showed a peak production in IgA and IgM by 11 days, which remained elevated for a prolonged period until 28 days post-infection. Expression of IgG was also found to be similar, with a peak at day 11 and an elevated response maintained until 28 days post-infection, though this rise was not found to be statistically significant. Volunteers who were infected, but remained well, demonstrated an intermediate antibody response in IgA, IgG and IgM.

Each immunoglobulin has an important role in the host's response to a *Campylobacter* infection (Wallis, 1994). In the intestine, specific IgA immobilises *Campylobacter* causing the cells to aggregate and activate the complement system by the Alternative pathway. The IgM antibodies act against the LPS of an infecting isolate. Lasting immunity against the infecting isolate is provided by IgG antibodies as demonstrated by Ruiz-Palacios *et al.*,

(1983), where the IgG status of infected children was monitored and found to remain raised.

Differences between the response of the host's immune system to particular strains of *Campylobacter* have been suggested to contribute to the different disease presentations observed in humans, for example, watery diarrhoea and severe inflammatory diarrhoea. A speculative model has been proposed by Ketley (1997) in an attempt to explain these observations. A lack of symptoms was suggested to occur when the host was re-exposed to the same isolate of *C. jejuni* which had previously colonised the intestinal epithelium, whose action was restricted through the pre-existing IgA activity of the host. Partial immunity was suggested to arise from previous exposure to *C. jejuni* of a different strain, which resulted in watery diarrhoea. This transpires when *C. jejuni* colonises and was able to partially invade the epithelium, and was inhibited by a partially protective IgA response by the host. However, when the immune system has not previously been exposed to *Campylobacter*, the IgA response was slow, allowing *C. jejuni* to colonise, invade and translocate to the lamina propria causing more severe inflammatory disease. Evidence has been reported in support of these views from volunteers who were re-challenged with the same *Campylobacter* strain; they were protected from illness but not intestinal colonisation (Black *et al.*, 1988).

Phagocytosis acts as secondary defence mechanism against infection by *Campylobacter*. The pathogen can stimulate phagocytosis by polymorphonuclear leukocytes (PMNs), which is enhanced if the bacterium has been opsonised previously by antibodies (Bearaowski *et al.*, 1989). Phagocytosis of *Campylobacter* and their eventual death was demonstrated to be less efficient without opsonisation (Walan *et al.*, 1992).

C. jejuni strains have been shown to survive for 6-7 days in macrophages (Kiehlbauch *et al.*, 1985). However, this study failed to stimulate the monocytes with cytokines to aid their differentiation into activated macrophages. Work by Wassenaar *et al.*, (1997) demonstrated that activated macrophages efficiently killed *C. jejuni* within 24-48 h, and superoxide dismutase (SOD) and catalase (*kat*) mutants (see section 1.13.2) were equally sensitive to macrophage killing as the wild type, suggesting that intra-phagocytic survival is not likely to be common.

The infrequent occurrence of *C. jejuni* bacteraemia is thought to be due to the serum bactericidal effect which develops in the host. On gaining entry to the deeper lying tissues of the host, beyond the gut mucosa, *C. jejuni* is exposed to the bactericidal and bacterostatic activity of human serum. This involves iron limitation which results from the action of iron binding proteins such as transferrin, and the complement system which attacks invading micro-organisms.

1.8 Pathogenesis

The molecular basis of the mechanisms by which *Campylobacter* causes disease remains unclear. Virulence determinants which promote disease can be multifactorial and may be linked to different environmental cues, for example calcium, osmotic stress and temperature (Mekalanos *et al.*, 1992). Many structures have been proposed to act as virulence factors for *Campylobacter*, including flagella, outer membrane proteins, lipopolysaccharides (LPS), toxins and most recently the fimbria (Ketley, 1995).

1.8.1 Toxins

These may contribute to the disease process of *Campylobacter*, and can be divided into two groups: exotoxins (soluble proteins) and endotoxins (lipopolysaccharide components of the outer membrane). Exotoxins which are heat labile, have been generally classified as: enterotoxins, which are secreted proteins able to bind to cellular receptors, enter the cell, elevating intracellular cyclic AMP levels and cytotoxins, which kill target cells and often form pores in target membranes and neurotoxins. *Campylobacter* has been suggested to produce two types of exotoxin – enterotoxin and cytotoxin (Wassenaar, 1997). Evidence of an enterotoxin was reported in 1983 where culture supernatants caused intra-luminal fluid secretion in the Rabbit ileal loop test (RILT) model (see section 1.12). A cell product was identified which elongated CHO cells (Wassenaar, 1997), though its expression and its role in pathogenesis has been questioned by a number of investigators.

A number of cytotoxins have been identified in *Campylobacter* including cytolethal distending toxin (CLDT) and haemolysin (Pickett *et al.*, 1996; Hossain *et al.*, 1993). CLDT production was first observed in 1988 causing HeLa and Vero cells to become distended over a two to four day period (Johnson & Lior, 1988). Screening of over 500 isolates found that 41 % produced CLDT, and its distribution was not sub-species specific. The genes encoding CLDT were recently sequenced and three genes, *cdtA*, *cdtB*, *cdtC*, were found to be essential for toxin activity (Pickett *et al.*, 1996). The toxin was found to cause an accumulation of the inactive phosphorylated form of CDC2, the catalytic subunit of the cyclin-dependent kinase (Whitehouse *et al.*, 1998), causing the eukaryotic cells to become blocked in the G_2 phase of the cell cycle, preventing cell division.

Haemolytic activity of *C. jejuni* was first identified by Hossain *et al.*, (1993), and was thought to be involved in iron acquisition *in vivo*. A two-step mechanism of action was

proposed, initially a temperature-independent binding reaction to the erythrocyte followed by a temperature-dependent lytic stage. In addition, more than one haemolysin molecule is probably required to lyse a single erythrocyte.

1.8.2 Flagella and Chemotaxis

The genome of *Campylobacter* was found to carry tandem repeat of the flagellin gene, *flaA* and *flaB*, each 1731 bp. These were found to contain different promoter structures, with *flaA* possessing a σ^{28} promoter and *flaB* one similar to the σ^{54} promoter (Miller *et al.*, 1993). The expression of the FlaB protein which forms a minor component of the flagellum, was found to be growth dependent and subject to environmental regulation, by factors such as temperature and pH (Alm *et al.*, 1993). Mutagenesis experiments have demonstrated that in the absence of *flaB*, *flaA* encodes a normal flagellum with reduced motility, whereas in the absence of *flaA*, *flaB* forms a truncated flagellum with poor motility. These experiments also demonstrated that *flaA* was essential for invasion into, and translocation across cultured cells of intestinal origin (Wassenaar *et al.*, 1991; Grant *et al.*, 1993). Further work has shown that motility was necessary for invasion into tissue culture cells, rather than the presence of FlaA protein. Cells with immobilised flagella were able to adhere to the INT407 cell monolayers, but failed to be internalised, suggesting that further adhesins were involved in the process (Yao *et al.*, 1994). Other genes involved in flagella assembly have been identified, including *ptmB* which was identified to be involved in the glycosylation of the flagellum (Guerry *et al.*, 1996), a motor function gene (Yao *et al.*, 1994), a gene encoding a hook protein, *flgE*, (Kinsella *et al.*, 1997) and *flhA*, involved in regulation of expression or biosynthesis of the flagella (Miller *et al.*, 1993).

The flagellum provides a means of motility for *C. jejuni*, but to determine the direction of movement *Campylobacter* must be able to sense and respond to the environment through a

chemosensory mechanism. Chemotaxis allows an organism to move towards attractants, such as nutrients, and away from repellents, such as toxic compounds (Silversmith & Bourret, 1999). A number of attractants have been recognised for *Campylobacter* including L-fucose, L-asparate, L-cysteine, L-glutamine, L-serine, fumarate, succinate and mucin (Hugdahl *et al.*, 1988). These compounds control the switch between flagella rotating in a counter clockwise rotation, producing smooth swimming or clockwise rotation which causes a tumbling action. Bacteria bias their switching frequency in response to specific attractants (Alon *et al.*, 1999), which causes them to tumble less and move up specific concentration gradient. However, a reduction in the concentration of the attractant can produce a rapid change in the bacteria's tumbling frequency allowing it to move away from the source.

At the molecular level the changes in the external environment are detected by membrane-spanning receptors, which are coupled to an intracellular sensor kinase, CheA. This protein undergoes autophosphorylation, which subsequently transfers the phosphate group to CheY, a cytoplasmic protein. Phosphorylated CheY moves through the cytoplasm and binds to the protein at the base of the flagellar motor causing the direction of rotation to change (Silversmith & Bourret, 1999). The system represents an example of a two-component regulatory system (see section 1.14). The gene encoding CheY, has been identified in *Campylobacter* (Yao *et al.*, 1997) and mutation of the gene was found to increase virulence. Further work by Marchant *et al.*, (1997) demonstrated that CheY was responsible for the tumbling action of *Campylobacter*. However, the recovery of chemotatic behaviour in some mutants suggests that alternative chemotaxis pathways may exist. Recent work by Gonzalez *et al.*, (1998) identified a methyl-accepting chemotaxis-like protein (TlpA) in *C. coli*. However, a *C. coli tlpA* mutant continued to demonstrate wild-type chemotaxis behaviour, suggesting that although *tlpA* may play a role in environmental

sensing and signalling, the gene may mediate signal transduction in pathways other than those involved in chemotaxis.

1.8.3 Other Surface Components

The most recently proposed virulence structure for *C. jejuni* has been the fimbriae, whose expression was reported to be promoted by the presence of bile salts, in particular sodium deoxycholate (Doig *et al.*, 1996b). Although mutation in an accessory gene produced an afimbriant mutant, it showed no alteration in its ability to adhere to, or invade INT407 cells and remained able to colonise ferrets producing less severe disease symptoms. However, there have been no further reports by other groups on the presence of such a structure.

The outer membrane of Gram-negative bacteria contains LPS, including that of *C. jejuni* (Ketley, 1995). In many micro-organisms LPS contributes to pathogenesis, including serum resistance, resistance to phagocytic killing, inflammatory response and cell toxicity (Ketley, 1997). In *C. jejuni* the molecule is composed of three regions, lipid A, a core oligosaccharide and an O-chain consisting of repeating oligosaccharide units. The LPS of *C. jejuni* contains a variable and unusual core of sugars, which may aid the molecular mimicry which occurs in LPS. Recently, many of the genetic loci involved in LPS biosynthesis were identified in *C. jejuni* (Wood *et al.*, 1999), though the contribution of LPS to pathogenesis remains unclear.

Other surface molecules have been suggested to contribute to the disease process. Fauchère *et al.*, (1986) identified two proteins of 27 and 29 kDa from a *Campylobacter* isolated from a patient with fever and diarrhoea, which were able to bind to epithelial cells. The 27 kDa protein was surface located and able to bind directly to epithelial cells (Kervella *et al.*, 1993). However, the 29 kDa protein was found not to be surface exposed and not

involved directly with adherence. In complementary studies by other groups, these proteins were named PEB-1 (28 kDa) and PEB-4 (31 kDa) with a further two proteins identified as PEB-2 (29 kDa) and PEB-3 (30 kDa) (Pei *et al.*, 1991). The roles of PEB-1 and PEB-4 were assigned following N-terminal analyses, with PEB-4 proposed to be an extracytoplasmic lipoprotein (Burucoa *et al.*, 1995) and PEB-1 proposed to have a role as an amino acid transporter (Pei & Blaser, 1993). However, further work has now demonstrated a role for PEB-1 in primary adherence. Mutation of the gene reduced adherence of *C. jejuni* to INT407 cells and reduced colonisation of the mouse intestine (Pei *et al.*, 1998). The role of PEB-2 remains unclear, and PEB-3 has been identified as having homology to a potassium-transport ATPase of *Mycobacterium tuberculosis* (Manning *et al.*, 1997).

Other studies have highlighted a 37 kDa and a 59 kDa protein of *Campylobacter* to be involved in binding to extracellular matrix proteins and soluble fibronectin (Konkel *et al.*, 1997; Moser *et al.*, 1997). The binding of *C. jejuni* to INT407 cells was also found to promote the synthesis of 14 proteins which may be involved in internalisation into the epithelial cells (Konkel & Cieplak, 1992). Further work has identified a protein CiaB which appears to be involved in protein secretion and internalisation in INT407 cells (Konkel *et al.*, 1999).

1.9 Epithelial Interactions

Many structures and molecules have been proposed to function as adhesins in *Campylobacter* (section 1.8), including flagella and proteins such as PEB-1. The invasion by *Campylobacter* into host cells has been observed in infected macaques (Russell *et al.*, 1993) and in the colon of infected patients (van Spreewel *et al.*, 1985). In an attempt to model this process *in vitro*, a range of cell lines of human origin have been used, for

example HEp-2 and INT407 (De Melo *et al.*, 1989; Konkel & Joens, 1989; Konkel *et al.*, 1992). Work by Everest *et al.*, (1992) has demonstrated the use of Caco-2 cells, an enterocyte cell line. The group reported a correlation between the ability of *Campylobacter* isolates to invade Caco-2 cells, with isolates associated with symptoms of colitis being more invasive than those isolates associated with watery diarrhoea. Considerable work is now carried out using Caco-2 cells (see section 3.3).

Adhesion of *Campylobacter* to INT407 cells was found to be enhanced following growth of the organism at 37°C compared to 30°C or 42°C. *C. jejuni* cells taken from a culture in early growth phase at 24 h was found to be more invasive than those harvested following 48 h or 72 h of growth (Konkel *et al.*, 1992a). Expression of *de novo* proteins was found to facilitate internalisation of *C. jejuni* into INT407 cells (Konkel & Cieplak, 1992), suggesting that *Campylobacter* syntheses proteins on contact with the epithelium. However, incubation of *C. jejuni* with INT407 monolayers at 4°C was found to reduce internalisation, suggesting this process also involved specific host receptors (Konkel *et al.*, 1992a). Reducing the incubation temperature may have decreased membrane fluidity and hence reduced receptor recycling.

Other host factors have been observed to contribute to the invasion process, including components of the cytoskeleton. Treatment with cytochalasin D (De Melo *et al.*, 1989; Konkel *et al.*, 1992a), which prevents actin polymerisation, was found to inhibit internalisation by HEp-2 cells and INT407 cells, suggesting microfilaments were involved in the uptake process. The presence of condensed actin has also been observed in association with sites of bacterial attachment (De Melo *et al.*, 1989; Konkel *et al.*, 1992), with host cell pseudopods interacting directly with attached *C. jejuni* cells. Recent work by Wooldridge *et al.*, (1996) has identified caveolae as having a role in invasion. These structures are non-

clathrin coated plasma membrane invaginations whose function can be inhibited by use of filipin III, a sterol binding agent which inhibits transport of ligands across endothelial cells, without affecting transport of other ligands. This caused a reduction in the ability of *C. jejuni* to enter Caco-2 cells, suggesting that caveolae may interact with *C. jejuni* through signal transduction pathways. Inhibition of host cell tyrosine kinases, hetrotrimeric G-proteins or phosphatidylinositol 3-kinase also prevented uptake of *C. jejuni* by Caco-2 cells, suggesting that signal transduction was required for uptake. However, other groups (Russell & Blake, 1994; Oelschlaeger *et al.*, 1993) found that uptake was not inhibited by cytochalsin D. Instead, inhibition of microtubule polymerisation and coated pit formation was found to prevent uptake of *C. jejuni* into INT407 cells. This mechanism may be specific for the cell line, as further work by Russell & Blake (1994) demonstrated that inhibitors of microtubule polymerisation, vincristine and vinblastine, failed to reduce uptake of *C. jejuni* by Caco-2 cells.

1.10 Epithelial Translocation

Campylobacters have been observed to translocate across the epithelial cell barrier (Everest *et al.*, 1992; Konkel *et al.*, 1992b; Grant *et al.*, 1993), allowing *Campylobacter* to translocate through the cell using a cytoplasmic pathway. Although evidence has shown that *C. jejuni* can also pass between tight junctions (Oelschlaeger *et al.*, 1993), which is known as paracellular translocation, without loss of tight junction integrity. The lack of disruption has been reported previously after monitoring the trans-epithelial resistance across the monolayer during *Campylobacter* translocation (Konkel *et al.*, 1992b; Ketley, 1997). This contrasts directly with the translocation of *Salmonella typhimurium* which causes a loss of tight junction integrity (Finlay & Falkow, 1990), which is thought to be due to the pathogen taking an intracellular route through the Caco-2 cell monolayers.

Campylobacter has also been observed to be associated with Peyer's patches in ligated rabbit ileal loops (Walker *et al.*, 1988). These structures are aggregates of lymphoid nodules where antigen and micro-organism sampling may lead to initiation of an immune response. Overlying these nodules are M-cells which efficiently transport antigens across the epithelial barrier to the underlying lymphoid tissue (Madara, 1997). In the ligated ileal loops, *C. jejuni* were found to be selectively attached to M-cells and in the process of translocating to the underlying lymphoid nodules. However, translocation via M cells during *Campylobacter* infection of macaque monkeys was not observed (Russell *et al.*, 1993).

1.11 Intracellular Survival

Following invasion of epithelial cells, campylobacters have been observed in membrane-bound vacuoles within cells (Konkel *et al.*, 1992; Russell & Blake, 1994). Initially *Campylobacter* was observed as a spiral form in the endocytic vacuole within HEp-2 cell monolayers, but after endosome-lysosome fusion the morphology of *Campylobacter* altered, becoming coccoid in appearance (de Melo *et al.*, 1989). The host and bacterial factors which determine the fate of internalised *Campylobacter* are not fully understood. Inhibition of endosome acidification by the addition of monesin did not appear to promote *C. jejuni* survival (Oelschlaeger *et al.*, 1993). However, intracellular reactive oxygen species do appear to affect survival, as intracellular survival of a *sodB* mutant of *C. jejuni* was reduced (Pesci *et al.*, 1994). This enzyme also appears to be important in chick gut colonisation, as a *sodB* mutant demonstrated a decreased colonisation potential in 1-day old chicks (Purdy *et al.*, 1999).

1.12 Animal Models of Infection

Animal models of human disease offer the potential to follow the disease process in an *in vivo* situation and to assay more fully how the pathogen interacts with the host. In addition, these models can be used to identify virulence determinants following genetic manipulation. Currently there is a lack of suitable animal models for campylobacterosis. A variety of models have been evaluated for use, but some, for instance, calves and ferrets were found to be cumbersome and difficult to work with (Firehammer & Myers, 1981; Bell & Manning, 1990). Others have proven expensive, for example primates (Fitzgeorge *et al.*, 1981), or produced inconsistent results between studies, for example, cats and dogs (Prescott & Kamali, 1978). Other models, such as those involving chickens, represent models of colonisation which do not develop the human disease symptoms (Nachamkin *et al.*, 1993). The RITARD (removable intestinal tie adult rabbit diarrhoea) model has been used successfully in demonstrating the changes in histology and immune responses during infection with *C. jejuni* (Caldwell *et al.*, 1983). Although infection was introduced orally in this model, the natural route of infection in man, the model was not able to assess fluid secretion in conjunction with tissue damage. This model has been refined in the RILT (rabbit ileal loop test), where although the infection does not occur through the oral route, the model does allow both tissues and secreted fluids to be collected for histological and biochemical analysis (Everest *et al.*, 1993). Work with this particular model revealed that the isolates of *C. jejuni* tested all induced inflammatory reactions, shortened villi, white cell infiltration and bleeding. However, host-derived secretions were also thought to contribute to the response.

A small rodent model would be ideal for pathogenicity studies, as fewer animals are used and a wide range of immunological reagents are available. Colonisation of laboratory mice at low levels has been reported previously, though levels can be improved following use of

antibiotics (Yrios & Balish, 1986). Recent work by Hodgson *et al.* (1998) has demonstrated high levels of colonisation (approximately 10^8cfu.g^{-1} of faeces) of adult immunodeficient mice, with 10-20 % of the mice becoming ill with diarrhoea. The clinical signs and histopathology observed were similar to those in humans.

1.13 Response of *Campylobacter* to environmental conditions

The ability of *Campylobacter* to persist in the environment outside the host, which may not represent its preferred micro-environment, has many implications. Most enteric campylobacters are unable to grow at ambient temperature or at atmospheric oxygen tensions, preventing replication on food items (Leach, 1997). However, *C. jejuni* must be able to survive these adverse conditions to remain viable and cause infection, though strains may vary in this respect. For example, strains of *Campylobacter* have been shown to differ in their ability to survive incubation in water in batch culture (Jones *et al.*, 1991). Further work has demonstrated that incubation of the water microcosm at 4°C or 10°C greatly enhanced survival compared to incubation at 22°C or 37°C (Buswell *et al.*, 1998). Pathogens can also be exposed to a number of stresses within the host, including extremes of pH, oxygen tensions, iron availability and temperature which must promote adaptation of metabolism to ensure survival.

On encountering certain unfavourable environments *Campylobacter* has been shown to alter its morphology from spiral to coccoid form (Rollins & Colwell, 1986; Boucher *et al.*, 1994; Moran & Upton, 1986; Jones *et al.*, 1993; Hazeleger *et al.*, 1995). This phenomenon also occurs in a number of other micro-organisms including *Helicobacter pylori* and *Vibrio vulnificus* (Benaissa *et al.*, 1996; Oliver *et al.*, 1991). The coccoid form of *Campylobacter* has not been found to be culturable, but may remain viable and possibly retains the capacity

to cause disease. This has lead to the term "viable but non-culturable form" (VNC) being applied (Rollins & Colwell, 1986).

The trigger for the switch in morphology remains unclear. Older cultures from water microcosms contain a higher proportion of coccoid cells, but a range of factors such as nutrient limitation, oxygen tension, pH and metabolic state will have been in a state of constant flux under these culture conditions. Other studies have identified additional potential triggers including exposure to light, aeration, nutrient limitation and toxic oxygen derivatives, such as superoxide anions and peroxides (Rollins & Colwell, 1986; Moran & Upton, 1987a; Boucher *et al.*, 1994).

Viability of the VNC form has been suggested with chemical indicators such as acridine orange and CTC which detect the presence of protein synthesis (Rollins & Colwell, 1986; Beumer, 1992; Boucher *et al.*, 1994). Other studies using indicators such as intra- and extra-cellular ATP levels, and cellular ultrastructure, suggested that the cells were degenerate forms. Similar discrepancies have been shown in studies of the infectious nature of the VNC form, with successful colonisation of mice and chickens being achieved by some workers (Jones *et al.*, 1991; Stern *et al.*, 1994), but no colonisation detected in chickens or rats by others (Medema *et al*, 1992; Beumer *et al.*, 1994).

1.13.1 Iron

Iron is essential for the growth of micro-organisms, acting as a key component of cytochromes and as a co-factor of several enzymes (van Vliet *et al.*, 1998). In the natural environment, iron availability is severely restricted, where it is often present as insoluble ferric hydroxide. Intracellular iron in animals is also tightly restricted, bound to protein molecules such as ferritin, or haemoglobin. The small amounts circulating extracellularly

are bound to host iron-binding and transport proteins, such as transferrin, an iron-binding glycoprotein, and in the blood by lactoferrin present in neutrophils (Otto, 1992). These molecules act to create an iron-limited environment within the host, where the amount of free iron is too small to sustain bacterial growth (Weinberg, 1990).

A number of bacterial mechanisms have been identified for acquiring iron including, siderophore-mediated uptake and the more direct method of non-siderophore mediated interactions. In response to iron limitation, many bacteria secrete siderophores, which possess high affinity ferric-specific ligands to bind extracellular iron. This iron-complex can then be taken up by specific membrane receptors and transport systems in the cell membrane (Neilands, 1981). Two broad types of siderophore have been identified; the phenolates and the hydroxamates, with many enteric bacteria able to synthesis a phenolate known as enterobactin, and a hydroxamate known as aerobactin. It is widely thought that *Campylobacter* does not synthesis its own siderophores (Baig *et al.*, 1986), but scavenges those produced by the indigenous microflora in the gastro-intestinal tract (Field *et al.*, 1986), which it then takes up by its own specific receptors.

Following acquisition of the iron molecule, the pathogen must transport the iron to the inside of the cell. After binding to outer membrane receptors iron is first transported into the periplasm. Transport across the cytoplasmic membrane then occurs via a periplasmic binding-protein-dependent system which utilises ATP as energy (Klebba *et al.*, 1993). In the case of siderophores, the iron is released from the complex in the cytoplasm where it can be utilised by the organism. This type of iron uptake system has been described in many micro-organisms, including *Escherichia coli* (Klebba *et al.*, 1993; Braun, 1995).

As an alternative mechanism, some bacteria can express specific cell surface receptors and obtain iron directly from iron-loaded transferrin or lactoferrin, as is the case for *Neisseria meningitidis* (Simmonson *et al.*, 1982). The lactoferrin or transferrin binds to the surface receptors in an energy dependent process, the iron being subsequently removed, releasing the transferrin (Otto *et al.*, 1992). However, transferrin and lactoferrin did not act as iron sources for a panel of *C. jejuni* strains tested by Pickett *et al.*, (1992).

As a further alternative, some bacteria are able to utilise haem compounds as iron sources, for example, *Neisseria gonorrhoeae*, *Shigella flexneri* and *Vibrio cholerae* (Mickelson & Sparling, 1981; Lawlor *et al.*, 1987; Stoebner & Payne, 1988). Haem compounds are released following tissue damage, whereby the freed erythrocytes are lysed with haemolysins produced by the bacteria. *C. jejuni* was shown to preferentially acquire iron from haemin or haemoglobin complexes (Pickett *et al.*, 1992), and was suggested to produce a haemolysin (Pickett *et al.*, 1992; Hossain *et al.*, 1993), though this was found to be strain dependent. More than one haemolysin molecule was reported to be required to be bound to a single erythrocyte to release the haem compound (Hossain *et al.*, 1993). The expression of the haemolysin was not regulated by the concentration of available iron in the environment, but was thought to be expressed constitutively.

In *Campylobacter*, several outer membrane proteins have been reported to be up-regulated by iron restriction, varying in molecular weight from 71-82 kDa (Field *et al.*, 1986; Goosen *et al.*, 1989; Schwartz *et al.*, 1994). An iron repressible 75 kDa protein, the product of the *cfrA* gene was thought to act as a receptor for siderophores (Guerry *et al.*, 1997). In *C. coli* an enterochelin uptake mechanism was identified with homology to the iron uptake system of *E. coli* (Richardson & Park, 1995). The system, in *C. coli*, consists of two hydrophobic integral membrane proteins, CeuB and CeuC, forming a cytoplasmic

membrane permease, an ATP-binding protein, CeuD, and a periplasmic substrate-binding protein, CeuE. A 36 kDa lipoprotein was identified in *C. jejuni* with homology to other periplasmic iron binding proteins of Gram-negative bacteria (Park & Richardson, 1995).

The availability of iron in the environment not only controls its own uptake mechanism, but allows the co-ordinated expression of virulence determinants, which may further facilitate survival in the host (Crosa, 1997). This is often achieved by the regulatory locus *fur* (ferric uptake regulator). The Fur protein dimerises in the presence of iron and then acts as a DNA binding protein, attaching to a specific sequence, known as a *fur* box, to prevent the transcription of the gene down-stream of the box. Conversely, in the absence of iron, *fur* is unable to dimerise or bind and transcription proceeds (Finlay & Falkow, 1997). This type of regulation has been described in a number of pathogens, including, *E. coli* (Bagg & Neilands, 1987), *V. cholerae* (Litwin *et al.*, 1992) and now *C. jejuni* (Wooldridge *et al.*, 1994). Up to 30 proteins were found to be controlled by *fur* in *E. coli*, whereas only a low number of *fur* regulated genes were identified in *C. jejuni* (van Vliet *et al.*, 1998).

1.13.2 Oxygen

Many pathogens possess mechanisms which act to scavenge and remove the reactive oxygen species produced by oxidative metabolism, and those specifically released during phagocytosis in the host. These include superoxide dismutases (SOD), catalases (Kat) and thiol peroxidases (Tpx) which act to minimise the effects of the reactive molecules as follows (Storz *et al.*, 1990):

$$3O_2^{\cdot-} + 2H_2 \rightarrow 2H_2O_2 + O_2 \text{ catalysed by SOD}$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \text{ catalysed by catalase}$$

$$H_2O_2 + RH_2 \rightarrow 2H_2O + R \text{ catalysed by thiol peroxidase}$$

When the defence mechanisms fail to work effectively the levels of the reactive oxygen species rise in the cell and oxidative stress results. Reactive oxygen species form intracellularly, through incomplete reduction of molecular oxygen, as indicated in the first equation (Imlay & Fridovick, 1992). Extracellular oxygen species may arise following exposure to oxidative bursts generated by PMNs (Grant & Park, 1995), as well as redox-cycling drugs including thiols and paraquat (Farr & Kogoma, 1991). The activities of oxidases and UV irradiation can also create hydrogen peroxide (H_2O_2) and hydroxyl radicals (Gort & Imlay, 1998). In the reduction of H_2O_2, metal ions may act as a catalyst leading to Fenton reactions and the production of damaging hydroxyl radicals, for example,

$$3H_2O_2 + 3Fe^{++} \rightarrow 2OH^- + 2H_2O_2 + 2Fe^{+++}$$

All of these reactive oxygen species are capable of eliciting oxidative damage in many sites. These include peroxidation of membrane lipids and reactions with amino acids converting them into derivatives which when they become incorporated into proteins form inactivated enzymes. DNA can also be damaged by oxygen radicals through strand breakages at purine and pyrimidine sites (Flahaut *et al.*, 1998).

Experimentally, oxidative stress can be induced by the addition of H_2O_2, superoxide radical generators such as paraquat, or by raising the partial pressure of O_2 in a culture (Farr & Kogoma, 1991). In both *E. coli* and *S. typhimurium* separate exposure of cultures to H_2O_2 and superoxide has demonstrated two distinct responses controlled by different regulators, *oxyR* and *soxR*. For example, exposure of *S. typhimurium* to high levels of H_2O_2 caused the expression of 30 proteins which were induced at different rates within 1 h of application (Christman *et al.*, 1989). However, of these proteins only nine of the early expressed proteins were found to be regulated by *oxyR*, acting as a positive transcriptional regulator. Several of the genes which are regulated by *oxyR* have been identified, including *katG* (hydrogen peroxidase 1), *gorA* (glutathione reductase) and *ahpFC* (NADPH-dependent

alkyl hydroperoxidase). OxyR was proposed to bind to the DNA target using a helix-turn-helix recognition motif, initiating transcription of genes (Demple, 1991). In the absence of oxygen, *oxyR* was found to be inactive, but in the presence of oxygen it was active and initiated transcription. Hence, *oxyR* functions in these circumstances as both the sensor and transducer of an oxidative stress signal.

In response to elevated levels of superoxide anions ($O_2^{\cdot-}$), produced by paraquat or menadione, the expression of 33 proteins was induced in *E. coli*, which differed from those expressed in response to H_2O_2 in *E. coli* (Greenberg & Demple, 1989). Further work identified 12 gene promoters which were induced via the *soxRS* system which transcriptionally induced, for example, *sodA* (Mn-SOD), *nfo* (endonuclease IV), *zwf* (glucose 6-phosphate dehydrogenase) and *fumC* (fumerase C)(Demple, 1996).

The proteins induced in *E. coli* and *S. typhimurium* by the two regulators, *soxR* and *oxyR*, act to minimise the damage caused by oxidative stress. SODA acts to reduce the level of superoxide, with glucose 6-phosphate dehydrogenase supplying the reducing power for SODA. Endonuclease IV repairs damaged DNA and fumerase C functions in place of the superoxide sensitive fumerase A (Demple, 1996). Similarly, catalase and AhpFC act to reduce the concentration of H_2O_2 in the cell and AhpFC also reduces the level of peroxidised cellular components, whereas the role of glutathione reductase during exposure to H_2O_2 remains unclear. Other genes and their products which were not under regulation of *soxR* or *oxyR*, were found to be involved in the response to oxidative stress including heat shock proteins and carbon starvation response proteins (Farr & Kogoma, 1991).

Campylobacters are unable to tolerate high oxygen tensions and must possess mechanisms to prevent the creation of oxidative stress and repair mechanism. Two anti-oxidant

enzymes have been identified in *Campylobacter*, SODB (Pesci *et al.*, 1994; Purdy & Park, 1994) and KatA (Grant & Park, 1995). Recent work has also identified the presence of GroEL (Takata *et al.*, 1995) and AhpC (van Vliet *et al.*,1998) which may function as further defence mechanisms in *Campylobacter*. However, it is likely that other mechanisms also exist for this microaerophilic organism (see section 4.1.2)

1.13.3 pH

Alkaline or acidic conditions in the environment can cause significant problems for many bacteria. Acid stress, in particular, can be exacerbated through the combined biological effects of low pH and the presence of weak organic acids in the environment (see section 4.1.2) (Bearson *et al.*, 1997). *C. jejuni* has been shown to survive exposure to pH 4.0, but higher levels of hydrogen ion concentration (pH 2 and pH 3) promoted cell death (Rotimi *et al.*, 1990). The acid stress response of *C. jejuni* has not yet been investigated in depth, but that of *S. typhimurium*, *E. coli* and *H. pylori* have been well studied. In particular, work on *S. typhimurium* has identified a two stage response.

S. typhimurium when grown to exponential phase at pH 7.7, was found to die when the culture pH was altered to pH 3.3. However, if *S. typhimurium* was first altered to pH 5.8, an acid tolerance response (ATR) was initiated, prolonging subsequent survival at pH 3.3 (Foster & Hall, 1990). Changes in protein expression allowed an inducible pH homeostasis mechanism to function within the cell with a proton antiporter system actively pumping out H^+ ions to maintain the balance of internal and external pH (Foster & Hall, 1991). However, in the absence of an effective ATPase the inducible pH homeostasis was found to be unable to function, suggesting that proton translocating ATPases were important in the acid tolerance.

Shifting of the pH from pH 7.7 to pH 4.5, was also found to evoke changes in the protein expression of *S. typhimurium*. These proteins were found to be distinct from those of the ATR and were called acid shock proteins (ASP), and were proposed to be involved in the prevention of the acid denaturation of proteins (Foster, 1991). The ATR has been found to consist of two stages, that is a pre-acid shock response induced at pH 5.8 and post-acid shock response induced at pH 4.5 and below, with the proteins expressed in both stages thought to require separate signals for induction (Foster, 1993).

Three regulatory proteins (RpoS, Fur and PhoP) have been identified, each controlling a distinct subset of ASPs (Bearson *et al.*, 1997). A *fur* mutant of *S. typhimurium* was found to lack an inducible pH homeostasis system which is essential for the induction of the ATR system (Foster & Hall, 1992). The alternate sigma factor (σ^s), encoded by *rpoS*, was found to be essential in sustaining an ATR response in *S. typhimurium*, and this factor was required for the expression of seven ASPs (Lee *et al.*, 1995). In this case, induction of σ^s appeared to be controlled by a second gene, mouse virulence gene, *mviA*, which encodes a 38 kDa protein (Bearson *et al.*, 1996). Regulation of the turnover of the *mviA* gene was found to control the expression of the σ^s and the σ^s in-dependent ASPs. PhoP forms part of a two-component regulatory system in *S. typhimurium* (Véscovi *et al.*, 1994). Mutation within *phoPQ* appeared to cause a reduction in survival in macrophages and an increase in susceptability to low pH (Foster & Hall, 1990), which suggests a role in the response to acidic conditions.

In some environments, the increases in acidity may be more gradual, causing the induction of acid resistance within the organism which does not relate to the ATR system of *S. typhimurium*. This alternative response has been termed acid habituation, involving phenotypic changes which allow survival in conditions that would normally be lethal in non-

habituated cells (Raja *et al.*, 1997). Induction of acid habituation in *E. coli* occurs through changes in the resistance of the outer membrane to the movement of hydrogen ions.

1.14 Genetic control of gene expression

The host-pathogen interactions which occur during infection are a dynamic process, as the pathogen encounters many different and changing environmental conditions (Miller *et al.*, 1989). These changes in location and environment induce adaptive responses by the bacterium, using many sets of genes and operons. The expression of virulence factors often involves global regulatory networks, and may also be categorised as random or non-random. A good example of random control is the antigenic variation of flagella (DiRita & Mekalanos, 1989). Non-random control involves the bacterium sensing its environment and altering the expression of a number of genes as a direct response.

A number of regulatory mechanisms are thought to contribute to the expression of virulence genes. These include the AraC DNA binding transcriptional regulators identified in *E. coli* which controls the arabinose operon. The proteins in this family bind to specific DNA sequences upstream of genes and initiate transcription of the gene (Finlay & Falkow, 1997). In *S. typhimurium*, expression of a transcriptional activator, HilA, was found to be modulated by oxygen, osmolarity, pH and PhoPQ. The level of expression of HilA was found to activate, directly or indirectly, the expression of genes necessary for invasion (Baja *et al.*, 1996). Other DNA binding proteins have been identified, including Fur (see section 1.13.1) and sigma factors, which also act to regulate gene expression (Finlay & Falkow, 1997). The level of supercoiling of bacterial DNA may also regulate gene expression (Dorman, 1991). DNA is normally isolated from a bacterial cell in a negatively supercoiled form, maintained by an ATP-dependent type II topoisomerase called DNA gyrase. The level of DNA supercoiling has been found to respond to environmental conditions, which

affects the transcription of promoters. For example, the level of supercoiling in *E. coli* and *S. typhimurium* was found to be increased following exposure to increased osmolarity and temperature. These types of responses allow pathogens to respond to multiple environmental influences simultaneously through the co-ordinate regulation of expression of many genes.

Another important method of controlling gene expression is the system of signal transduction (Dorman, 1991), which has been found to regulate many bacterial functions. The mechanism is based on phosphotransfer reactions between histidine and asparate residues. A model for this process is a membrane bound protein, possessing an extracellular domain which acts as a sensor to monitor the changes within the environment and an intracellular domain containing kinase activity. On receiving a signal the sensor undergoes autophosphorylation and transfers the phosphate from the phospho-histidine to an asparate residue located in an acidic pocket of the receiver domain (Perraud *et al.*, 1999). This second regulator protein acts as an effector protein which can mediate DNA binding and exerts transcriptional control of target genes (Finlay & Falkow, 1997). Such systems may contain feed back mechanisms which alter the sensitivity to the signal allowing the response to be terminated (Alex & Simon, 1994).

Expression of many bacterial virulence factors is regulated by such two-component systems. The PhoP/PhoQ system in *S, typhimurium* (Véscovi *et al.*, 1994) controls many pathogenic properties including intra-macrophage survival, resistance to host defence antimicrobial peptides and to acid pH. The gene *phoP* encodes the regulator and *phoQ* encodes the sensor protein. Expression of the system has been shown to be controlled directly by magnesium ions (Mg^{2+}). Low levels of these cations cause the sensor kinase, PhoQ, to activate the regulator, PhoP, by phosphorylation.

A similar system occurs in *V. cholerae* known as the ToxR/ToxS system (Skorupski & Taylor, 1997) with ToxS acting as the sensor and ToxR as the regulator. This system controls pathogenic properties such as toxin production, survival within the host and intestinal colonisation. Many different environmental factors have been found to modulate the expression of the ToxR regulon, including temperature, pH and osmolarity (Gardel & Mekalanos, 1994).

It is highly probable that two-component signal transduction systems function in *Campylobacter* enabling it to respond to changes in its environment. Work by Emery *et al.*, (1997) provided evidence for the presence of such a system which had similarities to OmpR of *E. coli*. Also work by Gonzalez *et al.*, (1998) identified a methyl-accepting chemotaxis-like protein which was proposed to function in signal transduction. Following the sequencing of the genome preliminary analysis suggested the presence of several two-component systems in *Campylobacter*. A temperature-dependent signalling pathway was identified through use of this information, designated RacR-RacS, which was found to control the expression of genes important for growth and survival in the intestinal tract of chickens (Brás *et al.*, 1999).

Objectives of this study

Campylobacter continues to cause many cases of gastroenteritis each year in the UK and elsewhere, creating an economic burden on both employers, the NHS and it is a major problem for the food industry. The understanding of how this pathogen causes disease has improved, but many aspects of the disease process remain unclear. Evidence for different virulence factors has been demonstrated both *in vitro* and *in vivo*. However, little work has been carried out on the role of environmental cues in triggering the expression of virulence determinants which may aid survival both within and outside the host, or promote invasiveness. Molecular genetic techniques for *Campylobacter* have improved allowing the creation of knock-out mutants which can be used to confirm the role of particular genes in the pathogenesis of this organism.

Campylobacter has been proposed to interact with epithelial cells in many different ways. However, the relative contribution of different mechanisms of invasion and translocation, and the extent of isolate variation, in these aspects has not been assessed quantitatively. Differences between isolates in the mechanism of epithelial penetration may contribute to different clinical presentations of the disease.

The objectives of this study were:

- To determine whether *Campylobacter* isolates demonstrate quantitatively different invasion phenotypes in Caco-2 cell monolayers.
- To determine the effect of repeatedly exposing *Campylobacter* isolates to Caco-2 cell monolayers, with reference to changes in invasiveness and any correlated changes in protein expression.

- To use well defined continuous culture experiments to determine the effects on *Campylobacter* of three environmental cues - iron limitation, oxidative stress and acid stress, with special reference to the effects on survival, protein expression and virulence.
- To develop knock-out mutants in putative survival and virulence determinants identified from above, and assess their role in environmental persistence and pathogenesis.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals were purchased from Sigma (UK), unless otherwise stated. Restriction endonucleases were purchased from New England Biolabs (UK) or Pharmacia Biotech (UK).

2.2 Antibiotics

When required, antibiotics were added to LB-broth, Mueller-Hinton broth, molten LB-agar or molten Mueller-Hinton agar. Stock solutions were prepared as follows: Ampicillin in sterile water (20 $mg.ml^{-1}$), used at a working concentration of 20 $\mu g.ml^{-1}$ in both broth and agar; Chloramphenicol in ethanol (20 $mg.ml^{-1}$), used at a working concentration of 20 $\mu g.ml^{-1}$ in both broth and agar.

2.3 Bacterial strains and plasmids

Isolates of *Campylobacter* utilised in this study are summarised in Table 1. These were gifts from Prof. T. Humphrey (Exeter PHLS) and Dr. R. Owen (CPHL). The isolates were stored on beads (Prolab) at -70°C. These were revived by plating onto Columbian bilayer plates (bilayer with 10 % (v/v) defribinated horse blood, Oxoid; CBA plates), and were cultured at 37°C with a controlled atmosphere of 4 % (v/v) O_2 and 5 % (v/v) CO_2 (Don Whitley CO_2 incubator). *E. coli* DH5α (Hanahan, 1983) was used as the host strain for cloning experiments, and was routinely cultured on LB-agar at 37°C. Plasmids pMTL20, pMTL23P (Chambers *et al.*, 1988) and pAV35 (gift from Dr D. Purdy) were used during genetic manipulation (see Table 2).

Table 1: Sources and serotypes of *Campylobacter* isolates used in this study

Isolate	Species	Source	Other Information	Serotype[a]
206	*C. jejuni*	Exeter PHL	Clinical isolate	50
235	*C. jejuni*	Exeter PHL	Clinical isolate	11
247	*C. coli*	Exeter PHL	Clinical isolate	56
799	*C. coli*	Exeter PHL	Clinical isolate	56
9519	*C. jejuni*	Exeter PHL	Clinical isolate	59
9752	*C. jejuni*	Exeter PHL	Clinical isolate	50
10392	*C. jejuni*	Exeter PHL	Clinical isolate	6
81116	*C. jejuni*	NCTC	NCTC 11828	6
C677	*C. jejuni*	NCTC	NCTC Letton Hall (outbreak)	27
CH1	*C. jejuni*	Exeter PHL	Chicken skin isolate	1
CH5	*C. jejuni*	Exeter PHL	Chicken skin isolate	1
81116	*C. jejuni*	NCTC	NCTC 11828	6
11168	*C. jejuni*	NCTC	NCTC 11168	2
81-176	*C. jejuni*	NCTC	NCTC 81-176	

[a]The serotypes of the isolates were determined by using the Laboratory of Enteric Pathogens scheme. This is modified from the Penner serotyping scheme for *C. jejuni* and *C. coli*, based on the use of absorbed antisera to heat-stable antigens and utilizing direct whole cell agglutination instead of passive haemagglutination as the detection system.

Table 2: Other bacteria and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/Reference
Strain		
E. coli DH5α	*supE44* Δ*lacU169* (Φ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi- relA1*	Hanahan (1983)
E. coli "One-shot" cells		Invitrogen (Netherlands)
Plasmids		
pMTL20	Ap^R	Chambers *et al.*, 1988
pMTL23	Ap^R	Chambers *et al.*, 1988
pAV35	Ap^R, Cm^R	Dr D Purdy (Yao *et al.*, 1989)
pCR2.1	Ap^R, Kan^R	Invitrogen (Netherlands)

Key: Ap^R, ampicillin resistance, Cm^R, chloramphenicol resistance; Kan^R, kanamycin resistance.

2.4 Growth conditions

2.4.1 Batch Culture

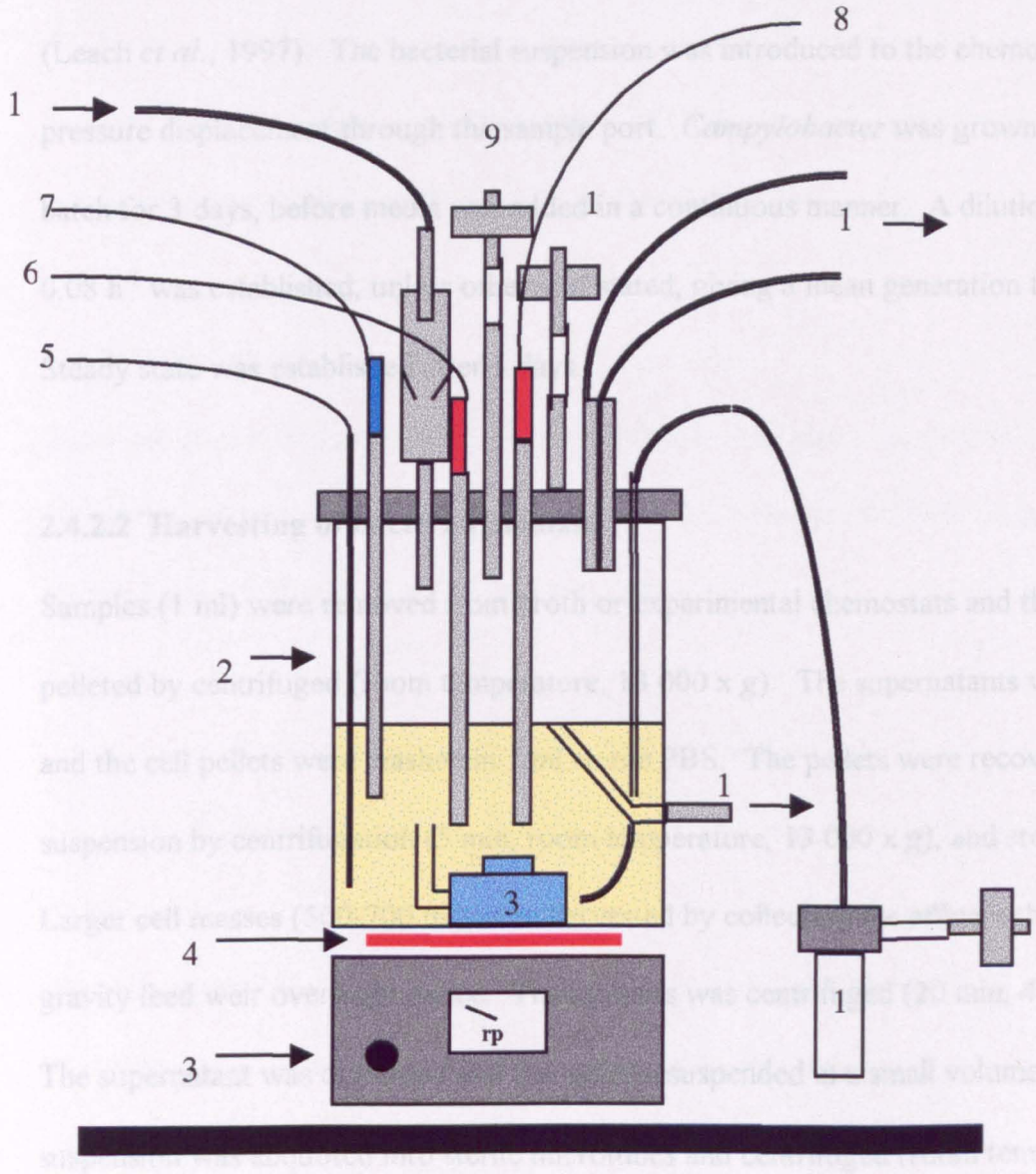
Campylobacter spp. were revived from beads (see section 2.3) and biomass was harvested to form the inocula and resuspended in 10 ml of Cpmod4 broth (see Appendix I) in a 100 ml conical flask, to give a starting optical density of 0.05 at A_{650nm} (see section 2.5). Cultures were incubated at 37°C with shaking (LH orbital shaker, 150 rpm) with a continuous supply of gas containing 5 % (v/v) O_2 in N_2. After 10 h the cultures entered late log phase and were removed to inoculate 10 ml of fresh Cpmod4 broth (1 in 10 dilution). The flasks were returned to the incubator and growth continued for a further 12 h under the same conditions.

2.4.2 Continuous culture

Continuous culture equipment (see Fig. 1) was based on Anglicon controllers (Brighton Systems, Newhaven, UK). Probes to monitor Eh and pH (Ingold, Leicester) were connected to the controller panel, following their calibration at pH 7.0 and pH 4.0, according to the manufacturer's instructions. The level of oxygen in the chemostat was monitored by a galvanic oxygen probe (Uniprobe, Cardiff), following conditioning by autoclaving, and calibration with the controller panel as described in the manufacturer's instructions. The temperature was monitored using a temperature probe connected to the controller panel and maintained at 37°C using a heating pad.

The culture volume was sustained at a working volume of 500 ml of ABCD media (see Appendix I), unless stated, by a gravity feed weir and re-circulated by a magnetic pump. The dissolved oxygen tension (DOT) was maintained at between 2-4 % (v/v), unless otherwise stated using a solenoid valve that allowed oxygen to be released into a continuous stream of anaerobic gas mix (80 % (v/v) N_2, 10 % (v/v) CO_2, 10 % (v/v) H_2).

Fig. 1: Showing the chemostat set up for continuous culture



Key: 1, Feed from medium reservoir; 2, culture vessel with titanium top plate; 3, recirculator pump; 4, heating pad; 5, temperature sensor; 6, redox electrode; 7, oxygen probe; 8, pH electrode; 9, air inlet; 10, vent; 11, acid/alkali addition; 12, effluent outlet to waste reservoir; 13, sample port.

2.4.2.1 Seed Preparation

Inoculum for continuous culture of *Campylobacter* spp. was prepared by harvesting biomass from two CBA plates and resuspending in 5 ml of pre-warmed ABCD medium (Leach *et al.*, 1997). The bacterial suspension was introduced to the chemostat using pressure displacement through the sample port. *Campylobacter* was grown in the vessel in batch for 3 days, before media was added in a continuous manner. A dilution rate of 0.08 h^{-1} was established, unless otherwise stated, giving a mean generation time of 8.7 h. Steady state was established after 5 days.

2.4.2.2 Harvesting of bacterial biomass

Samples (1 ml) were removed from broth or experimental chemostats and the cells were pelleted by centrifuged (room temperature, 13 000 x *g*). The supernatants were discarded and the cell pellets were washed in 1 ml sterile PBS. The pellets were recovered from suspension by centrifugation (5 min, room temperature, 13 000 x *g*), and stored at -20°C. Larger cell masses (500-700 ml) were harvested by collecting the effluent through the gravity feed weir overnight on ice. The biomass was centrifuged (20 min, 4°C, 7000 x *g*). The supernatant was discarded and the pellet resuspended in a small volume of PBS. The suspension was aliquoted into sterile microtubes and centrifuged (room temperature, 13 000 x *g*). The supernatant was discarded and the pellet was stored at -20°C.

2.5 Determination of Optical density

Samples were removed from experimental chemostats, or batch grown cultures and optical density was monitored using a 1 cm light path. Absorbency was read at 650 nm in a SP 6-550 UV-vis spectrophotometer (Pye-Unicam), having been previously blanked on sterile medium.

2.6 Cell counting techniques

2.6.1 Total colony forming units

The number of viable *Campylobacter* cells from a population were determined by plating on CBA plates. Samples (100 µl) were removed and serially diluted 10-fold in PBS. Aliquots (100 µl) were distributed across the surface of CBA plates using sterile spreaders. The plates were incubated for 48 h at 37°C, as described in section 2.3.

2.6.2 Total cell counts

Total cell counts of a population were determined using phase contrast microscopy. Samples were removed and diluted between 10-25 fold in sterile PBS. A small aliquot was counted using a Thoma counting chamber (BDH, UK).

2.6.3 Assay of metabolic activity (CTC/DAPI staining)

The method used was essentially that described previously (Rodriguez *et al.*, 1992). Samples of *C. jejuni* were removed and diluted (10-100) fold in PBS. To 800 µl of the diiluted suspension, 100 µl sodium pyruvate (5 % (w/v) in PBS), 100 µl CTC (4 mM in PBS; Polysciences USA) and 2 µl DAPI (1 $mg.ml^{-1}$; Polysciences USA) were added. The mixture was vortexed and incubated at 30°C for 2 h. An aliquot (100-500 µl, depending on cell density) was removed and mixed with 10 ml of pre-filtered (0.2 µm, Satorious, Germany) sterilised PBS. The resultant suspension was filtered through a Swinex housing containing a black filter (Type GTBP, pore size 0.2 µm; Millipore, UK). The filter was mounted under a coverslip in immersion oil and examined using an U.V. epifluorescence microscope (Nikon Labophot, Nikon, Telford, UK) with a x100 oil immersion objective. For visualisation of the DAPI and CTC labelling, a U.V. -2 A filter block (dichroic mirror, 400 nm; excitation filter 330-380 nm; barrier filters 420nm) and a G-1B filter block

(dichroic mirror, 580 nm; excitation filter 546/10 nm; barrier filters 590 nm) were used respectively. The number of labelled cells within 16 randomly selected calibrated eyepiece field areas (0.024 mm^2) was determined. By correcting the average count obtained by the ratio of the functional filter area (380 mm^2) to the field area, the number of labelled cells in the entire sample was determined.

2.7 Electron Microscopy Sample Preparation

Samples of *C. jejuni* were harvested from the chemostat and fixed in 2 % (v/v) formalin overnight. These were washed in PBS and then applied to formvar/carbon filmed 400 mesh copper specimen grids. The cells were then negatively stained with 1 % sodium silicotungstate, pH 7. Electron micrographs (Philips EM 400T, 100kV) were recorded on Ilford Technical film. EM was carried out by the Electron Microscopy Unit of CAMR Scientific Services.

2.8 *In vitro* assays

2.8.1 Cultured epithelial cells

Caco-2 cells (purchased from ECACC [ECACC no. 86010202]) were maintained in Minimal Essential Media (MEM) with 1 % (v/v) Non-essential amino acids (NEAA), 1 % (v/v) glutamine and 10 % (v/v) Foetal calf serum (FCS) without antibiotics. Cells were routinely grown in flasks at 37°C in a humidified atmosphere with 5 % (v/v) CO_2. The Caco-2 cells were seeded onto a 24-well tissue culture plate (Costar, MA), at a density of 2×10^5 $cell.ml^{-1}$ with the medium described previously. The cells were maintained for seven days in the environment stated previously, with three medium changes to reach confluency prior to use.

2.8.2 Invasion of Caco-2 cell monolayers

The method used was essentially that described by Everest *et al.*, (1992). *Campylobacter* cells were resuspended in MEM + NEAA + 1 % (v/v) FCS (hereafter known as MEM + 1 % FCS) to give 10^8 cell.ml^{-1}. The seeded Caco-2 cells (section 2.8.1) were infected with 0.5 ml of the *Campylobacter* suspension and the wells were incubated at 37°C within a controlled atmosphere (Don Whitley CO_2 incubator). After 3 or 6 h the wells were treated to determine the number of intracellular bacteria. The bacterial suspension was removed and the monolayer was incubated for 2 h with MEM + 1 % FCS containing 250 μg.ml^{-1} gentamicin to kill any extracellular bacteria. The monolayers were washed three times with PBS, to remove the gentamicin. The intracellular bacteria were released by lysing the monolayer with ice-cold 1 % Triton X-100 in PBS. Colony forming units were determined, as described section 2.6.1, on CBA plates. Colony forming units of the inoculum applied to the monolayers was also determined by the same means and the number of intracellular bacteria expressed as a proportion of the inoculum.

2.8.3 Translocation Assay

This assay was based on that of Finlay & Falkow (1990). Caco-2 cells were seeded on to the Transwell units following passage between 8 and 14 times, and maintained as described previously. The cells were added to the apical surface of each Transwell insert (0.4 μm pore, 12 mm diameter [tissue culture treated, Costar, MA]) at a density of 1 x 10^5 cell.ml^{-1} (see Fig. 2). Each insert was placed in a well containing 1.2 ml of fresh MEM + 1 % FCS. The 12-well tissue culture plates were then incubated at 37°C as described in section 2.8.1, with apical and basolateral medium changes every 3 days. Following 7 days of incubation, the monolayers were used to assess the movement of *Campylobacter* isolates.

C. jejuni isolates were grown in batch culture (section 2.4.1) and an inoculum of 10^8 cell.ml^{-1} was prepared in MEM + 1 % FCS. The medium, in the basal compartment of the Transwell unit, was removed and replaced with 1.2 ml of MEM + 1 % FCS. An inoculum of 0.5 ml of the bacterial suspension was added to the apical surface of the Caco-2 cells in duplicate Transwell inserts. The plates were incubated at 37°C as described in section 2.8.1. At regular intervals the Transwell inserts were removed for sampling.

2.8.4 Quantification of bacterial translocation

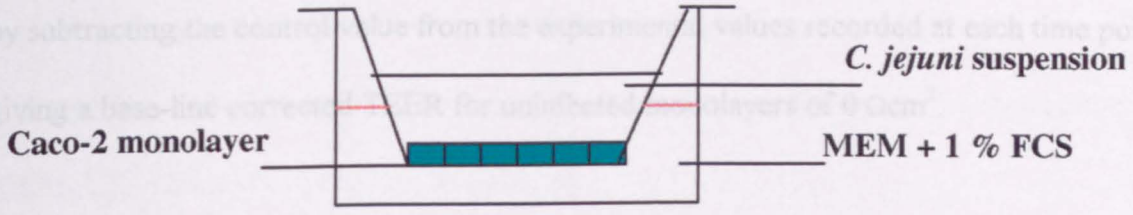
Samples (100 µl) of the tissue culture media were removed from the apical and basal compartments. Colony forming units were determined by serial dilution and plating on CBA plates (see section 2.6.1). The number of *C. jejuni* passing through the monolayer was expressed as a proportion of the bacterial counts in the upper chamber.

2.8.5 Trans-epithelial electrical resistance (TEER) measurements across the Caco-2 cell monolayers

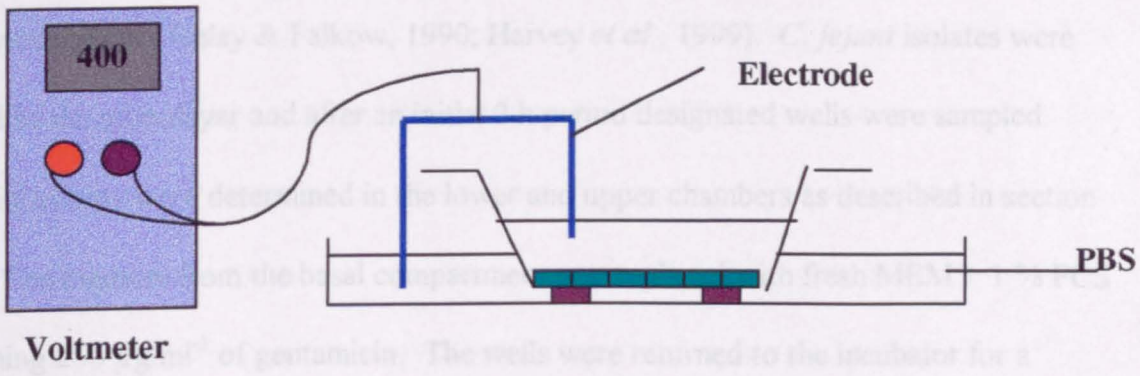
The Transwell inserts were removed at specific time intervals and the TEER was measured with a Milicell-ERS apparatus (Millipore, Bedford, MA) using the "STX-type" electrode (see Fig. 2). Different Transwells were set up in duplicate and used for each time point in order to avoid having to sample single wells repeatedly and risk damaging the monolayers. The TEER of the Transwell inserts was determined following replacement of the culture medium with PBS on both sides of the membrane. The probe legs were placed either side of the unit and each TEER value was measured in triplicate and corrected for filter area. Prior to the start of the experiment, control values of the TEER were determined by measuring uninfected Transwell inserts seeded with Caco-2 cells. Control values averaged 430 $\Omega cm^2 \pm 10$ (95 % CI [n=36]). The TEER of the experimental wells was measured only

Fig. 2: Schematic of sampling of the Transwell insert following infection with *C. jejuni*

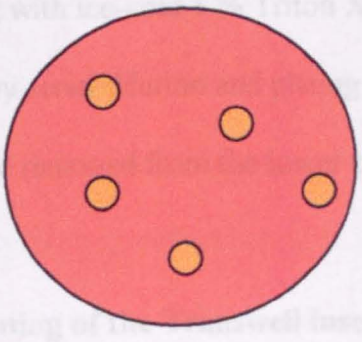
A) Transwell unit placed in a well



B) Measurement of TEER



C) *C. jejuni* recovered by plating on CBA



Note: A, shows the experimental set up of a Transwell unit with the apical surface incubated in the presence of *C. jejuni* and the basolateral surface incubated in medium. B, the TEER was measured across the membrane of the Transwell unit. The electrode legs were placed either side of the unit. C, samples of *C. jejuni* were removed from the apical and basolateral surfaces and recovered by plating on CBA plates

after the required period of incubation with *C. jejuni*, since repeated measurements on individual wells themselves were likely to interfere with monolayer integrity and the TEER. The effect of the *C. jejuni* isolates on the resistance across the monolayer was determined by subtracting the control value from the experimental values recorded at each time point, giving a base-line corrected TEER for uninfected monolayers of 0 Ωcm^2.

2.8.6 Recovery of *C. jejuni* from the Transwell inserts

The number of *C. jejuni* in the Caco-2 cell monolayer was determined by using a modified transcytosis assay (Finlay & Falkow, 1990; Harvey *et al.*, 1999). *C. jejuni* isolates were applied to the monolayer and after an initial 2 h period designated wells were sampled. Bacterial counts were determined in the lower and upper chambers as described in section 2.8.4. The medium from the basal compartment was replaced with fresh MEM + 1 % FCS containing 250 $\mu g.ml^{-1}$ of gentamicin. The wells were returned to the incubator for a further 2 h. After the incubation period the monolayers were washed twice with PBS, before lysing with ice-cold 1 % Triton X-100 in PBS. Colony forming units were determined by serial dilution and plating onto CBA plates (see section 2.6.1). Samples (100 µl) were removed from the lower chamber and plated on CBA plates.

2.8.7 Sectioning of the Transwell inserts

Transwell units infected with *C. jejuni* were removed at 2 and 6 h and fixed in 2 % glutaraldehyde in 0.1 M sodium phosphate buffer overnight. Uninfected wells were also fixed by the same process. The membranes were embedded in paraffin wax and stained with Haemotoxylin and Eosin for light microscopy.

2.8.8 Trypan Blue staining of Caco-2 cells

Trypan blue is used as a viablity stain for eukaryotic cells (Doyle & Griffiths, 1998), with the stain being excluded from viable cells. The Caco-2 cell monolayer was released from wells and lysed with Trypsin (0.05 %) and the cell suspension mixed with an aliquot of Trypan Blue, (0.004 % (w/v) in saline). The cells were counted using a haemocytometer (BDH, UK) using phase contrast microscopy.

2.9 Outer membrane preparations

Approximately 0.5 g (wet weight) of cell mass was resuspended in 1-2 ml of 0.05 mM Tris-HCl, pH 7.5 buffer (Newell *et al.*, 1984). Aliquots (1 ml) of the suspension were sonicated (Sonipreps, Fisons, Crawley) on ice, at maximum frequency for 30 s with a 30 s break; this process was repeated 4 times. The sonicates were centrifuged (20 min, 4°C, 5000 x *g*), the pelleted debris discarded, and the supernatant decanted for ultracentrifugation (2 h, 4°C, 100 000 x *g*). Following ultracentrifugation the supernatant containing the soluble intracellular proteins was decanted and stored at -20°C. The remaining pellet was resuspended in 7 mM EDTA pH 7.6, containing N-lauroylsarcosine, (sodium salt, Sarksoyl) to give a minimum protein:detergent ratio of 1:4. The suspension was incubated for 20 min at 37°C before ultracentrifugation (2 h, 4°C, 100 000 x *g*). The supernatant containing Sarksoyl-soluble inner membrane proteins was carefully removed and stored at -20°C and the Sarksoyl extraction procedure repeated on the pellet and the supernatant removed. The pellet was carefully rinsed with PBS and resuspended in 0.05 mM Tris-HCl, pH 7.5 buffer and ultracentrifuged (2 h, 4°C, 100 000 x *g*). The supernatant was discarded and the washing step repeated a further two times. The pelleted outer membrane preparation was finally resuspended in 300-400 µl of ultra-pure water (Elgast UHQ, High Wycombe) and stored at -20°C.

2.10 Polyacrylamide gel electrophoresis

2.10.1 1-dimensional [1-D] polyacrylamide gel electrophoresis

Electrophoresis of whole cell proteins was carried out allowing the individual proteins in a sample to be separated by molecular weight down a SDS gradient. Samples were analysed using pre-poured Excel Gel SDS gradient (8-18 %) gels and Excel Buffer strips, on a Multiphor II system (Pharmacia Biotech, St. Albans) following the manufacturer's instructions. The cellular material was diluted in water, to give a protein concentration of approximately 1 $mg.ml^{-1}$. Concentrated sample buffer (see Appendix II) was added to give a cell suspension to buffer ratio of 10:1. Freshly made DTT [1.5 µl of 80 $mg.ml^{-1}$ aqueous solution] was added to each sample. These were incubated for 3 min at 95°C. High and low molecular weight standards (Pharmacia Biotech) were similarly treated. The samples and standards were loaded onto application wicks (10 µl/wick) on the gel surface, and run for 75 min at 600 V, 50 mA, 30 W. The gel was stained with pre-heated (60°C) Coomassie Blue R250 (Appendix II) and preserved by covering in a gel preserving sheet for image analysis and photographed.

Electrophoresis of outer membrane preparations was carried out with modifications. Fractions containing the outer membrane preparation were cleaned by ultrafiltration (Ultrafree-MC 10000 NML filters, Millipore), using a microcentrifuge (MSE, Fisons). The volume of the retained high molecular weight protein components was restored with sterile water, and solubilised in sample buffer as described above.

2.10.2 2-Dimensional Electrophoresis [2-D] (Isoelectric Focusing and SDS PAGE)

The technique of 2-D gel electrophoresis allows the individual proteins to be separated on the basis of pI and molecular weight. Initially, samples were separated by their isolelectric

point, whereby a protein become electrically neutral along a linear pH gradient, and in the second dimension the proteins are separated by molecular weight by movement down a SDS gradient.

The Multiphor II system was used according to the manufacturer's instructions, with modifications to improve electrophoresis. The cells were solublized in Lysis Solution A (see Appendix II) to give a protein concentration of 200-600 $\mu g.ml^{-1}$. In the first dimension, isoelectric focusing (IEF) was carried out using Immobiline Dry Strips (pH 3-10L [Linear] 18 cm) (Pharmacia Biotech), which were reswelled in Rehydration Solution overnight (see Appendix II) and placed on the gel plate. Sample cups were positioned over the strips and pressed down to make contact onto the separate strips, and the gel plate was immersed in Silicon Oil (DC200, Fluka). The lysed samples were applied to the cups (50-100 µl) by under-laying and the power supply connected. The power was run initially for 1 h at 500 V, 1 mA and 5 W, followed a further 5 h at 500 V, 1 mA, 5 W and 11.5 h at 3500 V, 1 mA, 5 W.

After the IEF, the strips were either stored at -80°C, wrapped in foil, or equilibrated immediately for the 2nd dimension. The equilibration solution (see Appendix II) was modified to form Solution 1 by the addition of DTT (200 mg/10 ml). Each strip was incubated for 30 min in 20 ml Solution 1, followed by 30 min in 20 ml Solution 2 (Equilibration Solution containing 0.45 g Iodoacetamide/10 ml, Bromophenol blue). The strip was placed face down onto a pre-poured Excel Gel SDS gradient 8-18 % (Pharmacia Biotech) and was run for 30 min at 600 V, 20 mA, 30 W, after which the power was disconnected and the IEF strip removed. The power was reconnected and run for 5 min at 600 V, 50 mA, 30 W, after which the cathodic buffer strip was moved up to cover the

indentation left by the IEF strip. The power was reconnected and left to run for 70 min at 600 V, 50 mA, 30 W. The gel was stained with pre-heated (60°C) Coomassie blue R250 (Appendix II), and preserved by covering in a cellulose sheet for image analysis and photography.

2.10.3 Gel Image Analysis

The gels were analysed using image analysis equipment and software supplied by BioImage (Cheshire, UK). Images of the gels were captured using a Kodak camera and then analysed using "Whole Band Anaylze" and "2-D Anaylze" computer packages running on a Sun Sparc system. During whole band analysis of the 1-D gels, molecular weights were assigned to sample bands by the software through reference to the molecular weight markers. The integrated intensity of each band and each complete lane was calculated. The level of expression of each band was then normalised as a percentage of the integrated intensity of its corresponding lane.

The "2-D analyze" programme automatically detected all major protein spots and overlaid the gels to form a composite figure. To allow for slight shifts in the spot location between gels, well-spaced anchor points were assigned manually. From the composite gel, spots were then identified either as matched or novel to one of the gels. All of the spots on the composite were allocated unique composite numbers. The level of expression of each spot was recorded by area and normalised as a percentage of the integrated intensity of each spot.

2.10.4 N-terminal sequencing of proteins

Proteins were transferred onto Problot (PVDF) membranes by semi-dry electroblotting based on the method of Matsudaira (1987). Protein samples taken from *C. jejuni* following exposure to different environmental conditions were analysed by 2-D gel electrophoresis. Following separation in the second dimension, the gel was disassociated from its backing sheet using a Film Remover (Pharmacia Biotech), before incubation in Electroblotting Buffer for 5 min (see Appendix II). The Problot PVDF membrane (Applied Biosystems, Perkin Elmer, UK) was pre-soaked in methanol before being rinsed in Electroblotting buffer and placed on top of the gel. This sandwich (minus the backing sheet) was transferred to the blotter (TE77, Pharmacia Biotech) containing filter paper (Whatman No 3, Whatman, Maidstone, UK) pre-soaked in Electroblotting Buffer. Three additional sheets of filter paper, of the exact size of the gel, were placed on top of the Problot membrane, before the lid was secured. The proteins were transferred at 0.8 $mA.cm^{-2}$ of gel for 1-2 h. The Problot membrane was removed and stained with Amido Black (see Appendix II) and destained as required. The blot was allowed to air dry.

The N-terminal sequence of proteins of interest was determined by Edman degradation, by CAMR Scientific Services. The protein was excised from a maximum of 5 blots and was sequenced using a Protein Sequencer (ABI Y77A System with a 120B PTH Analyser, Applied Biosytems Inc.). The residues were identified with 610 Software. Database searches, using Swiss Prot and the genome sequence of *Campylobabacter* released on the Sanger center web page, were carried out on the sequences to identify any homologies.

2.11 DNA Analysis

2.11.1 Oligonucleotides

Degenerate oligonucleotides were synthesised on a DNA Synthesizer (ABI 380B, Applied Biosystems) by CAMR Scientific Services, as per manufacturer's instructions. These were designed for use in Southern blotting and PCR reactions.

2.11.2 Extraction of DNA

Genomic DNA was extracted from *Campylobacter* using the method of Pitcher *et al.*, (1989). Biomass was harvested from the *Campylobacter* grown on CBA plates (section 2.3) and resuspended in 1 ml PBS, to give 10^9 cell.ml^{-1}. The suspensions were microcentrifuged (5 min, room temperature, 13 000 x *g*) and the supernatant discarded. The pellets were then resuspended in 100 µl of TE buffer (see Appendix II) and the bacteria lysed with 0.5 ml of GES reagent (see Appendix II). These suspensions were vortexed briefly and checked for lysis after 10 min. Upon lysis, 0.25 ml of ice-cold 7.5 M ammonium acetate was added with mixing to the lysate, and the suspension was retained on ice for a further 10 min. An aliquot (0.5 ml) of a chloroform:2-pentanol (24:1) mixture was added to the suspension prior to centrifugation (20 min, room temperature, 13 000 x *g*). The upper aqueous layer was removed to sterile eppendorfs (1.5 ml) and 0.54 volumes of ice-cold 2-propanol added to precipitate the DNA, which was deposited by centrifugation (5 min, room temperature, 6 500 x *g*). The DNA pellets were washed twice in 70 % ice-cold ethanol, air dried and then resuspended in 100 µl of sterile water before being stored at 4°C.

2.11.3 Quantification of DNA

The amount of extracted DNA was determined by spectrometric methods. Aliquots of DNA were removed and diluted (25-50 fold) in sterile water. The samples were placed in

quartz cuvettes (Pye-Unicam) and the absorbancy read at A_{260nm} using a SP 6-550 UV-vis spectrophotometer (Pye-Unicam). A reading of 1.0 was equivalent to 50 $\mu g.ml^{-1}$ DNA (Sambrook *et al.*, 1989).

2.11.4 Agarose gel electrophoresis

DNA samples treated with restriction endonucleases (section 2.13.1) were analysed by agarose gel electrophoresis according to Sambrook *et al.*, (1989), and carried out using an LKB system 5 or 11-14 gel electrophoresis units (Gibco, UK). A 0.8 % (w/v) agarose gel (Gibco, UK) was prepared in 1x TAE buffer (see Appendix II), containing ethidium bromide (0.5 $\mu g.ml^{-1}$). The gel was submerged in 1x TAE buffer, prior to sample loading. Sample buffer (30 % (w/v) glycerol, 1 % BPB) was added to the samples before loading into the gel by underlaying. In addition molecular weight markers, 1 kb ladder (Gibco, UK) and fluorescein labelled lambda-*Hin*dIII (Amersham, UK), were included. Electrophoresis was carried out generally at 10 Vcm^{-1} for 1½ h. The DNA on the gel was visualised under ultraviolet light, using a Transluminator (Ultraviolet Products Inc) and the image was captured on a Mitsubishi video copy processor or photographed using a Polaroid hand camera containing black and white Polaroid instant film (Sigma).

2.12 Non-radioactive hybridization

2.12.1 Southern blotting of DNA agarose gels

This technique allows the transfer of digested DNA from the agarose gel to a charged nylon membrane (Sambrook *et al.*, 1989). The agarose gel (see section 2.11.4) was prepared by depurination of the DNA in 0.25 M HCl for 20 min, and then denaturation of the DNA in 0.4 M NaOH for 20 min, followed by neutralisation in Neutralisation buffer for 30 min (see Appendix II). Between each incubation the gel was washed with 20x SSC. The DNA was

transferred to Hybond-N+ membrane (Amersham, UK) overnight by capillary action, using 20x SSC and Quick Draw blotting paper (see Fig. 3). The membrane containing the transferred DNA was washed briefly in 5x SSC, prior to fixation of the DNA onto the membrane by laying the membrane on filter paper (Whatman No.3), soaked in 0.4 M NaOH, for 10 min. The blot was then rinsed briefly in 2x SSC, and could be stored at 4°C wrapped in SaranWrap.

2.12.2 Detection with fluorescein labelled oligonucleotides

Oligonucleotides (see Table 16, section 5.2.1; section 2.11.1) including a positive control probe based on the conserved sequence of the 16s rRNA gene (5'-TTA CCG CGG CTG CTG GCA CGT-3') were labelled using the ECL kit (3'-oligolabelling system, Amersham, UK), following the manufacturer's instructions. Briefly, all of the reagents except terminal transferase, were defrosted on ice. In a 160 µl reaction volume the components were added in the following order; oligonucleotide (100×10^{-12} M), 10 µl Fluorescein-11-dUTP, 16 µl cacodylate buffer, 16 µl terminal transferase with water added to make up the final volume (Appendix IV). The solutions were mixed gently by pipette action and incubated for 90 min at 37°C. The labelled probe was stored at -20°C.

The membrane was treated according to the manufacturer's instructions in preparation for hybridization with the labelled probe. The stored membrane was pre-hybridized for 30 min, at 42°C in a hybridization oven (Biometra Hybridization Oven) in Hybridization buffer (see Appendix II) allowing 0.125 $ml.cm^{-2}$ of buffer. An aliquot of the buffer was removed and the labelled oligonucleotide was added at a concentration of 20 $ng.ml^{-1}$, prior to returning the buffer to the tubes. Hybridization was performed for 2 h at 42°C. The membrane was washed twice in an excess of 5x SSC, 0.1 % (v/v) SDS for 5 min at room temperature.

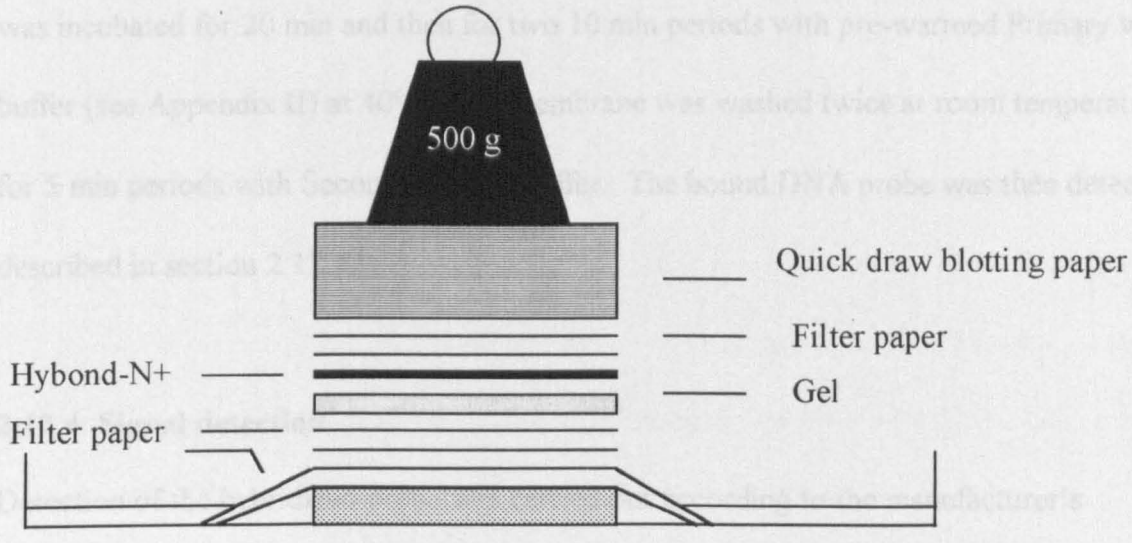
Stringency of the hybridization was controlled through salt concentration during the wash steps. The membrane was incubated for two 15 min periods, with pre-warmed stringency wash buffer (see Appendix II) allowing 0.25 $ml.cm^{-2}$ of membrane, at 42°C in the hybridization oven.

The remaining incubations were then carried out at room temperature. The membrane was rinsed in Buffer 1 allowing 0.25 $ml.cm^{-2}$ of buffer (see Appendix II) for 1 min, before incubating for 30 min in Buffer 1, containing a 20-fold dilution of Liquid Block (Amersham). After rinsing briefly with Buffer 1, the membrane was incubated for 30 min in Buffer 2 allowing 0.25 $ml.cm^{-2}$ of buffer (see Appendix II) containing 0.5 % BSA and 100-fold dilution of anti-fluorescein-HRP conjugate (Amersham, UK). To remove non-specifically bound antibody the membrane was rinsed for 5 min in Buffer 2 repeated for a futher three washes, before detecting any bound labelled oligonucleotide as described in section 2.12.4.

2.12.3 Detection with a peroxidase labelled DNA probe

Probe DNA was amplified from template DNA using specific primers (see section 2.13.8). The DNA was labelled using the ECL kit (Direct Nucleic Acid Labelling System, Amersham, UK), following the manufacturer's instructions. The DNA sample (100 ng) was denatured by heating for 5 min in boiling water, before snap cooling on ice for 5 min. The DNA labelling reagent (10 µl) (Amersham, UK) was added with mixing. The glutaraldehyde solution (10 µl) was added with further mixing and the reaction mixture was incubated at 37°C for 20 min. The labelled probe was held on ice for up to 15 min prior to use.

Fig. 3: Schematic of Southern blotting using the Capillary method



DNA was transfer to the charged nylon, by capillary action. The reservoir was filled with 20x SSC

The DNA on fixed blots was detected using a non-radioactive method developed by Amersham, UK. The blot was treated according to the manufacturer's instructions. The fixed blot was pre-hybridized for 1 h at 40°C in a hybridization oven (Biometra Hybridization Oven) in Hybridization Buffer II, allowing 0.125 $ml.cm^{-2}$ of buffer. An aliquot of the buffer was removed and the labelled DNA probe was added, prior to returning to the tube. The labelled probe was hybridized overnight at the same temperature, after which the membrane was washed twice with 5x SSC at 40°C. Stringency was controlled through the salt concentrations during the following wash steps. The membrane was incubated for 20 min and then for two 10 min periods with pre-warmed Primary wash buffer (see Appendix II) at 40°C. The membrane was washed twice at room temperature for 5 min periods with Secondary wash buffer. The bound DNA probe was then detected as described in section 2.12.4.

2.12.4 Signal detection

Detection of the hybridized probe was carried out according to the manufacturer's instructions. Equal volumes of Detection Reagents 1 and 2 (Amersham, UK) were mixed allowing 0.125$ml.cm^{-2}$. The detection reagents were applied to the side of the membrnae which had been in contact with the gel and left to incubate for 1 min. The membrane was wrapped in SaranWrap prior to exposure to Hyperfilm (Amersham, UK), a blue light sensitive film, for 1 h for the fluorescein-labelled probe and between 5-20 min for the peroxidase labelled probe. The film was developed using an Automated Developer Unit (Compact x 4; Malmesbury, UK).

2.13 Recombinant DNA Techniques

2.13.1 Digestion with restriction endonuleases

C. jejuni DNA was digested with restriction endonucleases according to the manufacturer's instructions. The reaction was carried out as follows unless otherwise stated in 20 µl reaction volume containing 0.5 $\mu g.ml^{-1}$ DNA, 1 Unit restriction enzyme and 2 µl restriction enzyme buffer. The suspensions were mixed and incubated in a water bath according to the manufacturer's specified temperature. The mixture was incubated for a minimum of 5 h, or overnight and stopped by heat shock.

2.13.2 Purification of DNA

DNA was purified from agarose gels (see section 2.11.4) using the Sephaglas BandPrep kit (Pharmacia Biotech, UK) according to the manufacturer's instructions. The gel fragment of interest was excised using a scalpel and the required volume of Gel Solubilizer (1 $\mu l.mg^{-1}$ agarose) was added to the agarose plug and the mixture was incubated for 10 min at 60°C. Sephaglas BP (10 µl) was added to the suspension and incubated for 5 min at room temperature with vortexing every minute, before the suspension was microcentrifuged (5 min, room temperature, 13 000 x *g*) and the supernatant removed. The pellet was washed three times with Wash Buffer (100 µl), containing 90 ml of ethanol. The resulting pellet was left to air dry, after which the DNA was eluted following incubation with 20 µl of Elution buffer. The resulting solution was recovered by microcentrifugation (5 min, room temperature, 13 000 x *g*) and stored at -20°C.

2.13.3 Klenow Reaction

In some instances digestion with certain restriction endonucleases created a recessed 3' termini. To allow ligation (see section 2.13.5) with other gene fragments the recess can be

filled through the action by DNA Polymerase I (Klenow fragment), to form a blunt ended fragment. In the presence of 33 mM of dATP, dTTP, dCTP, dGTP (Perkin Elmer, UK) and 1U of Klenow fragment (T4 DNA polymerase, (Boehringer Mannheim, UK) the digested DNA fragment was incubated for 15 min at 25°C. The reaction was stopped by the addition of 0.5 M of EDTA.

2.13.4 Dephosphorylation

To prevent the linearised vectors, such as pMTL20, from self-ligating in the ligation reaction (see section 2.13.5) the 5'phosphate groups are removed from the single stranded DNA. *E. coli* alkaline phosphatase (1U) (Boehringer Mannheim, UK) was added to the digested sample of vector back bone and incubated at 65°C for 1 h.

2.13.5 Ligation

DNA ligase is used to ligate together two or more fragments of DNA. This enzyme catalyses the formation of a phosphodiester bond between adjacent 5'-P and 3'-OH termini of double stranded DNA. Purified foreign DNA fragments can be ligated into linearised plasmid vector using T4 DNA Ligase (Boehringer Mannheim). The reaction was incubated overnight at 14°C in Ligase Buffer (see Appendix IV).

2.13.6 Transformation

2.13.6.1 Compentent *E. coli* prepared by Calcium chloride

Competent *E. coli* DH5α cells were prepared as described by (Sambrook *et al.*, 1989). A single *E. coli* DH5α colony was harvested from LB-agar plates (see Appendix I) and used to inoculate 10 ml of LB-broth (see Appendix I). The culture was incubated overnight at 37°C in a shaking incubator (LH orbital shaker, 150 rpm). An aliquot (1 ml) of the bacterial

suspension was used to inoculate 50 ml of fresh LB-broth which was returned to the incubator and grown until an optical density, (see section 2.5), of between 0.6-0.9 (A_{450nm}) was achieved. The bacterial suspension was placed on ice for 5 min prior to centrifugation (5 min, 4°C, 3 000 x *g*). The supernatant was discarded and the pellet resuspended in 25 ml 0.1 M magnesuium chloride. The suspension was immediately centrifuged (5 min, 4 °C, 3 000 x *g*) and the supernatant discarded. Finally, the pellet was resuspended in 2.5 ml of 0.1 M calcium chloride and held on ice for up to 24 h.

An aliquot (100 µl) of competent *E. coli* DH5α was placed in a sterile microtube and the ligated suspension was added and held on ice for 30 min. The microtube was transfered to to a pre-heated water bath and incubated for 2 min at 42°C. The microtube was removed and chilled for a further hour. An aliquot of LB-broth (100 µl) was added to the microtube and the suspension was incubated for 1 h at 37°C with shaking to allow expression of the antibiotic marker encoded by the plasmid. Recovery of host cells containing the cloning vectors was carried out by plating onto LB-agar containing a selection antibiotic, IPTG (200 $mg.ml^{-1}$) and X-Gal (20 $mg.ml^{-1}$) and incubated at 37°C.

Selectional plating using an antibiotic marker allows selection of those transformed cells which contain the plasmid. However, colourmetric selection allows colonies containing recombinant plasmids to be distinguished from those which are not in the correct position. Within the polylinker region of the pMTL vectors (Chambers *et al.*, 1988) part of the β-galactosidase gene (*lacZ*) was located, which is not enzymatically active, though whose expression can be induced by IPTG. The host *E. coli* cell, for example *E. coli* DH5α, is also capable of expressing part of the β-galactosidase gene which is unactive. These two forms can become associated to form an enzymatically active protein via intra-allelic

exchange or α-complementation, which in the presence of X-Gal forms a blue colony. However, if the plasmid has been inserted into the polylinker site in the vector, it is unable to express the *lacZ* gene and can not under go α-complementation, causing the colonies formed to be white in colour. Hence those colonies which are grow in the presence of the selectional antibiotic and are white in colour contain a recombinant plasmid.

2.13.6.2 Electro-transformation

Electroporation was carried out according to the method of Wassenaar *et al.*, (1993). *C. jejuni* 11168 and 81-176 were washed four times in Electroporation Buffer (see Appendix II) before a final resuspension in Electroporation Buffer to give 10^{10} cell.ml^{-1}. The suspension was aliquoted (50 µl) into sterile microtubes and stored on ice or at -80°C. To reduce the ion concentration of the plasmid DNA aliquots (see section 2.13.10) were dialysed on a filter disc (0.025 µm pore; Millipore) placed on the surface of sterile water. An aliquot of the plasmid was mixed with *C. jejuni* (50 µl) before adding to a cooled electroporation cuvette (Gene Pulser, 0.1 cm gap, BioRad). Plasmid DNA was electro-transformed into *C. jejuni* using the Gene Pulser Electroporator (BioRad) at 2.5 kV, 200 ohms, 25 µF. Pre-filter sterilsed (0.4 µm, Millipore) Muller-Hinton broth (Oxoid) was then added to the cuvette and the resuspended bacteria were plated onto Muller-Hinton agar and incubated for 5 h at 37°C in microaerophilic conditions. Bacterial growth was harvested by resuspension with 200 µl of Muller-Hinton broth (Oxoid, UK) and used to inoculate Muller-Hinton plates containing a selectional antibiotic. The plates were returned to the incubator for up to one week.

2.13.7 Polymerase chain reaction (PCR)

PCR allows amplification of a segment of DNA between two known regions of DNA sequence. Two oligonucleotide primers are designed which flank the region of DNA to be amplified and are complementary to opposite strands of template DNA. These primers extend from their 3' termini in the presence of deoxyribonucleotides (dNTPs) and a thermostable DNA polymerase, such as Taq to produce a single copy of the gene. Initially, the DNA is denatured by heating to form single stranded templates and then cooled to approporiate conditions to allow the primers anneal. The primers extend with DNA polymerase. The cycle repeats itself with the temperature being raised to denature the primer from its target sequence and is lowered to allow annealing of the primers and extension with DNA Polymerase.

PCR was carried out based on the methods of Mullis & Faloona (1987). *C. jejuni* genomic DNA (see section 2.11.2) from isolates 81-176, 11168 and 81116 were used as templates for amplification. The PCR reaction mixture contained 100 pM of each unique primer (see section 2.11.1; Appendix III, Table 23), 0.2 mM of each dATP, dTTP, dCTP and dGTP (Perkin Elmer), 10 mM Taq Buffer and 2.5 U Taq DNA Polymerase (Perkin Elmer). The reaction was made up to 50 µl with sterile water and gently mixed. A control using all of the components except the genomic DNA which was replaced by water was included. In general the cycling parameters were as described below, unless otherwise stated.

Cycle number	Temperature (°C)	Time
1	94	10 min
2	94	30 s
3	50	30 s
4	72	2 min
5	Repeat stages 2-4 for 30 cycles	
6	72	8 min
7	4	extended time period

2.13.8 Cloning PCR products into plasmid vectors

The PCR products were ligated into the *Eco*RI site of pCR2.1TA, due to the creation of poly A tail in the final stage of the PCR cycle, using the TOPO TA coloning kit (Invitrogen, Version D; Leek, The Netherlands) according to the manufacturer's instructions. Briefly, all of the reagents were defrosted on ice. A total volume of 5 µl contained the following; 10 ng.µl^{-1} of PCR product, sterile water (to give a volume of 4 µl) and 1 µl pCR2.1 (TOPO vector). This mixture was placed on ice whilst 2 µl of β-mercaptoethanol was added to the competent "One-shot" *E. coli* cells, provided with the kit. An aliquot (2 µl) of the reaction mixture was added to the competent cells and gently mixed using a pipette tip. The reaction was held on ice for 30 min, and incubated at 42°C for 30 s. The reaction was returned to ice for a further 2 min, before the additon of an aliquot of SOC media (250 µl), provided with the kit was added to the reaction and incubated for 30 min at 37°C with shaking for 30 min to allow expression of the amplicillin resistance gene. Aliquots were plated onto agar containing ampicillin and X-Gal and grown overnight at 37°C.

2.13.9 Plasmid Purification

Plasmid DNA was isolated from *E. coli* cells using the Wizard Plus Minipreps DNA-Purification System (Promega, UK), following the Durham modification (Promega) to generate sequence grade plasmid DNA. Briefly, a single colony was used to inoculate 15 ml of LB-broth containing an appropriate antibiotic, and at 37°C with shaking overnight. The suspension was centrifuged (5 min, 4°C, 3 000 x *g*) and the supernatant was discarded. The pellet was resuspended in Resuspension Buffer (300 µl), (Promega, UK), prior to the addition of 300 µl of Lysis Buffer (Promega, UK). The suspension was mixed gently by inversion, before the addition of 300 µl of Neutralisation Buffer (Promega, UK) and pelleted by centifugation (5 min, room temperature, 13 000 x *g*). The supernatant was

removed as 2 x 400 µl aliquots into separate tubes. Wizard Resin (500 µl) (Promega, UK) was added to each tube, and the plasmid was collected on the column by vacuum filtration. The column was washed with Column Wash solution (Promega, UK) containing 95 % ethanol, with the remaining wash solution removed by microcentifugation of the column (1 min, room temperature, 13 000 x *g*). The plasmid DNA was recovered by microcentifugation (1 min, room temperature, 13 000 x *g*), after incubating the column for 1 min with warmed sterile water on the bench. The resulting solution was re-applied to the column and re-centrifuged. The purified plasmids were analysed by agarose gel electrophoresis and then stored at -20°C.

2.13.10 DNA Sequencing

Plasmids were sequenced using an Automated DNA Sequencer with an ABI Prism Dye Terminator Cycle Sequence ready reaction kit (Perkin Elmer, UK). Primers were synthesised as before (see section 2.12.1). Computer analysis of the sequence data was carried out using Lasergene software (DNASTAR, Perkin Elmer).

2.14 Statistical Methods

The data sets collected through each experiment were used to calculate the mean and standard deviations. A parametric test, ANOVA with Scheffe's Method, was used to determine the significance of the differences in invasive ability following exposure to different environmental conditions.. A non-parametric test, Kruskal-Wallis, was used to determine the significance of the differences in the invasion, or translocation ability between the *Campylobacter* isolates. Paired comparisons of isolates were made by the Mann Whitney U test. A p value of <0.05 was considered significant.

Chapter 3
Interaction of *Campylobacter* spp. with Caco-2 cell monolayers

3.1.1 Introduction

Enteric pathogens vary greatly in their interactions with mammalian intestinal cells in the process of causing disease in the host (Polotsky *et al.*, 1994). Many of these pathogens have to evade the host defence systems and penetrate the epithelial cell barrier in order to cause disease. This allows these pathogens to exert their physiopathological effects and interact with the mucosa-associated lymphoid tissues (Kernéis *et al.*, 1997). The epithelial barrier can be infiltrated by these invasive enteropathogens by a variety of methods including toxin release, invasion or translocation, or a mixture of all three. The process of translocation involves the pathogen passing through the enterocyte cell layer either by moving through the enteroctye cell itself or by moving between the tight junctions, giving access to the underlying tissues. Many enteroinvasive pathogens have demonstrated an ability to translocate including *S. typhimurium* (Finlay & Falkow, 1990), enteropathogenic *E. coli* (Canil *et al.*, 1993) and *Listeria monocytogenes* (Czuprynski & Balish, 1981).

3.1.2 Penetration of the epithelium

The extent of the disease caused by *C. jejuni* and *C. coli* is thought to be caused by a number of factors, including the state of the host's immune system and the virulence determinants of the pathogen (see section 1.7 and 1.8). It has been proposed that *C. jejuni* adheres to, colonises and invades the small intestinal and colonic muscosa (Allos & Blaser, 1995). Penetration of the intestinal mucosa by intracellular invasion has been reported in infected monkeys (Russell *et al.*, 1993). This resulted in inflammation and infiltration of the lamina propria by neutrophils and bacteraemia, suggesting invasion is an important pathogenic mechanism for *C. jejuni* (Russell *et al.*, 1993). Additional invasive pathogenic mechanisms may also be involved in *Campylobacter* infections since observations by

electron microscopy have demonstrated *C. jejuni* translocating both through and between epithelial cells in culture, with *C. jejuni* located in junctional spaces in some instances (Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993). This suggests both transcellular and paracellular routes are important in *Campylobacter* invasion, though the relative importance of these different routes is not clear (see section 1.9).

Host cells interact with pathogens during infection, for example, it has been demonstrated that *C. jejuni* binds fibronectin, an extracellular matrix protein, via a 37 kDa outer membrane protein known as CadF (Konkel *et al.*, 1997). Metabolically inactived *C. jejuni* have been shown to be able to bind to host cells, but failed to be internalised. Hence, internalisation of *C. jejuni* by host cells probably requires *de novo* protein synthesis (Konkel & Cieplak, 1992). Recent work has identified a 73 kDa protein, CiaB, which was involved in protein secretion by *Campylobacter* during co-cultivation with INT407 cells (Konkel *et al.*, 1999).

Repeated exposure of *Campylobacter* isolates to the intestinal epithelium of the host may cause adaptation to enhance their survival or virulence, through the induction of genotypic, or phenotypic changes. An avirulent strain of *Campylobacter*, LOPEZ, when repeatedly exposed to the chorio-allantoic membrane of chickens, became more able to survive *in vivo* (Field *et al.*, 1993). The colonisation potential of *C. jejuni* 81116 in the chick model was found to be enhanced following a single passage through the avian gastro-intestinal tract (Cawthraw *et al.*, 1997). This suggests that adaptation to an *in vivo* system may enhance characteristics that promote survival and virulence in susceptible hosts.

3.1.3 Cell culture models

Various cell systems have been used to study the mechanisms of *Campylobacter* infection, specifically those of binding, internalisation and translocation (see section 1.9). Such systems have included HEp-2 and INT407 (Konkel & Joens, 1989; Oelschlaeger *et al.*, 1993). However, the enterocyte-like Caco-2 cell line, derived from a human colonic carcinoma is probably more relevant as it represents closely the site of invasion, *in vivo*, in both architecture and function (Grant *et al.*, 1993). Polarised Caco-2 cells differentiate to give characteristic apical and basolateral surfaces, and possess microvilli and markers' characteristic of small intestinal cells (Hidalgo *et al.*, 1989). For example, the expression of the enterocyte marker, alkaline phosphatase, has been found to increase between 3-18 days post confluence (Engle *et al.*, 1998). Caco-2 cells grown on non-permeable tissue culture wells have been used previously for bacterial invasion studies (Conte *et al.*, 1996), including those of *Campylobacter* spp. (Everest *et al.*, 1992). However, when grown on porous membranes, the Caco-2 cells probably model the columnar intestinal barrier more closely, with the cells and intercellular tight junctions separating the medium surrounding the apical surface from that surrounding the basolateral surface (Hidalgo *et al.*, 1989). This model has been used to investigate epithelial cell translocation by a number of pathogens including *Salmonella* (Finlay & Falkow, 1990) and *C. jejuni* (Konkel *et al.*, 1992b).

3.1.4 Aims

Though previous studies have demonstrated the ability of *C. jejuni* to translocate across Caco-2 monolayers (Everest *et al.*, 1992; Konkel *et al.*, 1992), neither the mechanism by which *Campylobacter* invades these cells, nor the relative contribution of paracellular translocation to epithelial penetration in these systems is completely understood. It has also been shown that the more invasive and translocating strains may correspond to those that cause colitis and more severe disease (Everest *et al.*, 1992). However, the relative

importance of the different putative translocation mechanisms was not been clearly quantified. Four different possible interactions between *Campylobacter* and epithelial cells have been proposed (Ketley, 1995), these include non-invasive, invasive without translocation, invasion with translocation and translocation without invasion. To assess the relative importance of the different potential translocation mechanisms used by *Campylobacter* spp., a number of isolates were quantitatively compared for translocation and invasive abilities.

Further, the ability of *Campylobacter* to survive *in vivo* has been shown to be enhanced following passage of strains in two different chicken models. Adaptations that occur during passage may result in phenotypic changes that alter the intrinsic invasive characteristics of an isolate. However, the effect of repeatedly exposing *Campylobacter* isolates to Caco-2 monolayers, which is more relevant to model human disease, has not been investigated. To assess the effects of passage on the intrinsic invasive nature of *Campylobacter*, a number of isolates were repeatedly exposed to Caco-2 cells and their invasive abilities and phenotypic characteristics compared.

3.2 Results

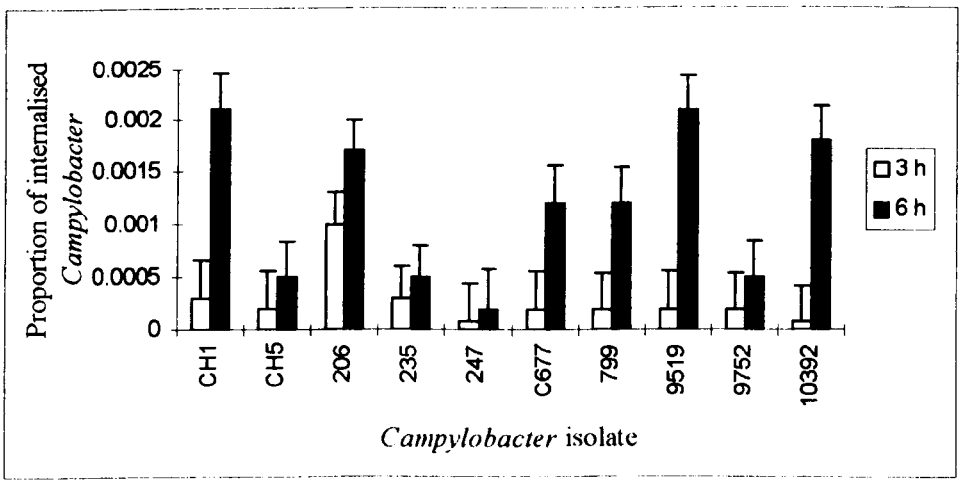
3.2.1 Invasiveness of *Campylobacter* isolates into Caco-2 cell monolayers on non-permeable tissue culture wells

The ability of a panel of *Campylobacter* isolates grown in batch culture (see section 2.4.1) to invade Caco-2 cell monolayers was assessed as described in section 2.8.2. During the 6 h invasion assay, the proportion of *Campylobacter* cells internalised by the Caco-2 cells and protected from the gentamicin, increased with time, with all of the isolates examined (see Fig. 4). The invasive potential of the *C. coli* isolates, 247 and 799, did not differ when compared with *C. jejuni* isolates over the time course. However, by 6 h the isolates differed significantly from one another in their invasiveness, as determined by Kruskal-Wallis ($p=0.002$). Two groups were apparent, one group (CH1, 799, 9519, 10392, C677, 206) was more invasive than the other (CH5, 235, 247, 9752). The kinetics of invasion was also found to differ between the isolates. Some isolates showed a greater proportional increase between 3 and 6 h, for example, 9519 and 10392 than between 0 and 3 h. In contrast, isolate 206 invaded the monolayers to a higher level by 3 h.

3.2.2 Bacterial Translocation across Caco-2 monolayers

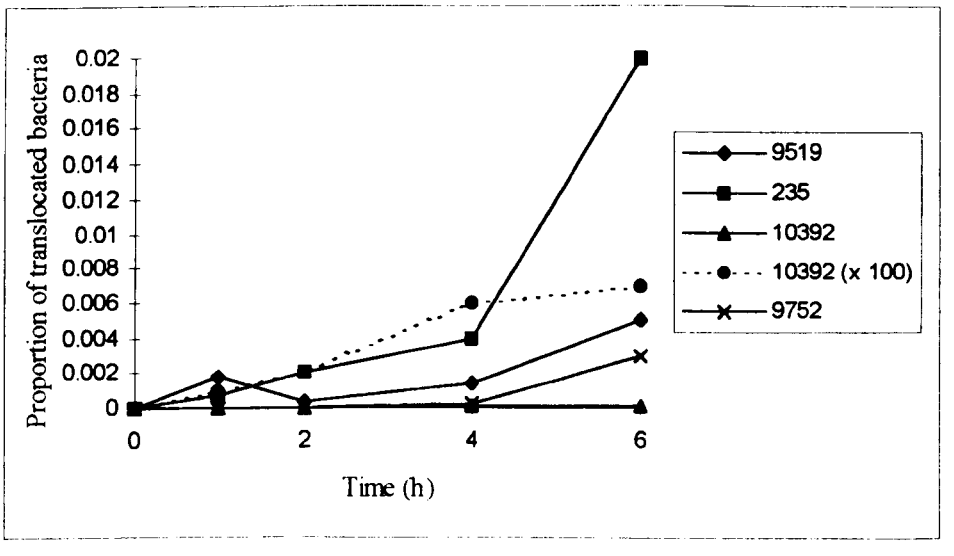
The ability of four isolates (235, 9519, 9752 and 10392) with contrasting invasive ability, were compared in their ability to translocate across the monolayer as described in section 2.8.3. The four isolates examined showed slight growth in MEM + 1 % FCS, the growth rate constants averaging 1.3 h^{-1} for isolate 9519, 1.1 h^{-1} for isolate 235, 0.89 h^{-1} for isolate 9752 and 1.2 h^{-1} for isolate 10392. All of the four isolates translocated across the monolayer into the lower chamber throughout the 6 h of the assay (see section 2.8.4), but to significantly different extents (see Fig. 5). A higher proportion of *C. jejuni* 235 was recovered in the lower chamber by 6 h than any of the other isolates. *C. jejuni* isolates 9519

Fig. 4: The invasiveness of different *Campylobacter* isolates in Caco-2 cell monolayers cultured on non-permeable supports



Data represents the proportion of the inoculum internalised by the Caco-2 cells. Bars represent means ± S.E. (12 separate determinations from three separate experiments). Differences between isolates were highly significant (p=0.002, Kruskal-Wallis non-parametric analysis).

Fig. 5: Comparison of the ability of four isolates of *C. jejuni* of differing invasiveness to translocate across polarised Caco-2 cell monolayers cultured on semi-permeable supports.



Data represents the proportion of the inoculum recovered in the lower chamber. Data points are mean values from six separate determinations from three separate experiments. Differences between isolates were highly significant (p=0.002, Kruskal-Wallis non-parametric analysis).

and 9752 were recovered with 75 % and 85 % respectively fewer cells translocating by comparison to isolate 235 at the same time point. By contrast, isolate 10392 showed a much poorer ability to transolcate, with 99.7 % fewer cells translocating than isolate 235 by 6 h. Analysis of the data using the Kruskal-Wallis test demonstrated that isolates differed significantly in their abilities to translocate (p=0.002). Individual pairs which differed significantly were 10392 vs. 9752 (p=0.02), 10392 vs. 235 (p=0.05), 9752 vs. 9519 (p=0.005), 9519 vs. 235 (p= 0.005) as determined by the Mann Whitney U test.

3.2.3 Changes in the trans-epithelial electrical resistance (TEER) in response to *Campylobacter* infection

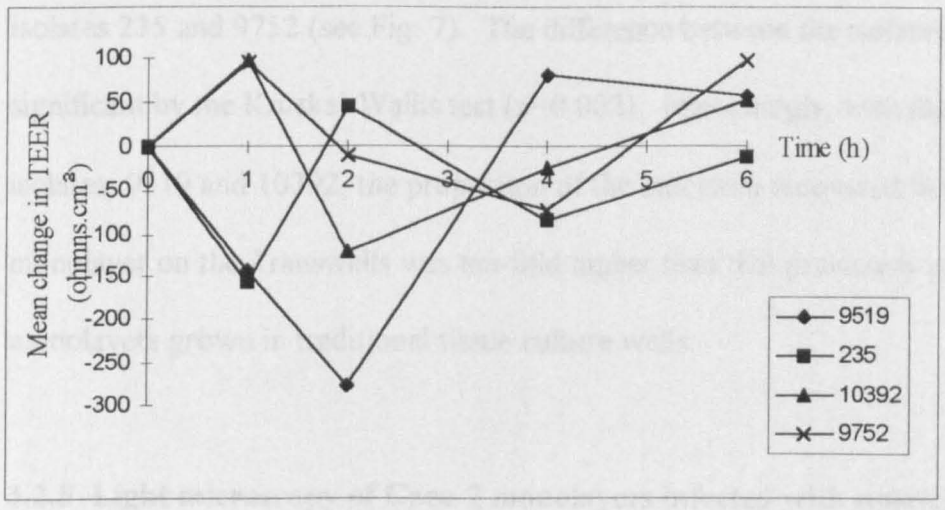
The TEER across the Caco-2 monolayer was monitored as described in section 2.8.5. Infection of the polarised Caco-2 cell monolayer with *Campylobacter* isolates caused some alterations in the electrical resistance across the monolayer (see Fig. 6), indicating the monolayer integrity had altered. The variation within the individual data sets for each isolate prevented statistically significant conclusions being drawn from the measurements.

However, one interesting trend was observed. Infection with isolate 9519 caused a rapid decline in TEER across the monolayer by 2 h, followed by recovery over the remaining 4 h, this being the only consistent trend between replicate experiments. With the other isolates, the TEER appeared to change less dramatically showing smaller fluctuations around the base-line TEER recorded across the non-infected monolayers.

3.2.4 Recovery of *C. jejuni* from within Caco-2 monolayers grown on permeable filters

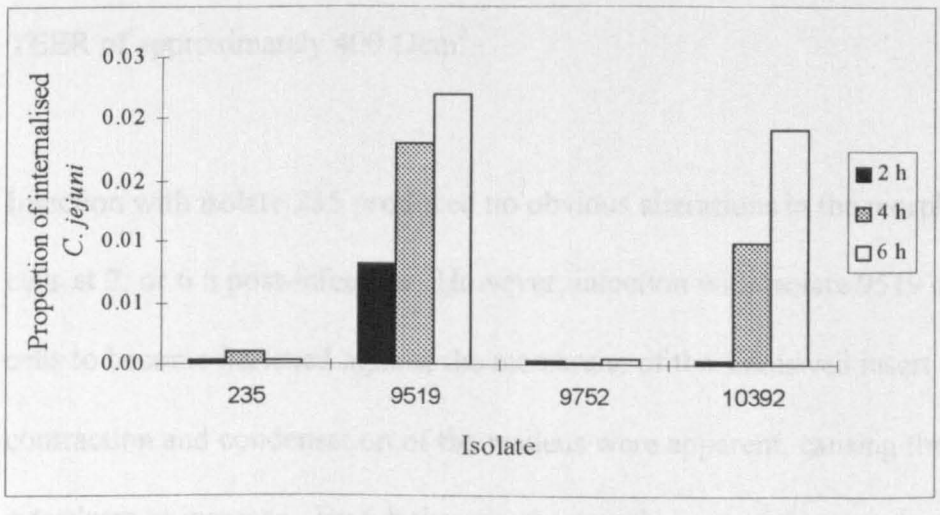
C. jejuni were recovered from the Caco-2 cell monolayers grown on Transwell inserts as described in section 2.8.6. The inserts were incubated with gentamicin for 2 h prior to

Fig. 6: Changes in TEER across polarised Caco-2 cell monolayers cultured on semi-permeable supports following infection with four isolates of *C. jejuni* of contrasting invasiveness.



Data represents the mean of 18 replicate determinations from three separate experiments, corrected for the TEER of control uninfected monolayers

Fig. 7: Comparison of the intracellular invasion of Caco-2 cells cultured on semi-permeable supports by the four different isolates of *C.jejuni*



Data represents the proportion of the inoculum recovered from within the Caco-2 monolayer. Differences between isolates were very highly significant (p=0.002, Kruskal-Wallis non-parametric analysis).

sampling to remove any extracellular bacteria. The number of internalised bacteria recovered was expressed as a proportion of the inoculum. A higher proportion of *C. jejuni* isolates 9519 and 10392 were recovered from within the monolayers by comparison with isolates 235 and 9752 (see Fig. 7). The difference between the isolates was statistically significant by the Kruskal-Wallis test ($p=0.002$). Interestingly, with the more invasive isolates, 9519 and 10392, the proportion of the inoculum recovered from within the monolayer on the Transwells was ten-fold higher than that previously recorded from the monolayers grown in traditional tissue culture wells.

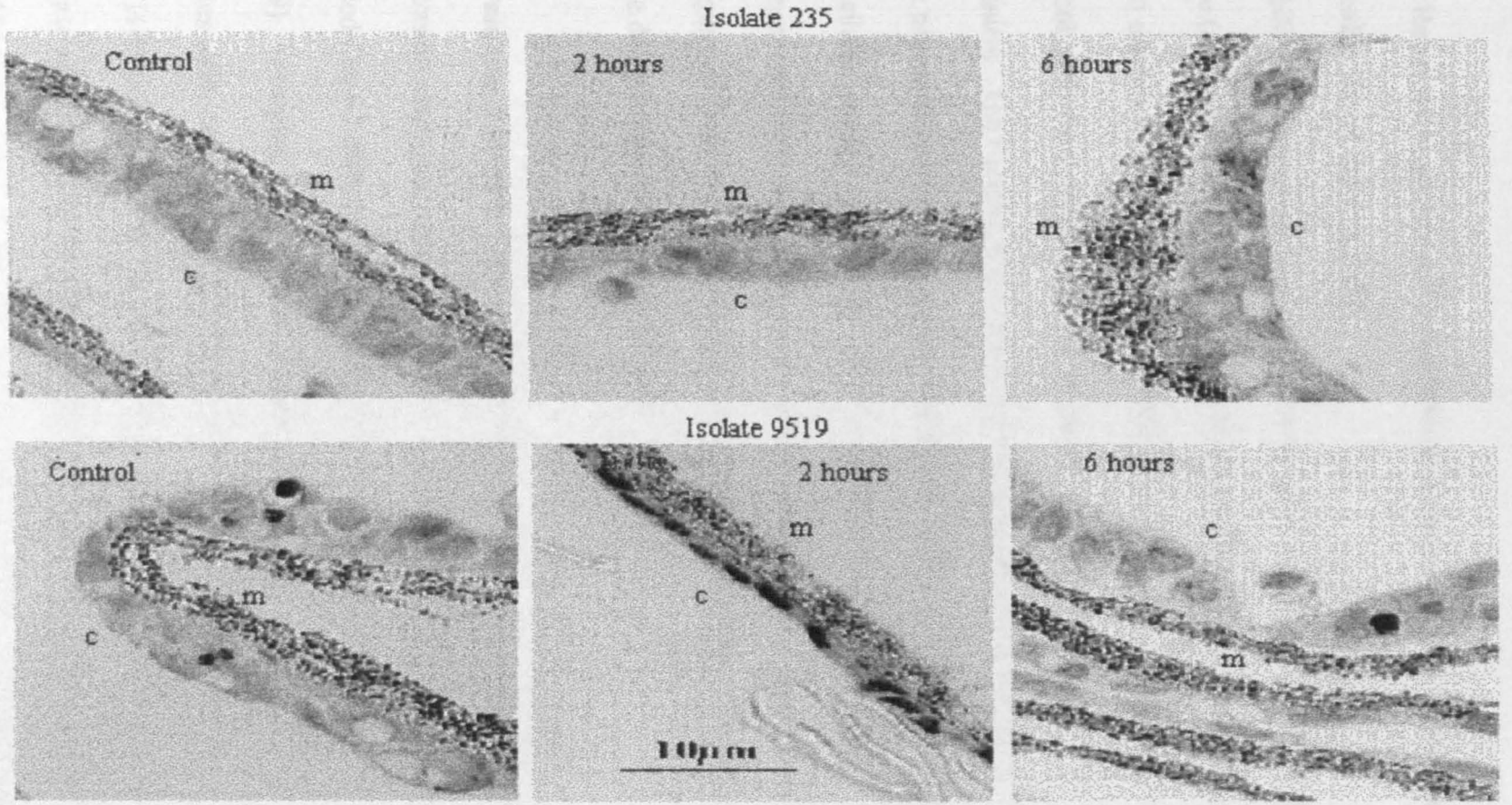
3.2.5 Light microscopy of Caco-2 monolayers infected with isolates 235 and 9519

The Transwell inserts seeded with Caco-2 cells were removed and stained with Haemotoxylin and Eosin to observe changes in morphology of the enterocytes as described in section 2.8.7. The Caco-2 cells, incubated with sterile medium, adhered to the permeable filters forming confluent monolayers. The cells exhibited a closely-juxtaposed columnar morphology consistent with that described for the polarised epithelium (see Fig. 8), with a TEER of approximately 400 Ωcm^2.

Infection with isolate 235 produced no obvious alterations in the morphology of the Caco-2 cells at 2, or 6 h post-infection. However, infection with isolate 9519 caused the Caco-2 cells to become flattened against the membrane of the Transwell insert by 2 h. Cytoplasmic contraction and condensation of the nucleus were apparent, causing the ratio of nucleus to cytoplasm to increase. By 6 h though, the morphology of the monolayer had recovered to its original state.

Monolayer confluency and integrity, as determined by light microscopy from the apical side, appeared to remain complete during infection with both of the isolates throughout the assay

Fig. 8: Comparison of changes in Caco-2 cell morphology during translocation by *C. jejuni* isolates 235 and 9519



Representative micrographs of sections across Transwell insert membranes (m) showing the typical columnar morphology of control and infected cells (c) in all cases except for isolate 9519 at 2 h. Here significant flattening of cells and condensation of nuclei in cells was observed.

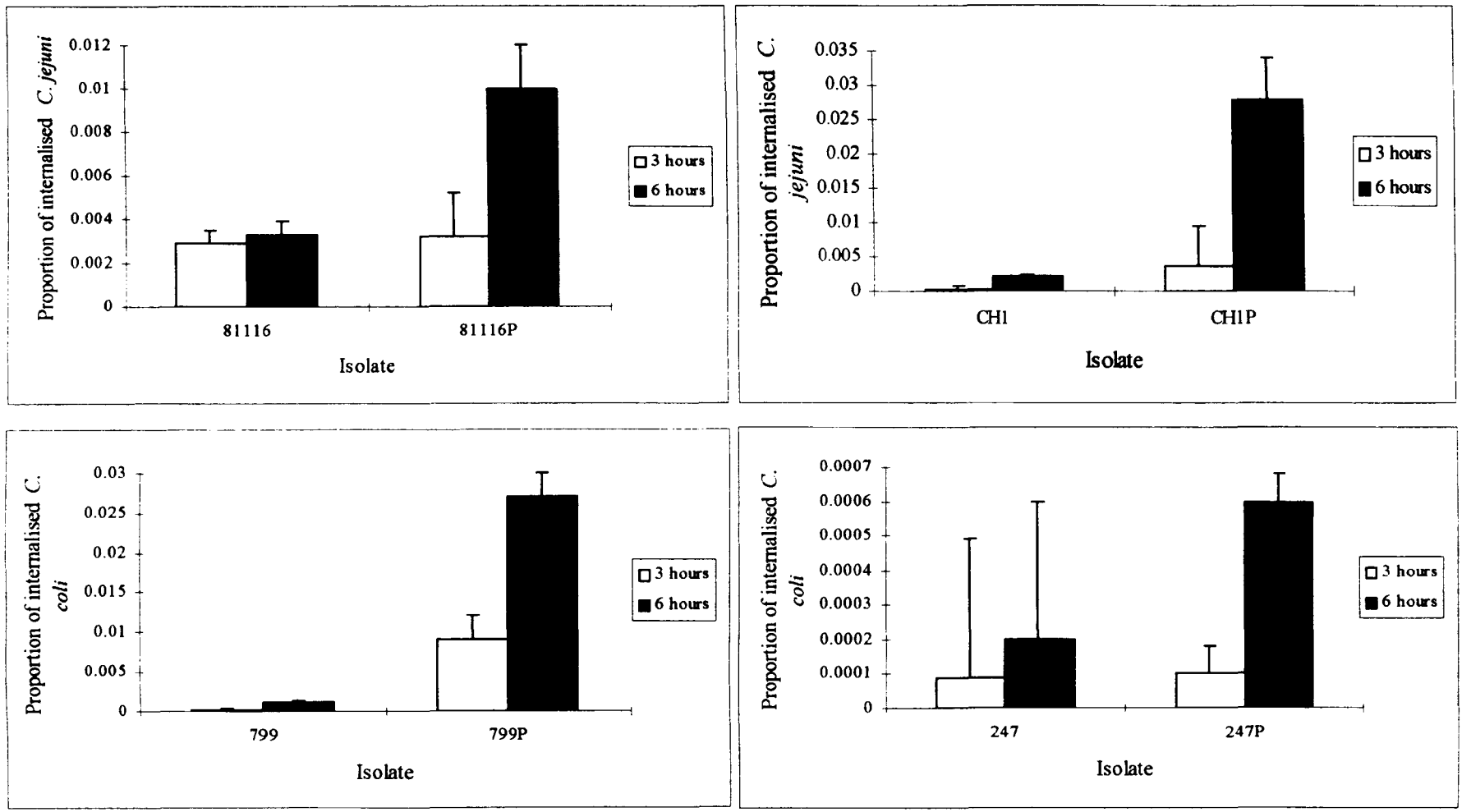
period. Staining of the infected Caco-2 cells with Trypan Blue (see section 2.8.8) resulted in only 5 % uptake of the stain in all cases. This demonstrated that the proportions of viable Caco-2 cells recovered from the monolayers at each time point during infection, with either isolate, did not change significantly throughout the experiment.

3.2.6 Repeated exposure of *Campylobacter* isolates to Caco-2 monolayers on non-permeable tissue culture wells

The ability of four isolates of *Campylobacter* (81116, CH1, 799, 247) grown in batch culture (see section 2.4.1) to repeatedly invade and be recovered from Caco-2 cells, was carried out based on the method described in section 2.8.2. Modifications were made to the protocol, with the isolate being recovered after 3 h of invasion, and 2 h incubation with gentamicin. The bacteria were recovered on CBA plates and frozen on Prolab beads. These passaged isolates were subsequently used to form the inoculum for further intracellular passage experiments. This was repeated until each isolate had been exposed and recovered from the monolayer for a total of eight times. Passaged isolates were distinguished from the non-passaged isolates by the addition of a trailing "P" to the original isolate designation, for example 81116P.

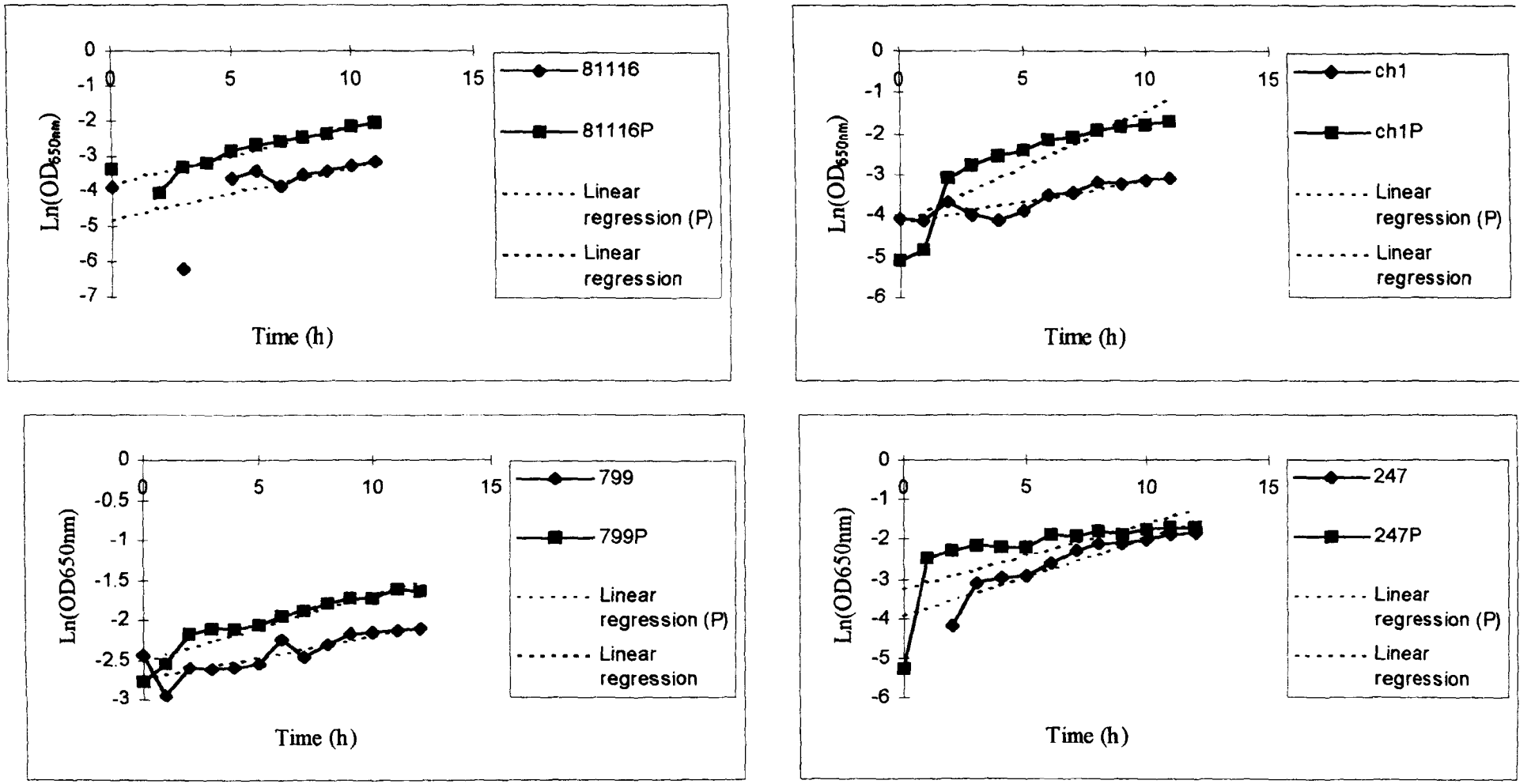
The resulting passaged *Campylobacter* isolates were compared with the original isolates in an invasion assay (as described in section 2.8.2). Both passaged and original isolates invaded Caco-2 cells and were recovered in increasing proportions over the 6 h period (see Fig. 9). Passaging was observed to significantly increase the invasive ability of two of the isolates when compared to the original isolates as determined by Kruskal-Wallis test (CH1 vs. CH1P, $p=0.0006$; 799 vs. 799P, $p=0.04$). The other two isolates were not found to increase significantly in their invasive ability (247 vs. 247P, $p=0.07$; 81116 vs. 81116P, $p=0.4$).

Fig 9: Comparison of the invasive ability of the orginal and passaged *Campylobacter* isolates in Caco-2 cell monolayers



Data represents the proportion of the inoculum internalised by the Caco-2 cells. Bars represent ± SE (3 separate experiments). The difference between the invasive ability of isolates was found to be significant by ANOVA for CH1 ($p=0.0006$) and 247 ($p=0.04$).

Fig 10: Growth of original and passaged *Campylobacter* isolates in Cpmod4.



Note: The increase in biomass was monitored by optical density with absorbance read at A_{650nm}. Data ln transformed, with a linear regression line plotted to show growth rate of the culture.

3.2.7 Growth curves of passaged *Campylobacter*

Liquid batch cultures of the original and passaged campylobacters were prepared in Cpmod4 broth, as described in section 2.4.1, with the growth of the cultures monitored by changes in optical density as described in section 2.5. The isolates demonstrated a slight variation in growth rate in Cpmod4, with the growth rate constants averaging $1.16h^{-1}$ for 81116, 1.17 h^{-1} for 81116P, $1.11h^{-1}$ for CH1, 1.32 h^{-1} for CH1P, 1.22 h^{-1} for 247, 1.18 h^{-1} for 247P, 1.06 h^{-1} for 799 and 1.09 h^{-1} for 799P (see Fig. 10). Microscopic observation of all of the isolates demonstrated motile cultures with rapid swimming.

3.2.8 Changes in protein expression following passage of *Campylobacter* isolates

Whole cell proteins of original and passaged isolates of *Campylobacter* were analysed by 2-D gel electrophoresis and stained with Coomassie Blue as described in section 2.10.2. The gels were analysed by the BioImage system in corresponding pairs, i.e. original and passaged isolate, as described in section 2.10.3.

Many proteins were identified as novel either to the original or to the passaged isolates. However, only proteins with an integrated intensity greater than 0.1 were considered further in this analysis, as proteins of lower integrated intensity were not always clearly resolved on the gels. Under these more stringent conditions 11 novel proteins were identified in CH1 (composite nos. 27, 30, 43, 92, 95, 109, 143, 172, 187, 192, 222) and 11 novel proteins were identified in CH1P (composite nos. 225, 253, 265, 266, 271, 301, 343, 344, 354, 366, 375) (see Fig. 11; Table 3). In isolate 799, 18 novel proteins were identified (composite nos. 8, 68, 72, 94, 95, 127, 141, 143, 149, 152, 153, 160, 161, 232, 259, 277, 311, 319) and 18 novel proteins were identified in 799P (composite nos. 345, 346, 354, 357, 362, 373, 374, 394, 408, 409, 416, 422, 436, 440, 451, 456, 466, 489) under the same stringent conditions (see Fig. 12; Table 4). Seventeen proteins were identified as novel in isolate 247

(composite nos. 1, 4, 8, 16, 95, 115, 120, 138, 142, 168, 170, 174, 222, 276, 280, 281, 283) and eight proteins in isolate 247P (composite number nos. 297, 301, 333, 335, 337, 347, 360, 363) (see Fig. 13; Table 5). In isolate 81116, four novel proteins were identified (composite nos. 18, 19, 82, 137) and five proteins in 81116P (composite nos. 202, 204, 219, 230, 245) (see Fig. 14; Table 6).

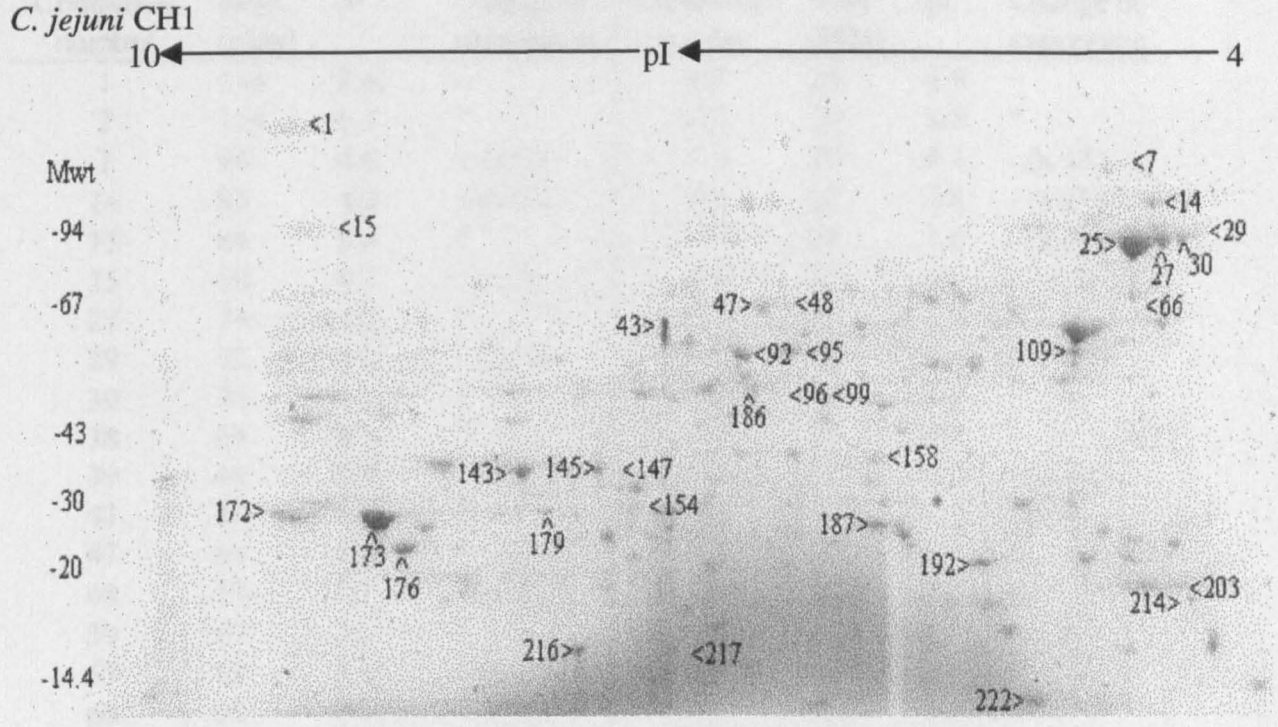
The remaining proteins were matched between the pairs of original and passaged isolates. These matched proteins also responded to passage by up- and down-regulation of their level expression. Of the 12 proteins matched between isolates 81116 and 81116P, nine were up-regulated between 2-3 fold (including composite nos. 34, 66, 149). Seven proteins were identified as up-regulated between 3-4 fold (composite nos. 31, 80, 89, 90, 129, 132, 179) and five proteins were up-regulated more than 4-fold (composite nos. 30, 68, 148, 150, 168). Six proteins were down-regulated between 2-3 fold (including composite nos. 94, 116, 147) and three proteins were down-regulated more than 3-fold (composite nos. 1, 5, 95). The remaining proteins (91) did not alter in their expression (including composite nos. 8, 14, 55, 72, 130, 141, 140).

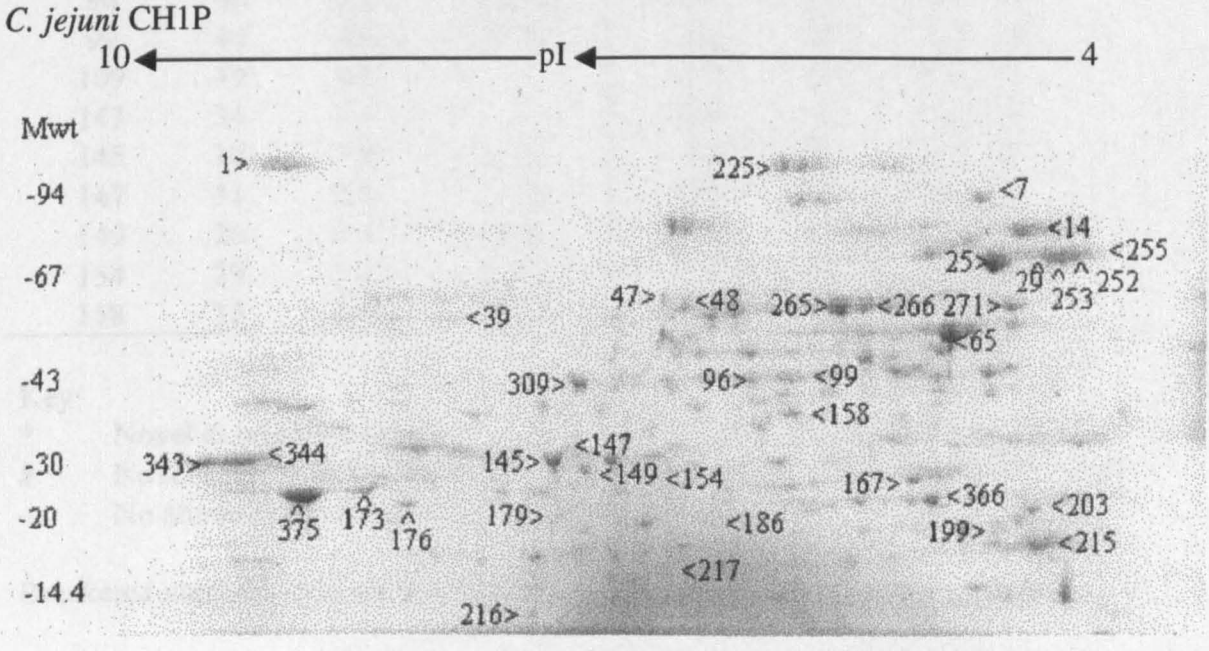
The 61 proteins matched between isolates CH1 and CH1P also demonstrated a similar response. One protein (composite no. 25) was up-regulated between 2-3 fold. Five proteins were up-regulated between 3-4 fold (composite nos. 7, 14, 29, 99, 145) and a further five proteins were up-regulated more than 4-fold (composite nos. 47, 96, 147, 158, 215). Proteins were also down-regulated, including four proteins which were down-regulated 2-3 fold (including composite nos. 48, 186). Two proteins were down-regulated between 3-4 fold (composite nos. 66, 154) and seven proteins were down-regulated more than 4-fold (composite nos. 173, 176, 179, 203, 214, 216, 217). The remaining 36 matched proteins did not alter in expression.

A total of 61 matched proteins were identified from isolates 799 and 799P. Seven proteins were up-regulated between 2-3 fold (including composite nos. 182, 300). Six proteins were up-regulated between 3-4 fold (composite nos. 35, 126, 183, 226, 253, 304) and five proteins were up-regulated more than 4-fold (composite nos. 91, 124, 154, 159, 193). Down-regulation of between 2-3 fold was observed in five proteins (including composite nos. 66, 309). Three proteins were down-regulated 3-4 fold (composite nos. 229, 297, 308) and eight proteins were down-regulated more than 4-fold (composite nos. 147, 168, 228, 231, 279, 284, 298, 312). The remaining 27 matched proteins were unaltered in their expression following passage.

Of the 156 matched proteins of isolates 247 and 247P, 18 were up-regulated between 2-3 fold (including composite nos. 132, 155). Four proteins were up-regulated between 3-4 fold (composite nos. 152, 172, 216, 246) and one protein was up-regulated more than 4-fold (composite no. 150). Nine proteins were down-regulated between 2-3 fold (including composite nos. 139, 247, 265) and one protein was down-regulated between 3-4 fold (composite no. 37). The remaining 123 matched proteins remained unaltered in their expression.

Fig 11: 2-Dimensional gel electrophoresis of whole cell proteins of original and passaged isolates of *C. jejuni* CH1





Whole cell proteins were harvested from cultures of both *C. jejuni* populations and were focused in the first dimension on IEF strips pH 3 –10. Proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system and protein composite numbers were assigned. Mwt markers (kDa) and pI are indicated on the gels.

Table 3: Comparison of proteins identified by 2-D gel electrophoresis of *C. jejuni* CH1 before and after passage

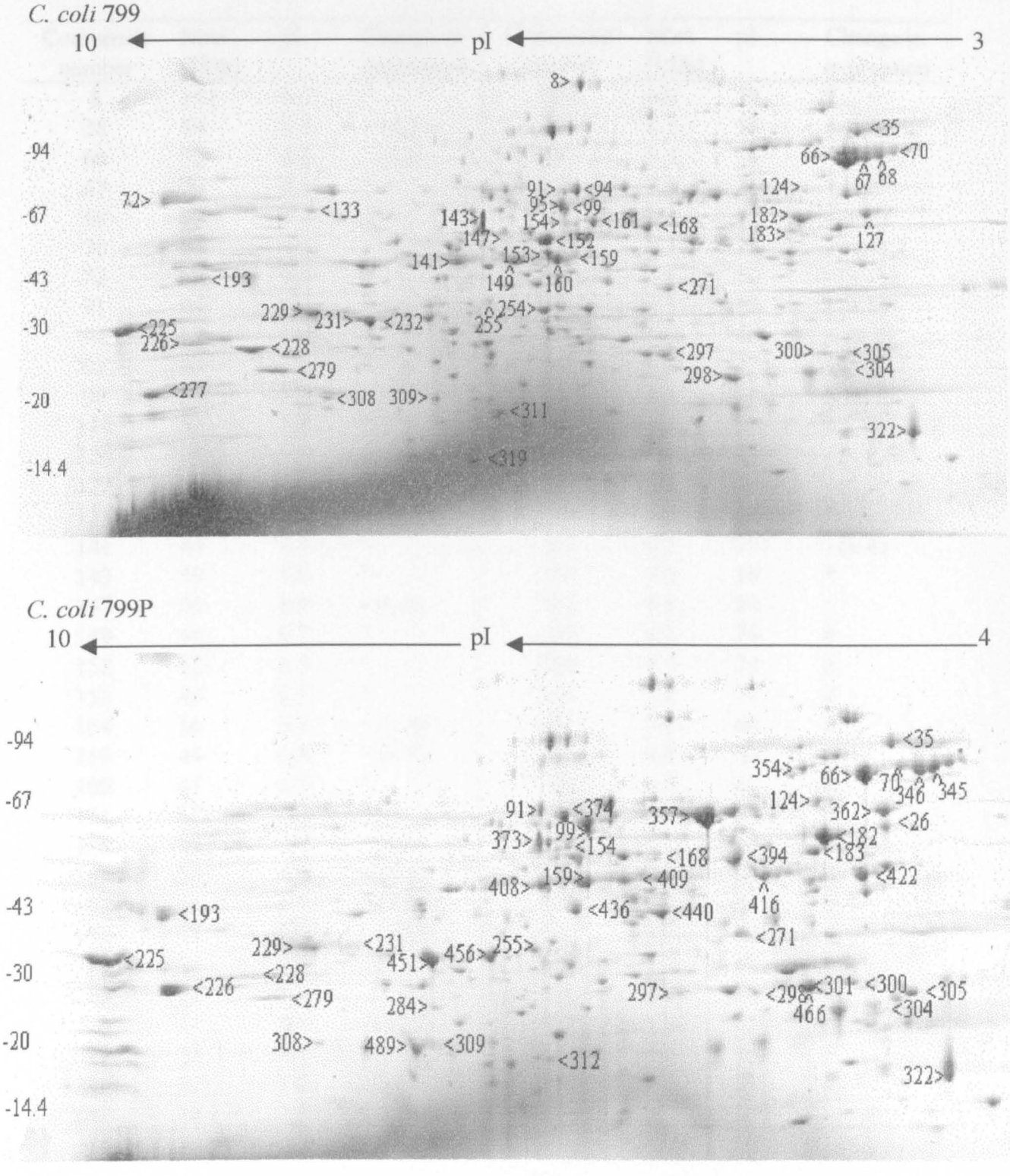
Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
1	116	8.6	~	167	28	4.9	~
2	114	6.1	*	172	30	8.6	*
7	94	4.6	+ (x 3)	173	27	8.1	- (x 11)
14	81	4.3	+ (x 3)	176	20	7.8	- (x 29)
15	84	8.6	*	179	23	7.0	- (x 6)
25	50	4.7	- (x 2)	186	23	6.1	+ (x 2)
27	74	4.2	*	187	27	5.7	*
29	72	4.2	+ (x 3)	192	23	5.1	*
30	74	4.1	*	196	30	5.0	~
38	64	8.2	*	199	23	4.4	~
39	49	7.4	- (x 2)	203	25	4.2	+ (x 6)
43	53	6.8	*	214	20	4.4	- (x 4)
47	60	6.4	+ (x 4)	215	19	4.1	*
48	59	6.3	- (x 2)	216	14.4	7.0	#
59	67	5.6	*	217	17	6.6	- (x 4)
65	59	5.0	~	222	14.4	4.9	*
66	60	4.5	- (x 3)	225	111	5.7	#
82	46	7.1	*	253	72	4.1	#
92	47	6.3	*	252	81	4.0	#
95	47	6.1	*	261	30	4.8	#
96	40	6.1	+ (x 14)	265	57	5.4	#
99	40	5.9	+ (x 3)	266	58	5.2	#
109	49	4.6	*	271	58	4.4	#
143	34	7.5	*	301	39	6.9	#
145	34	7.0	+ (x 3)	343	30	9.0	#
147	31	6.9	+ (x 5)	344	30	8.9	#
149	26	6.7	+ (x 2)	354	30	6.7	#
154	29	6.4	+ (x 3)	366	27	4.8	#
158	35	5.8	+ (x 15)	375	25	8.5	#

Key:

*	Novel in original isolate	+	up-regulated as a result of passage
#	Novel in passaged isolate	-	down-regulated as a result of passage
~	No alteration in expression		

Bracketed numbers indicate the level of change in expression

Fig 12: 2-Dimensional gel electrophoresis of whole cell proteins of original and passaged isolates of *C. coli* 799



Whole cell proteins were harvested from cultures of both *C. coli* populations and were focused in the first dimension on IEF strips pH 3 –10. Proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system and protein composite numbers were assigned. Mwt markers (kDa) and pI are indicated on the gels.

Table 4: Comparison of proteins identified by 2-D gel electrophoresis of *C. coli* 799 before and after passage

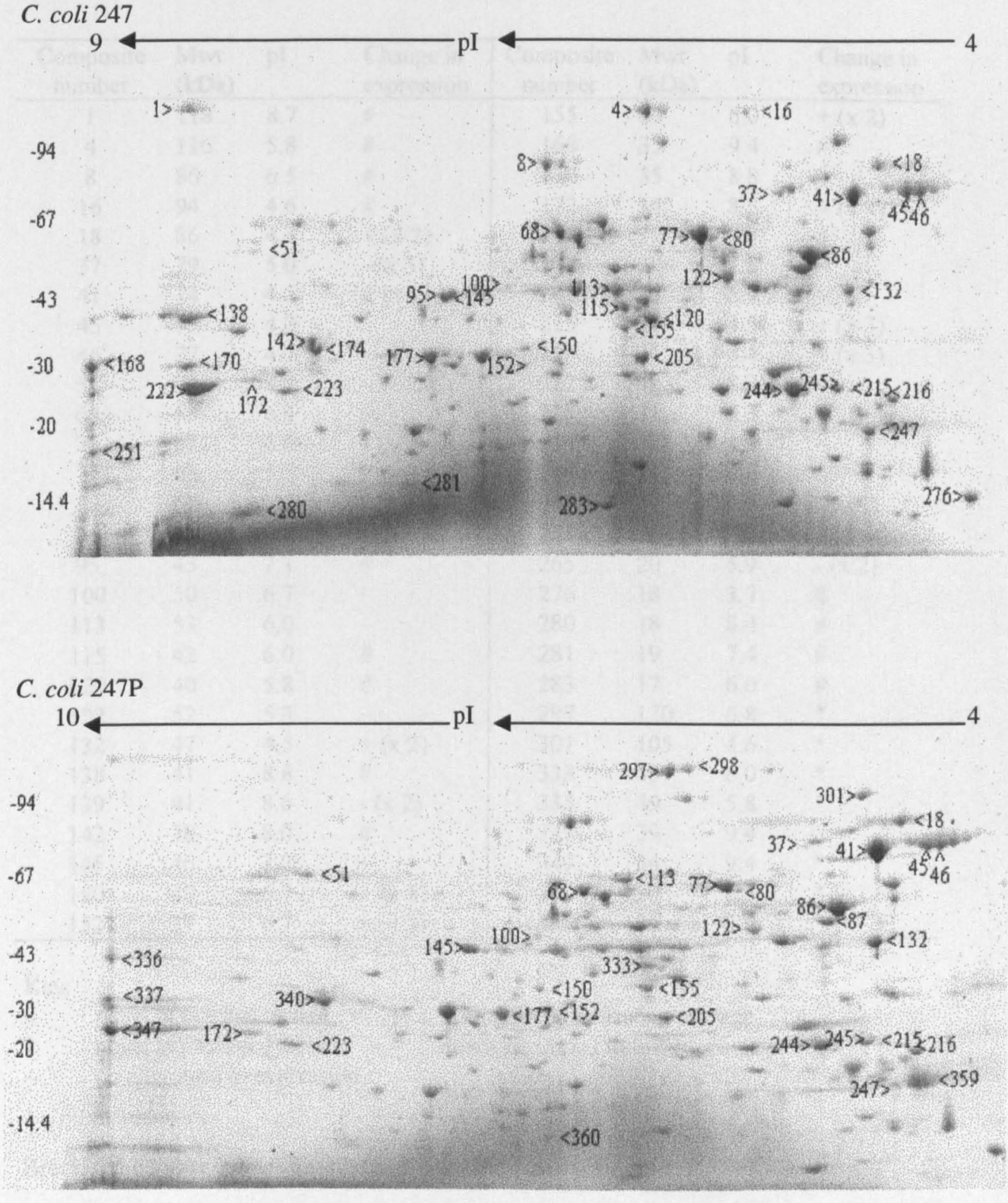
Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
8	118	6.3	*	254	6.6	38	*
35	84	4.5	+ (x 3)	255	6.7	37	+ (x 3)
66	72	4.6	- (x 2)	271	5.4	38	~
67	79	4.5	*	277	9.2	26	*
68	79	4.4	*	279	8.3	31	- (x 7)
70	80	4.3	+ (x 2)	284	7.3	29	- (x 4)
72	67	9.0	*	297	4.8	30	- (x 3)
91	64	6.6	+ (x 11)	298	5.3	30	- (x 11)
94	67	6.4	*	300	5.3	46	+ (x 2)
95	61	6.4	*	301	4.8	27	~
99	59	6.5	+ (x 13)	304	4.4	28	+ (x 3)
114	93	5.9	*	305	4.3	30	~
124	64	4.9	+ (x 4)	308	8.0	25	- (x 2.5)
127	60	4.4	*	309	7.3	25	- (x 2)
133	60	8.1	*	311	6.8	24	*
141	44	7.1	*	312	6.7	23	- (x 4)
143	59	7.0	*	319	7.0	18	*
147	54	6.9	- (x 4)	322	4.1	20	~
149	44	6.7	*	345	4.2	74	#
152	52	6.5	*	346	4.3	74	#
153	49	6.5	*	354	5.0	77	#
154	56	6.5	+ (x 4)	357	5.6	60	#
159	44	6.4	+ (x 5)	362	4.5	63	#
160	47	6.5	*	374	6.5	61	#
161	57	6.2	*	394	6.2	67	#
168	52	5.9	- (x 5)	409	6.1	46	#
182	54	4.9	+ (x 2)	416	5.3	46	#
183	53	4.9	+ (x 3)	422	4.6	44	#
193	41	9.0	+ (x 5)	436	6.4	40	#
225	33	9.4	~	440	5.9	40	#
226	31	8.9	~	451	7.3	34	#
228	34	8.6	- (x 32)	456	6.9	35	#
229	37	8.1	- (x 3)	466	5.0	30	#
231	37	7.8	- (x 4)	489	7.4	24	#
232	36	7.7	*				

Key:

#	novel in passaged isolate	+	up-regulated following passage
*	novel in original isolate	-	down-regulated following passage
~	no alteration in expression		

Bracketed numbers indicate the level of change in expression

Fig 13: 2-Dimensional gel electrophoresis of whole cell proteins of original and passaged isolates of *C. coli* 247



Whole cell proteins were harvested from cultures of both *C. coli* populations and were focused in the first dimension on IEF strips pH 3 –10. Proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system and protein composite numbers were assigned. Mwt markers (kDa) and pI are indicated on the gels.

Table 5: Comparison of proteins identified by 2-D gel electrophoresis of *C. coli* 247 before and after passage

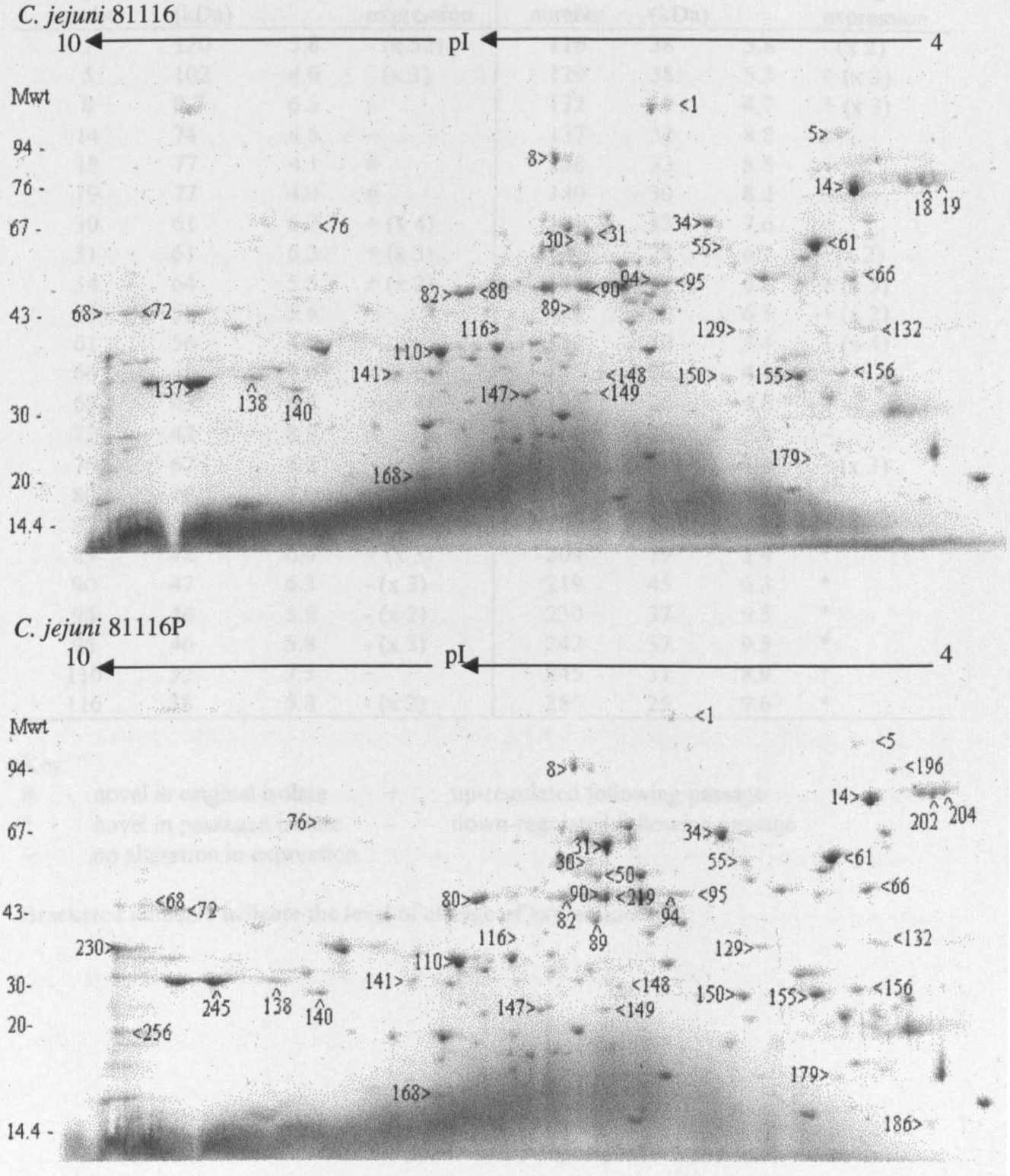
Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
1	118	8.7	#	155	39	6.0	+ (x 2)
4	116	5.8	#	168	35	9.4	#
8	86	6.5	#	170	35	8.8	#
16	94	4.6	#	172	34	8.5	+ (x 4)
18	86	4.4	+ (x 2)	174	37	7.9	#
37	79	5.0	- (x 3)	177	38	7.8	~
41	74	4.5	+ (x 2)	205	35	5.9	~
45	77	4.2	~	215	31	4.5	+ (x 3)
46	77	4.1	~	216	31	4.4	+ (x 3)
51	70	8.2	~	222	32	8.7	#
68	63	6.4	~	223	31	8.3	~
75	67	6.0	- (x 2)	244	30	4.9	~
77	63	5.5	~	245	31	4.6	
80	67	5.4	- (x 3)	247	20	4.3	- (x 2)
86	56	4.8	~	251	26	9.4	#
95	45	7.1	#	265	20	5.9	- (x 2)
100	50	6.7	~	276	18	3.7	#
113	53	6.0	~	280	18	8.4	#
115	42	6.0	#	281	19	7.4	#
120	40	5.8	#	283	17	6.0	#
122	52	5.3	~	297	120	5.8	*
132	47	4.5	+ (x 2)	301	105	4.6	*
138	41	8.8	#	333	43	6.0	*
139	41	8.6	- (x 2)	335	49	5.8	*
142	38	8.0	#	337	39	9.4	*
145	46	7.2	~	347	34	9.4	*
150	40	6.7	+ (x 5)	360	20	6.6	*
152	37	6.7	+ (x 3)	363	20	5.9	*

Key:

#	novel in original isolate	+	up-regulated following passage
*	novel in passaged isolate	-	down-regulated following passage
~	no alteration in expression		

Bracketed number indications the level of change in expression.

Fig 14: 2-Dimensional gel electrophoresis of whole cell proteins of original and passaged isolates of *C. jejuni* 81116



Whole cell proteins were harvested from cultures of both *C. jejuni* populations and were focused in the first dimension on IEF strips pH 3 –10. Proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system and protein composite numbers were assigned. Mwt markers (kDa) and pI are indicated on the gels.

Table 6: Identification of proteins identified by 2-D gel electrophoresis of *C. jejuni* 81116 before and after passage

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
1	120	5.8	- (x 52)	116	38	5.8	- (x 2)
5	102	4.6	- (x 3)	129	38	5.3	+ (x 3)
8	8.7	6.5	~	132	39	4.7	+ (x 3)
14	74	4.6	~	137	32	8.8	#
18	77	4.1	#	138	33	8.5	~
19	77	4.0	#	140	30	8.2	~
30	61	6.2	+ (x 4)	141	33	7.6	~
31	61	6.3	+ (x 3)	147	28	6.7	- (x 2)
34	64	5.5	+ (x 2)	148	32	6.2	+ (x 5)
55	56	5.4	~	149	30	6.3	+ (x 2)
61	56	4.8	~	150	30	5.4	+ (x 4)
66	48	4.6	+ (x 2)	155	26	4.7	~
68	43	9.4	+ (x 4)	156	26	4.6	~
72	42	8.9	~	168	22	7.4	~
76	67	8.2	~	179	23	4.8	+ (x 3)
80	46	7.0	+ (x 3)	196	87	4.4	*
82	47	7.1	#	202	77	4.2	*
89	42	6.4	+ (x 3)	204	79	3.4	*
90	47	6.3	- (x 3)	219	45	6.3	*
94	46	5.9	- (x 2)	230	37	9.5	*
95	46	5.8	- (x 3)	242	37	9.5	*
110	32	7.3	~	245	31	8.9	*
116	38	5.8	- (x 2)	256	25	9.6	*

Key:

#	novel in original isolate	+	up-regulated following passage
*	novel in passaged isolate	-	down-regulated following passage
~	no alteration in expression		

Bracketed numbers indicate the level of change of expression.

3.3 Discussion

On infection of human hosts, *Campylobacter* colonises the intestine before damaging epithelial cell function inducing diarrhoea and in many instances acute inflammatory enteritis. Interaction between the pathogen and the epithelium probably involves cellular invasion or production of toxins (Ketley, 1995). The process of cell invasion is not fully understood, though a number of virulence factors have been proposed including the flagella (Wassenaar *et al.*, 1991). *De novo* bacterial protein synthesis is also thought to be involved in internalisation (Konkel *et al.*, 1990), but the specific protein components have yet to be identified, though secretion of proteins has been shown to be involved (Konkel *et al.*, 1999). The mechanisms by which campylobacters penetrate the epithelium are unclear, possibly involving communication between the micro-organisms and the epithelial cells. This communication, or cross-talk promotes uptake by either microfilament-dependent or microtubule-dependent processes, possibly involving clathrin-coated pits or caveolae, G-proteins and membrane ruffling (see section 1.9).

The extent to which particular isolates of *Campylobacter* invade Caco-2 cells has been reported to correlate with the severity of disease observed in humans (Everest *et al.*, 1992). Isolates from patients with colitis symptoms were found to be more invasive than those isolates from patients with watery diarrhoea. Similarly, the *Campylobacter* isolates in this study were also found to differ significantly in their invasiveness and in their kinetics of cellular invasion, roughly dividing into two groups, one more invasive than the other. *C. jejuni* have also been shown previously to possess the capacity to translocate across polarised Caco-2 cell monolayers (Konkel *et al.*, 1992b; Everest *et al.*, 1992; Ketley, 1995). In separate electron microscopy studies *C. jejuni* have been shown to gain access to junctional spaces between INT407 cells (Oelschlaeger *et al.*, 1993). Correlations between the invasive ability of *Campylobacter* isolates and their potential to translocate across

epithelial cell monolayers have been drawn from these studies, but comparative quantitative and kinetic data have been lacking. The relative contribution of cellular invasion to monolayer translocation is therefore unclear.

The ability of *C. jejuni* isolates to translocate across polarised epithelial membranes was compared and contrasted with their ability to invade the same cell line. Four isolates were selected for their contrasting invasiveness, determined in the initial part of this study, and these isolates were found to vary in their ability to translocate, but this did not correlate with their invasive ability.

All of the *C. jejuni* isolates were detected in the lower chamber after 1 h of incubation, similar to that reported by Konkel *et al.*,(1992b), and continued to translocate for up to 6 h as noted previously by Ketley (1995). Isolate 235 was found to be the most effective translocator though it was identified in the earlier experiment as having poor invasive ability. Another poor invader, isolate 9752, showed only a modest ability to translocate. Interestingly, the highly invasive isolate 10392 was found to be a particularly poor translocating strain, whilst isolate 9519, another highly invasive strain, was found to be able to translocate reasonably well. Konkel *et al.*, (1992b) also demonstrated a similar spectrum of translocative ability between *C. jejuni* isolates, but invasive ability was not compared.

The proportion of the *C. jejuni* inoculum recovered from within the monolayers during the translocation assay was considerably higher than that recovered from the standard invasion assay. This suggests that the physiological state of the Caco-2 cells was different when grown on the semi-permeable Transwell units, increasing their susceptibility to invasion. Changes in Caco-2 cell physiology have previously been reported to depend on cell age and culture conditions (Hidalgo *et al.*, 1989; Engle *et al.*, 1998). However, the distinction

between the more invasive isolates, 9519 and 10392, and the less invasive, 235 and 9752, remained the same. This demonstrates that the relative invasiveness of the isolates had not differed in the two assay formats and that factors, other than invasion, contributed to the extent of translocation. This suggests that different isolates of *Campylobacter* penetrate the Caco-2 monolayers by different mechanisms, some involving intracellular invasion and others not. An alternative explanation could be that the different levels of intracellular bacteria are due to differences in intracellular survival. However, this seems to be an unlikely explanation for many of the observed discrepancies between invasion and translocation. For example, the high levels of translocation by the poorly invasive isolate, 235, might have indicated poor intracellular survival, yet in order to translocate intracellularly across the monolayers the organism would have to remain in a viable state. The reverse argument would apply to isolate 10392, which survived comparatively well within the monolayers, as determined by its invasiveness and yet it did not translocate well across the monolayers. Another possibility would be that different intracellular invasion and transport mechanisms are involved which have different efficiencies of bacterial transfer across the cell. As discussed above, different host pathways have previously been suggested involving, alternatively, microtubules or microfilaments. If more efficient translocation mechanisms were operating then this would result in fewer bacteria within the cells and an apparently poorer level of invasiveness, similar to that seen with isolate 235.

The proposition that different mechanisms of translocation are utilised by different isolates, either paracellular vs. intracellular, or intracellular with different efficiencies of transfer, is further corroborated by the other responses of the Caco-2 monolayers with isolates 235 and 9519. The invasive isolate, 9519, caused a large drop in the TEER at 2 h, and light microscopy indicated a corresponding flattening of the Caco-2 cells. A similar response did not occur with the less invasive isolate 235. Since there were no corresponding changes in

Caco-2 cell viability, nor microscopic evidence of loss of monolayer confluence, it seems unlikely that either isolate 235 or 9519 was able to translocate because of degeneration of the monolayers. This supports the suggestion that isolate 9519 followed an intracellular pathway involving invasion and cytoskeletal rearrangement resulting in alteration in cell shape. This may have contributed to the disruption of the tight junction integrity as indicated by the drop in TEER. Alternatively, the integrity of the tight junctions may have remained unaffected, and the drop in the TEER may be a consequence of a disruption to a transcellular pathway affecting ion transport mechanisms, as has been suggested to be the case with enteropathogenic *E. coli* (Canil *et al.*, 1993).

By contrast, isolate 235 caused no consistent changes in TEER or cell morphology. A similar lack of damage following infection with another *C. jejuni* isolate, M129, was reported in a previous study (Konkel *et al.*, 1992b). It seems more likely that isolate 235 translocated across the epithelial cell monolayers due to movement through the tight junctions, that is a paracellular route, or alternatively a more efficient intracellular pathway involving less disruption to the cytoskeleton. The relative lack of fall in the TEER with this isolate also indicates that the integrity of the monolayer was not compromised significantly. If translocation was by paracellular passage in this case it may have involved the resealing of tight junctions following the transit of the organism. This phenomenon has been previously reported for *S. typhimurium* by Takuechi (1967) and suggested to be the case for *C. jejuni* M129 (Konkel *et al.*, 1992).

Konkel *et al.*, proposed that *Campylobacter* passes through the epithelial cell monolayers by both paracelluar and intracellular routes. Based on this four separate phenotypes have been suggested to exist within clinical isolates of *Campylobacter* (Ketley, 1995), including non-invasive (without translocation), invasive (with translocation), and invasive without

translocation. The data presented here provide quantitative evidence to substantiate distinct phenotypes. Isolate 9752 showed poor invasive and modest translocation ability, whereas isolate 10392 appeared to penetrate by invasion only and translocated 100 times less well than the other isolates. Isolate 9519 translocated modestly and also invaded well. However, isolate 235 translocated very efficiently with evidence of low levels of intracellular invasion. These levels of invasion and translocation were comparable to those described in earlier studies (Konkel *et al.*, 1992b).

Passaging of *Campylobacter* through different models, for example, the chorio-allantoic membrane (CAM) of chicks (Field *et al.*, 1993) and the gastrointestinal tract of chickens (Cawthraw *et al.*, 1997), has been shown to enhance their survival characteristics. However, the response of *Campylobacter* isolates to *in vivo* conditions which are relevant to the human host has not been investigated. Caco-2 cells are thought to represent more closely the target site in the initial stage of invasion in humans, that is enterocyte invasion.

Four isolates of contrasting invasive ability were passaged for a total of eight times through Caco-2 cell monolayers. On comparison with their non-passaged originals, a higher proportion of the inoculum was recovered from all of the passaged isolates in subsequent invasion assays. This suggests that the repeated exposure acted to enhance the expression of survival and/or virulence determinants in each isolate. However, the experimental procedure was selectional, based on survival of the isolate within the monolayer, followed by recovery on plates and storage. This selectional process may have allowed the survival of a more invasive sub-group of each population. A similar selectional process was carried out by Field *et al.*, (1993) who passaged a non-virulent human isolate, LOPEZ, in the CAM of chicks to form a virulent variant isogenic form which had an increased ability to invade and survive.

Comparison of the passaged isolates with one-another revealed that two of the passaged isolates, *C. jejuni* CH1P and *C. coli* 799P, had significantly increased their invasive ability compared to isolates *C. coli* 247P and *C. jejuni* 81116P. This demonstrated that invasiveness could be increased but did not correlate to species type, in contrast to Field *et al.*, (1993) who found that the majority of *C. coli* isolates were less invasive in the CAM of chicks than *C. jejuni*.

The adaptation of the isolates to Caco-2 cell monolayers acted to enhance their virulence *in vitro*. This enhancement may have arisen from phenotypic switching, induced *in vivo* altering the intrinsic invasive ability. A similar phenomenon has been demonstrated with enhanced colonisation of chickens with passaged *C. jejuni* 81116 (Cawthraw *et al.*, 1997) and increased survival in neutrophils following passage of *Salmonella choleraesuis* (Roof *et al.*, 1992). Repeated passage did not enhance the growth rate of the passaged isolates when compared to the original isolates. This suggests that the enhanced recovery was not due to the passaged isolates being able to replicate more rapidly within the monolayer, but may be due solely to an increase in virulence. Similarly, there was no increase in growth rate observed in *C. jejuni* LOPEZ (Field *et al.*, 1993), nor in passaged *Legionella pneumophila* (Horwitz, 1987).

Passaging appeared to caused extensive alterations in protein expression which correlated with enhanced invasive ability. The increased expression of particular proteins observed may have arisen through phenotypic up-regulation or genotypic changes in constitutive expression of particular virulence determinants. A similar correlation of increased virulence with numerous specific changes in protein synthesis has been reported with *Naegleria fowleri*, an amoeboflagellate (Hu *et al.*, 1991).

Protein expression was compared between the passaged and original isolates. It was noted that overall the number of proteins detected in *C. jejuni* CH1P and 81116P was greater than the number detected in *C. coli* 799P and 247P. This does not appear to relate to invasive ability, but to the species involved. The two isolates, CH1P and 799P, which increased most in their invasiveness also synthesised the greatest number of proteins following passage. The proteins identified as novel in CH1P and 799P may represent virulence determinants, for example toxins, adhesion proteins or involved in the secretion of other proteins involved in the internalisation process. Similarly, some of the matched up-regulated proteins may contribute to such processes. Protein expression was altered in a less marked manner in the less invasive passaged isolates, 247P and 81116P. This suggests that passaging was less able to alter the intrinsic nature of these isolates, and that the enhanced invasive ability was probably due to phenotypic changes which occurred following passage.

A set of four proteins was consistently identified in each passaged isolate (for example, CH1 composite nos. 25, 253, 252, 255), all with an approximate molecular weight of 70 kDa. These proteins were identified to be flagellin (see section 4.3.5). Interestingly, there was a shift in the pI of this group of proteins following passage of the isolates CH1P and 799P, which may contribute to the antigenic variation demonstrated by *C. jejuni* (Harris *et al.*, 1987) or possibly to adhesion or motility functions. There was little or no alteration in the level of expression of GroEL (detected in section 4.3.5) following passaged of the isolates (reference to Table 6, protein composite no. 14) (see section 3.2.9), suggesting that the passaging did not represent a stressful environment. However, there was an increase in the level of expression of translation elongation factor and DnaK (Table 6, protein composite no. 61), which may have contributed to the increase in protein expression. PEB-1, a known adhesin was not found to be substantially altered in its expression (Table 3, protein

composite no. 176), suggesting that the isolates may have been using an alternative adhesion mechanism.

Passage of the four isolates induced changes in invasive ability and protein expression, but had no obvious effect on growth rate. However, passaging failed to substantially increase the invasiveness of the less invasive isolates, or their growth rate. The increase in invasive ability was associated with a substantial alteration in protein expression, suggesting that passaging may have enhanced invasiveness through adaptation and genotypic switching. It is possible that the increased invasive ability may be due to increased resistance to gentamicin, and caused the alteration in protein expression. The intrinsic characteristics of each isolate is pre-determined in its genotype but through environmental influences the phenotype and genotype can adapt to the environmental niche within the enterocyte. In this instance, isolates CH1P and 799P appeared to have undergone phenotypic switching allowing increased survival, whereas isolates 247P and 81116P did not possess the genotypic scope to allow such alterations.

In summary, the differences identified here in the invasiveness of isolates, the effects of passage and the mechanisms used to penetrate enterocyte monolayers, suggests that many elements contribute to strain variation in virulence. In the small sample of isolates investigated here, it appears that the invasive ability and the mechanism of penetration probably contribute to the disease presentation, and the number of times the isolate has been exposed to such a host may also contribute to the severity of disease. However, as stated by Smith (1956) the behaviour of micro-organisms *in vivo* is determined by both genetic make-up and environment. *Campylobacter* is regarded as a food borne pathogen and has to be able to survive many adverse conditions before reaching the site of infection in the host.

Exposure to these environments and the adaptations that occur may cause further phenotypic changes that enhance the ability of *Campylobacter* to cause disease.

Chapter 4

Effects of different environmental conditions on the survival, protein expression and virulence of *Campylobacter*

4.1.1 Introduction

Pathogenic bacteria are exposed to many environments during their life cycle, causing them to alter gene expression, to ensure their survival, retention of viability and the ability to replicate. *Campylobacter*, a fastidious organism, was found to be unable to grow in food or on food preparation surfaces, hence its ability to persist under the hostile conditions, for example, low temperature, starvation and extremes of pH (Leach, 1997) acts to ensure successful transmission. However, once *Campylobacter* has gained entrance to the host, it is exposed to other stresses, such as reactive oxygen molecules, restricted iron availability and temperature. To allow infection and colonisation, these stresses must be overcome.

Exposure of pathogens to these environmental cues may trigger expression of virulence determinants (Mekalanos, 1992). For example, recent work has demonstrated expression of the virulence gene *invG* in *S. typhimurium* was controlled by oxygen tensions, pH and the phase of growth which the organism was in prior to infection (Leclerc *et al.*, 1998). To allow co-ordinated response to the numerous cues, the signal transduction system of the organism acts to fine tune the expression of virulence determinants and may similarly function in *C. jejuni* (Ketley, 1997) (see section 1.14).

4.1.2 Response to environmental conditions

Iron has been described as an essential element for microbial growth, though its availability can be severely restricted in the environment, either as insoluble ferric hydroxide or in humans, tightly bound to protein molecules (see section 1.13.1). Bacteria have evolved

mechanisms to acquire iron to ensure their survival, including the use of siderophores, or direct interactions with iron-carrying molecules of the host. Following the capture of iron molecules by the siderophore dependent systems, iron is transported via periplasmic-binding-protein-dependent systems. These consist of a specific outer membrane protein, a periplasmic siderophore-binding protein, a permease within the cytoplasm and an ATP-binding protein (Park & Richardson, 1995). Such a system has been described in *E. coli*, for example the TonB-dependent system (Braun, 1995), and a similar mechanism has been proposed to function in *C. jejuni* (Richardson & Park, 1995; Park & Richardson, 1995; Guerry *et al.*, 1998).

The levels of iron within the cell can regulate the expression of iron scavenging mechanisms and virulence determinants through the activities of the Fur protein (see section 1.13.1). A *fur*-like responsive gene has been identified in *C. jejuni*, which, by analogy, with other pathogens, may regulate the expression of iron scavenging mechanisms and virulence determinants (Wooldridge *et al.*, 1994; Chan *et al.*, 1995).

Oxidative stress is experienced by many bacteria, including *Salmonella* spp. and *E. coli*, created by reactive oxygen species including superoxide anions, hydrogen peroxide and hydroxyl radicals following the reduction of oxygen (Farr & Kogoma, 1991). Alternatively, oxidative stress can be encountered extracellularly following exposure to radiation, light, redox active drugs or from oxidative bursts released by polymorphonuclear leukocytes.

To withstand exposure to elevated levels of oxygen many bacteria, including *Campylobacter*, have been found to possess enzyme defence mechanisms which act to remove radical oxygen molecules, including superoxide dismutase (SOD) and catalase (Kat) (Purdy & Park, 1994; Pesci *et al.*, 1994; Grant & Park, 1995) (see section 1.13.2). The

presence of thiol peroxidase has also been identified in a number of pathogens including *E. coli, Haemophilus influenzae, H. pylori* and *V. cholerae* (Wan *et al.*, 1997) and is involved in Fenton's reaction (see section 1.13.2).

Pathogenic bacteria can be exposed to extremes of acidity in the natural environment, in food or within the host. Exposure can arise in the natural environment through chemically compromised water, and in food processing to control growth and survival of spoilage-causing and pathogenic micro-organisms. Infection of the host can cause enteric pathogens to be exposed to lethal acidic conditions during movement through the stomach (pH 3-5) and within degradative cellular organelles, for example, macrophage phagolyosomes (pH 4 - 5) (Rathman *et al.*, 1996). To survive the rise in hydrogen ion concentration, many pathogens including *L. monocytogenes*, *Shigella* spp., *H. pylori* and *S. typhimurium* have been shown to induce an acid tolerance response (ATR) (see section 1.13.3). This acts to maintain pH homeostasis through induction of proton antiporters and changes in protein expression.

4.1.3 Microbial Growth

Bacterial growth is defined as an orderly increase in all of the cellular chemical components, with the rate of increase in their number, at a particular time point, proportional to the number of cells present (Griffith & Park, 1990). However, bacterial growth can vary depending on the surrounding environment.

When grown in a closed system, such as batch culture, initially an adaptive (lag) phase occurs, dependent on the size and state of the inoculum. This is followed by exponential growth of the organism where the cells grow at a constant, maximal rate, as described by

the equation (Stanbury & Whitaker, 1989), where by x, equals concentration of microbial biomass; t, equals time in hours; μ, equals specific growth rate;

$$\frac{dx}{dt} = \mu x$$

on integration this gives:

$$x_t = x_0 e^{\mu t}$$

where x_t, equals biomass concentration after time interval t; e, equals base of the natural log.

Exponential growth continues until the nutrients become depleted, or secondary metabolites formed by the bacteria act to inhibit growth (Gilbert, 1985). As the growth declines, stationary phase is entered and at this point the cells may be different in chemical composition to those in exponential phase.

Prolonged growth of a bacterial population can be achieved by addition of fresh media to the culture vessel, until the culture vessel is full. If, though, the added medium displaces an equal volume of culture from the vessel then a continuous production of cells would be maintained. Steady state is achieved by balancing the formation of new biomass with the loss of cells from the vessel. The rate of formation of new biomass is determined by the rate of addition of fresh medium to the vessel. This addition is termed the dilution rate and is related to the volume of the vessel:

$D = \frac{F}{V}$ (D = dilution rate; F= flow rate; V= volume)

The net change in cell concentration over a time period may be expressed as:

$$\frac{dx}{dt} = \mu x - Dx$$

Where μx represents growth and Dx represents output. Under steady state conditions the cell concentration remains constant, thus:

$\frac{dx}{dt} = 0$ and $\mu x = Dx$ and $\mu = D$

Hence under steady state conditions the specific growth rate is controlled by the dilution rate. The biomass of the culture is normally limited by the concentration of usually a single nutrient in the medium. In the instance of the experiments described here with *C. jejuni* grown in ABCD, serine was found to be the limiting nutrient (Leach *et al.*, 1997).

Continuous culture provides a constant source of cells at exponential phase growth, with no alterations to the physio-chemical environment of the cells. The growth rate of the bacterial population can be kept constant, whilst at the same time allowing the physio-chemical environment to be adjusted to determine the response to one environmental change.

4.1.4 Aims

The aim of the work here was to investigate the specific cellular responses to three specific environmental stresses, iron availability, oxidative stress and acid stress. Continuous culture techniques were employed in these studies not so much as to mimic the *in vivo* situation, but to permit the independent control of growth rate to allow inferences from the study to be attributed with greater certainty to the effects of each stress.

4.2 Effects of iron limitation

4.2.1 Changes in growth and morphology

C. jejuni 81116 was established in continuous culture under iron replete conditions in ABCD medium (see section 2.4.2; Appendix I). Iron limitation was subsequently induced by with-holding the iron bearing components of the medium; haemin (2 $g.l^{-1}$) and ferrous sulphate (4 $g.l^{-1}$) which removed 148 μM of available iron. The remaining parameters of continuous culture were retained.

As a consequence of iron restriction, cell numbers in the chemostat declined from 2.1×10^9 $cfu.ml^{-1}$ when iron replete (iron conc. of spent medium, 90 μM) to 3.4×10^8 $cfu.ml^{-1}$ (iron conc. of spent medium 0.051 μM) [see Table 7]. Cellular morphology also altered in response to iron restriction with the cells becoming filamentous (mean cell length 14 μm), compared to the predominantly short spirals (mean cell length 3.7 μm) found in the iron replete population.

4.2.2 Effect of iron on whole cell protein expression

Whole cell proteins of *C. jejuni* were analysed by 1-D gel electrophoresis as described in section 2.10.1. The profiles were dominated by the major outer membrane protein located at 43-46 kDa irrespective of the iron concentration (see Fig. 15) with other common bands identified, such as that at 38 kDa. Fewer protein bands were apparent under iron limitation, with the loss of bands at 26, 36, 52, 68, 71, 80 and 92 kDa. However, unique bands were detected at 24, 31, 54, 66 and 75 kDa when iron limited.

Iron replete and iron limited whole cell proteins were analysed by 2-D gel electrophoresis (see section 2.10.2) and stained with Coomassie Blue (see Appendix II). Modifications

Table 7

Fig. 15

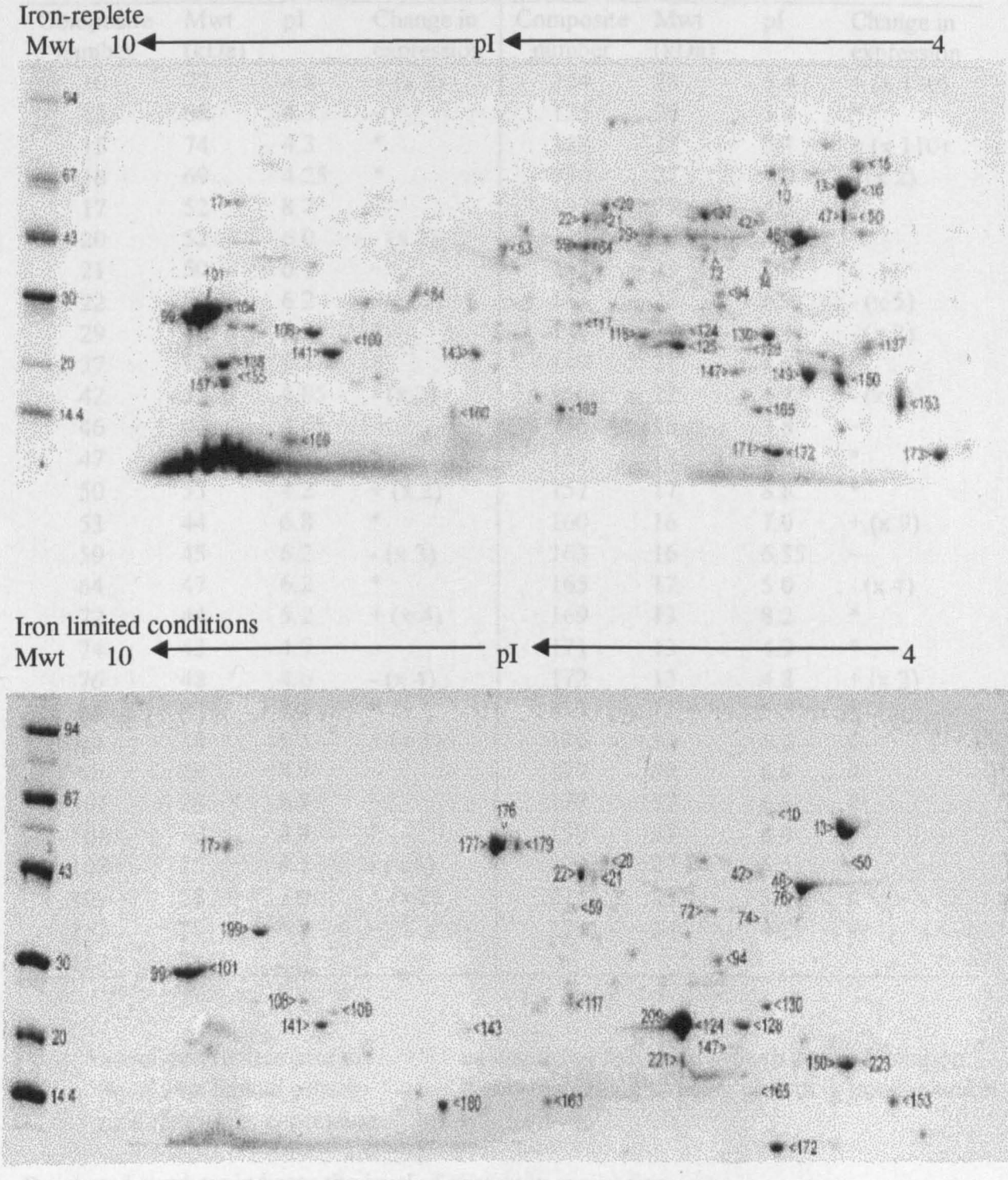
were made to the sample preparation as the iron limited proteins were unable to focus in the 1st dimension. The small molecular components of the cell lysates (lysed in Solution A - see Appendix II) were removed by ultrafiltration (Ultrafree-MC 10 000NML filters, Millipore). The volume of the solution of the retained higher molecular weight material was restored with Lysis solution B (see Appendix II) prior to loading of the sample.

The BioImage system merged the iron replete and limited 2-D gels to produce a normalised composite (see section 2.10.3). Nine anchor points were assigned to allow the gels to be overlaid correctly.

Under iron limitation (see Fig. 16) 99 proteins were detected, compared to 173 under iron replete conditions. Of these, 46 proteins were matched on the two gels and the remaining proteins, 53 under iron limitation and 127 under iron replete conditions, were identified as novel. The fainter novel proteins with integrated intensities of less than 5 were not included in any further analysis, as these were not always clearly resolved in both gels. Under these more stringent conditions (see Table 8) seven novel proteins (composite nos. 176, 177, 179, 199, 209, 221, 223) were therefore reliably detected under iron limitation and 19 novel proteins were detected under iron replete condition (composite nos. 15, 16, 29, 37, 47, 53, 64, 84, 104, 118, 125, 137, 138, 149, 155, 157, 169, 171, 173).

The matched proteins also demonstrated a marked response to iron availability. Iron limitation caused between a 2-3 fold up-regulation in six proteins (composite nos. 10, 22, 50, 94, 109, 172). In addition, a greater than 3-fold up-regulation was identified in four proteins (composite nos. 72, 124, 128, 160). Iron limitation also caused a number of proteins to be down-regulated, with nine proteins (composite nos. 20, 42, 59, 74, 101, 130, 141, 147, 150) showing between a 2-3 fold decrease, and four showing a greater than 3-

Fig 16: 2-D gel electrophoresis of whole cell proteins of *C. jejuni* 81116 following growth under iron-replete and iron-limited conditions



Whole cell proteins harvested from chemostat cultures grown in iron-replete and iron-limited conditions were focused in the 1st dimension on IEF strips pH 3-10. The proteins were stained with Coomassie blue and the gels were analysed by the BioImage system. Protein composite numbers were assigned, some of which are indicated on the gels. Mwt markers (kDa) and pI are indicated on the gel.

Table 8: Comparison of protein expression of iron replete and iron limited *C. jejuni* 81116 cells by 2-D gel electrophoresis

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
10	72	4.8	+ (x 2)	124	28	5.4	+ (x 130)
13	68	4.3	~	125	26	5.4	*
15	74	4.3	*	128	27	5.1	+ (x 110)
16	69	4.25	*	130	27	4.9	- (x 2)
17	52	8.7	~	137	27	4.2	*
20	52	6.0	- (x 2)	138	20	8.8	*
21	50	6.1	~	141	23	8.0	~
22	50	6.2	+ (x 2)	143	23	6.9	- (x 5)
29	48	5.8	*	147	24	5.1	- (x 2)
37	52	5.3	*	149	21	4.6	*
42	52	4.85	- (x 2)	150	22	4.3	- (x 2)
46	46	4.65	~	153	18	3.8	~
47	53	4.3	*	155	18	8.8	*
50	53	4.2	+ (x 2)	157	17	8.8	*
53	44	6.8	*	160	16	7.0	+ (x 9)
59	45	6.2	- (x 3)	163	16	6.35	~
64	47	6.2	*	165	17	5.0	- (x 4)
72	44	5.2	+ (x 4)	169	13	8.2	*
74	43	4.9	~	171	13	4.9	*
76	48	4.6	- (x 4)	172	13	4.8	+ (x 2)
84	34	7.4	*	173	13	6.5	*
94	38	5.1	+ (x 2)	176	53	6.7	#
99	29	8.9	~	177	50	6.8	#
101	28	8.8	~	179	57	6.6	#
104	30	8.8	*	199	37	8.4	#
108	26	8.1	- (x 4)	209	27	5.5	#
109	25	7.9	+ (x 2)	221	23	5.4	#
117	28	6.2	~	223	24	4.2	#
118	28	5.7	*				

Key:

*	novel iron replete protein	+	up-regulated following growth in iron limitation
#	novel iron limited protein	-	down-regulated following growth in iron limitation
~	no alteration in expression		

Bracketed numbers indicate the level of change in expression

fold decrease (composite nos. 76, 108, 143, 165) in expression. The remaining 23 proteins showed little alteration in their integrated intensities (including composite nos. 13, 17, 46, 74, 99, 101, 117, 163).

4.2.3 Identification of protein homologues

Edman degradation (see section 2.10.4) was used to characterise the N-termini of selected proteins identified from the proteome generated under both iron replete and iron limited conditions. The proteins were selected based on their high integrated intensities assigned during analysis of the 2-D gels (see section 4.2.3) and in certain instances due to their expression solely under iron limitation or iron replete conditions.

Sequence data and homologies were assigned for 14 proteins (see Table 9). The N-termini were screened through the data released from the *Campylobacter* genome sequence (http://www.sanger.ac.uk/Projects/C_jejuni/) on the Internet. This allowed the complete encoding region to be identified and the theoretical pI and Mwt to be determined, which were found to be very similar to the experimental pI and Mwt calculated from the 2-D gels shown here. One protein (composite no. 179) could not be sequenced, possibly due to post-translational modification of the N-terminus. Proteins with homology to the N-teminal of protein composite no. 2 could not be identified despite extensive searching of protein data bases. This was probably due to insufficient sequence being recovered from the protein.

The commonest starting residue of the N-termini was methionine and those lacking a methioine residue at the start, for example composite no. 15 and 46, were found to be initiated with methionine, following analysis of the gene sequence from the genome project. This suggests that some of the first residues were lost during the Edman degradation

Table 9: N-terminal sequences identified by Edman degradation from proteins excised from 2-D gels of iron replete and limited *C. jejuni* 81116.

Composite number	N-terminal sequence	Mwt[a]	pI[a]	Protein homology, (contig number)	Organism	Theoretical Mwt[c]	Theoretical pI[c]
2	GFTQY	-	-	N/D	-	-	-
15	QKVIGIDLGTTNSXV	74	4.3	Dnak-type molecular chaperone (Cj 0759)	*C. jejuni* (Y17165)	67.4	4.98
18	KVDQEEQVN	50	7.9	[S]Protease (Cj 0511)	*H. pylori* (genome sequence)[b]	Full protein, 49; Truncated, 45	Full protein, 8.5: Truncated, 7.8
22	MEYREVEHDTMGEVKV	50	6.2	Fumarate hydratase, *fumC* (Cj 1364c)	*C. jejuni* (Y16882)	50.7	6.12
29	MFGAKKNNTEIIEKL	48	5.8	[S]Methyl-accepting chemotaxis protein (Cj 1110c)	*B. subtilis* (genome sequence)[b]	48.4	6.0
37a	MEVKAKQLDFVNATK	52	5.3	Trigger factor (Cj 0193c)	*C. jejuni* (X85954)	50.9	5.69
37b	TDMANMEQNNMMEKER	52	5.3	Glutamine synthetase (Cj 0699c)	*H. pylori* (genome sequence)[b]	53.9	5.6
46	GKEKFSRNKPHVNIG	46	4.65	Translation elongation factor (Cj 0470)	*H. pylori* (genome sequence)[b]	43.6	5.11
53	MLTKRSQVLE	44	6.8	[S]Aspartate aminotransferase (Cj 0762c)	*Bacillus subtillis* (Z99115)	43.1	8.26
84	SIKVNKNTKVIVQGF	34	7.4	Succinyl coA synthetase (Cj 0534)	*Coxiell burnetii* (X77919)	30	7.65
176	MKKLTN	53	6.7	Catalase (Cj 1385)	*C. jejuni* (X85130)	58.4	7.74

177	MKKLTNDFGNIIADN	50	6.8	Catalase (Cj 1385)			
179	No sequence	57	6.6	-	-	-	-
209	MIVTKKALDFTAPAVLGN	27	5.5	Alkyl hydroperoxidase (Cj 0334)	*H. pylori* (genome sequence)[b]	21.9	5.66
221	MSVTIKQLLQMQADAKKL	23	5.4	[S]Neutrophil activating protein/ Bacteroferrittin (Cj1534c)	*H. pylori* (U16121)	17.2	5.55
223	GEVPIGD	24	4.2	[S]Periplasmic protein (Cj 1659)	*C. jejuni* (genome sequence)	17.5	Full protein, 5.23; Truncated protein, 4.97

[a], The molecular weight and pI indicated in these two columns were calculated directly from the 2-D gel.

[b], Homology assigned from sequences taken from the genome sequence projects of *H. pylori* and *B. subtilis*.

[c], The theoretical molecular weight and pI indicated in these two columns were calculated using ExPASy (www.expasy.ch/tools/pi_tool.html).

[S], indicated that the sequence was identified solely from the *Campylobacter* sequencing project carried out by the Sanger Center.

process. Two of the proteins (composite no. 18 and 223) appeared to contain leader sequences, which were identified in the genome sequence data, suggesting those encoded proteins were exported to sites outside of the cytoplasm.

Of the 14 proteins identified, five were found to have homologies to *C. jejuni* proteins previously registered with EMBO database, including composite no. 15, 22, 37a, 176 and 177. Interestingly, protein composite no. 37 generated two amino acid signals, whose theoretical pI and Mwt were similar to that of the actual pI and Mwt. This suggests that the proteins co-migrated under the conditions of gel electrophoresis used here. In addition, protein composite no. 176 and 177 were both identified as having homology to catalase, differing from each other in both molecular weight and pI. This mis-match between the theoretical and experimental pI and Mwt assigned, suggests post-translational modification may have occurred. The remaining sequences were found to have homology to proteins identified in *E. coli*, *H. pylori* and *Bacillus subtilis*.

4.2.4 Effects of iron on protein expression in subcellular fractions

The outer membrane proteins were extracted from iron-replete whole cell proteins as described in section 2.9. Profiles of iron replete membranes (see Fig. 15, Lane 4) contained bands at 21.5, 68 and 92 kDa, which were not apparent under iron limitation. However, novel bands at 24, 54, 66, 69-70 and 75 kDa were detected when the culture was iron limited (see Fig. 15, Lane 3).

The inner membrane bound proteins (sarkosyl soluble) showed several differences in their banding profiles in response to iron availability. In iron-limited culture unique bands at 32, 40.5, 43, 57 and 74 kDa predominated (see Fig. 15, Lane 5), whilst profiles of iron replete cultures showed unique components at 21, 25, 36, 44 and 80.5 kDa (see Fig. 15; Lane 6).

4.2.5 The response of an iron limited culture to a pulse of iron (Fe^{++})

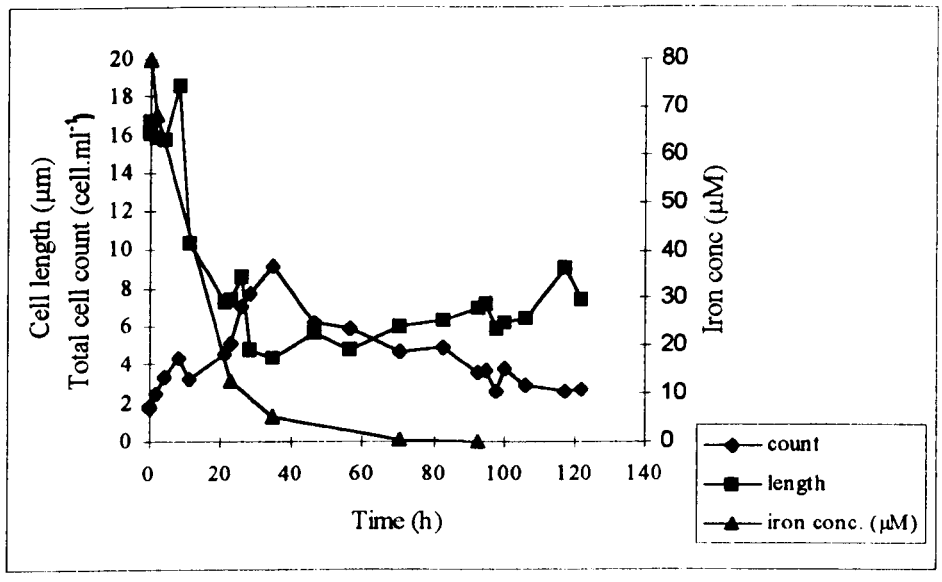
Steady-state in an iron limited chemostat was disturbed by the addition of a pulse of iron (5 ml of 10 mM ferrous chloride solution; filter sterilised). This caused the population to increase from the steady state value of 3.4×10^8 cell.ml^{-1} to a maximum density of 9×10^9 cell.ml^{-1} over 35 h (see Fig. 17). As the concentration of iron was then depleted over 122 h, the total cell count in the chemostat steadily declined to 2.7×10^9 cell.ml^{-1}.

The previously filamentous iron-limited cells became gradually shorter on the addition of iron until by 34.5 h the cells had reduced from a mean length of 14.1 μm to 4.4 μm (see Fig. 17). As iron subsequently became the limiting nutrient again within the population, *C. jejuni* began to increase in length, and by 122 h had a length of 8.8 μm.

All of the bands specific to iron limitation in the whole cell 1-D protein profiles were lost by 2 h following the pulse of iron, except for the band at 66 kDa (see Fig. 19). By 34 h, the culture had developed a profile characteristic of iron replete conditions including acquisition of bands at 21.5, 26, 36 and 80 kDa. However, by 106 h the protein profile returned to one more characteristic of iron limitation, with the up-regulation of bands at 24 and 75 kDa. However, some bands characteristic of iron replete conditions were still present at 36, 80 and 92 kDa.

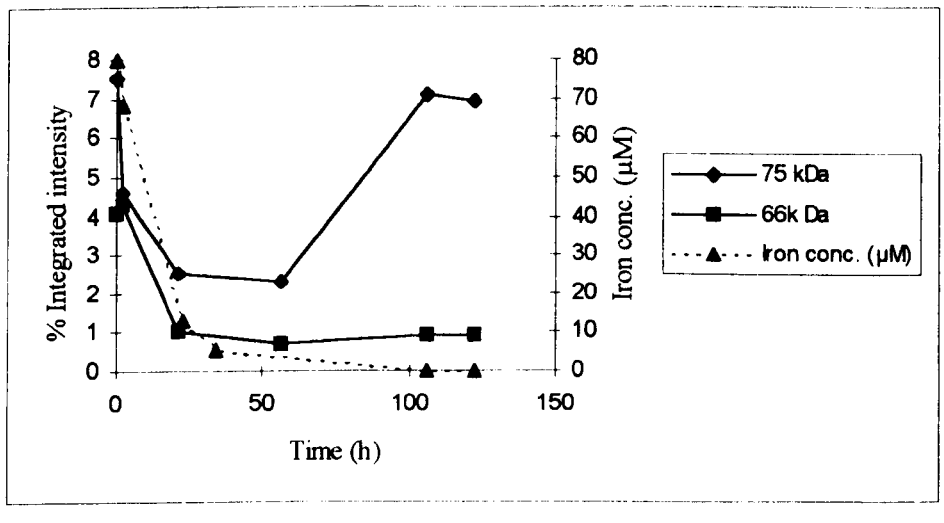
The outer membrane profiles also altered with the changes in iron concentrations (see Fig. 20). By 2 h (iron conc. spent medium, 68 μM), there was a loss of some bands seen previously under iron limitation (31 and 54 kDa). Bands unique to iron replete outer membranes were detected by 56 h including those bands at 22 and 92 kDa. A further shift in expression was noted at 106 h (iron conc. of spent medium 0.051μM), with an increased expression of the iron limited bands at 24 and 75 kDa. The 75 kDa protein remained

Fig. 17: Response of a representative continuous culture of iron-limited *C. jejuni* 81116 to an iron pulse



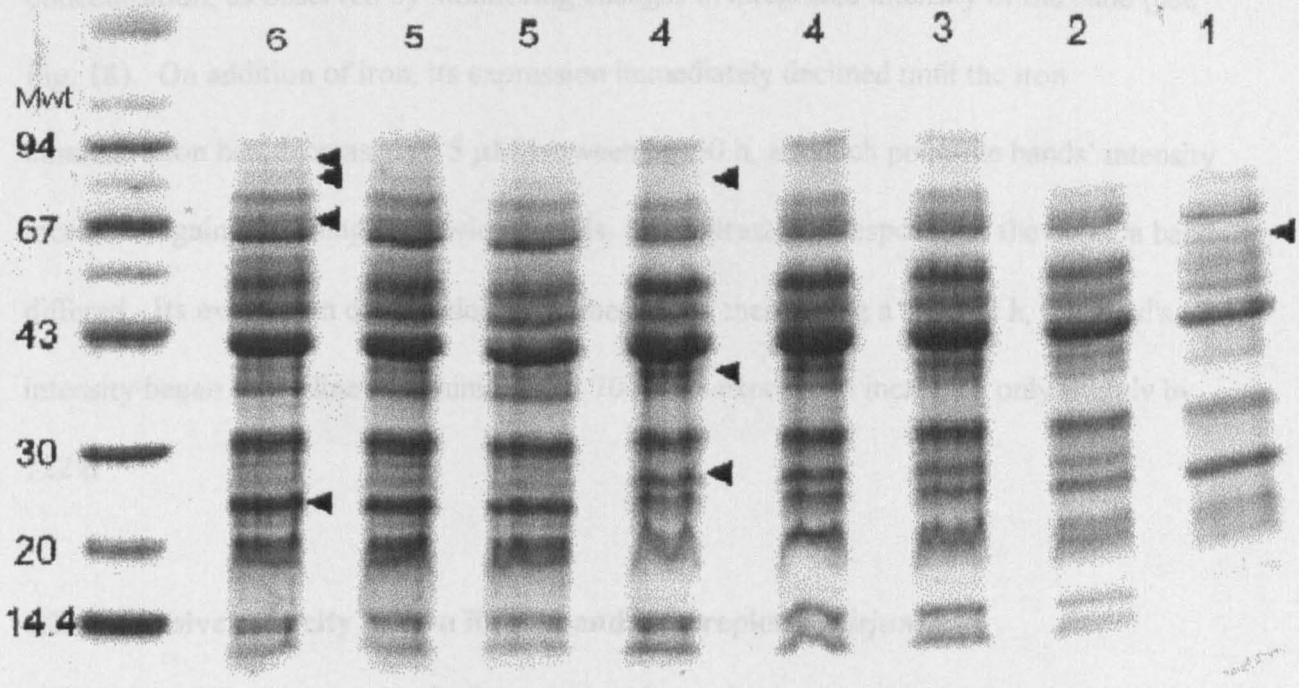
Note: At t=0, 100 µM ferrous chloride (filter sterilized) was added to the chemostat and the changes in cell length, total cell count (x 10^9 cell.ml^{-1}) and iron concentration (µM) were monitored over 122 h.

Fig. 18: Fluctuations in protein band expression during the iron pulse



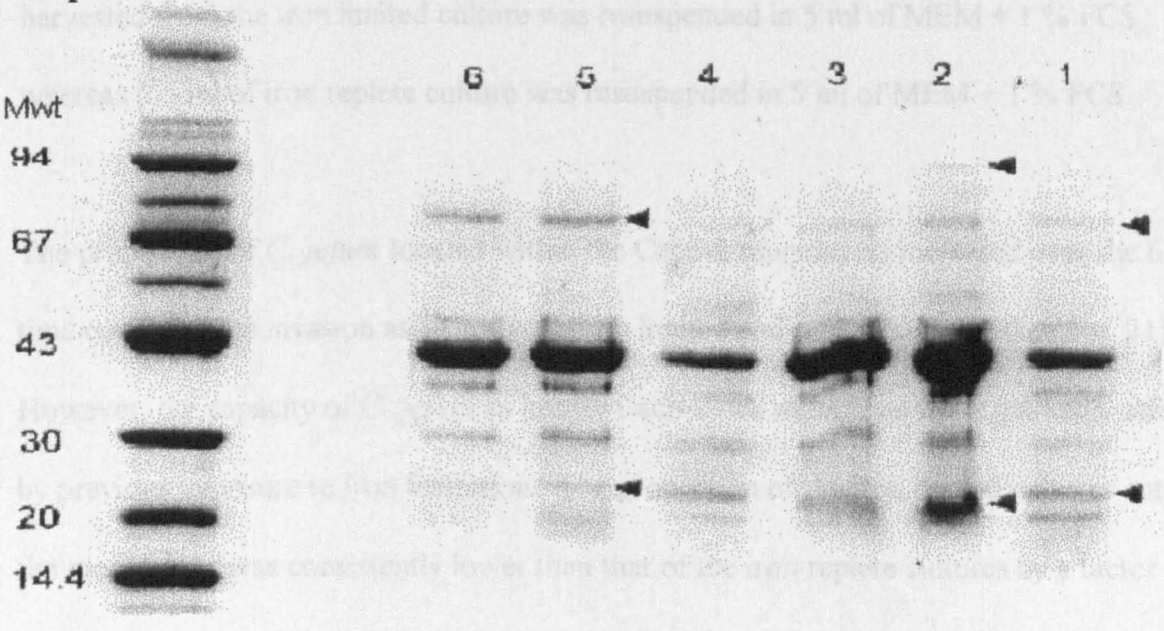
Note: The percentage (%) integrated intensity represents the level of intensity of the band normalised against the remainder of the bands within the lane.

Fig 19: SDS-PAGE showing whole cell protein profiles of *C. jejuni* during the iron pulse



Note: Lane 1, 2 hours; Lane 2, 21 hours; Lane 3, 28 hours; Lane 4, 34 hours; Lane 5, 92 hours; Lane 6, 106 hours. Arrows heads indicate bands highlighted in section 4.2.5. Mwt markers are indicated on the left hand side.

Fig 20: SDS PAGE showing the outer membrane protein profiles of *C. jejuni* through the iron pulse



Note: Proteins were stained with Coomassie Blue and analysed by the BioImage system. Arrow heads indicate bands highlighted in section 4.2.5. Lane 1, 2 hours; Lane 2, 21 hours; Lane 3, 56 hours; Lane 4, 84 hours; Lane 5, 106 hours; Lane 6, 122 hours. Mwt markers are indicated on the left hand side.

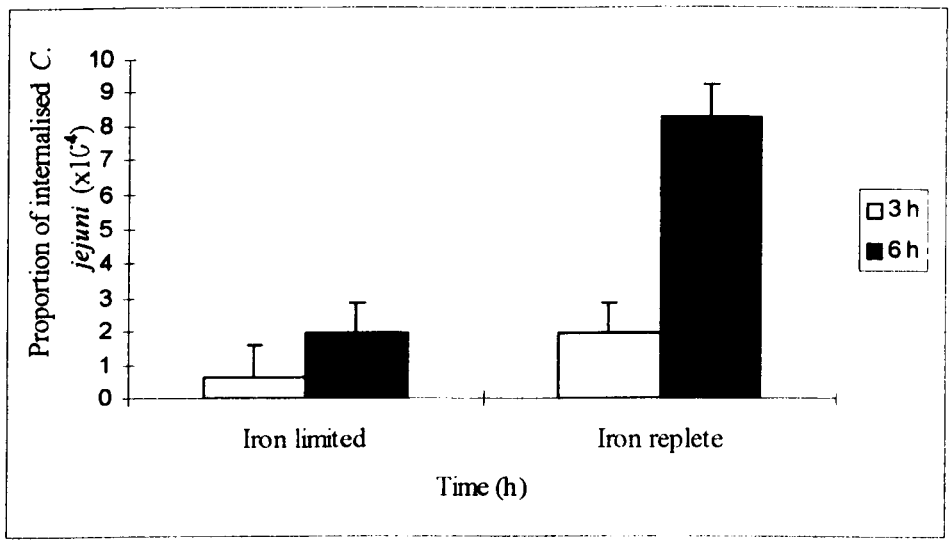
detectable throughout the pulse, though the expression varied with time and iron concentration, as observed by monitoring changes in integrated intensity of the band (see Fig. 18). On addition of iron, its expression immediately declined until the iron concentration had decreased to 5 μM between 20-60 h, at which point the bands' intensity increased again, returning to previous levels. In contrast, the response of the 66 kDa band differed. Its expression did not decline immediately, there being a lag of 2 h, the band's intensity began to decline to a minimum at 70 h. Its expression increased only slightly by 122 h.

4.2.6 Invasive capacity of iron limited and iron replete *C. jejuni*

The ability of *C. jejuni* to invade Caco-2 monolayers having previously been grown under iron replete and iron limited conditions was assessed as described in section 2.8.2. Minor alterations were made in the inoculum preparation to ensure the same number of iron limited *C. jejuni* were used to challenge the Caco-2 monolayers. Biomass (8 ml) was harvested from the iron limited culture was resuspended in 5 ml of MEM + 1 % FCS, whereas 0.5 ml of iron replete culture was resuspended in 5 ml of MEM + 1 % FCS.

The proportion of *C. jejuni* located within the Caco-2 monolayers increased over the 6 h time course of the invasion assay for both iron limited and replete cultures (see Fig. 21). However, the capacity of *C. jejuni* to invade Caco-2 cell monolayers was markedly affected by previous exposure to iron limitation. The proportion of the iron limited cultures entering the monolayers was consistently lower than that of the iron replete cultures by a factor of between three and four at 3 and 6 h, respectively. The difference between the two culture conditions was significant by ANOVA ($p=0.0002$).

Fig. 21: Comparison of the invasive ability of iron-limited and iron-replete *C. jejuni* 81116 in Caco-2 cell monolayers



Note: Data represents the proportion of the inoculum internalised by the Caco-2 cells. Bars represent means ± SE (six separate experiments). The difference between the invasive ability of iron-replete and iron-limited cultures was significant by ANOVA ($p=0.0002$).

Section 4.2.7 Discussion

C. jejuni 81116 was able to sustain growth in the iron-limited ABCD medium with the same mean generation time of 8.7 h, irrespective of the iron concentration of the medium. Iron limitation resulted in a six-fold reduction in the viable cell count, which was restored during an iron pulse, confirming that iron was the limiting nutrient. Interestingly, cellular morphology was altered by iron limitation, with cells becoming longer and filamentous, a feature previously observed for batch grown iron-limited cultures, suggesting that iron may influence cell division and hence cell length (Field *et al.*, 1986). However, a lengthening effect was not reported in the *fur* mutant of *C. jejuni* (van Vliet *et al.*, 1998). The pulse of iron to the iron-limited culture caused a gradual decline in cell length over 30 h, during which time the iron concentration had fallen 1000-fold. However, by 92 h with an iron concentration of 0.051 μM in the medium, *C. jejuni* had not extended in length to the levels observed previously. This would suggest that the extracellular levels of iron were not regulating cell length and division directly, though intracellular levels of iron may have been influencing cell length more directly.

Elongation of *C. jejuni* has also been described during mid-stationary phase and attributed to differential gene expression in response to an unspecified environmental or nutritional stimulus (Griffiths, 1993). A similar response was caused by carbon and energy excess conditions at low growth rates in continuous culture (Leach *et al.*, 1997). Similarly here, the conditions imposed of iron-limitation on *C. jejuni* may have created a carbon and energy excess environment, formed by the reduced population of the chemostat not consuming all of the available carbon, which may promote cell lengthening. Interestingly, protein composite no. 37a, identified as having homology to the protein trigger factor 2, was detected under iron-replete conditions, but not under iron-limited conditions. This protein was thought to have a role in cell division in *E. coli* and *C. jejuni*, acting as a chaperone to

proteins involved in septation (Guthrie & Wickner, 1990; Griffiths *et al.*, 1995). Depletion or overproduction of trigger factor by enhancement of the promotor caused filamentation in *C. jejuni* (Griffiths *et al.*, 1995). Similarly here, the pulse of iron may have increased transcription of trigger-factor, causing the cells to shorten. However, the slow increase in cell length after the utilisation of the iron suggests that the proposed trigger factor protein was not under such immediate iron regulation.

In the 1-D whole cell profiles of iron-replete *C. jejuni*, proteins of molecular weight 21.5, 26, 36, 71, 68, 80 and 92 kDa were up-regulated, by comparison with iron-limited profiles. The protein bands at 21.5, 68 and 92 kDa appeared to be associated with the outer membrane and the 26, 36 and 80 kDa with the inner membranes. The location of the 71 kDa protein, was not clear, and was thought to be cytoplasmic or loosely associated with the membrane. Previous workers have described *C. jejuni* proteins apparently specific to growth in iron-supplemented medium with similar molecular weights (Schwartz *et al.*, 1994).

Iron limitation resulted in the up-regulation of proteins of 24, 31, 40.5, 57, 66, 70 and 75 kDa. The 24, 70 and 75 kDa proteins were apparently associated with the outer membrane, with the 40.5 and 57 kDa proteins associated with the inner membranes. The remaining proteins at 31 and 66 kDa, were assumed to be cytoplasmic or only loosely associated with the membrane. The 75 kDa band was probably that described previously at 74 kDa, 75 kDa and 76 kDa in previous studies (Field *et al.*, 1986; Schwartz *et al.*, 1994; Goosen *et al.*, 1989). This variation in size may be due to different sample preparations techniques and different running conditions of electrophoresis. In *C. jejuni* a gene encoding an iron regulated protein of similar molecular weight, CfrA, has been identified, showing homology to a putative ferric siderophore receptor of *Bordetella bronchiseptica*

and *V. cholerae* (Guerry *et al.*, 1997). This protein, CfrA, was also identified in a *fur* mutant of *C. jejuni* 11168 and found to be under *fur* regulation (van Vliet *et al.*, 1998). Southern blot hybridization of different strains demonstrated that *cfrA* may not be present in all *Campylobacter* isolates, including *C. jejuni* 81116 (Guerry *et al.*, 1997). It has been speculated that in these strains another allele of *crfA* with a distinct product may possibly be present. The observation of an iron-regulated 75 kDa protein band in *C. jejuni* 81116 here would support such a view, although the identification of the protein remains to be established. Attempts were made to identify this protein by Edman sequencing from 1-D gels but were unsuccessful, possibly due to a blocked N-terminus.

The 70 kDa outer membrane protein may be similar to that described previously at 70 kDa which was found in *C. jejuni* 11168 to be under the control of the Fur protein (van Vliet *et al.*, 1998). This protein was identified as ChuA, and was proposed to function as a ferric siderophore receptor. However, there have been no previous reports of the up-regulation of the 24, 31, 40.5, 57 or 66 kDa proteins under iron limitation.

The addition of the pulse of iron to the chemostat evoked both rapid and gradual adaptations of protein expression. Initially, there was a prompt reduction on the amount of the 31, 54 and 75 kDa bands present, suggesting that these were under more immediate iron regulation. The level of expression of the 75 kDa protein was reduced until the iron concentration reached 5 μM, after which, its expression began to be rapidly up-regulated. Similar control of the 74 kDa product of the *cir* gene in *E. coli* has been reported which was also critically influenced by iron-siderophore concentrations around 5 μM (Klebba *et al.*, 1982). This suggests that the 75 kDa protein may be functioning as a receptor for iron bearing molecules. In contrast, the repression of the 66 kDa band occurred more slowly

suggesting an alternative mode of regulation, less immediately associated with the availability of iron.

2-D gel electrophoresis demonstrated more fully the complexity of the response of *C. jejuni* to iron. Interestingly, few proteins were expressed under either environment and resolved by this technique to have a Mwt greater than 74 kDa. Work here and that of previous workers had identified iron-regulated proteins of 82 - 71 kDa using 1-D gels, which were not apparent here. This suggests that conditions of urea denaturation followed by treatment by SDS and DTT may have disrupted the proteins, causing the subunits to run out as smaller components. Alternatively, the proteins may not have moved out of the IEF strip.

Fewer proteins were expressed overall by iron-limited than iron-replete cultures of *C. jejuni*, despite equivalent protein loading on the gels. Of the proteins included in this analysis, with an integrated intensity greater than five, 13 proteins were found to be down-regulated under iron limitation and 19 proteins and possibly as many as 127 were absent. In addition, possibly only seven but perhaps as many as 53 unique proteins were expressed and a further ten proteins were up-regulated. However, in *C. jejuni* 11168 only a small proportion of the proteins were found to be regulated by iron or Fur by van Vliet *et al.*, (1998). This suggests that the small genome of *C. jejuni* may not be able to use a wide variety of iron compounds. In contrast, iron limitation in *S. typhimurium* and *V. cholerae* has been reported to result in an increase in the number of proteins expressed and was correlated with an increase in virulence in *V. cholerae* (Foster & Hall, 1992; Litwin & Calderwood, 1993; Litwin & Calderwood, 1994).

The N-terminal amino acid sequences of some of the proteins expressed under iron-replete and iron-limited conditions were determined. Protein composite no. 15 showed significant

homology to DnaK of *H. pylori* and *E. coli*. This protein is a member of the highly conserved heat shock 70 protein family, and has the ability to recognise nascent chains of polypeptides which can interact with unfolded polypeptides (Langer *et al.*, 1992). The novel expression of this protein under iron-replete conditions suggests a role in protein synthesis in *Campylobacter* and that it may be iron regulated. Protein composite no. 37a was identified as another molecular chaperone, trigger factor 2, with homology to trigger factor of *H. pylori*, *B. subtilis*, *H. influenzae* and *E. coli*. As mentioned previously this protein has also been shown to be involved in cell division. Further work has suggested that trigger factor of *E. coli* carries a catalytically active domain for protein folding (Stoller *et al.*, 1996).

Protein composite no. 46 showed homology to the translocation elongation factor (TEF) protein of *H. pylori*, *H. influenzae* and *E. coli*. This protein can account for 8-9 % of total cell protein and acts to promote efficient binding of aminoacyl tRNA molecules to the correct site on the ribosome during the elongation step of protein synthesis (Nierhaus, 1996). The level of expression of TEF was not significantly altered by the availability of iron, which suggests that transcription of the protein itself was not iron regulated. Overall, protein synthesis was found to be substantially up-regulated under iron-replete conditions, which suggests that the increased level of expression was not controlled by the activity of TEF. The two molecular chaperones - DnaK and trigger factor were unique proteins under iron-replete conditions and may act to increase the rate and fidelity of protein folding, under the stressful conditions. However, these molecular chaperones may have an additional role, as Lewthwaite *et al.*, (1998) proposed that they may also act as virulence factors, for example the Hsp70 molecule of *Mycobacterium avium* was able to bind to eukaryotic cells (Ratnakar *et al.*, 1996). The presence of such molecules may contribute to the increased invasiveness of *C. jejuni* into Caco-2 cell monolayers.

Proteins composite no. 22, 53 and 84 were identified as fumarate hydratase, aspartate aminotransferase and succinyl coA synthetase, respectively, which are all involved in the tricarboxylic acid (TCA) cycle. Three fumarate hydratase (Fum) genes A, B and C, have been identified in *E. coli* (Guest *et al.*, 1985). Here, FumC was identified, showing homology to the product of the *fumC* gene of *C. jejuni*, and also *B. subtilis* and *E. coli*. This enzyme acts to catalyse the reversible inter-conversion of fumarate and L-malate in the TCA cycle, and in *E. coli* has been shown to function during environmental stress (Park & Gunsalus, 1995). Interestingly, *fur*-binding sites have been located up-stream of *fumC* in *Pseudomonas aeruginosa* (Hassett *et al.*, 1997), and in this study this protein was up-regulated under iron-limitation, suggesting possible regulatory control by *fur*.

Protein composite no. 53 was identified as aspartate aminotransferase through the data from the *Campylobacter* genome sequencing project with homology to the aspartate aminotransferase of *B. subtilis*. In other organisms this enzyme was involved in the transamination of many amino acids, requiring pyridoxal 5'phosphate as a co-factor. A sequence similar to succinyl coA synthetase was identified from protein composite no. 84 by homology to the protein in *E. coli* and *B. subtilis*. This key enzyme of the TCA cycle functions to form succinyl coA, releasing ADP and orthophosphate as by-products. These three enzymes have an important role in maintaining the TCA cycle to provide *Campylobacter* with energy. Although FumC, appears to be iron-regulated here, it was not identified to be under the control of Fur in the *fur* mutant of *C. jejuni* 11168 (van Vliet *et al.*, 1998). Aspartate aminotransferase and succinyl coA synthetase were up-regulated under iron-replete conditions, suggesting that these may be functioning to provide energy allowing growth until the bacteria reached the maximal steady state equilibrium determined by the concentration by the limiting nutrient in the ABCD media.

Protein composite no. 37b was identified to have homology to glutamine synthase, an enzyme involved in amino acid biosynthesis. This was identified under iron-replete conditions and appeared to co-migrate with trigger factor protein (protein composite no. 37a). This enzyme is the sole means of synthesising glutamine, and is responsible for ammonia assimilation in low external nitrogen concentrations (Klose & Mekalanos, 1997).

Protein composite no. 209 and the doublet 176 and 177 were identified as alkyl hydroperoxide reductase (AhpC) and catalase (KatA) respectively, which have been identified to be involved in responding to oxidative stress (see section 4.1.2). AhpC, identified by homology to the AhpC protein of *H. pylori* and *E. coli*, acts as a repair mechanism for oxidatively damaged proteins, nucleic acids and lipids. KatA, identified through homology to published *C. jejuni*, *Bordetella pertuissis* and *H. influenzae* sequences, catalyses the dismutation of H_2O_2 to H_2O and O_2. The expression of KatA was identified solely under iron-limitation, and AhpC appeared to be substantially up-regulated, which suggests that they were both iron-regulated, possibly involving *fur*-independent mechanisms due to their detectable presence in the *fur* mutant of *C. jejuni* 11168 (van Vliet *et al.*, 1998). A possible *fur*-independent mechanism has been recently identified as homologue of Fur, PerR (peroxidase stress regulator), which regulates the expression of AhpC and KatA in *C. jejuni* (van Vliet *et al.*, 1999). The up-regulation of these proteins under iron-limited conditions may be related to their protective role, to prevent the scavenged iron coming into contact with reactive oxygen intermediates causing damage within the cell. In such instances KatA would act to remove hydrogen peroxide and AhpC would act to repair any damage from generation of oxygen radicals in iron-dependent Fenton reactions.

Protein composite no. 221 was identified by homology to neutrophil activating protein A of *H. pylori*. This protein was thought to be involved in the adhesion of *H. pylori* to host cells (Evans *et al.*, 1995a). However, further work identified the protein to act as a bacterioferritin, which may have been under the transcriptional control of *fur* (Evans *et al.*, 1995b). This protein, which was identified as novel under iron limitation in *C. jejuni*, and may also be functioning as an iron storage molecule, minimising the contact of free iron with oxygen radicals in the cell. A ferritin molecule was previously identified in *C. jejuni* (Wai *et al.*, 1995) and was proposed to have a role in iron storage and protection against iron-mediated oxidative stress (Wai *et al.*, 1996). However, it appears that *C. jejuni* has more than one type of iron storage molecule.

A methyl accepting chemotaxis protein was identified from the amino acid sequence of protein composite no. 29, by homology with sequences from *B. subtilis* and *H. pylori*. This may contribute to the chemotactic response mechanism of *Campylobacter*, allowing modulation of flagellar rotation, following signal transduction from chemotaxis receptors and movement along concentration gradients of nutrients. The motility of *C. jejuni* has been suggested to be important in attachment to the intestinal epithelium (Ferrero & Lee, 1988) and hence, may contribute to virulence. Under iron-replete conditions this protein was up-regulated and its role may be reflected in the greater invasiveness of iron-replete cultures and contribute to the pathogenesis of *Campylobacter*.

The 19 kDa periplasmic protein was also identified in iron-limited cells (protein composite no. 223), which was similar in amino acid sequence to that described by van Vliet *et al.*, (1998). Since there were no homologues in the sequence databases, the role of this protein remains unclear, though it does appear to be iron-regulated. As an interesting note this protein does appear to possess a leader signal of 21 amino acids suggesting that the protein

was exported from the cytoplasm. Similarly, protein composite no. 18 was identified to have a leader signal of 34 amino acids and was probably exported. This protein was identified to have homology to a protease of *H. pylori* and *Bartonella bacilliforms*, which is thought to have a role in the processing of the carboxyl-terminals of proteins. Its expression under iron-replete conditions may reflect the shift to lower levels of free amino acids in the medium and the up-regulation of the systems designed to utilise more complex substrates.

Iron limitation acted to significantly reduce the ability of *C. jejuni* to invade the human enterocyte cell line, Caco-2. This was an unexpected finding, as iron limitation is more usually associated with the up-regulation of virulence determinants in some pathogens (Litwin & Calerwood, 1994). However, this response was not unique, as other pathogens have demonstrated a reduction in virulence traits as a consequence of iron limitation. For example, *L. pneumophila*, having previously been exposed to iron-limited conditions, was less able to invade macrophages and cause disease in guinea pigs (James *et al.*, 1995). Similarly, *L. monocytogenes* was found to be less invasive in Caco-2 cells having previously been grown in an iron-limited medium (Conte *et al.*, 1996).

The results presented here with *C. jejuni* may reflect the influence of iron availability in one of the earliest stages of disease, that is, enterocyte invasion. It has been suggested that gastrointestinal pathogens do not necessarily experience iron limitation in the gut lumen, as there may be significant levels of assimilable iron in the small intestine (Griffiths, 1989). Further, a major iron regulated outer membrane protein of *V. cholerae*, IrgA, has been reported not to be expressed in either the mouse small intestine or the rat ileal loop, whereas it was expressed in the peritoneal cavity of mice, which does represent an iron-limited environment (Camilil *et al.*, 1994).

In the later stages of the disease process, as the organism spreads to sites outside of the gut lumen, iron-limited conditions here may trigger another set of virulence determinants. Thus, some of the proteins up-regulated under iron-limited conditions, as seen in the 2-D gel analysis, may be important virulence determinants, but perhaps only once the organism has invaded. Other proteins, such as AhpC and KatA may also have an important role in the iron-limited environments in the host in avoiding the potential damage which may be caused by iron-mediated Fenton reactions, such as may occur if host iron reserves are suddenly released on cell lysis. These proteins may have relevance to pathogenesis in deeper lying host tissues

Conversely, exposure to iron-replete conditions stimulated the invasive ability of *C. jejuni* into the Caco-2 monolayers. Iron-replete conditions may be experienced during the initial invasion process from the gut, and the proteins up-regulated under iron-replete conditions and identified under 2-D gel analysis may represent novel virulence determinants. Of those which were sequenced here, including the methyl-accepting chemotaxis protein, the protease and the molecular chaperones, DnaK and trigger factor may contribute to pathogenesis. The methyl-accepting chemotaxis protein may increase the targeting of the motile *C. jejuni* to the eukaryotic cell surface, the chaperones may be involved in adhesion or other aspects of virulence and the extracellular protease may be involved in the degradation of defence molecules or structural host proteins.

In summary, the work here demonstrated that *C. jejuni* can detect and respond to changing iron concentrations. The use of continuous culture ensured that the responses were specific to iron limitation and not due to growth rate effects. Cellular morphology altered extensively, with iron limitation promoting the lengthening of the cells. Protein expression changed dramatically with the up-regulation of many proteins identified in 1-D gel

electrophoresis. Different patterns of regulation of protein expression by iron were observed, with some iron responsive proteins more immediately influenced by iron availability than others. Through the use of 2-D gel electrophoresis, protein expression was shown to be influenced extensively by iron, with many proteins lost on exposure to iron limitation.

Iron-replete cultures were found to be more invasive in the enterocyte cell-line Caco-2. Proteins unique to these conditions may include virulence determinants in the early stages of disease. As the disease progresses proteins unique to iron-limited conditions may become more significant in the disease process. Of those proteins sequenced with identified homologies, none were obvious virulence determinants. However, further studies using classical genetic techniques may clarify their possible roles. Of particular interest would be gene knockout studies on the chemotaxis protein, the two molecular chaperones and the protease.

4.3 Effects of oxidative stress

4.3.1 Application of oxidative stress to continuous cultures

C. jejuni isolates 81116 and 9519 were established separately in continuous culture (as described in section 2.4.2) in ABCD medium in which the serine level had been reduced from 2 $g.l^{-1}$ to 1 $g.l^{-1}$ (ABCD ½ serine). Oxidative stress was induced by raising the level of dissolved oxygen (DOT) in the chemostat from 2-4 % to 100-120 % by changing the set point of the oxygen controller and gas flows.

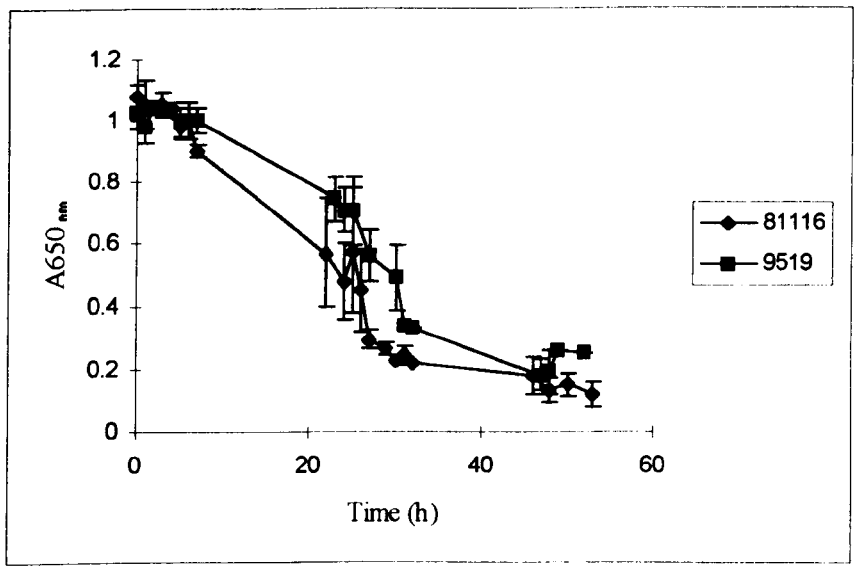
4.3.2 Effects of oxidative stress on cell numbers

Increasing the DOT reproducibly caused, after a short lag phase, a decline in the biomass of both isolates as recorded by changes in optical density as described in section 2.5 (see Fig. 22). The decline in the density of the cultures of *C. jejuni* 81116 and 9519 had a decay constant of 0.057 h^{-1} which was less than the set dilution rate of 0.08 h^{-1}, suggesting that the population was not being washed out of the chemostat.

The initial 24 h period of oxidative stress caused a 50 % reduction in total cell counts (see section 2.6.2) of both isolates, which corresponded to the decline in the number of *C. jejuni* staining with DAPI (see section 2.6.3) (see Figs. 23 and 24). The culturability of both *C. jejuni* isolates also declined over the same period (see section 2.6.1), with *C. jejuni* 81116 and *C. jejuni* 9519 both reduced by 99 %. The number of *C. jejuni* cells staining with the metabolic indicator CTC (see section 2.6.3) also diminished in this period, with a 56 % decline for *C. jejuni* 81116 and 99 % decline for *C. jejuni* 9519.

With application of a further 24 h of oxidative stress, cultures of both *C. jejuni* isolates continued to decrease in cell counts. The total cell count, and those staining with DAPI, declined in number further by 70 % for both isolates, and the number of *C. jejuni* detected

Fig. 22: Decline in biomass of *C. jejuni* 81116 and *C. jejuni* 9519 whilst grown in continuous culture and exposed to oxidative stress



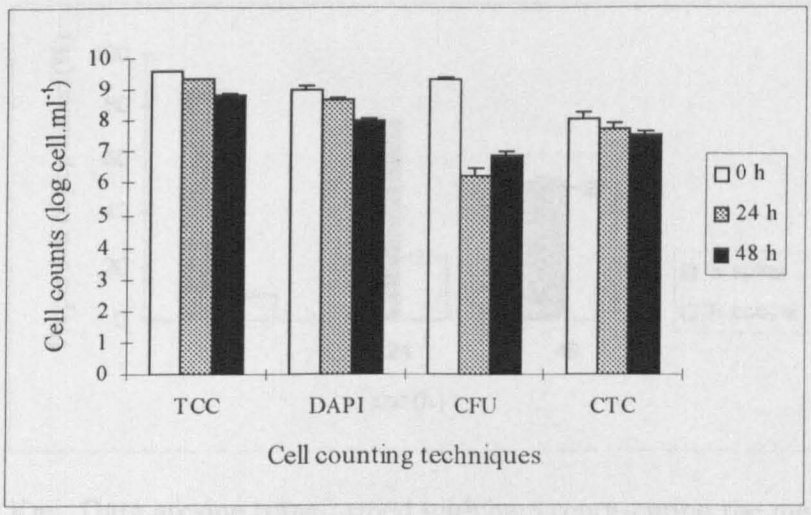
Note: Both *C. jejuni* isolates were established in continuous culture in ABCD ½ serine medium and exposed to oxidative stress through elevation of oxygen tension in the chemostat. Samples were removed from the chemostat and the optical density monitored at an absorbancy of A_{650nm}. The data points are the mean ± SE (three separate experiments).

by CTC staining was also reduced by a further 42 % with *C. jejuni* 81116 and by 2 % with *C. jejuni* 9519. Interestingly, the number of culturable *C. jejuni* increased during this period, by 80 % with isolate 81116 and by 28 % with isolate 9519.

4.3.3 Changes in cellular morphology

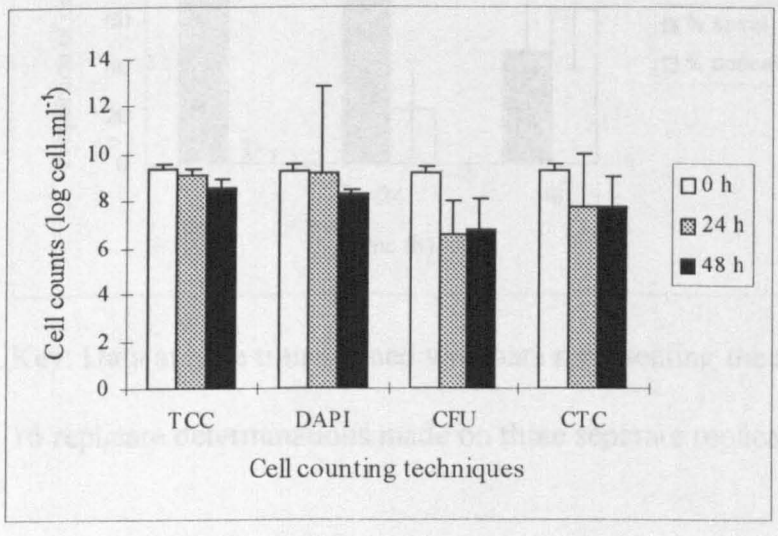
The morphology of the cells in the cultures of both *C. jejuni* 81116 and 9519 was observed by light microscopy and classified as either spiral or coccoid (spherical) in shape. Both isolates, whilst grown under microaerobic conditions contained a predominantly spiral cell population, with a low number of coccoid cells present (see Figs. 25 and 26). Electron micrographs (see section 2.7) of the microaerobic cultures of *C. jejuni* 81116 also confirmed that the cells were mainly intact spirals and approximately 7 μm long (see Fig. 27).

Fig. 23: The effect of oxidative stress on cell number and culturability of *C. jejuni* 81116 whilst grown under reduced carbon concentration in continuous culture



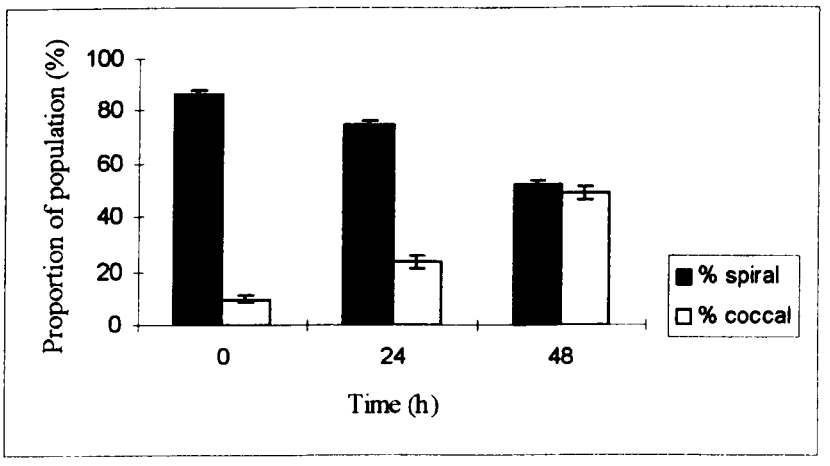
Key: TCC, total cell count; DAPI, staining with DAPI; CFU, colony forming units; CTC, staining with CTC. Data $\log_{10}$ transformed, with bars representing the mean ± SE. Values are means of 16 replicate determinations made on seven separate replicate chemostat experiments.

Fig. 24: The effect of oxidative stress on cell number and culturability of *C. jejuni* 9519 whilst grown under reduced carbon concentration in continuous culture



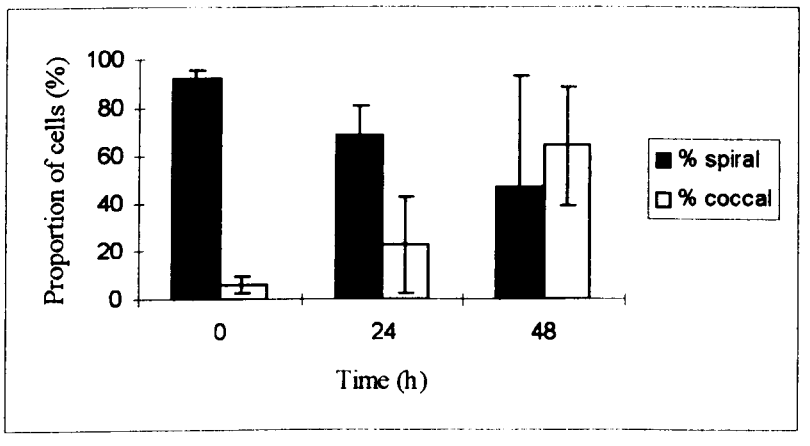
Key: TCC, total cell count; DAPI, staining with DAPI; CFU, colony forming units; CTC, staining with CTC. Data $\log_{10}$ transformed, with bars representing the mean ± S. E. Values are means of 16 replicate determinations made on three separate replicate chemostat experiments.

Fig. 25: The effect of oxidative stress on the formation of coccal cells of *C. jejuni* 81116 grown under reduced carbon concentration in continuous culture



Key: Data arcsine transformed with bars representing the mean ± S. E.. Values are means of 16 replicate determinations made on seven separate replicate chemostat experiments.

Fig. 26: The effect of oxidative stress on the formation of coccal cells of *C. jejuni* 9519 grown under reduced carbon concentration in continuous culture



Key: Data arcsine transformed with bars representing the mean ± S. E. Values are means of 16 replicate determinations made on three separate replicate chemostat experiments.

Fig. 27: Electron micrographs of microaerobically grown spiral cells of *C. jejuni* 81116

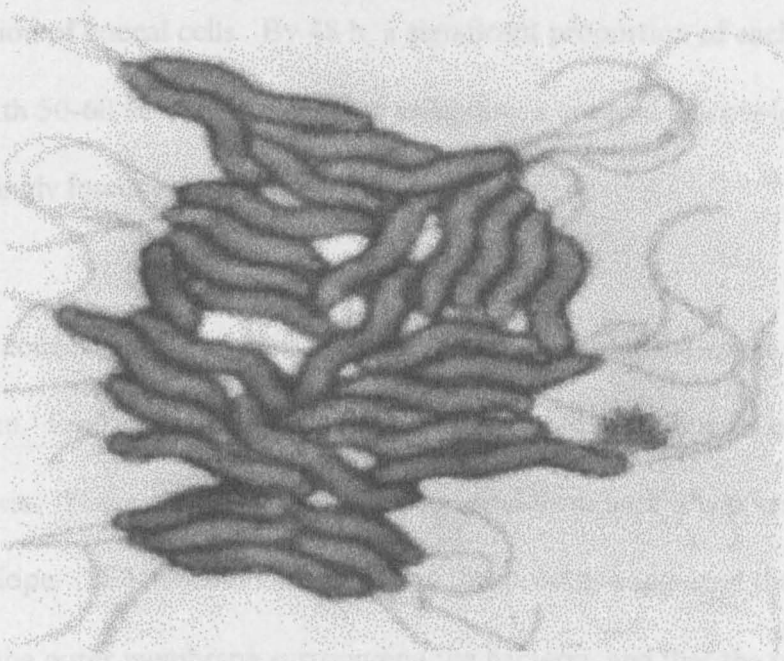
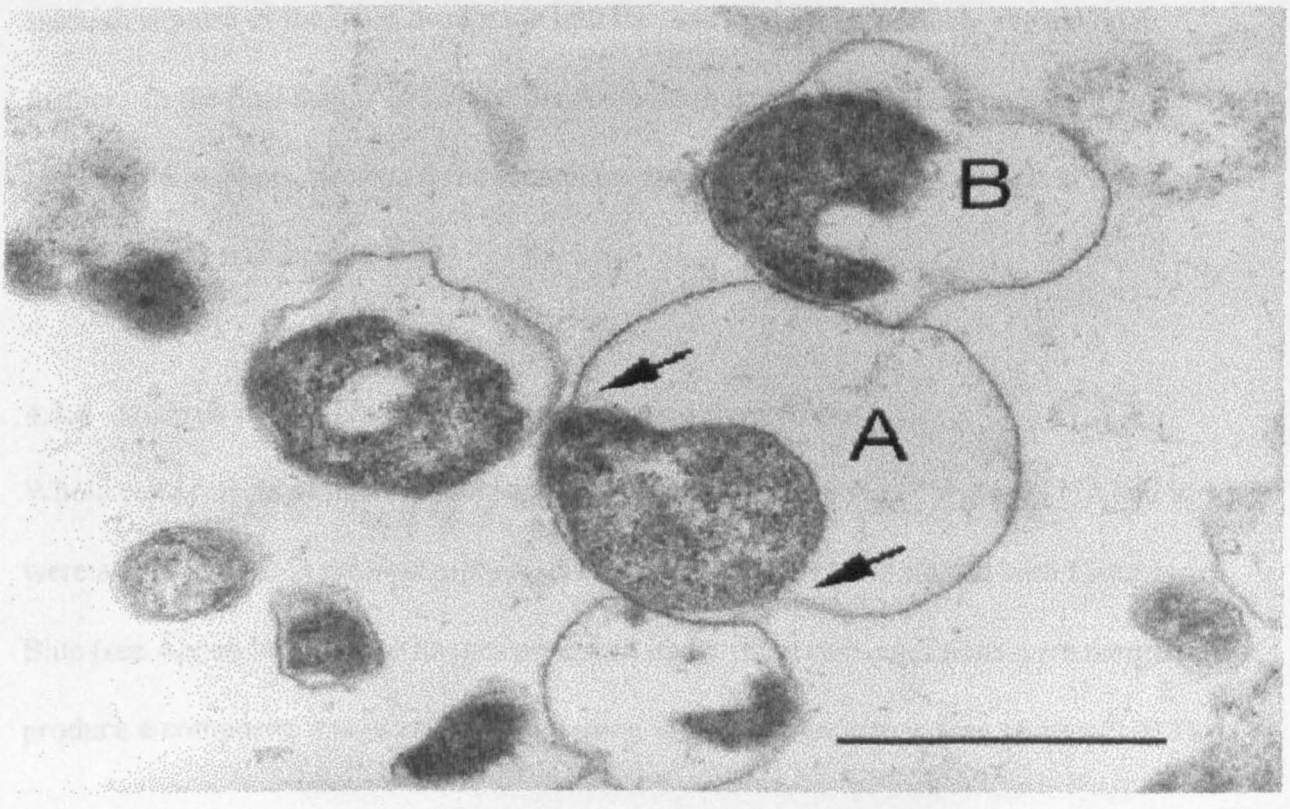


Fig. 28: Electron micrographs of oxidatively stressed coccoid cells of *C. jejuni* 81116



Arrow heads indicate where the inner and outer membranes are separating; cell A shows ballooning periplasmic space but with an intact inner membrane while cell B shows ruptured inner membrane and leaking cytoplasmic contents into enlarged periplasmic space. Bar =1 μm

Elevation of the DOT in the chemostat induced changes in the morphology of the cultures of both isolates (see Figs. 25 and 26). During the first 24 h there was a gradual increase in the proportion of coccal cells. By 48 h, a significant proportion of each culture was affected, with 50-60 % of the population exhibiting a coccoid morphology and a correspondingly lower proportion of spiral cells.

EMs of the coccal cells were used to monitor the collapse of the spiral shape into the coccoid form. Protrusions usually appeared at one pole of the spirals on the outer surface of the bacteria. These appeared to engulf the spiral form until it was contained within the coccal envelope. TEM sections taken during this process suggested that the protrusions arose from the outer membrane surrounding the bacteria, and that the periplasmic space had expanded (see Fig. 28). The cytoplasm of the cells, appeared to disintegrate and be released through rupture of the inner membrane into the "sac" structure, possibly expanding it further. In the final stages of cellular break-down the cytoplasm, which contained some electron dense spots, became a rounded mass, though it did remain in contact at one place with the outer membrane.

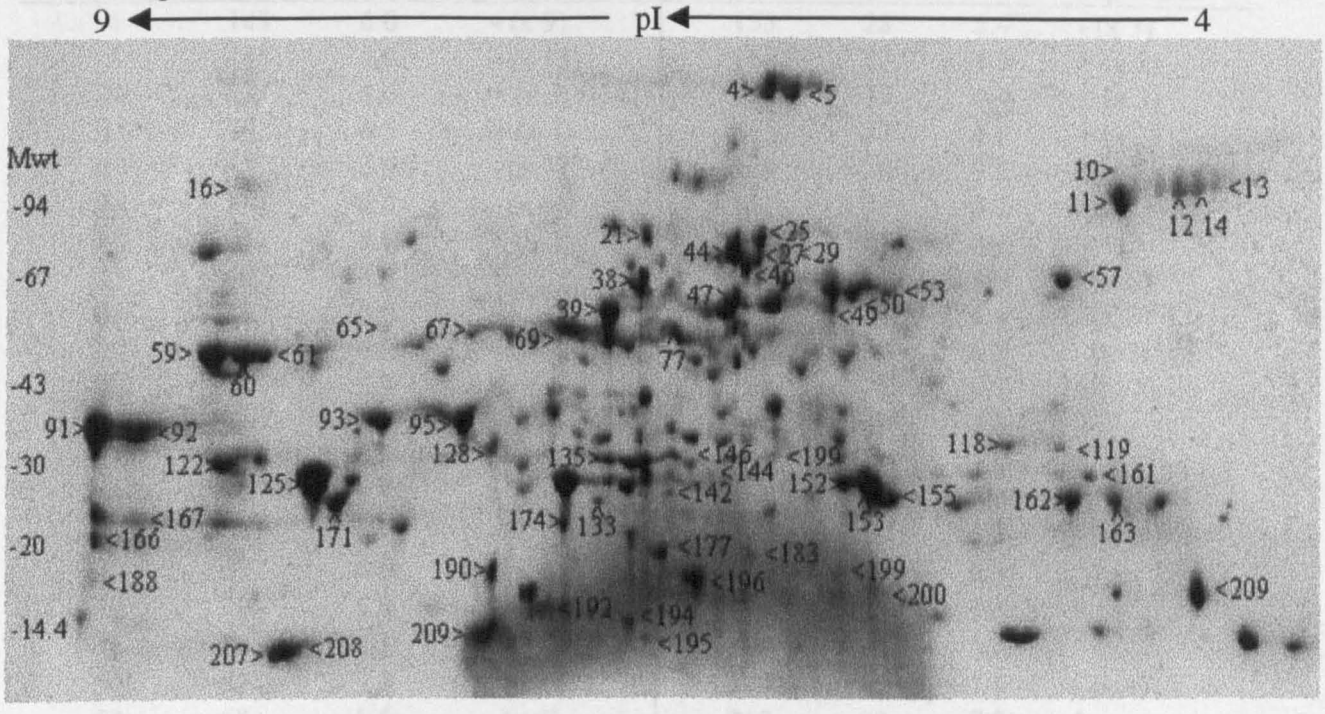
4.3.4 Analysis of protein expression under oxidative stress

Whole cell proteins of microaerobic and 48 h oxidatively stressed *C. jejuni* 81116 and 9519 were analysed by 2-D gel electrophoresis (see section 2.10.2) and stained with Coomassie Blue (see Appendix II). The images produced under these two conditions were merged to produce a composite image and analysed using the BioImage system (see section 2.10.3).

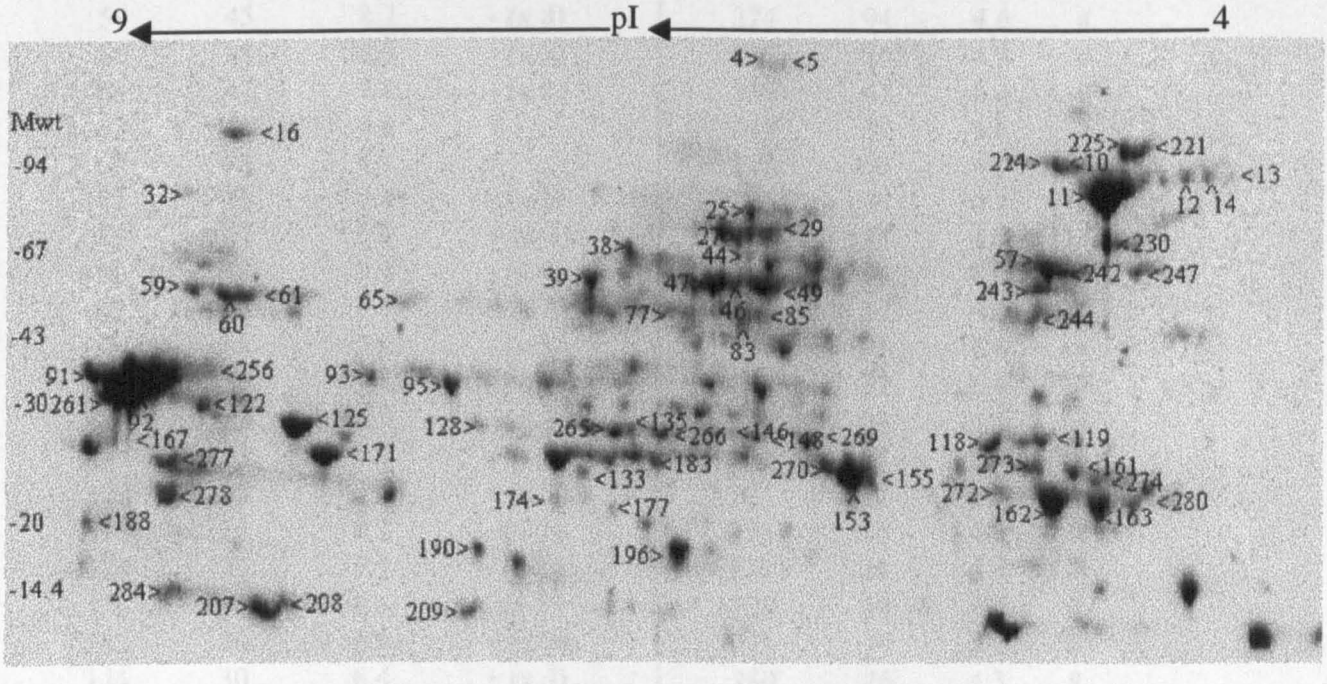
Following this analysis, 193 proteins were detected from *C. jejuni* 81116 grown under oxidative stress, compared to 219 proteins under microaerobic conditions (see Fig. 29). Of these proteins, 124 were matched between the two environments and the remaining spots,

Fig 29: 2-Dimensional gel electrophoresis of whole cell proteins of *C. jejuni* 81116 grown in ABCD ½ serine under microaerophilic conditions and oxidative stress

Microaerophilic conditions



Oxidative stress



Whole cell proteins harvested from chemostat cultures grown under microaerobic and oxidative stress were focused in the 1st dimension on IEF strips pH 3-10. The proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system. Protein composite numbers were assigned, some of which are indicated on the gels. Mwt markers (kDa) and pI are indicated on the gels.

Table 10: Comparison of protein expression of microaerobic and 48 h oxidatively stressed *C. jejuni* 81116 by 2-D gel electrophoresis

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
4	145	6.0	- (x 9)	155	28	5.4	- (x 3)
5	145	5.9	- (x 15)	161	32	4.5	+ (x 2)
10	87	4.6	+ (x 3.5)	162	27	4.4	+ (x 3)
11	76	4.5	+ (x 4)	163	27	4.6	+ (x 3)
13	83	3.96	- (x 3)	166	25	9.2	*
14	85	4.0	- (x 3)	167	32	9.2	- (x 8)
16	85	8.6	+ (x 2)	171	27	8.1	~
21	69	6.6	*	174	23	6.9	- (x 5)
25	69	6.0	~	177	24	6.6	- (x 3)
27	67	6.0	~	183	17	6.0	- (x 3)
29	65	5.9	+ (x 4)	188	21	9.2	+ (x 2)
32	74	9.0	- (x 9)	190	19	7.3	~
53	58	5.5	- (x 8)	192	18	7.0	*
29	68	5.9	+ (x 4)	194	16	6.6	*
32	68	8.7	- (x 14)	195	17	6.6	- (x 2)
38	57	6.6	- (x 4)	198	20	6.3	*
39	49	6.8	- (x 2)	199	20	5.5	*
46	54	6.3	+ (x 3)	200	23	5.6	*
50	58	5.7	- (x 2)	209	15	7.6	- (x 4)
53	58	5.5	- (x 8)	210	20	7.7	*
57	44	4.7	+ (x 2)	221	103	4.2	#
59	45	8.7	- (x 8)	224	94	4.6	#
60	45	8.5	- (x 3)	225	103	4.3	#
65	47	7.8	- (x 2)	230	67	4.4	#
67	48	7.4	*	242	59	4.6	#
69	48	7.1	*	243	53	4.7	#
77	46	6.4	- (x 2)	244	49	4.7	#
83	47	6.0	+ (x 4)	247	58	4.2	#
91	35	9.2	- (x 4)	256	36	8.9	#
92	34	8.9	+ (x 7)	261	32	9.0	#
93	36	7.8	- (x 5)	265	31	6.6	#
95	36	7.4	- (x 2)	266	32	6.4	#
118	33	4.9	+(x 3)	269	28	5.6	#
119	35	4.7	+ (x 4)	270	28	5.7	#
122	33	8.6	+ (x 2)	272	27	4.8	#
125	28	8.2	- (x 2)	273	30	4.6	#
128	33	7.3	- (x 3)	274	28	4.4	#
135	33	6.6	- (x 5)	277	23	8.7	#
142	30	6.6	+ (x 8)	278	19	8.7	#
144	30	6.4	+ (x 4)	280	26	4.3	#
146	34	6.2	- (x 2)	284	16	9.0	#
148	34	6.0	+ (x 4)				
152	31	5.6	*				
153	28	5.5	~				

* novel protein under microaerobic conditions

\# novel protein under oxidative stress

~ no alteration in expression

\+ up-regulated following growth under oxidative stress

\- down-regulated following growth under oxidative stress

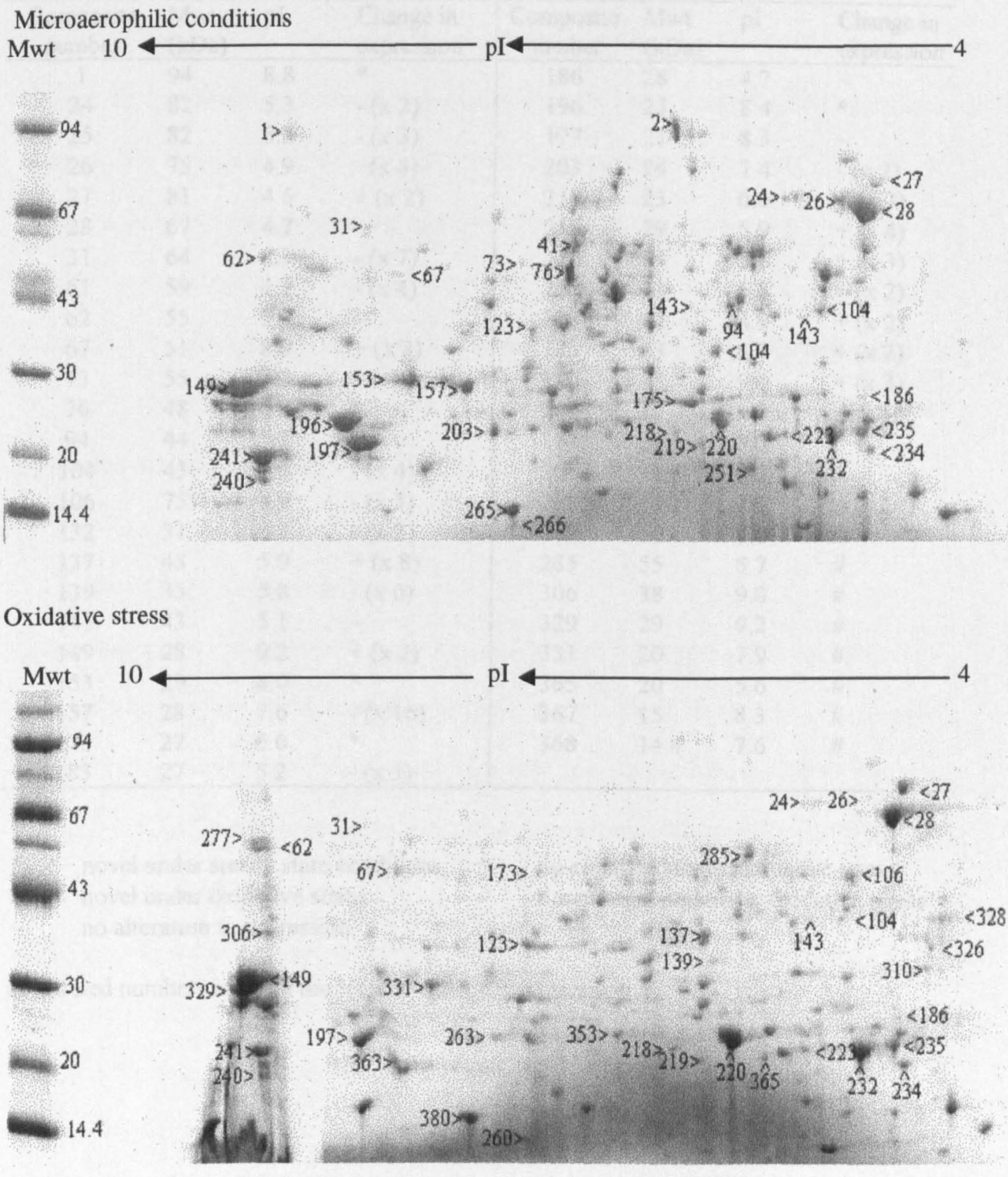
Bracketed numbers indicate the change in expression

69 under oxidative stress and 95 under microaerobic conditions were novel to their respective environmental conditions. In this instance, proteins which had an integrated intensity of less than 0.7 were not included in any further analysis as they were not always reliably resolved on the gels. Under these more stringent conditions (see Table 10), 21 novel proteins were identified under oxidative stress (composite nos. 221, 224, 225, 230, 242, 243, 244, 247, 256, 261, 265, 266, 269, 270, 272, 273, 274, 277, 278, 280, 284), and 11 proteins were identified as novel under microaerobic conditions (composite nos. 21, 67, 69, 152, 166, 192, 194, 198, 199, 200, 210).

Oxidative stress also caused alterations in the level of expression of the matched proteins of *C. jejuni* 81116. Eight proteins were up-regulated between 2-3 fold (including composite nos. 16, 46, 57, 118, 188). Eight proteins were up-regulated between 3-4 fold (composite nos. 10, 11, 29, 83, 119, 148, 162, 163) and three proteins were up-regulated more than 4-fold (composite nos. 92, 142, 144). However, a high proportion of the matched proteins were down-regulated in their expression. Sixteen proteins were down-regulated between 2-3 fold (including composite nos. 13, 14, 39, 50, 60, 65, 77, 95, 122, 128, 146). Four proteins were found to be down-regulated between 3-4 fold (composite nos. 38, 91, 177, 209) and ten proteins were down-regulated more than 4-fold (composite nos. 4, 5, 32, 38, 53, 59, 93, 135, 167, 174). The remaining 55 proteins were unaltered in their expression following exposure to oxidative stress (including composite nos. 25, 27, 125, 153, 171, 190).

A similar analysis of *C. jejuni* 9519 demonstrated the presence of 269 proteins under microaerobic growth and 164 proteins under oxidative stress. Of these, 57 were matched and the remaining proteins, 212 under microaerobic growth and 107 under oxidative stress, were identified as novel (see Fig. 30). Under the same more stringent detection conditions,

Fig 30: 2-Dimensional gel electrophoresis of whole cell proteins of *C. jejuni* 9519 grown in ABCD ½ serine under microaerophilic conditions and oxidative stress



Whole cell proteins were harvested from chemostat cultures grown under microaerophilic conditions and oxidative stress and focused in the 1st dimension on IEF strips pH 3-10. The proteins were stained with Coomassie Blue and the gels analysed by the BioImage system. Protein composite numbers were assigned, some of which are indicated on the gels. Mwt markers (kDa) and pI are indicated on the gels.

Table 11: Comparison of protein expression of microaerobic and 48 h oxidatively stressed *C. jejuni* 9159 by 2-D gel electrophoresis

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
1	94	8.8	*	186	28	4.7	~
24	82	5.3	- (x 2)	196	23	8.4	*
25	82	5.2	- (x 3)	197	21	8.3	~
26	75	4.9	- (x 4)	203	24	7.4	- (x 2)
27	81	4.6	+ (x 2)	218	23	6.0	+ (x 3)
28	67	4.7	~	219	29	5.9	+ (x 4)
31	64	8.3	- (x 7)	220	24	5.8	+ (x 3)
61	59	4.7	- (x 4)	223	23	5.3	+ (x 2)
62	55	9.0	~	232	22	4.9	+ (x 2)
67	51	8.0	+ (x 2)	235	23	4.7	+ (x 2)
73	55	7.2	~	240	18	9.0	+ (x 3)
76	48	6.8	*	241	19	9.0	- (x 2)
94	44	5.7	*	251	19	5.7	*
104	43	5.0	- (x 4)	265	14	7.2	*
106	75	4.9	- (x 3)	266	13	7.2	+ (x 10)
132	31	6.3	- (x 2)	277	56	9.0	#
137	43	5.9	+ (x 8)	285	55	5.7	#
139	35	5.8	- (x 6)	306	38	9.0	#
143	43	5.1	~	329	29	9.2	#
149	28	9.2	+ (x 2)	331	20	7.9	#
153	29	8.0	*	365	20	5.6	#
157	28	7.6	- (x 16)	367	15	8.3	#
175	27	6.0	*	368	14.4	7.6	#
183	27	5.2	- (x 3)				

Key:

*	novel under steady state conditions	+	up-regulated under oxidative stress
#	novel under oxidative stress	-	down-regulated under oxidative stress
~	no alteration in expression		

Bracketed numbers indicate the level of change in expression.

as described before, 31 novel proteins were identified under oxidative stress (including composite nos. 277, 285, 306, 329, 331, 365, 367 368) and 40 novel proteins were identified under microaerobic conditions (including composite nos. 1, 76, 94, 153, 175, 196, 251, 265) (see Table 11). The matched proteins also responded to the oxidative stress through up- and down-regulation of their expression. Oxidative stress caused 13 proteins to increase in expression between 2-3 fold (including composite nos. 67, 149, 223, 232, 235). Six proteins were up-regulated more than 3-fold (composite nos. 149, 218, 219, 220, 240, 266). Five proteins were down-regulated between 2-3 fold (composite nos. 24, 25, 132, 203, 241) and eight proteins were down-regulated more that 3-fold (composite nos. 26, 31, 61, 104, 106, 139, 157, 183). The remaining 25 proteins remained unaltered in their expression (including composite nos. 28, 62, 73, 143, 186, 197).

4.3.5 Identification of protein homologues

Edman degradation (see section 2.10.4) was used to identify the amino acid sequence of proteins from *C. jejuni* 81116 following exposure to oxidative stress for 48 h. These were selected based on their high integrated intensities detected during 2-D gel analysis (see section 4.3.4) and in certain instances due to their expression solely under one condition.

Sequence data and homologies were assigned for 13 proteins (see Table 12). The completed genome sequence of *Campylobacter* allowed the complete encoding region to be identified for each protein and the theoretical pI and Mwt to be determined. These were found to be very similar to the experimental pI and Mwt calculated from the 2-D gels shown here.

In Table 12 it can be seen that few of the sequenced proteins were found to start with a methionine residue. However, following searches of the genome sequence for the complete

Table 12: N-terminal sequences identified by Edman degradation from proteins excised from 2-D gels of *C. jejuni* 81116 after 48 h exposure to oxidative stress

Composite number	N-terminal sequence	Mwt[a]	pI[a]	Protein homology; [contig number]	Organism	Theoretical Mwt[c]	Theoretical pI[c]
11	AKEIIFSDEARNKL	76	4.5	GroEL, molecular chaperone [Cj 1221]	*C. jejuni* (L23798)	57.9	5.02
13	GFEINTRA			Flagellin protein [Cj 1339c]	*C. jejuni* (J05635)	59	5.59
14	GFRINT	85	4.0	Flagellin protein [Cj 1339c]	*C. jejuni* (J05635)	59	5.59
57	TLEKFSRI	44	4.7	[S] Translation elongation factor [Cj 0470]	*E. coli* (X57091)	43.6	5.11
92	AVVAYVGS	34	8.9	PEB-4 [Cj 0596]	*C. jejuni* (X84703)	Full protein 30.5 Truncated 28.38	Full protein 9.23 Truncated 9.09
125	AEGKLEEIK???QLIVGD	28	8.2	PEB-1 [Cj 0921c]	*C. jejuni* (L13662)	28.7	9.04
153	VIVTKKALDFAPAVLN	28	5.5	Alky hydroperoxide reductase (AhpC) [Cj 0334]	*H. pylori* (M55507)	21.9	5.66
162	STVNFKGNPVKLKG	27	4.4	[S] Thiol peroxidase [Cj 0779]	*E. coli* (U33213)	18.4	5.13
163	GTVNFKGNPVKLK	27	4.6	[S] Thiol peroxidase [Cj 0779]	*E. coli* (U33213)	18.4	5.13
171	KA?LNKAHT?DN	27	8.1	[S]High temperature requirement B protein	*E. coli* (X61000)	35.4	9.29

261	ATVAMANGKSIXDL		9.0	PEB-4 [Cj 0596]	*C. jejuni* (X84703)	Full protein 30.5 Truncated 28.38	Full protein 9.23 Truncated 9.09
277	VSFKE??LK	23	8.7	[S] Hypothetical protein [Cj 0998c]	*H. pylori*[b]	Full protein 20.5 Truncated 18.5	Full protein 9.11 Truncated 8.89
278	MKVLLIKLIKALGQ	19	8.7	[S] 50s ribosomal protein [Cj 0664c]	*H. pylori*[b]	16.2	8.82

[a], The molecular weight and pI indicated in these two columns were calculated directly from the 2-D gel.

[b], Homology assigned from sequences taken from the genome sequence project of *H. pylori*.

[c], The theoritical molecular weight and pI indicated in these two columns were calculated using ExPASy (www.expasy.ch/tools/pi_tool.html).

[S], indicated that the sequence was identifed solely from the *Campylobacter* sequencing project carried out by the Sanger center.

encoding region, all of the proteins were found to be initiated with a methionine residue. This suggests that the starting residue was lost during the sequencing process. In certain cases, protein composite no. 92/261 and 277, the proteins were found to possess leader sequences of 21 and 34 amino acids respectively, suggesting that these proteins were exported from the cytoplasm.

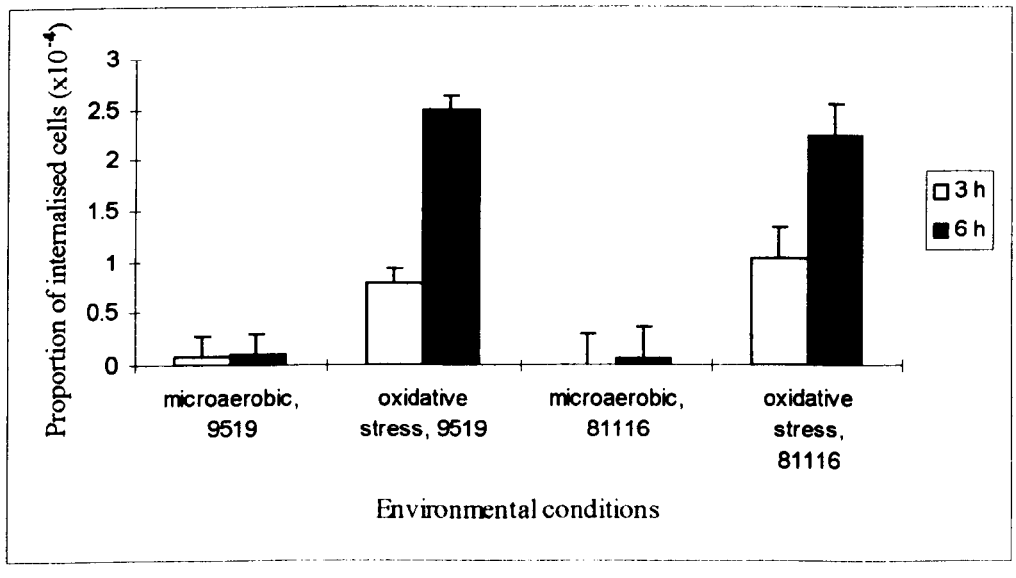
Of the 13 proteins identified, four were found to have homologies to *C. jejuni* proteins previously registered in the non-redundant sequence databases, including protein composite no. 11, 13/14, 92/261 and 125. Protein composite no. 13 and 14 were both identified as flagellin protein, and were separated by a small change in pI suggesting post-translational modification had occurred. The remaining sequences had homology to proteins identified in *E. coli* or *H. pylori* and to sequences in the *Campylobacter* genome sequencing data bases where it was possible to identify and confirm homologies. Interestingly, protein composite no. 162 and 163 were both identified as thiol peroxidases. These proteins were separated by 0.2 of a pH unit, suggesting post-translational modifications had occurred.

4.3.6 Invasive ability of *C. jejuni* grown under oxidative stress

The ability of microaerobic and oxidatively stressed cultures of *C. jejuni* 81116 and 9519 to invade Caco-2 monolayers was compared over 6 h, as described in section 2.8.2. Minor alterations were made in the inoculum preparation of oxidatively stressed *C. jejuni* to allow for the lower numbers of viable cells present in the cultures. Therefore, 8 ml of oxidatively stressed culture and 0.5 ml of microaerobic culture were resuspended, independently, in 5 ml of MEM + 1 % FCS. The experiment was then continued as described in section 2.8.2.

The proportion of the inoculum recovered from within the monolayers increased over the time course with cells from cultures of both *C. jejuni* isolates. However, prior exposure to oxidative stress dramatically increased the ability of both isolates to invade the Caco-2 monolayers (see Fig. 31). By 6 h, the proportion of oxidatively stressed cells of *C. jejuni* 81116 recovered was 30-fold higher than that of the microaerobically grown cultures. Interestingly, there was a more substantial increase in the invasiveness of oxidatively stressed *C. jejuni* 9519, with a 200-fold increase in the number of cells recovered from the monolayers compared to microaerobically grown *C. jejuni*. The difference between the invasive abilities of the microaerobic and oxidative stressed populations, of both isolates, was found to be highly significant by ANOVA (p=0.001).

Fig. 31: Comparison of the invasive ability of microaerobic and 48 h oxidatively stressed *C. jejuni* 81116 and *C. jejuni* 9519 in Caco-2 cell monolayers



Note: Data represents the proportion of the inoculum internalised by the Caco-2 cells. Bars represent means ± SE (six separate experiments). The difference between invasive ability of microaerobic and 48 h oxidatively stress cultures of both *C. jejuni* 81116 and *C. jejuni* 9519 was significant by ANOVA (p=0.001, p=0.001 respectively).

Section 4.3.7 Discussion

C. jejuni was able to maintain its growth in the modified ABCD medium with a reduced concentration of carbon source, with only a slightly lower biomass than that observed with ABCD (see section 4.2.1). Oxidative stress was induced uniquely by raising the oxygen levels in the chemostat. This resulted in a marked decline in the number of *C. jejuni* recovered by plating, suggesting oxidative damage had occurred. However, both *C. jejuni* isolates continued to replicate within the chemostat, maintaining the population at a reduced level during the 48 h of exposure to oxidative stress.

Over the 48 h period cultures of both isolates were found to decline significantly in cell number, as observed by total cell counts and staining with DAPI. Interestingly, simultaneous to these changes, the morphology of the cells altered, going from an approximate ratio of 3 spiral:1 coccoid, to at 24 hours to 1 spiral:1 coccoid by 48 hours. These morphological changes may have reflected other cellular changes taking place which also resulted in changes in culturability.

Although a large decrease in culturability occurred in *C. jejuni* 81116 in the initial 24 h of exposure to oxidative stress, only a relatively small reduction in the numbers of metabolically active cells was observed as indicated by staining with CTC. In the following 24 h, the number of metabolically active cells of this isolate was further reduced, although the number of culturable cells increased. This occurred in conjunction with a substantial rise in the proportion of coccal cells observed in the culture. This would suggest that isolate 81116 has undergone a period of adaptation, within the first 24 h, causing the isolate to become more resistant to oxidative stress in the ensuing 24 h. This increased resistance was unlikely to be due to the selection of a sub-population of the chemostat culture, as the

response occurred rapidly within approximately 3 doublings of the bacterial population given the mean generation time of 8.7 h^{-1} as determined by the dilution rate.

A similar decline in culturability was accompanied in *C. jejuni* 9519 by a large decrease in the number of metabolically active cells in the initial 24 h period. In the ensuing 24 h, the number of culturable and metabolically active cells of *C. jejuni* 9519, both continued to decline, although at a much slower rate than in the initial period. Concurrent to these changes, the proportion of coccoid cells in the culture increased significantly and to a greater extent than that observed for *C. jejuni* 81116. Together this data suggests that *C. jejuni* 9519 was less able to respond to compensate for high oxygen tensions and may be more oxygen sensitive than *C. jejuni* 81116. The demonstration of the up-turn in culturability of oxidatively stressed cultures of *C. jejuni* 81116 and to a lesser extent *C. jejuni* 9519 may be a feature particular to continuous culture where fresh nutrients are continuously added. It has not usually been possible to demonstrate an equivalent phenomenon in batch culture experiments. However, a better general recovery of *C. jejuni* 81116, by comparison with other isolates has been noted by previous authors in batch experiments and attributed to laboratory adaptation causing this isolate to be better able to with-stand adverse conditions (Cawthraw *et al.*, 1997).

Previously, aerobic adaptation of *C. jejuni* had also been demonstrated to occur following prolonged incubation in humidified air on blood agar plates (Jones *et al.*, 1993). Prior to adaptation, culturability was poor on agar plates, but then improved, which was similar to the apparent recovery observed with the two isolates used here.

Similar differences between the number of cells which were culturable and those which retained metabolic activity have been previously reported in other work with *C. jejuni*. A

discrepancy was noted between plate and direct counts following incubation of *C. jejuni* in water microcosms (Rollins and Colwell, 1986). Additional work in this study using acridine orange, a marker of protein synthesis, revealed that protein synthesis continued after *C. jejuni* failed to be cultured suggesting the retention of metabolic activity and possible viability in non-culturable cells. This inconsistency was proposed to be due to the adoption of a VNC form by *Campylobacter* which was thought to be represented by the coccoid form.

The combination of a reduced carbon source and oxidative stress induced coccoid morphology in both isolates of *C. jejuni*, until at 48 h approximately 50 % of the bacterial cultures was coccoid. Previous workers have created coccoid cell populations of 100 % in less well-controlled batch culture experiments, and have suggested many factors may be responsible for triggering the morphology changes including exposure to light and the presence of hydrogen peroxide (Moran and Upton, 1987). Boucher *et al.*, (1994) also identified aeration and nutrient limitation to contribute to the conversion to coccoid forms, with culturability declining over a 90 h period, whilst metabolic activity remained detectable by CTC staining.

Electron micrographs of coccoid cells of *C. jejuni* 81116 following exposure to oxidative stress for 48 h demonstrated that they were degenerate, with visible cytoplasmic masses contained within separating double membranes, suggesting break-down of internal structures. In some instances though, the presence of spiral forms was still observed within the separating inner and outer membrane, suggesting that this morphological form was intact and may represent a VNC state. A similar response has been previously observed in *H. pylori* and thought to be induced by the tension created by the ballooning of the surrounding spherical outer membrane (Benaissa *et al.*, 1996; Kusters *et al.*, 1997). Hence,

the engulfed spiral forms observed here with *C. jejuni* 81116 may represent earlier stages of the cellular break-down process. Previous workers have also reached a similar conclusion that the coccoid cells of *C. jejuni* are degenerate, including Moran and Upton (1987), who found that cytoplasmic contents and nucleic acids were present in lower concentrations in the coccoid forms than in the spiral form. Similarly, Boucher *et al.*, (1994) found that the coccoid forms were unable to sustain their existence under adverse conditions. Changes in protein expression have been investigated by others during these alterations in morphology in *Campylobacter*, suggesting that the conversion to the coccoid form was a passive process. The addition of protein synthesis inhibitors did not prevent coccoid cell formation (Boucher *et al.*, 1994; Hazelaeger *et al.*, 1995), suggesting that *de novo* protein synthesis was not required for this conversion.

Alterations in protein expression demonstrated that the response of *C. jejuni* to oxidative stress was complex. Overall, fewer proteins were detected in both *C. jejuni* isolates, 81116 and 9519, as a consequence of oxidative stress when compared to microaerobic cultures. Oxidative stress in isolate 81116 resulted in the down-regulation of 30 proteins, with a further 11 and possibly as many as 95, proteins lost. However, expression of 19 proteins was up-regulated, with a further 21, and possibly as many as 69 other proteins induced. In isolate 9519, 13 proteins were down-regulated, with a further 31 and possibly as many as 212, proteins absent following exposure to oxidative stress. In contrast, 19 proteins were up-regulated, with 40, and possibly as many as 107, proteins induced. Those proteins whose expression did not alter may represent products of house keeping genes. Both isolates demonstrated a similar level of alteration in protein expression in response to oxidative stress. The coccoid cells of the population have been shown here and by previous workers , to be degenerate. Boucher *et al.*, (1994) and Hazeleger *et al.*, (1995) found that *de nova* protein synthesis was not required for the transformation of morphology, and that it

may be a passive process. Therefore, the proteins identified in the 2-D gels of both *C. jejuni* 81116 and 9519 were expressed in response solely to oxidative stress, not due to changes in morphology. Alternatively, the substantially alteration in protein expression may reflect the microaerophilic nature of *Campylobacter* and that many mechanisms are functioning to maintain culturability in this extreme environment.

In other pathogens oxidative stress has been induced, in batch culture, by the addition of hydrogen peroxide (H_2O_2) or from superoxide radical generators, such as paraquat or menadionine. Incubation of *S. typhimurium* with H_2O_2 induced the expression of 30 proteins (Christman *et al.*, 1985). Exposure of *E. coli* to superoxide radicals induced 40 proteins (Greenberg *et al.*, 1990). However, in both examples fewer proteins were affected by oxidative stress than reported here in *C. jejuni*. The substantial changes in protein expression in *C. jejuni* observed in response to oxidative stress induced by increasing the dissolved oxygen tension in the chemostat, may reflect the involvement of more than one oxidative stress regulon. For example, two distinct regulons have been identified in *E. coli*, *oxyR*, responsive to H_2O_2, and *soxR*, responsive to superoxide radicals which act to co-ordinate induction at particular promoters of genes allowing transcription of antioxidant enzymes and repair molecules (Demple, 1996) (see section 1.13.2). The extensive response of *C. jejuni* observed here may reflect the involvement of several different regulators, though no homologues to *oxyR* or *soxR* have currently been identified in *C. jejuni*. However, given the large response, the families of proteins may be under control of a variety of regulatory networks. The detection of networks will be enhanced by the genome sequencing project which will allow identification of oxidative stress regulators in *C. jejuni*.

The N-terminal sequencing of selected proteins from *C. jejuni* 81116 expressed after 48 h exposure to oxidative stress were determined. Proteins corresponding in *C. jejuni* 9519 to

the sequenced proteins are indicated in brackets. Protein composite no. 153 (220) showed significant homology to alkyl hydroperoxide reductase C (AhpC) of *H. pylori*. This protein acts to remove oxygen radicals and repair damage by the removal of peroxide groups from peroxidized cellular components. The response of this protein to oxidative stress differed between the two isolates with no alteration in expression of *C. jejuni* 81116, and up-regulation in *C. jejuni* 9519. This difference may reflect the increased sensitivity of *C. jejuni* 9519 to oxidative stress and an increased need for AhpC as more proteins are damaged during exposure to oxidative stress. However, the small increase observed in *C. jejuni* 81116 may indicate that the isolate was not experiencing oxidative stress.

The up-regulated doublet, protein composite no. 162 and 163 (232, 235) were both identified as having significant homology to thiol peroxidase (Tpx) of *E. coli*. The two proteins were separated by 0.2 pH units possibly suggesting two forms of the protein are encoded in the genome. However, there is evidence of only one *tpx* gene in the genome sequence of *C. jejuni* 11168. Alternatively, the proteins may have undergone post-translational modification causing the shift in pI. This protein was located periplasm of *E. coli* and acts as a defence mechanism against reactive oxygen species in the immediate location. In *E. coli*, synthesis of the protein was found to increase in response to oxygen stress (Cha *et al.*, 1995), and a specific promoter was also identified up-stream of the encoding. This suggests that oxygen-related transcriptional regulatory proteins were involved in its transcription (Kim *et al.*, 1996). The substantial up-regulation of this protein in *C. jejuni* would suggest that it is acting to remove reactive oxygen molecules.

Protein composite no. 11 (28) was identified to have homology to GroEL of *C. jejuni*. This protein has been proposed to have a role in protection against oxidative stress (Takata *et al.*, 1995). GroEL is a highly conversed protein and a member of the Heat Shock Protein

(HSP) 60 family and functions as a chaperone maintaining proteins in their pre-folded state allowing their export and facilitating protein assembly (Langer *et al.*, 1992). GroEL was up-regulated under oxidative stress in *C. jejuni*, though the level of expression did not alter in *C. jejuni* 9519, which can not be accounted for given its oxygen sensitive nature. The relatively high proportion of GroEL present in the cell may reflect the protective role and the organism acting to minimise the number of mis-folded proteins caused by oxidative damage. The possible involvement of HSP in promoting virulence has been mentioned previously (see section 4.2.7). Of particular interest GroEL was up-regulated in *Haemophilus ducreyi* following exposure to oxidative stress which was found to promote survival and adherence to INT 407 cells, suggesting a possible role in virulence in this pathogen (Parsons *et al.*, 1997).

The up-regulated protein composite no. 57 (100) was identified as having significant homology to translocation elongation factor of *H. pylori*. This was previously identified in section 4.2.3. This protein acts to promote efficient binding of an aminoacyl tRNA molecule to the correct site on the ribosome during the elongation of protein synthesis. Protein composite no. 278 (240) was found to have homology to the 50S Ribosomal protein L9 of *H. pylori*. This protein forms' part of the large subunit, and in *E. coli* was thought to bind the 3' 12S fragment of the 23S RNA domain near L11 (Adamski *et al.*, 1996). The role of the L9 site in the ribosome was thought to position the P-site of t-RNA during translation allowing the anti-codon:codon interaction. Both of these proteins were found to be substantial up-regulated under oxidative stress in both *C. jejuni* 81116 and *C. jejuni* 9519. This may reflect the organisms demand for protein synthesis as it adapts to oxidative stress allowing survival.

Interestingly, two proteins were identified as having homology to PEB-4 of *C. jejuni*, which were separated by 0.1 pH units. These were protein composite no. 92 (149) which was up-regulated following oxidative stress and protein composite no. 261 (329), a novel protein under oxidative stress. PEB-4 was initially isolated from an acid glycine extraction of *C. jejuni*, following its possible involvement in adhesion to eukaryotic cells (Kervella *et al.*, 1993). Subsequently this protein was shown not to be surface exposed and could not function as an adhesion nor directly involved in adherence. The gene, *peb4A*, found to have homology to a gram-positive extracytoplasmic lipoprotein involved in processing export proteins (Burucoa *et al.*, 1995). There was evidence of only one *peb4A* gene in the genome of *C. jejuni*, and the presence of two PEB-4 proteins in the proteome of oxidatively stressed *C. jejuni* does suggest post-translational modification has occurred, or that the protein may have been substantially up-regulation under oxidative stress. The protein sequence has been shown to contain a leader sequence of 21 amino acids suggesting the protein was exported from the cytoplasm. The role of PEB-4 under oxidative stress remains unclear, though it may be involved in the secretion of other proteins.

The protein composite no. 125 (196) was identified as having significant homology to PEB-1. This protein was first identified in an acid glycine cell extract, where it was found to be surface located and was thought to be involved in adherence to HeLa cells (Kervella *et al.*, 1993). Sequencing of the encoding gene, *peb1A*, identified homology to an amino acid transport protein (Pei *et al.*, 1993). However, further work has demonstrated that it plays an important role in epithelial cell interactions and in intestinal colonisation in a mouse model and may represent an important virulence determinant (Pei *et al.*, 1998). Interestingly, here the protein was down-regulated in both isolates in response to oxidative stress, suggesting that it may not be an essential adhesion molecule, as oxidative stress was found to enhance the invasive ability of *C. jejuni* into Caco-2 cell monolayers in this study.

Protein composite nos. 13 and 14 (not detected in isolate 9519) were identified to have significant homology to the flagellin protein of *C. jejuni*. This protein is a major component of the flagella, which is used for motility and invasion (Wassenaar *et al.*, 1991) by *C. jejuni*. These two proteins were separated by 0.1 pH units suggesting post-translational modification of the flagellin protein had occurred. Post-translational modification of flagellin has been reported previously by glycosylation (Doig *et al.*, 1996; Guerry *et al.*, 1996). The flagellin protein was substantially down-regulated following here under oxidative stress. This was unexpected as this structure was thought to be involved in invasion and under these conditions the invasive ability of *C. jejuni* into Caco-2 cell monolayers was substantially up-regulated.

No homology was identified for protein composite no. 277 (241) after searching the Campylobacter genome and non-redundant sequence databases. It was proposed to have similarity to a hypothetical protein of 20 kDa from *H. pylori*. However, its function remains unclear.

The protein composite no. 171 (197) was identified to have homology to the high temperature requirement B protein of *E. coli*. This protein was found to aid viability at high temperatures (Karow & Georgopoulos, 1991), and may be involved in the synthesis and/or maintenance of the cell wall (Karow *et al.*, 1991). In *C. jejuni* 81116 and *C. jejuni* 9519 the protein was found to be up-regulated under oxidative stress, which may reflect an increase in cell wall synthesis during the development of the protrusions from *C. jejuni* outer surface during the change in morphology. However, the role of HtrB in *C. jejuni* remains unclear

The invasive capacity of both *C. jejuni* isolates, 81116 and 9519, was significantly enhanced following prior exposure to oxidative stress. The much larger increase in invasive ability demonstrated for *C. jejuni* 9519 was unexpected, but may be due to the it being a fresh isolate rather than laboratory adapted, with more of its virulence determinants being expressed, or that *C. jejuni* 9519 was a clinical isolate, rather than an environmental isolate, such as *C. jejuni* 81116.

Oxygen tensions are considered to be low in the gut, and have been reported to enhance the invasive capacity of *S. typhimurium* (Ernst *et al.*, 1990; Lee & Falkow, 1990). Potential virulence determinants are expressed under low oxygen tensions in *S. typhimurium* have been identified including *orgA* and *invG* (Jones & Falkow, 1994; Lelerc *et al.*, 1998). Invasiveness was not similarly influenced in *C. jejuni* since higher oxygen concentrations and oxidative stress were conditions which significantly increased invasive ability.

However, the levels of oxygen that *C. jejuni*, a microaerophile, registers in the gut may be not be as high, relative to those perceived by *Salmonella* spp., which is a facultative anaerobe. It was thought unlikely that *C. jejuni* would encounter conditions of oxidative stress in the initial stages of enterocyte invasion, but these conditions may be encountered in the later stages of the disease process. Similarly high oxygen tensions induced the expression of the *ail* gene of *Yersinia enterocolitia* which mediates bacterial entry into the CHO cell line (Pederson & Pierson, 1995). This gene has now been suggested to have a role in the latter stages of the disease process, instead of entry into the host.

C. jejuni has been shown to elicit a host inflammatory response becoming exposed to oxidative bursts created by polymorphonuclear leukocytes (PMNs) infiltrating the intestinal epithelium (Russell *et al.*, 1989). Superoxide molecules are released, for example, by the

PMNs raising the oxidative potential in the bacteria's immediate environment. This acts as part of the hosts' defence mechanism against invading bacteria (Roggenkamp *et al.*, 1997). *C. jejuni* has been shown to stimulate oxidative bursts from PMNs (Walan *et al.*, 1992), but have been shown to be unable to survive for extended period in macrophages (Waasenaar *et al.*, 1998). The antioxidant enzymes of *C. jejuni*, including SOD and catalse, would act to remove the reactive molecules produced in these oxidative bursts. The SodB mutant of *C. jejuni* has been shown to be less invasive into the INT407 cell line, implicating the possible role of superoxide dismutase in the intracellular survival of *C. jejuni* in INT407 cells (Pesci *et al.*, 1994). However, the mutation in SodD and KatA did not alter the survival of *C. jejuni* in macrophages (Waasenaar *et al.*, 1998).

In summary, *C. jejuni* has been shown to sustain growth under oxidative stress. Culturability of the bacteria was found to decline in the first 24 h, though metabolic activity was retained in a proportion of the cells. Recovery of the VNC cells may have contributed to the increase in plate counts observed between 24h and 48 h. Oxidative stress in conjunction with a reduced carbon source was also found to promote coccoid cell formation. These structures were shown to be degenerate.

Protein expression altered significantly under oxidative stress, some of which were identified by Edman degradation. Protein homologues involved in protection against oxidative damage were identified including GroEL, AhpC and Tpx. Other protein homologues may contribute to maintaining cell functions under these conditions including the chaperone GroEL, translation elongation factor and the ribosomal protein, L9. These proteins may reflect an increased protein turnover, replacing those proteins damaged by oxidative stress. These changes in protein expression may represent an attempt by *C. jejuni* to maintain viability under oxidative stress and may contribute to the organism's persistence

in the environment outside of the gut. However, other proteins may be involved in the invasion into Caco-2 cell monolayers which was substantially increased under oxidative stress.

Of those proteins sequenced with identified homologues, two with virulence characteristics, flagella and PEB-1 were down-regulated in expression. However, further studies using classical genetic techniques may expand the roles of other identified proteins. Of particular interest would be gene knock-out studies on PEB-4, HtrB and Tpx.

4.4 Effects of acid stress

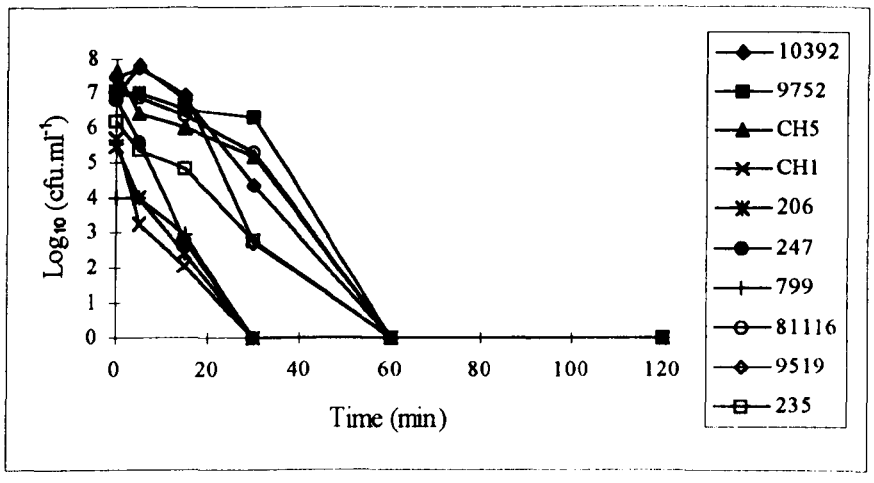
4.4.1 Assessing the variation in the ability of *Campylobacter* isolates to survive exposure to pH 3.5

Campylobacter isolates were grown in batch cultures (see section 2.4.1) and an aliquot removed to give 10^8 cell.ml^{-1} in 5 ml of Cpmod4 broth, pH adjusted to pH 3.5 using 2M HCl. Over a 2 h period samples were removed at regular intervals and colony forming units determined (see section 2.6.1) to assess the effect of exposure to pH 3.5 on the isolates' ability to survive.

All of the *Campylobacter* isolates were rapidly killed following exposure to this extreme pH (see Fig. 32). However, the time taken to kill the *Campylobacter* isolates was found to vary between the isolates. One group (CH1, 206, 247 and 799) failed to be cultured after 15 min of exposure to pH 3.5. The remaining group (CH5, 235, 9752, 81116, 9519 and10392) remained culturable until at least 30 min of exposure, with only a small decline in culturability observed in the first 15 min of exposure.

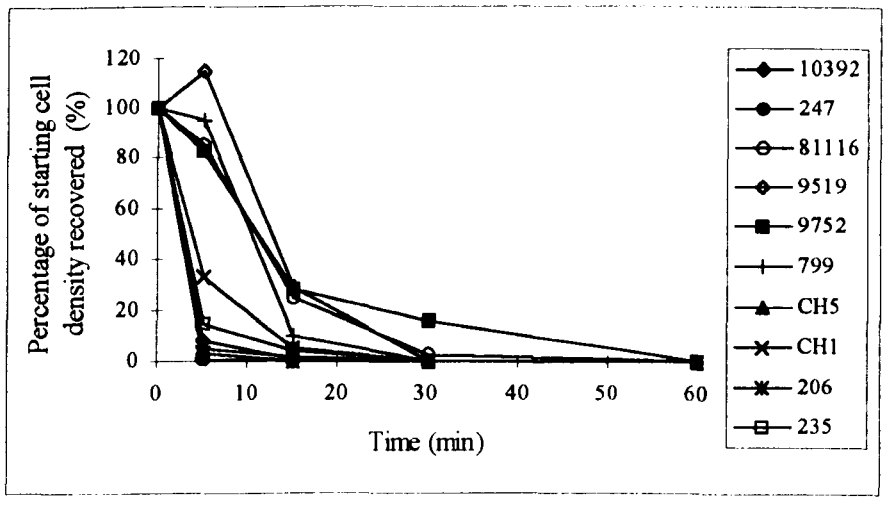
However, a strong correlation was indicated by Spearman Rank Correlation (p=0.05), between starting biomass and the ability of the isolates to resist acid shock. Re-plotting of the data, as a proportion of the starting cell density at t=0, demonstrated the presence of two groups, containing different members (see Fig. 33). One group (799, 9519, 9752 and 81116) was more able to resist acid shock that the other (CH1, CH5, 206, 235, 247 and 10392). The difference between these two groups was found to be significant at t=15 min by the t-test (p=0.05).

Fig. 32: Comparison of the ability of different *Campylobacter* isolates grown in batch culture in Cpmod4 broth to survive acid shock of pH 3.5.



Note: Each data point represents a single determination.

Fig. 33: Comparison of the ability of different *Campylobacter* isolates to survive acid shock of pH 3.5, with the recovered cells expressed as a proportion of the starting cell density.



Note: *Campylobacter* isolates were grown in batch culture in Cpmod4 and exposed to acid shock of pH 3.5. Data points represent single determinations.

4.4.2 Assessing the ability of chemostat grown *Campylobacter* to survive exposure to pH 3.5

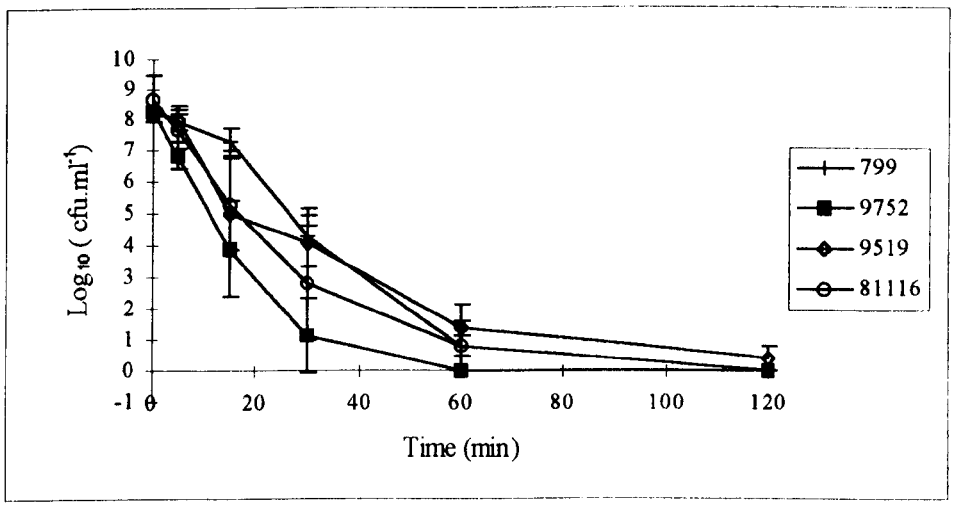
The difference between the isolates, to survive exposure to pH 3.5 in batch culture has been shown to be affected by the starting biomass. To standardize the cells used in subsequent experiments, four *Campylobacter* isolates, 9519, 799, 9752 and 81116 (selected for their differing ability to survive acidic conditions, as shown in Fig. 32) were grown independently in continuous culture in ABCD medium (see section 2.4.2). The pH of each steady state culture was allowed to reach its equilibrium pH of between pH 7.0 - 7.2. These cultures were then exposed to pH 3.5 over a 2 h time course as described in section 4.4.1. A decline in the number of culturable *Campylobacter* was observed (see Fig. 34), with the ability of the isolates to with-stand exposure to the acid shock found to vary. However, the isolates' responses were not found to differ significantly from one another as indicated by ANOVA ($p=0.05$). Isolate 9752 failed to be cultured after 30 min, isolates 799 and 81116 remained culturable following 1 h of exposure, and isolate 9519 remained culturable at low levels after 2 h.

4.4.3 Adaptation of *C. jejuni* to acidic environments whilst growing in continuous culture

C. jejuni 81116 was established in continuous culture in ABCD medium, with the metabolic activity of the bacteria poising the pH of the culture at pH 7.1 (see section 2.4.2). The pH of the cultures was shifted to pH 4.0 by adjusting the set points of the acid and alkali controllers. Peristaltic pumps were used to add 2 M HCl and 2 M NaOH to create the acidic conditions pre-set on the Anglicon controller panel.

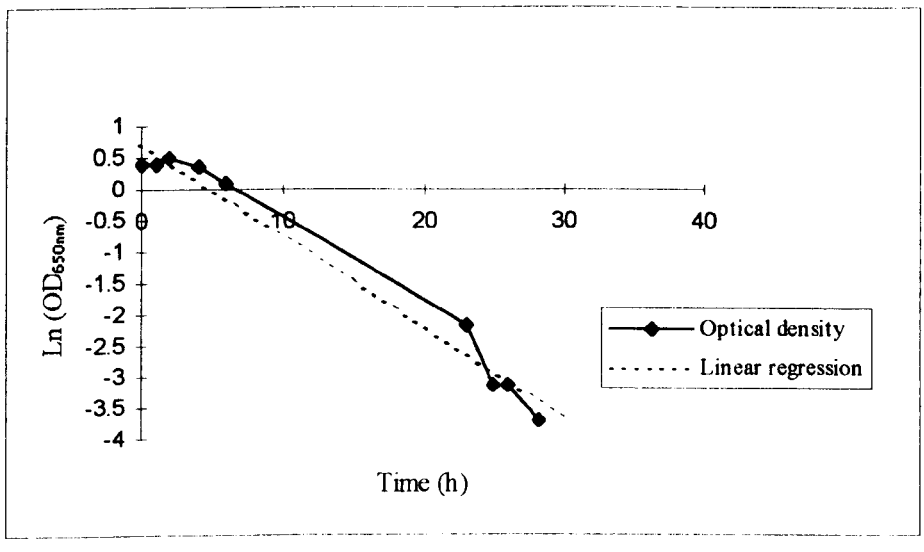
Over a 30 h period there was a steady decline in biomass as determined by optical density within the chemostat. The decay constant of the optical density of the culture was found to

Fig. 34: Comparison of the ability of four *Campylobacter* isolates grown in continuous culture in ABCD media to survive exposure to pH 3.5



Note: *Campylobacter* isolates were grown in continuous culture and during steady state conditions an aliquot was removed and resuspended in 5 ml of pre-acidified ABCD media (pH 3.5) to give 10^8 cell.ml^{-1}. Samples were removed at intervals and plated on CBA plates. Data points represent means ± SE (based on three replicate experiments).

Fig. 35: Response of *C. jejuni* 81116 grown in continuous culture to adjusting the chemostat to pH 4.0



Note: The change in biomass was monitored by optical density with absorbance read at A_{650nm}. Data was transformed by natural log with a linear regression line plotted (y=-0.1471x +0.7356) to show the wash out rate of the culture.

be 0.15 h^{-1} (see Fig. 35), which exceeded the dilution rate of the culture (0.08 h^{-1}) and confirmed that the culture was dying and washing out.

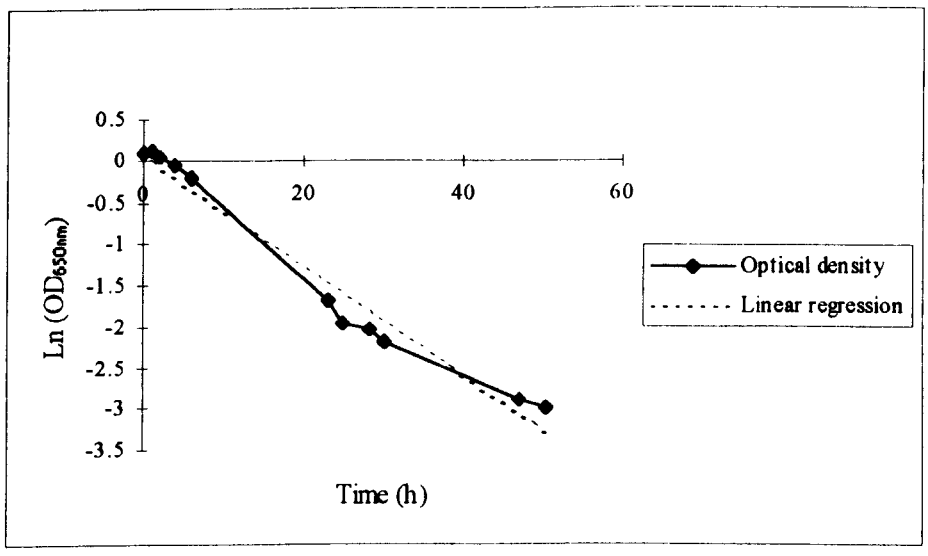
C. jejuni 81116 was re-established in continuous culture at between pH 7.0 - 7.2 before the pH was altered to pH 5.0 as described above. This caused a gradual decline in the optical density of the chemostat over a 52 h period (see Fig. 36). Again the decay constant of the optical density of the culture (0.07 h^{-1}) demonstrated that the bacteria were unable to replicate at the set dilution rate.

C. jejuni 81116 was re-established in continuous culture and the pH of the culture was shifted to pH 5.5. This also caused a decline in cell numbers, as indicated by optical density measurements (see Fig. 37). The rate of decline gave an overall decay constant of 0.05 h^{-1} over 50 h, which did not exceed the set dilution rate (0.08 h^{-1}) of the culture. However, the response of the culture was bi-phasic (see Fig. 37). In the initial 24 h period optical density declined with a decay constant of (0.05 h^{-1}) which did not exceed the dilution rate of the vessel (Fig. 38a). In the ensuing period the decline in optical density slowed (0.009 h^{-1}), wash-out ceased and continuous culture was established (Fig. 38b). A similar response was observed with isolate 9519, where adaptation to pH 5.5 caused a bi-phasic response (see Fig. 39). In the initial 48 h period there was a decline in optical density, though the decay constant of 0.04 h^{-1} did not exceed the set dilution rate (0.08 h^{-1}), washout ceased after 50 h and continuous culture was established.

4.4.4 Effects of acid adaptation on *C. jejuni*

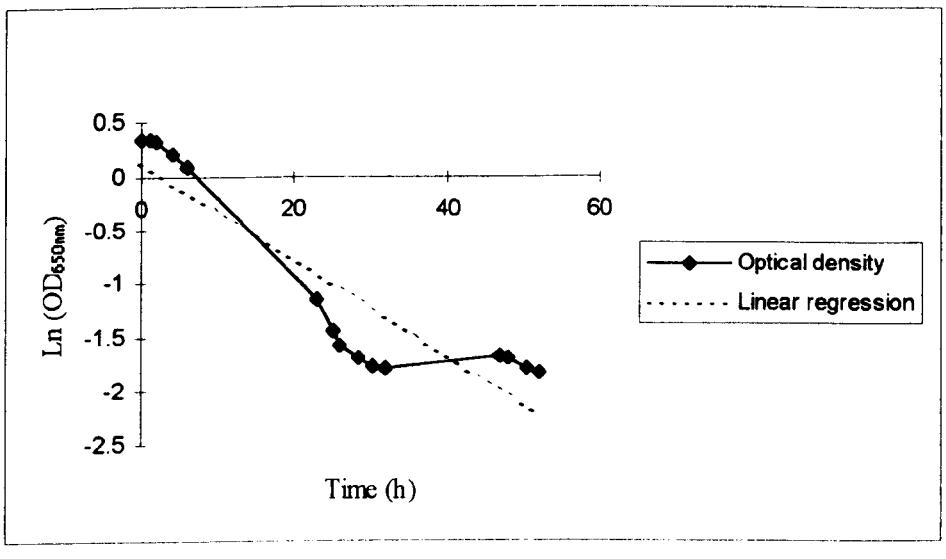
As a consequence of growth at pH 5.5, the viable count of *C. jejuni* 81116 declined 10-fold over the 48 h period from 1.78 x 10^9 cfu.ml^{-1} to 1.7 x 10^8 cfu.ml^{-1} (See Table 13). A less marked reduction was observed in total cell count over the same period.

Fig. 36: Response of *C. jejuni* 81116 grown in continuous culture to adjusting the chemostat to pH 5.0.



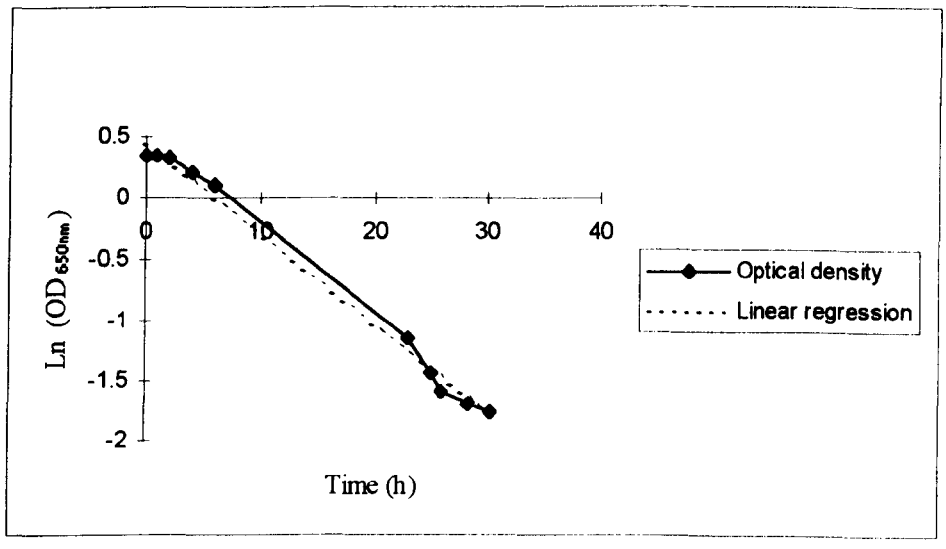
Note: The change in biomass was monitored by optical density with absorbance read at A_{650nm}. Data was transformed by natural log with a linear regression line plotted (y=-0.0669x + 0.0705) to show the wash-out rate of the culture.

Fig. 37: Response of *C. jejuni* 81116 grown in continuous culture to adjusting the chemostat to pH 5.5.



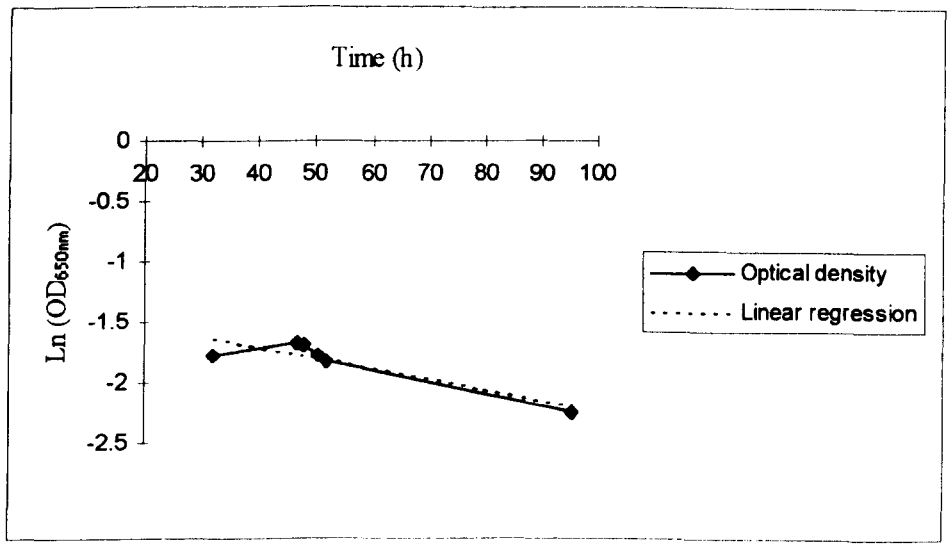
Note: The change in biomass was monitored by optical density with absorbance read at A_{650nm}. Data was transformed by natural log with a linear regression line plotted (y=-0.0453x + 0.1311) to show the wash-out rate of the culture.

Fig. 38a: Response of *C. jejuni* 81116 grown in continuous culture to adjusting the chemostat to pH 5.5 over the intial 24 h period.



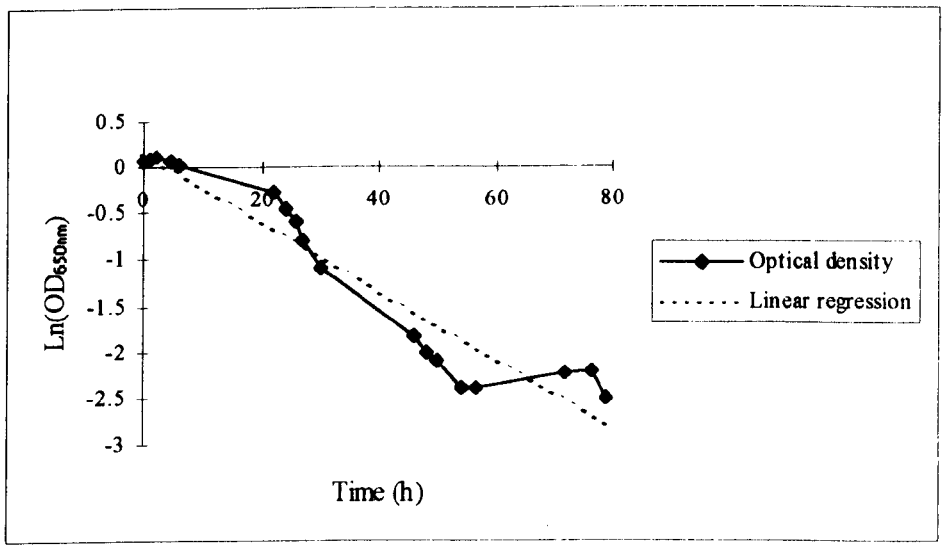
Note: The change in biomass over the initial 24 hours was monitored by optical density with absorbance read at A_{650nm}. The data points are natural log of the optical density with a linear regression line (y=-0.0453x + 0.1311) inserted to show the wash-out rate of the culture.

Fig. 38b: Response from 24 -95 h.



Note: The change in biomass was monitored by optical density with absorbance read at A_{650nm}. The data points are natural log of the optical density with a linear regression line (y=0.0087x - 1.1354) inserted to show the wash-out rate of the culture.

Fig. 39: Response of *C. jejuni* 9519 grown in continuous culture to adjusting the chemostat to pH 5.5.



Note: The change in biomass was monitored by optical density with absorbance read at A_{650nm}. Data was transformed by natural log with a linear regression line plotted (y=-0.0375x + 0.1645) to show the wash-out rate of the culture.

Table 13: Summary of the response of *C. jejuni* 81116 to acidic conditions (pH 5.5) in continuous culture

Parameter	*C. jejuni* 81116 grown at pH 7.1	95 % C.I	*C. jejuni* 81116 grown at pH 5.5	95 % C.I
Steady state optical density (A_{650nm})	1.3	ND	0.109	ND
Total cell count (cell.ml^{-1})	3.02×10^9	3.3×10^9 - 2.7×10^9	5.1×10^8	6.5×10^8 - 4.0×10^8
Colony forming units (cfu.ml^{-1})	1.78×10^9	2.2×10^9 - 1.5×10^9	1.7×10^8	2.1×10^8 - 1.4×10^8

Key: 95 % C.I, 95 % confidence intervals; ND, not determined.

4.4.5 Acid killing of acid adapted chemostat cultures

C. jejuni 81116 and 9519 were removed from continuous culture after adaptation at pH 5.5 for 24 h and 48 h and exposed to pH 3.5 over a 2 h period, as described previously (see section 4.4.1). *C. jejuni* 81116 following 24 h of adaptation did not substantially alter its ability to survive exposure to pH 3.5, with 38 % of the starting cell density remaining culturable after 30 min, similar to the proportion remaining after challenge of non-adapted bacteria (see section 4.4.2). After 48 h of adaptation at pH 5.5, *C. jejuni* 81116 was more able to with-stand acid shock at pH 3.5, with 78 % of the starting cell density remaining culturable after 30 min (see Fig. 40a). However, there was not a statistically significant difference between the survival abilities of the non-adapted and 48 h adapted cultures.

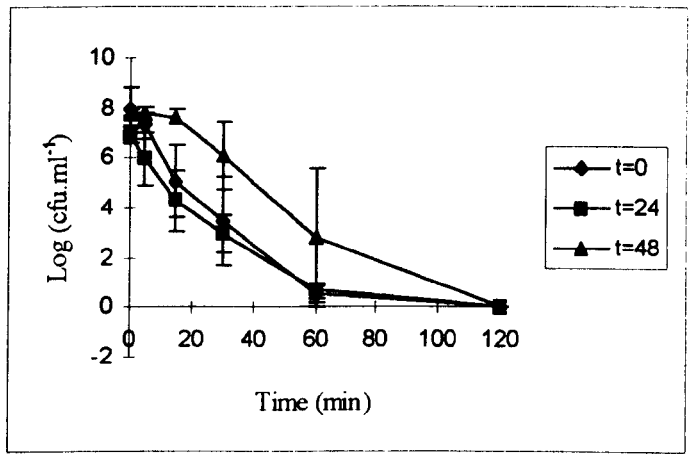
After 24 h of adaptation to pH 5.5, *C. jejuni* 9519 showed an increased ability to survive exposure to pH 3.5, with 68 % of the starting cell density recovered at 30 min compared to the non-adapted culture (see Fig. 40b). In addition, *C. jejuni* 9519, adapted for 24 h remained detectable at low levels up until the end of the 2 h acid shock. Following 48 h of adaptation at pH 5.5, 99 % of the starting cell density was recovered after 30 min of exposure, and a decline in culturability was only detected after 60 min of exposure. The significance of the increased survival demonstrated following growth at pH 5.5 could not be tested, due to these being only one sample for the group t=48 h.

4.4.6 Changes in protein expression following growth at pH 5.5

Proteins of *C. jejuni* 81116 and 9519 grown at pH 7.1 and adapted to pH 5.5 for 48 h were analysed using 2-D gel electrophoresis (see section 2.10.2) and stained with Coomassie Blue (Appendix II). The gels were matched and compared using the BioImage system (see section 2.10.3).

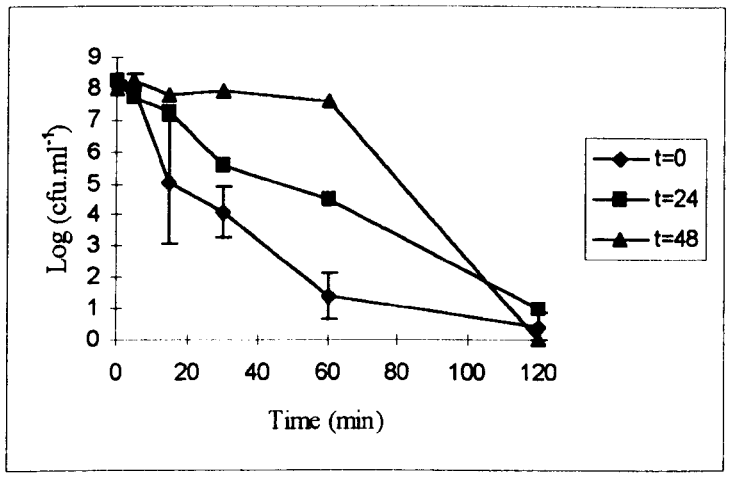
Fig. 40: Showing the ability of *C. jejuni* 81116 and 9519 to survive acid shock before and during acid adaptation.

a) Response of *C. jejuni* 81116



C. jejuni 81116 was harvested from the acid adapted chemostat at t=0, 24 and 48 h, and resuspended in 5 ml of pre-acidified ABCD media (pH 3.5) to give 10^8 cell.ml^{-1}. Samples were removed at intervals and plated on CBA plates. No significant difference was found between the non-adapted and 48 h adapted cultures.

b) Response of *C. jejuni* 9519



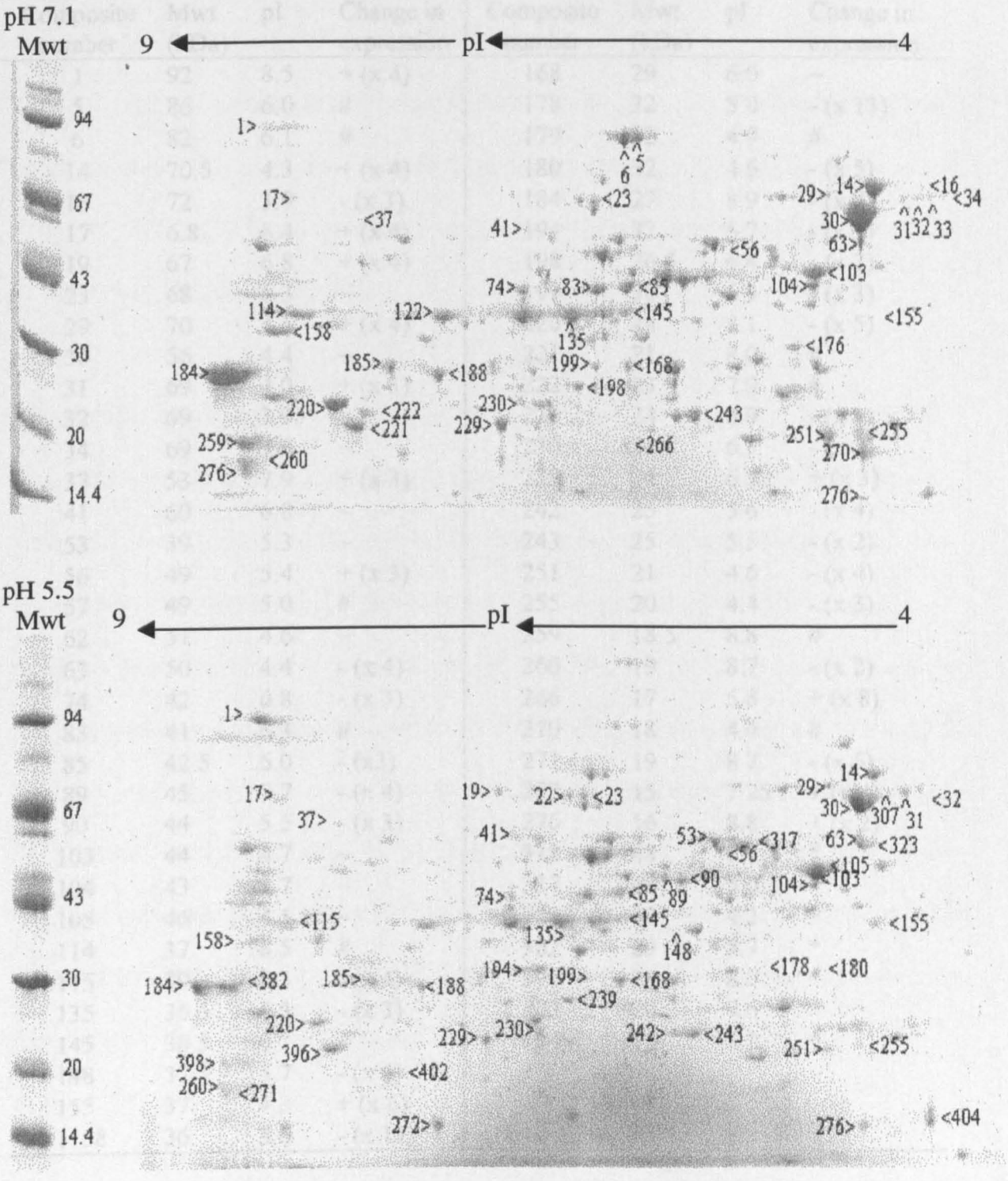
C. jejuni 9519 was harvested from the acid adapted chemostat at t=0, 24 and 48 h, and resuspended in 5 ml of pre-acidified ABCD media (pH 3.5) to give 10^8 cell.ml^{-1}. Samples were removed at intervals and plated on CBA plates. Statistical tests could not be carried out due to not enough repeats being carried out.

A total of 292 proteins were identified in *C. jejuni* 81116 grown at pH 5.5, and 277 in cultures grown at pH 7.1 (see Fig. 41). Of these, 163 were matched between the two conditions. The remainder, 114 after growth at pH 7.1 and 129 after growth at pH 5.5, were identified as novel to each particular growth condition. However, as lower abundance proteins were not always reliably identified by the BioImage system, novel proteins of integrated intensity less than 0.7 were not included in any further analysis. Hence, under these more stringent conditions, 10 novel proteins were identified from cultures grown at pH 7.1 (composite nos. 5, 6, 57, 83, 114, 179, 221, 222, 259, 270) and 7 novel proteins were identified from cultures grown at pH 5.5 for 48 h (composite nos. 312, 317, 323, 382, 396, 402, 404) (see Table 14).

Some of the matched proteins, were up- or down-regulated in their expression as a consequence of growth at pH 5.5. The majority of the proteins (42 %) did not alter in expression (including composite nos. 23, 30, 34, 41, 53, 62, 103, 104, 105, 145, 168). Twenty-seven proteins were up-regulated between 2-3 fold following the adaptation to pH 5.5 (including composite nos. 1, 276), and seven were up-regulated between 3-4 fold (composite nos. 17, 19, 31, 37, 56, 239 and 272). A total of three proteins (composite nos. 29, 155, 266) were up-regulated more than 4-fold. In contrast, many proteins were down-regulated following adaptation to pH 5.5 with 38 proteins (including composite nos. 85, 198, 230) down-regulated between 2-3 fold. Ten proteins were down-regulated between 3-4 fold (composite nos. 14, 63, 89, 90, 115, 135, 194, 199, 229 and 242), and eight were down-regulated more than 4-fold (composite nos. 148, 158, 178, 180, 184, 220, 251, 271).

More proteins were detected in cultures of *C. jejuni* 9519 than *C. jejuni* 81116, with 318 proteins detected following 48 h growth at pH 5.5 and 343 proteins detected at pH 7.1, which may reflect loading differences or species-specific proteins. In isolate 9519 under the

Fig 41: 2-Dimensional gel electrophoresis of whole cell proteins of *C. jejuni* 81116 grown at pH 7.1 and pH 5.5



Whole cell proteins were harvested from chemostat cultures grown at pH 7.1 and pH 5.5 and focused in the 1st dimension on IEF strips pH 3-10. The proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system. Protein composite numbers were ssigned, some of which are indicated on the gels. Mwt markers (kDa) and pI are indicated on the gels.

Table 14: Comparison of proteins identified following 2-D gel electrophoresis of *C. jejuni* 81116 grown at pH 7.1 and pH 5.5

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
1	92	8.5	+ (x 4)	168	29	6.0	~
5	86	6.0	#	178	32	5.0	- (x 13)
6	82	6.1	#	179	32	4.9	#
14	70.5	4.3	+ (x 4)	180	32	4.6	- (x 5)
16	72	3.9	- (x 3)	184	27	8.9	- (x 8)
17	6.8	8.4	+ (x 4)	194	32	6.7	- (x 3)
19	67	6.8	+ (x 4)	198	26.5	6.4	- (x 2)
23	68	6.3	~	199	30	6.3	- (x 3)
29	70	4.6	+ (x 4)	220	24	8.1	- (x 5)
30	56	4.4	~	221	21	8.0	#
31	69	4.0	+ (x 3)	222	23	7.9	#
32	69	3.95	~	229	24	6.9	- (x 3)
34	69	3.8	~	230	24.5	6.3	- (x 2)
37	53	7.9	+ (x 3)	239	24	6.3	+ (x 3)
41	60	6.8	~	242	25	5.6	- (x 4)
53	39	5.3	~	243	25	5.5	- (x 2)
56	49	5.4	+ (x 3)	251	21	4.6	- (x 4)
57	49	5.0	#	255	20	4.4	- (x 3)
62	51	4.6	~	259	18.5	8.8	#
63	50	4.4	- (x 4)	260	19	8.7	- (x 2)
74	42	6.8	- (x 3)	266	17	5.8	+ (x 8)
83	41	6.3	#	270	18	4.4	#
85	42.5	6.0	- (x3)	271	19	8.7	- (x 5)
89	45	5.7	- (x 4)	272	15	7.25	+ (x 4)
90	44	5.5	- (x 3)	276	16	8.8	+ (x 2)
103	44	4.7	~	312	48	6.1	*
104	43	4.7	~	317	49	5.0	*
105	46	4.5	~	323	49	4.2	*
114	37	8.5	#	382	29	8.7	*
115	39	8.3	- (x 4)	396	23	8.0	*
135	36.5	6.3	- (x 3)	402	20	7.6	*
145	38.5	6.1	~	404	15	3.2	*
148	37.5	5.7	- (x 6)				
155	37	4.3	+ (x 6)				
1158	36	8.6	- (x 17)				

Key:

* novel following growth at pH 5.5
\# novel following growth at pH 7.0
~ no alteration in expression
\+ up-regulated following growth at pH 5.5
\- down-regulated following growth at pH 5.5

Bracketed numbers indicate the level of change in expression

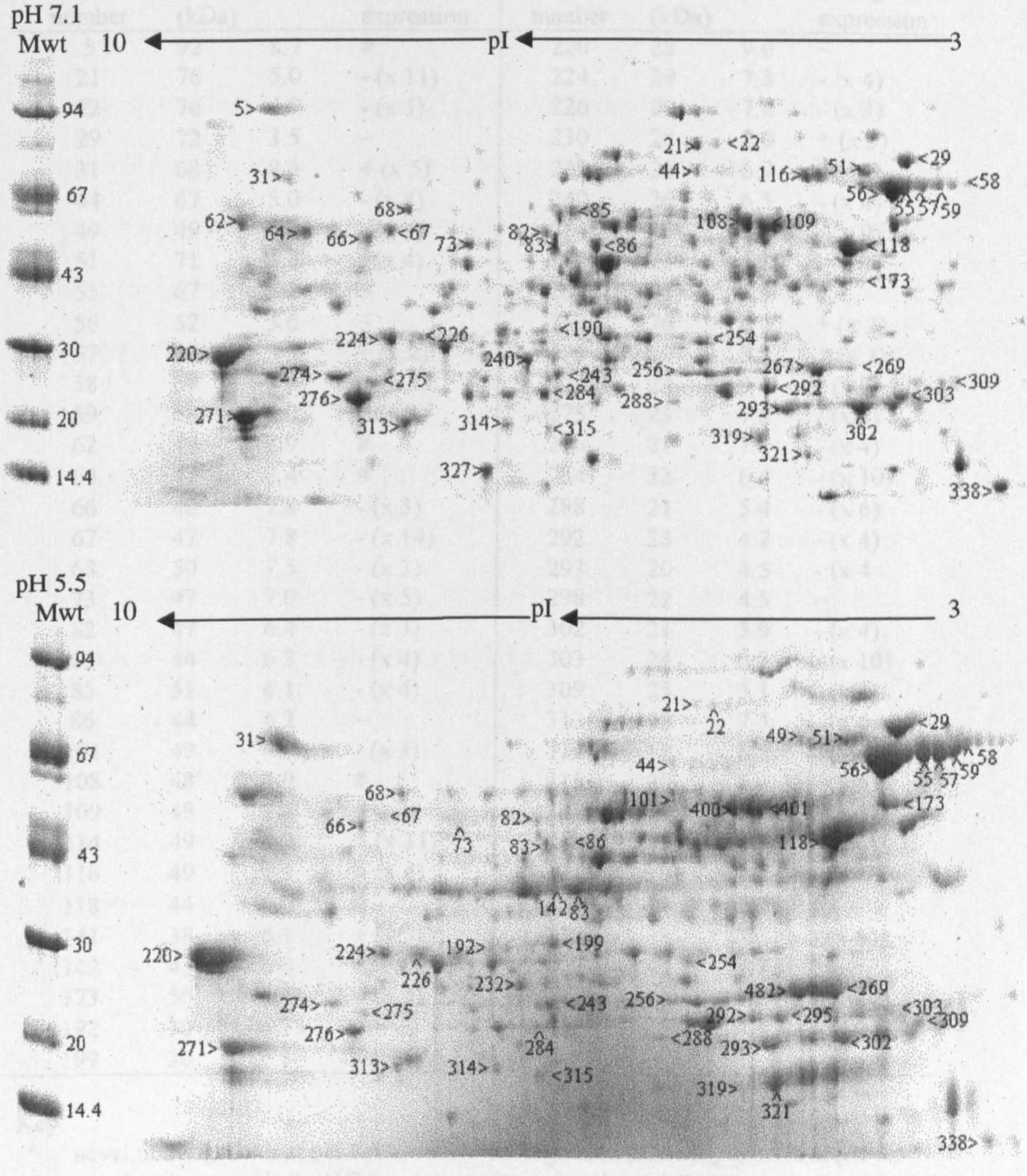
same conditions, 144 proteins were matched. The remaining proteins were classified as novel; that is 174 proteins from growth at pH 5.5 and 197 at pH 7.1 (see Fig. 42). However, as previously mentioned some of the lower abundance proteins were not reliably identified by the BioImage system and in this instance proteins of integrated intensity less than 0.3 were not included in the further analysis. Under these more stringent conditions, seven novel proteins were identified following growth at pH 7.1 (composite nos. 5, 62, 64, 108, 109, 267 and 327) and three novel proteins after growth at pH 5.5 (composite nos. 400, 401 and 482) (see Table 15).

The matched proteins also responded to growth at pH 5.5 by up- or down-regulation of their expression by comparison to cultures grown at pH 7.1. Nine proteins (including composite nos. 57, 58, 59, 269) were up-regulated between 2-3 fold and one protein was up-regulated between 3-4 fold (composite no. 116). A total of six proteins were up-regulated more than 4-fold (composite nos. 31, 114, 141, 199, 230, and 321). Twenty-one proteins were down regulated between 2-3 fold (including composite nos. 66, 271 and 188). Fourteen proteins were down-regulated between 3-4 fold (composite nos. 22, 51, 68, 82, 83, 85, 101, 224, 226, 232, 302, 309, 313 and 314) and a total of 24 proteins were down-regulated more than 4-fold (composite nos. 21, 44, 49, 67, 73, 86, 142, 192, 240, 254, 256, 274, 275, 276, 284, 288, 292, 293, 298, 303, 315, 317, 319 and 338). The remainder of the proteins (66), did not alter in their level of expression (including composite nos. 29, 55, 56, 118 and 220).

4.4.7 Invasive ability of acid adapted *C. jejuni* into Caco-2 monolayers

The ability of *C. jejuni* 81116 grown in continuous culture at pH 7.1 and pH 5.5 to invade Caco-2 cell monolayers was compared over 6 h. The invasion assay was carried out as described in section 2.8.8, with modification in the inoculum preparation for acid adapted

Fig 42: 2-Dimensional gel electrophoresis of whole cell proteins of *C. jejuni* 9519 grown at pH 7.1 and pH 5.5



Whole cell proteins were harvested from chemostat cultures grown at pH 7.1 and pH 5.5 and focused in the 1st dimension on IEF strips pH 3-10. The proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system. Protein composite numbers were assigned, some of which are indicated on the gels. Mwt markers (kDa) and pI are indicated on the gels.

Table 15: Comparison of proteins identified following 2-D gel electrophoresis of *C. jejuni* 9519 grown at pH 7.1 and pH 5.5

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
5	92	8.7	#	220	28	9.0	~
21	76	5.0	- (x 11)	224	29	7.8	- (x 4)
22	76	4.9	- (x 3)	226	29	7.4	- (x 3)
29	72	3.5	~	230	28	7.0	+ (x 5)
31	68	8.4	+ (x 5)	232	26	6.9	- (x 4)
44	67	5.0	- (x 4)	240	26	6.5	- (x 4)
49	49	4.7	- (x 4)	254	29	5.2	- (x 9)
51	71	3.8	- (x 4)	256	25	5.2	- (x 4)
55	67	3.2	~	267	20	4.6	#
56	52	3.6	~	269	26	4.0	+ (x 2)
57	67	3.3	+ (x 2)	271	19	8.8	+ (x 3)
58	69	3.0	+ (x 3)	274	24	8.0	- (x 7)
59	68	3.0	+ (x 3)	275	23	7.8	- (x 4)
62	49	9.9	#	276	21	7.8	- (x 4)
64	47	8.4	#	284	22	6.4	- (x 10)
66	46	7.8	- (x 3)	288	21	5.4	- (x 6)
67	47	7.8	- (x 14)	292	23	4.7	- (x 4)
68	50	7.5	- (x 3)	293	20	4.5	- (x 4
73	47	7.0	- (x 5)	298	22	4.5	~
82	47	6.4	- (x 3)	302	21	3.9	- (x 4)
83	44	6.3	- (x 4)	303	24	6.3	- (x 10)
85	51	6.1	- (x 4)	309	25	3.1	- (x 3)
86	44	6.2	~	313	18	7.5	- (x 4
101	49	4.9	- (x 3)	314	18	6.5	- (x 4)
108	48	4.9	#	315	20	6.4	- (x 6)
109	48	4.8	#	317	14.4	6.0	- (x 5)
114	49	4.2	+ (x 11)	319	17	4.7	- (x 6)
116	49	4.3	+ (x 4)	321	16	4.5	+ (x 7)
118	44	4.0	~	327	12	7.0	#
141	38	6.1	+ (x 5)	338	~8	3.0	- (x 6)
142	43	6.0	- (x 12)	400	49	4.7	*
173	50	3.5	+ (x 4)	401	48	4.6	*
192	30	6.75	- (x 6)	482	25	4.3	*
199	30	6.3	+ (x 5)				

Key:

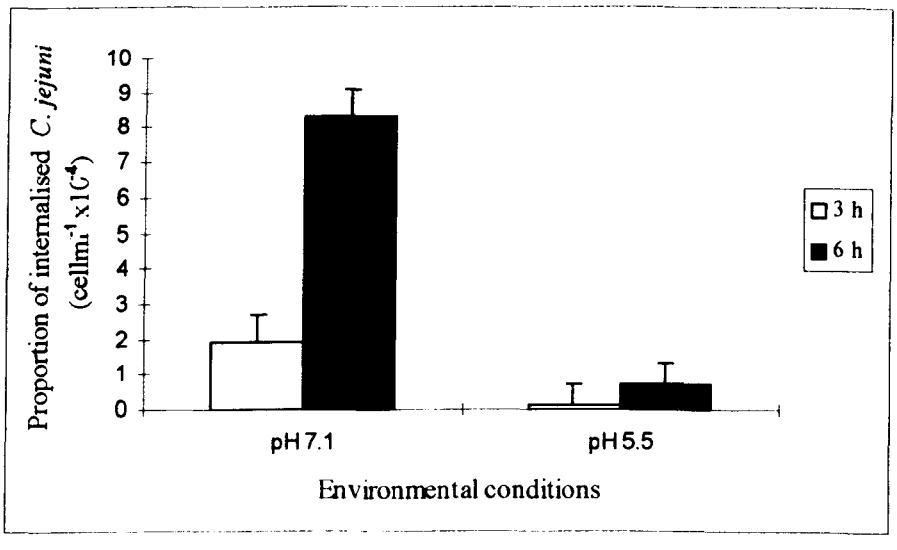
* novel under growth at pH 5.5
\# novel under growth at pH 7.1
~ no change in expression
\+ up-regulated following growth at pH 5.5
\- down-regulated following growth at pH 5.5

Brackets indicate the level of changes in expression

cultures to account for the lower cell density. Biomass (6 ml) was harvested from the acid adapted chemostat cultures, and 0.5 ml was harvested from the non-adapted culture, in order to be able to challenge the monolayers with 10^8 cell.ml^{-1}.

The proportion of the inoculum recovered from within the monolayers was found to increase with time with both *C. jejuni* 81116 grown at pH 7.1 and pH 5.5 (see Fig. 43). However, acid adaptation for 48 h was found to dramatically reduce the ability of *C. jejuni* 81116 to invade the cell monolayers. By 6 h, the proportion of *C. jejuni* 81116 grown under acid adaptation which was recovered from the cell monolayers was 10-fold lower than that of the cultures grown at pH 7.1. The difference between the invasiveness of these two types of culture was found to be highly significant by ANOVA (p=0.001).

Fig. 43: Comparison of the invasive ability of *C. jejeuni* 81116 into Caco-2 cell monolayers after growth at pH 7.1 and pH 5.5.



Note: Data represents the proportion of the inoculum internalised by Caco-2 cells. Bars represent means ± SE (three separate experiments). The difference between the invasive ability of cultures grown at pH 7.1 and pH 5.5 was significant by ANOVA (p=0.001).

Section 4.4.8 Discussion

Acid shock experiments were carried out at pH 3.5 to determine the extent of acid resistance amongst the panel of clinical and environmental *Campylobacter* isolates. This pH was selected as previous work with *Campylobacter* had demonstrated its ability to survive exposure to pH 2 and 3 for only 10 min, but was able to remain viable at pH 4 for 70 min (Rotimi *et al.*, 1990). The ability of *Campylobacter* spp. to resist exposure to lethal pH after growth in complex medium was found to vary between the isolates. However, initial analysis demonstrated that the concentration of biomass of *Campylobacter* challenged at pH 3.5 affected the survival. Although it was intended to compare stationary phase cultures, it was possible that the less dense cultures were still in exponential phase, which may have been more susceptible to acid shock than the denser stationary phase cultures. Compensating for differences in the starting biomass, the data demonstrated differences in acid resistance amongst the isolates tested, some being more resistant to acid shock than others. After 60 min of exposure, even the most acid resistant isolates failed to be recovered suggesting that *Campylobacter* had poor constitutive acid resistance compared to other enteric bacteria.

Resistance to low pH has been demonstrated in other enteric bacteria grown to stationary phase. For example, *Shigella dysenteriae* was able to survive exposure to pH 6 for up to 40 min, but at lower pHs (pH 2-5) it was found to be increasingly susceptible (Rotimi *et al.*, 1990). However, 75 % of *Shigella* and 80 % of *E. coli* isolates were acid resistant to exposure to pH 2.5, whereas *Salmonella* spp. were unable to survive exposure to pH 2.5, following growth in complex medium (Gorden & Small, 1993). Large variations can be observed between different strains of an organism which was demonstrated with different strains of *E. coli* (Brown *et al.*, 1997), following exposure to pH 3.0

To overcome influences of varying starting cell densities and to investigate the response of growing *Campylobacter* to acid shock, four isolates were established in continuous culture. *Campylobacter* isolates demonstrated a similar response to an acid shock of pH 3.5 as observed for batch grown cells. One isolate 9751 failed to be cultured after 30 min, two of the isolates, 81116 and 799, failed to be cultured after 60 min, and isolate 9519 was detected at low levels after 120 min of exposure to pH 3.5, suggesting that it was the most acid resistant isolate of those tested. The mode of bacterial growth was not found to significantly affect the ability of *Campylobacter* to withstand acid shock at pH 3.5, with those cells in stationary phase only slightly more resistant than the growing cells from the chemostat.

One mechanism able to protect against acid shock is the bacterial membrane (Chang & Cronon, 1999). During exponential phase growth the membrane is composed largely of unsaturated fatty acids (UFA) which offer little protection and may contribute to the poor acid resistance observed in some of the *Campylobacter* isolates tested. As bacteria enter stationary phase, a proportion of the UFA become converted by CFA synthetase into cyclopropane fatty acid (CFA) which decreases the leakage of protons across the cell membrane when the external proton concentrations are high (Jorden *et al.*, 1999). Interestingly, the phospholipid profile of *C. jejuni* 81116 whilst grown in ABCD medium in continuous culture has been shown to consist of a high proportion of CFA, depending on growth rate (Leach *et al.*, 1997). Hence, the membrane profile of *C. jejuni* grown in continuous culture here may be comparable to stationary phase membranes. Although resistance to acid shock was not increased substantially, some isolates did appear to be more able to withstand acid shock following growth in continuous culture which may be due to the presence of CFA.

Other mechanisms may contribute to acid resistance in enterobacteria. In *E. coli* and *Salmonella* spp. the presence of RpoS, an alternative sigma factor, has been shown to regulate many genes during stationary phase in response to nutrient-limiting conditions (Bearson *et al.*, 1997). It can promote expression of stress response proteins increasing resistance to environmental stresses such as low pH (Nickenon & Curtiss, 1997). However, the presence of RpoS has not been detected in *Campylobacter*, and no homologue has been identified in the genome sequence, which may offer further explanation for the poor acid resistance exhibited by this pathogen.

C. jejuni 81116 and 9519 were able to adapt to acidic conditions of pH 5.5 and sustain growth in continuous culture. This pH value appeared to be the minimum for growth as replication was not maintained at either pH 4.0 or pH 5.0. A new lower steady state cell density was established after six cell doubling times within the chemostat. This was unlikely to be a sufficient period to select for an acid resistant sub-population, and the bacteria most probably represent phenotypically acid-adapted *Campylobacter*. This type of acid adaptation in continuous culture has been reported for *Streptococcus mutans* (Belli & Marquis,1991). *H. pylori* has been shown to be unable to replicate when exposed to pH 5, but was able to replicate at pH 6. However, this organism's ability to grow at pH 5.5 was not investigated (Karita & Blaser, 1998). Both *Campylobacter* isolates were more able to resist acid shock of pH 3.5 after 48 h of growth at pH 5.5 compared to growth at pH 7.1. At the earlier stage of adaptation, 24 h, isolate 81116 did not show any marked improvement in acid resistance, though isolate 9519 did increase its resistance. Similar responses of increased resistance to acid shock following growth at a moderately acid pH has been demonstrated in *S. typhimurium* (Foster & Hall, 1990), *E. coli* (Goodson & Rowbury, 1989; Jorden *et al.*, 1999), *L. monocytogenes* (Davis *et al.*, 1996) and *H. pylori* (Karita & Blaser, 1998).

The ability to resist acid shock was not found to be enhanced when *Campylobacter* was grown in continuous culture. The *C. jejuni* isolates were more resistant when they became acid adapted to pH 5.5 over the 48 h peroid in continuous culture. The kinetics of death suggest that different molecular mechanisms may be involved in the adapted and the unadapted cultures. The unadapted cells may have a higher level of CFA present in their membranes which would protect them from acid shock (Leach *et al.*, 1997; Brown *et al.*, 1999). The increased resistance observed in the acid adapted cells may have arisen by other changes in membrane protein composition or permeability, for example active efflux via K^+/H^+ transporters or ATPases.

Acid adaptation has been observed in *L. monocytogenes* and *H. pylori* and researched most extensively in *S. typhimurium*, and is known as the acid tolerance response (ATR) (Foster & Hall, 1990). This occurs in log and stationary phase cultures, after a shift to mildly acidic conditions (pH 5.5 - pH 6.0), which causes an inducible pH homeostasis to function. The cytoplasm becomes alkalinised during exposure to acid conditions protecting the cells for several hours (Foster & Hall, 1991). This mechanism utilises amino acid decarboxylases, for example, lysine decarboxylase (CadA) in *S. typhimurium*. Lysine is decarboxylated within the cell by CadA using one proton to form cadaverine. Fresh lysine enters the cell in exchange for the cadaverine via the CadB antiporter, which acts to increase the alkalinity of the cytoplasm (Park *et al.*, 1998). A similar ATR appears to function but rather poorly in *Campylobacter*. However, currently no amino acid decarboxylases have been reported in *Campylobacter*, which may act as such a mechanism for protection, though homologues to the amino acid decarboxylases are apparent in the genome.

Adaptation of *E. coli* to acidic conditions also occurs through log or stationary phase ATR mechanisms, though these differ from those of *S. typhimurium* (Foster, 1999). Stationary

phase *E. coli* has been shown to be more acid resistant in complex media than *S. typhimurium*. This is thought to be due to RpoS and cAMP receptor protein-dependent systems, glutamate and the putative glutamate/gamma amino butyric acid antiporter and arginine decarboxylase. In *E. coli* log phase ATR to pH 5.0 has been termed acid habituation (Goodson & Rowbury, 1989). This has been found to occur in low phosphate medium, otherwise phosphate competes with hydrogen ions to cross the PhoE porin (Rowbury & Goodson, 1993). Owing to the high levels of phosphate in the ABCD medium used to grow *Campylobacter*, the ability to grow at pH 5.5 was not thought to be due to this mechanism.

Expression of proteins in response to acid stress has been shown to enhance survival in *S. typhimurium* (Bearson *et al.*, 1997). Acid adaptation of both *C. jejuni* 81116 and 9519 at pH 5.5 was found to cause alterations in protein expression as detected by 2-D gel electrophoresis. Growth of *C. jejuni* 81116 at pH 5.5 caused 56 proteins to be down-regulated and 10, and possibly as many as 114 were absent. In addition, possibly only 7, or as many as 129 proteins were uniquely expressed and a further 38 were up-regulated. A similar response was observed in *C. jejuni* 9519 with 59 proteins down-regulated and 3, and possibly as many 197 proteins absent. Also, possibly only 7, or as many as 174 were uniquely expressed and a further 18 were up-regulated. Acid responsive proteins have been identified in other organisms. For example, 12 proteins were induced and 6 repressed during the ATR of *S. typhimurium* (Foster & Hall, 1990). In *L. monocytogenes*, induction of the ATR by HCl caused the expression of 36 novel and up-regulated proteins and the down-regulation of eight proteins (Davies *et al.*,1996). The larger number of proteins expressed in *C. jejuni* suggests that it was undergoing substantial changes to allow survival to the acid stress. This may include repair mechanisms for proteins and DNA, or changes in protein composition within the membrane.

In addition to adaptation to moderate acidic conditions in S. *typhimurium*, lowering the adaptive pH further to pH 4.5 was found to induce the expression of 50 other proteins (Foster, 1993), known as acid shock proteins (ASPs). These may aid survival against the extreme acid conditions. Analysis of ASP expression has shown that some are induced in response to internal pH, whereas others respond to external pH (Foster, 1999). Expression of these proteins may also be controlled by the alternative sigma factor RpoS, Fur and the two component signal transduction system PhoP.

These three regulatory proteins have been identified in *S. typhimurium* and shown to exert an effect on the ATR and the proteins which are expressed in response (Foster, 1999). These form both a RpoS-dependent ATR and a RpoS-independent ATR. The RpoS-dependent response probably does not function in *Campylobacter*, due to the absence of this molecule. However, the response of *Campylobacter* to acid stress may be controlled though the RpoS-independent mechanisms, involving Fur and a system similar to PhoP of *S. typhimurium*.

The role of Fur in the ATR was first identified by Hall and Foster (1992), in *S. typhimurium*. Fur was found to control the expression of eight acid shock proteins (ASP), aiding survival at pH 3.0 and below. The action of Fur was not thought to be mediated through iron levels, but by Fur sensing iron and pH separately (Bearson *et al.*, 1997). The Fur protein and several of its putative binding sites have been identified in *C. jejuni* and may similarly modulate gene transcription in response to pH to aid survival at low pHs (Wooldridge *et al.*, 1994; Chan *et al.*, 1995).

The two-component regulator, PhoP, of *S. typhimurium* (see section 1.14) acts as an auto-induced ASP which induces three further ASPs. Mutations in *phoP* prevented the expression of four ASPs, and no ATR was observed (Bearson *et al.*, 1997). Such a

mutation caused a reduction in survival in macrophages and increased killing at low pH (Foster & Hall, 1990), suggesting a role in the response to acidic conditions. Further work has now demonstrated that the PhoP/Q system may be important in tolerance to inorganic acid stress and appears to sense H^+ ions directly in the external environment. The presence of a two-component system similar to PhoPQ in *Campylobacter* has not currently been reported, though analysis of the genome sequence may reveal similar systems.

Adaptation to acidic conditions of pH 5.5 significantly reduced the ability of *C. jejuni* 81116 to invade Caco-2 cell monolayers, when compared to *C. jejuni* 81116 grown at pH 7.1. This was not unexpected as exposure of *S. flexneri* to pH 3.0 caused a loss of invasive ability into HEp-2 cells (Gorden & Small, 1993). In contrast, prolonged exposure of *L. monocytogenes* to acidic conditions generated acid tolerant mutants, which were found to be more virulent in the mouse model (O'Driscoll *et al.*, 1996). This may have been due to their increased ability to resist the macrophage phagosome activity. Similarly, resistance of *S. typhimurium* to low pH has been shown to be essential for *Salmonella* pathogenicity (Rathman *et al.*, 1996) to ensure survival in acidified macrophages.

The loss of invasive ability of *Campylobacter* following growth at pH 5.5 may reflect *Campylobacter* sensing specific environmental cues, for example, the level of H^+ ions to determine its location. Previous work by Symanski *et al.*, 1995 had demonstrated that *C. jejuni* were non- motilite at pH 5.0, and this characteristic has been shown to be essential for the invasion process (Wassenaar *et al.*, 1991). An acidic environment would be encountered in the stomach (pH 1-3), which is not generally the site of invasion for *Campylobacter*. Hence, it would seem likely that *C. jejuni* uses a membrane bound receptor to detect changes in H+ ion concentration by signal transduction and gene expression is altered to prevent expression of virulence determinants. Such as system of

environmental stimulus triggering signal transduction processes has been reported in *S. typhimurium* with PhoP, which acts as a magnesium-sensing two-component regulator. Alternatively, acid pH may affect gene expression indirectly by altering cAMP levels in *C. jejuni*, which has been demonstrated for the expression of *hya* and *aniC* genes of *S. typhimurium* (Park *et al.*, 1998).

In summary, *Campylobacter* isolates demonstrated poor constitutive acid resistance which was not influenced extensively by growth phase. *Campylobacter* isolates were able to adapt within 48 h and sustain growth to a minimum pH of pH 5.5. This suggests that at the set mean generation time of 8.7 h^{-1} adaptation did not arise through selection of a mutant population, but through phenotypic adaptation. Growth at the acidic pH enabled *Campylobacter* to become more resistant to subsequent acid shock possibly through changes in the CFA of the membrane, and probably involved a system similar to the ATR of *S. typhimurium*. Adaptation to pH 5.5 resulted in changes in protein expression, and some of these proteins may represent ASPs which aid survival and repair damage. This would ensure survival in acidic niches created both in the natural environment and in human hosts. Exposure to low pH did not increase invasive ability into Caco-2 cell monolayers, which suggest that those proteins induced under low pH were not involved in invasion processes.

Chapter 5

Molecular Characterisation

5.1.1 Introduction

In causing an infection, a pathogen must be able to persist and multiply successfully either on, or within the host. The organisms' ability to cause disease can be influenced by the genetic make-up of the bacteria and by the environment that it finds itself within. These two factors contribute to the expression of virulence determinants of the pathogen, defining a bacterial product or strategy which contributes to virulence and influences the expression of virulence factors. Virulence determinants can be classified into those that promote colonisation and infection, and those which damage the host. In *Campylobacter* several virulence determinants have been proposed of both types including the flagella (Wassenaar *et al.*, 1991), chemotaxic gene - *cheY* (Yao *et al.*, 1997), fimbria (Doig *et al.*, 1996) and toxins (Pickett *et al.*, 1996).

Cellular macromolecules which are thought to act as virulence determinants promoting disease, can be identified using a number of techniques including column chromatography, western immunoblotting, ELISAs and two-dimensional gel electrophoresis. However, to prove association with virulence, Koch's molecular postulates dictates that, for a particular phenotype to be encoded by a specific gene, three conditions must be met (Salyers & Whitt, 1994):

1. specific mutants deficient in the phenotype of interest must be isolated
2. the wild-type virulence gene must be cloned
3. the wild-type phenotype must be restored through re-introduction of a specific mutant to the wild-type.

However, fulfilling these criteria can be difficult and often virulence-associated genes are identified through less stringent conditions.

5.1.2 *Campylobacter* genetics

Campylobacter chromosomal DNA has 70 % A+T content (see section 1.2), and a relatively small genome of 1, 641, 481 bp (Parkhill *et al.*, 2000). However, only a small number of the genes have been identified and their sequence deposited with GenBank (Ketley, 1997). Many of these are classified as general house-keeping genes which have homology with other species. The low number of proteins identified in *Campylobacter* are attributed to the difficulties encountered in cloning of *Campylobacter* gene sequences. This arises through the high AT content of *Campylobacter* DNA, which may be recognised in *E. coli* as promoter-like sequences. Incorrect accessory genes required for translation are present in *E. coli*, as well as different patterns of methlylation and codon usage.

However, progress has been made in the genetic manipulation of *Campylobacter* through the development and adaptation of techniques. Suicide and shuttle vectors for *Campylobacter* have been formed following work by Labigne-Roussel *et al.*, (1987). A hybrid shuttle vector was constructed from pBR322 and a plasmid from *C. coli* containing *oriT* (origin of transfer) and a kanamycin resistance gene which was able to confer kanamycin resistance to *Campylobacter* recipients (see section 1.3). Suicide vectors have also been constructed, lacking the *Campylobacter* replicon allowing gene replacement mutagenesis. Additional shuttle vectors have been constructed each containing a selective marker, in some cases the antibiotic genes were bracketed with polylinker regions to contain unique restriction sites. Plasmids can be transferred from *E. coli* to *Campylobacter* either by conjugation or electroporation (see section 1.2).

The sequencing of the *Campylobacter* genome has recently been undertaken by the Sanger Centre (see section 1.3). The shotgun approach allowed the insertion of *C. jejuni* 11168 into 2-3 kb fragments into the vector pUC18 forming a library (Karlyshev *et al.*, 1999).

Each clone was sequenced and aligned until sufficient overlaps were generated, to ensure the correct sequence being assigned. A BLAST search against non-redundant combined protein data-bases revealed that 54 % of the open reading frames had significant match with known genes, 22 % showed similarity to hypothetical open reading frames and 24 % showed no matches (Karlyshev *et al.*, 1999). Sequencing of the genome will allow identification of sets of genes, including virulence determinants, through database searches and sequence alignments with virulence determinants of other pathogens. Although alignments may give an indication of the biochemical function of the gene product, it does not increase the understanding of the gene's precise role in bacteria. The use of molecular tools allows the true role of the gene to be identified using mutation and gene replacement techniques.

5.1.3 Gene Identification

Recent developments in chemiluminescence chemistry have allowed use of non-radioactive techniques to identify specific regions of DNA by probing with an oligonucleotide labelled with a tail of fluorescein-11-dUTP. The bound probe can be detected through an anti-fluorescein horseradish peroxidase conjugate, which is reduced releasing luminol. The molecule breaks down, causing light to be emitted as it passes through an excited intermediate stage, which is detected on blue light sensitive film.

As an alternative route, specific primers can be used to amplify the gene by the Polymerase Chain Reaction (PCR). The template DNA can be copied in the presence of an excess of oligonucleotides and a heat stable DNA polymerase, isolated from *Thermus aquaticus* (Taq). During the continuous cycling of heating and cooling the gene product is amplified.

5.1.4 Aims

Under different environmental cues, such as iron replete, iron limitation and oxidative stress conditions, *C. jejuni* was found to increase in invasive ability. Analysis of the proteome of *C. jejuni* under these cues by 2-D gel electrophoresis highlighted cellular macromolecules whose appearance correlated with an increase in invasive ability into Caco-2 cell monolayers and these were identified by N-terminal sequencing. The role of some of the proteins was investigated further by genetic techniques to determine their possible contribution to survival and virulence.

Before the release of the *Campylobacter* genome on the internet, protein composite no. 278 (section 4.3.5: N-terminal sequence of MKVLLIKLIKALGQ) showed no homology to proteins in the protein databases searched. Classical techniques of Southern blotting and probing with fluorescein labelled probes were used to identify the gene encoding this protein. In the post- genomic era, the genes encoding three other proteins with proposed homology to the proteins PEB-4, high temperature requirement gene B (HtrB) and thiol peroxidase (Tpx) were amplified by PCR. Attempts were made to inactivate these genes through inserting a chloramphenicol gene into the gene of interest. The disrupted gene was returned to *Campylobacter* by electroporation and the phenotype of the resulting mutants identified.

5.2 Results

5.2.1 Homology of probes

Two degenerate oligonucleotide probes, DOT1a and DOT1b, were designed to identify a novel protein induced under oxidative stress (composite no. 278) (see Table 16). Two probes were generated due to the extensive coding range for the amino acid leucine, which accounted for $^1/_3$ of the coding amino acids. To determine the probe with the greatest homology, chromosomal DNA was blotted onto Hybond-N+ and under conditions of high stringency, 42°C and 1x SSC black dots were detected with probes DOT1b and KK, the positive control (section 2.11), as described in section 2.12.2 and 2.12.4. Probe DOT1a failed to produce a strong signal and was not used in the ensuing experiments.

Table 16: Codon usage in the probes designed to identify protein composite no. 278.

The N-terminal sequence of protein composite number 278 (section 4.3.5):

M K V L L **I K L I K A L G Q**

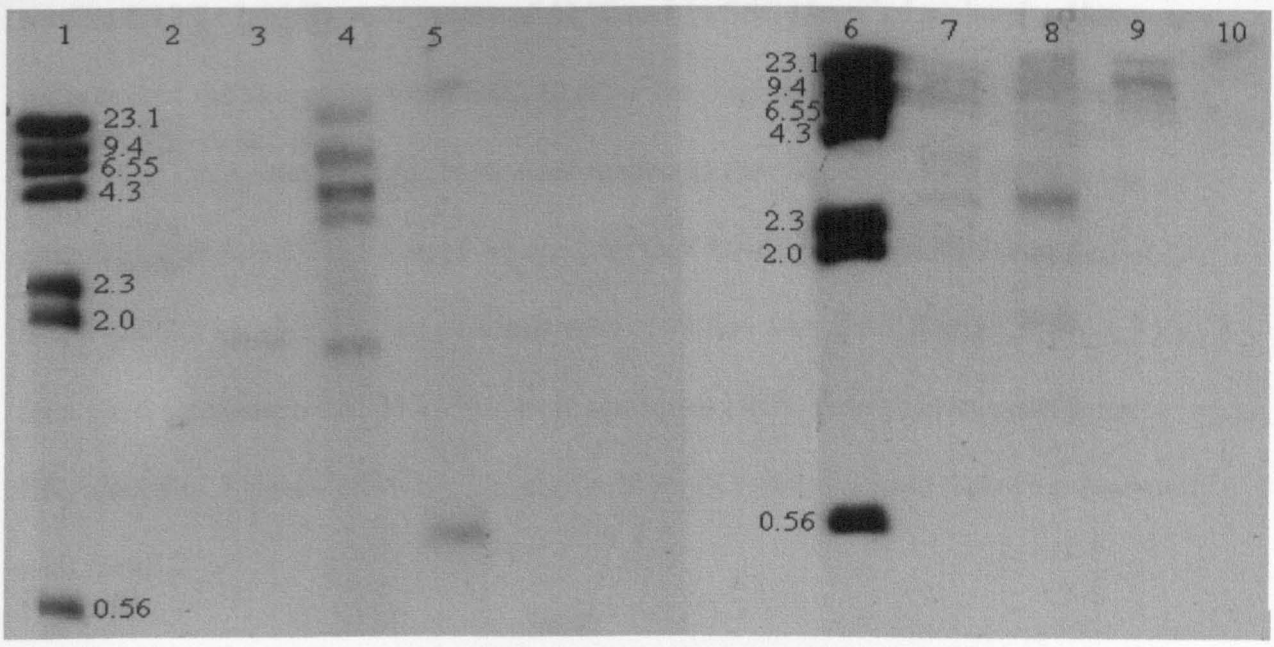
Two degenerate oligonucleotide probes were designed to the highlighted nine amino acid residues.

Amino Acid	I	K	L	I	K	A	L	G	Q
DOT 1a	AT$^A/_T$	AAA	TTA	AT$^A/_T$	AAA	GC$^A/_T$	TTA	GC$^A/_T$	CAA
DOT 1b	AT$^A/_T$	AAA	CTG	AT$^A/_T$	AAA	GC$^A/_T$	CTG	GG$^A/_T$	CAA

5.2.2 Hybridization of DOT1b to genomic digests of *C. jejuni*

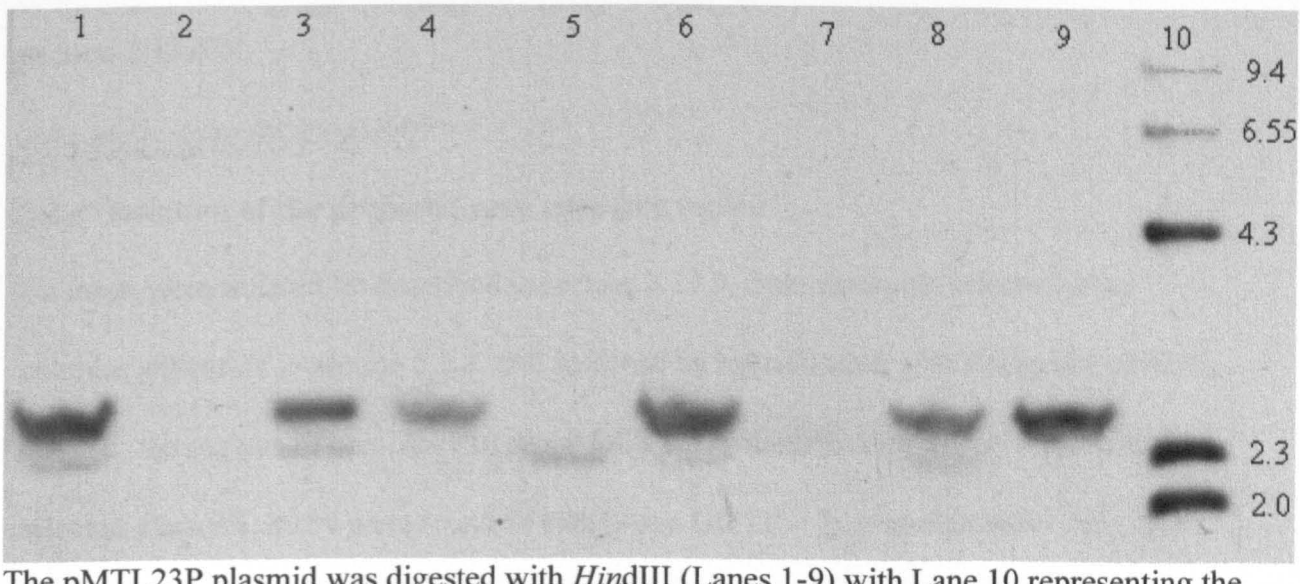
Chromosomal DNA was extracted from *C. jejuni* 81116 as described in section 2.11.2 and digested with restriction endonucleases as described in section 2.13.1. The digested DNA was transferred by Southern blotting to a charged nylon membrane as described in section 2.12.1. The blot was hybridized with fluorescein labelled DOT1b and KK, as described in

Fig 44: Southern blot analysis of chromosomal DNA of *C. jejuni* 81116



The chromosomal DNA of *C. jejuni* 81116 (Lanes 2, 3, 4, 5, 7, 8, 9, 10) was digested with *Bcl*I (Lanes 2 and 7), *Hin*dIII (Lanes 3 and 8), *Eco*RV (Lanes 4 and 9) and Lanes 5 and 10 represent undigested DNA. The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridisation with DOT1b (Lanes 7, 8 and 9) or KK (Lanes 2, 3 and 4) as DNA probes. Lanes 1 and 6 show a labelled DNA marker, band sizes indicated on the figure.

Fig 45: Southern blot analysis of plasmid DNA containing 100 colonies with a 2-3 kb insert of *C. jejuni* 81116 chromosomal DNA.



The pMTL23P plasmid was digested with *Hin*dIII (Lanes 1-9) with Lane 10 representing the labelled DNA marker. The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridisation with probe DOT1b as a DNA probe.

section 2.12.2. Initially a stringency of 42 °C and 1x SSC identified no bands. However, reduction of the stringency to 6x SSC, to allow the degenerate oligonucleotide probe to bind to the DNA, identified different sized fragments (see Fig. 44). Two bands were identified with *Eco*RV at 23 and 9 kb and four faint bands were identified with *Bcl*I at 23, 6.5, 4 and 2.3 kb. Three stronger bands were identified with *Hin*dIII at 23, 9 and 2.3 kb. A faint band was detected at 25 kb in the chromosomal DNA. The positive control probe, KK, identified 5 bands following digestion with *Eco*RV and one band following digestion with *Hin*dIII.

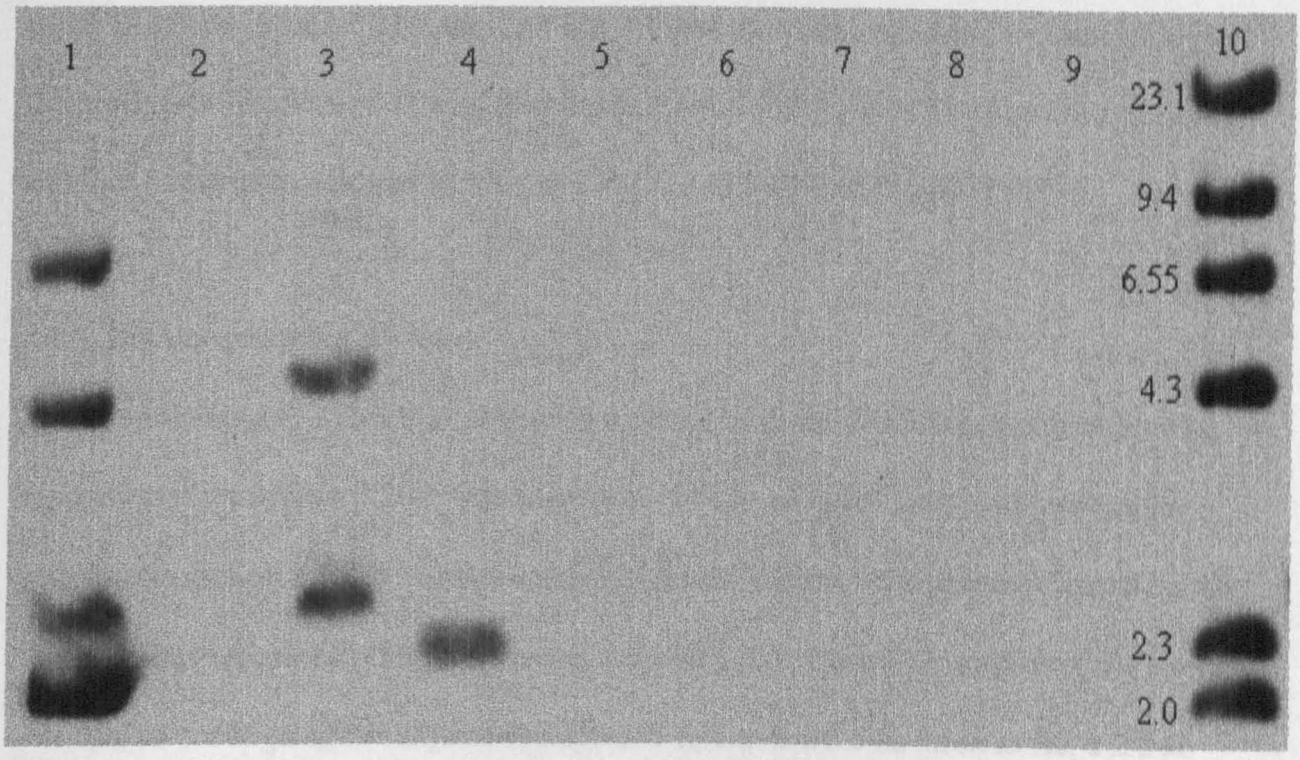
5.2.3 Cloning of the *Hin*dIII fragment from *C. jejuni*

A 2-3 kb region of DNA released by *Hin*dIII, which was identified previously was purified by agarose gel electrophoresis and ligated into the dephosphorylated *Hin*dIII site of plasmid pMTL23P, as described in section 2.13.2, 2.13.4 and 2.13.5. The ligated plasmid was transformed into *E. coli* DH5α and approximately 2000 white colonies were recovered following selection on LB-agar plates containing ampicillin, X-Gal and IPTG as described in section 2.13.6.1.

5.2.4 Isolation of the proposed gene encoding region

Plasmids were isolated, as described in section 2.13.9, from randomly selected white colonies generated in section 5.2.3, and screened by hybridization with stringency of 42°C, 6x SSC, for regions of homology to probe DOT1b, as described in section 5.2.2. Of the selected plasmids, 80 % were found to bind probe DOT1b. In some instances, two bands were apparent with a stronger band at 2.3kb and a fainter band underneath at 2.2 kb in some of the positive colonies (see Fig. 45). Further screening of a subset of plasmids from group 6a 5-6 (see Fig. 46, Lane 3) with DOT1b, as described in section 5.2.2, demonstrated that the fainter band did not contain regions of homology to the DNA probe.

Fig 46: Southern blot analysis of individual plasmid DNA containing 2-3 kb insert of *C. jejuni* 81116 chromosomal DNA.



The pMTL23P plasmid containing the *C. jejuni* insert was digested with *Hin*dIII (Lanes 1-9) with Lane 10 containing labelled DNA marker. The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridisation with probe DOT1b.

Within one group [6_{10}] (see Fig. 45, Lane 9) smaller subsets of plasmids were screened as described above until four individual clones were identified with homology to DOT1b. Clones 6_{10}A6 (see Fig 46. Lane 4), 6_{10}A7, 6_{10}A9 and 6_{10}G5 were found to produce strong bands at 2.3 kb. Two of the clones, A7 and A9, had more than one band with homology to the probe DOT1b, with two bands detected in A7 at 4.3 and 2.4 kb (see Fig. 46, Lane 3) and four bands were detected in A9 at 6.5, 4.3, 2.3, 2.0 kb (see Fig. 46, Lane 1).

5.2.5 DNA sequencing of clones

The three clones A6, A7 and G5, that were detected by probe DOT1b in Southern blotting experiments (see section 5.2.4) were sequenced initially using M13 universal primers as described in section 2.13.10. Subsequently, additional primers were generated based on the sequence data recovered (Oligo5 Software; Appendix III). Computer analysis of the inserts was carried out aligning the sequenced fragments to create a 2.6 kb insert in A6, a 2.2 kb insert in A7 and a 2.7 kb insert in G5 (see Appendix V). Short regions of homology to probe DOT1b were identified in the three clones of four to eight amino acids long. However, there were no extended regions of homology in any of the three clones. In the post-genomic era, searching of the *Campylobacter* genome sequence for the encoding region of protein 278 revealed the sequence had homology to L9, a subunit of the 50S rRNA protein.

An extensive search was made of each insert for open reading frames (ORFs), with potential ORF's translated and then used to search protein databases (see Table 17). Several ORFs were identified, some preceded by a possible Shine-Dalgarno sequence, encoding a range of proteins from alkaline phosphatase, phosphoenolypyruvate phosphormutase (*fomI*) and proteins involved in the regulation of flagellum assembly (for alignments see Appendix V).

Table 17: Summary of the gene homologies following protein database searches of the encoding region of clones A6, A7 and G5.

Clone	Gene homology	Region of homology in the ORF	Organism	Activity
A6	*dedA*	773-883	*Bacillus cereus*	Alkaline phosphatase
	apl	773-883	*L. lactis*	Alkaline phosphatase
	slr0509	773-883	*Synechocystis* spp.	Alkaline phosphatase
	slr0232	773-883	*Synechocystis* spp.	Alkaline phosphatase
	mdh	2301	*L. lactis*	Malate dehydrogenase
	ldh	2301	*L. lactis*	Lactate dehydrogenase
A7	*siaB*	65-240	*H. influenzae*	CMP NeuAc synthatase
	neuA		*Haemophilus ducreyi*	CMP NeuAc synthatase
	ylnC	245-445	*B. subtilis*	adenosine 5'-phosphosulphate
	orf1	455-730	*Pseudomonas aeruginosa*	hypothetical protein
	fom1	780-2210	*Strephomyces wedmorensis*	CMP NeuAc synthatase
G5	*fliD*	420-870	*H. pylori*, *P. aeruginosa*	Flagellar hook - associated protein 2
	fliS	2370-2660	*H. pylori*, *B. subtilis*	Flagellar protein, transcriptional regulator

Regions of homology were identified in the open reading frames of the sequence data

5.3 Identification of the role of proteins in the post-genome era

5.3.1 PEB-4 Protein

5.3.1.1 Amplification of the gene *peb4A* from *Campylobacter*

The protein PEB-4 was identified from its N-terminal sequence following Edman degradation (see section 4.3.5) and the gene, *peb4A*, appears in GenBank under the accession number X84703 (see Fig. 48)(Burucoa *et al.*, 1995). The entire sequence was taken from the *C. jejuni* genome sequencing project and primers were designed (see Fig. 47; Appendix II), within *peb4A* and within the gene *fdaA*, which is transcribed from the same operon.

Slight modifications were made to the PCR protocol described in section 2.13.7, by reducing the primer concentration to 50 pM and raising the annealing temperature to 53°C. Chromosomal DNA from *C. jejuni* 81116, 11168 and 81-176 was extracted as described in section 2.11.2, and used as templates to amplify an 800 bp product of *peb4A* (see Fig. 49). A discrete band was detected with all of the templates at 800 bp, and the fragment from *C. jejuni* 81-176 was ligated into plasmid pCR2.1-TOPO, as described in section 2.13.8. Its presence in pCR2.1 was confirmed following its release from the *Eco*RI site on the vector back-bone where the PCR product was inserted.

Fig. 47: Schematic for the amplification of the gene *peb4A*

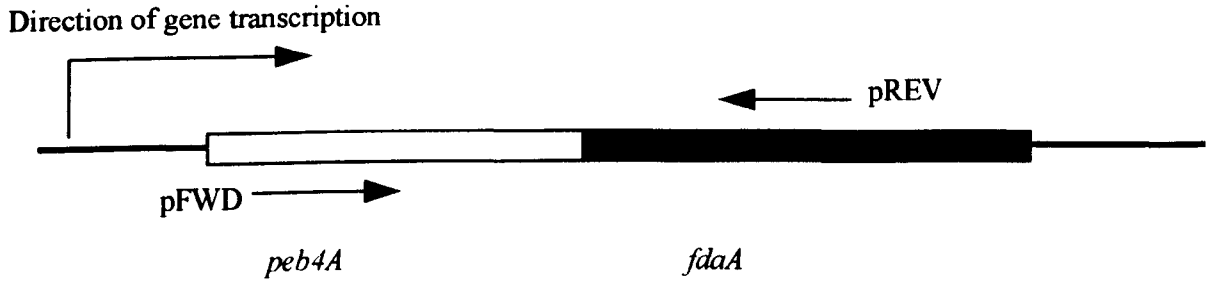


Fig. 48: Amino acid sequence alignment of PEB-4

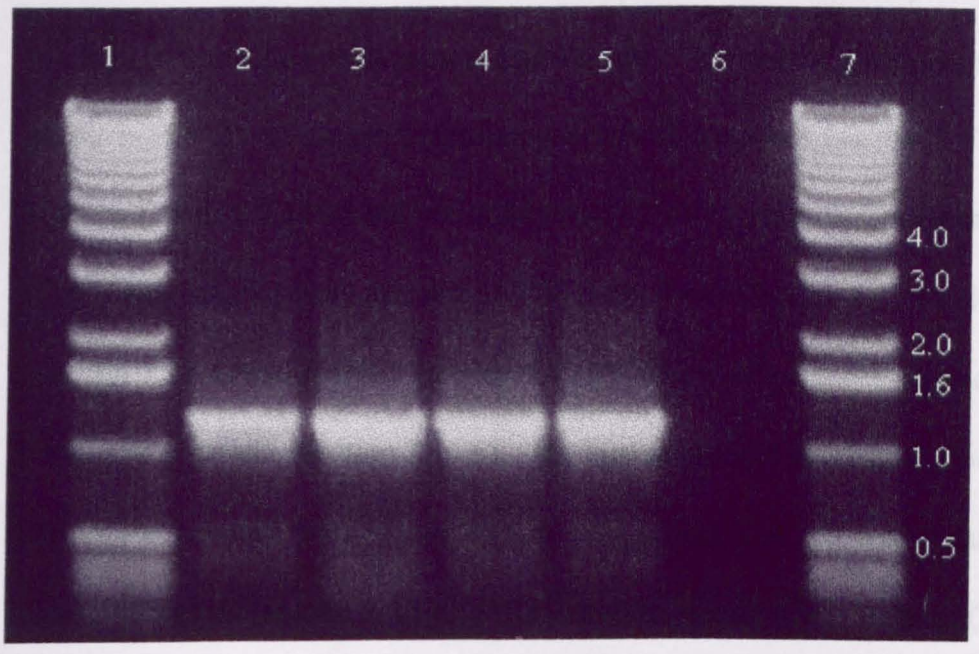
Amino acid no.	1																18
11168	M	K	K	F	S	L	V	A	A	T	L	I	A	G	V	V	L	V
81-176	M	K	K	F	S	L	V	A	A	T	L	I	A	G	V	V	L	N
92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
261	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PrsA	M	K	K				I	A	A	A	I	T	A	T	S	I	L	A
PrtM	M	K	K	K	M	R	L	K	V	I	L	L	A	S	T	A	T	A

Amino acid no.	19																36
11168	N	V	N	A	-	-	-	-	-	-	-						
81-176	N	V	N	A	-	-	-	-	-	-	-	A	T	V	A	T	V	N
92	-	-	-	-	-	-	-	-	-	-	-	A	V	V	A	Y	V	
261	-	-	-	-	-	-	-	-	-	-	-	A	T	V	A	M	A	N
PrsA	L	S	A	C	S	S	G	-	-	-	-	D	K	E		V	I	K
PrtM	L	L	L	L	L	S	G	C	Q	S	N	Q	T	D	Q	T	V	A

Amino acid no.	37														41		
11168																	
81-176	G	K	S	I	S	D	T	V	S	E	F	F	A	P	M		
92	G	-	S														
261	G	K	S	I	X	D	L										
PrsA	T	D	A	G	D	V	T	K	G	E	L	Y	T	N	M		
PrtM	T	V	A	T	Y	S	G	G	K	V	T	E	S	S	L	Y	

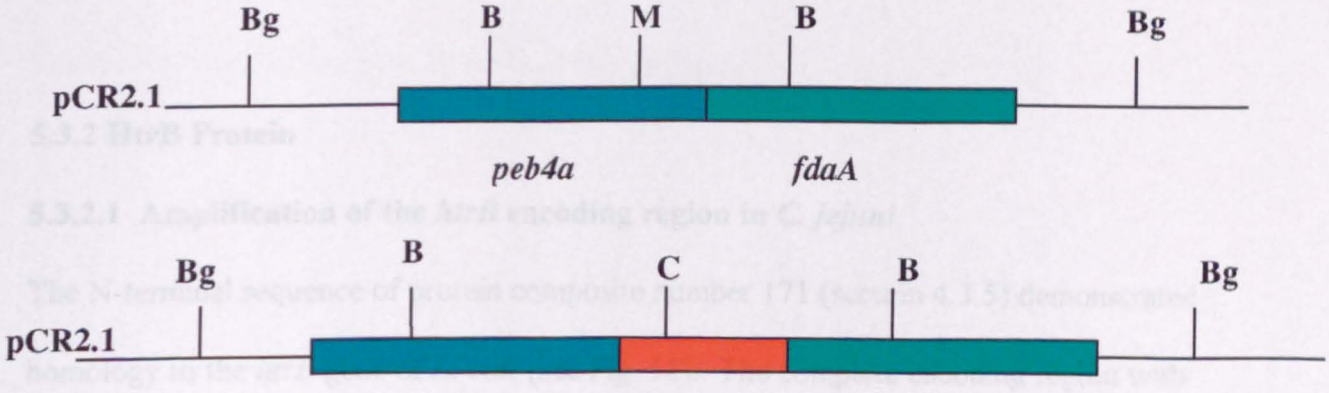
The amino acid sequences for PEB-4 of *C. jejuni* 81-176, gene *prsA* of *B. subtilis* and *prtM* of *L. lactis* were taken from Burucoa *et al.*, (1995). Amino acid sequences of proteins 92 and 261 were taken from Table 12 (section 4.3.5) and sequence for *C. jejuni* 11168 was taken from the Sanger centre.

Fig 49: Amplification of *peb4A* from *C. jejuni* isolates



Chromosomal DNA of *C. jejuni* 81116 (Lane 2), 81-176 (Lane 3) and 11168 (Lane 4 & 5) was used as templates in PCR reaction with *peb4A* specific primers – pREV and pFWD. Lane 6 demonstrated the control lane and Lanes 1 & 7 indicate marker of kb ladder.

Fig 50: Restriction map of *peb4A* in *C. jejuni*



Key: Restriction endonuclease sites are indicated in the diagram. Bg, *Bgl*II; B, *Bcl*I; M, *Mun*I.

5.3.1.2 Isolation of a *peb4A* insertionally inactivated mutant of *C. jejuni*

To determine the biological relevance of the protein PEB-4 in *C. jejuni*, attempts were made to construct a *peb4A* mutant (see Fig. 50). The *peb4A* gene present in plasmid pCR2.1-TOPO was cut with restriction endonuclease *Mun*I (section 2.13.1), treated with Klenow (section 2.13.3) and purified (section 2.13.2). The chloramphenicol-resistance gene (Cm^R) was isolated from plasmid pAV35 using *Pvu*II (section 2.13.1), dephosphorylated (section 2.13.4) and purified (section 2.13.2). The Cm^R gene was ligated into the blunted *Mun*I site of *peb4A* (section 2.13.5). The ligated plasmid was transformed into *E. coli* DH5α and colonies were recovered following selection on LB-agar containing chloramphenicol (section 2.13.6.1). The resulting plasmid was isolated (2.13.9) and returned to *C. jejuni* isolates 81-176 and 11168 by electroporation, as described in section 2.13..6.2. No viable *peb4A*-Cm^R transformants of *C. jejuni* 81-176 or 11168 were recovered following electroporation. Concentration and re-extraction of the *peb4A*-Cm^R plasmid and modification of the incubation times following electroporation also failed to produce viable colonies, although transformant *C. jejuni* were recovered when a positive control plasmid was returned by electroporation.

5.3.2 HtrB Protein

5.3.2.1 Amplification of the *htrB* encoding region in *C. jejuni*

The N-terminal sequence of protein composite number 171 (section 4.3.5) demonstrated homology to the *htrB* gene of *E. coli* (see Fig. 51). The complete encoding region with homology to this protein was identified from the *Campylobacter* genome and two primers were designed up- and down-stream from the encoding region (see Fig. 53; Appendix II). The gene was amplified by PCR using the template DNA of *C. jejuni* 81116, 11168 and 81-176 as described in section 2.13.7. A discrete 1 kb product was detected from all of the templates (see Fig. 53) and the product of 81-176B was ligated into plasmid pCR2.1-

Fig. 51: Alignment of the N-terminal of the HtrB protein

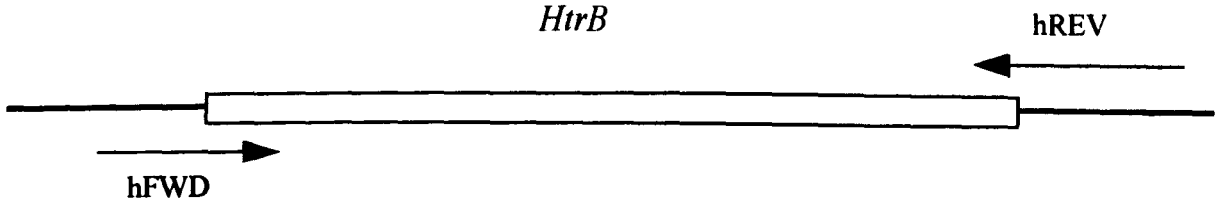
Amino acid no.	1											13
171	-	-	K	A	-	L	N	K	A	H	T	-	D
11168	L	P	K	A	Y	L	M	K	N	S	D	R	I
E. coli	M	T	Y	K	E	R	L	I	H	E	K	Q	D

Amino acid no.	14										24
171	N	-	-	-	-	-	-	-	-	-	-
11168	L	S	L	Y	Y	I	L	K	F	F	V
E. coli	D	K	G	F	K	T	E	L	R	I	L

The amino acid sequence for *E. coli* was taken from Karow & Georgopoulos (1991).

Amino acid sequence for 171 was taken from Table 9 (section 4.3.5) and the sequence for 11168 was taken from the *Campylobacter* genome project.

Fig. 52: Schematic of the proposed *htrB* gene in *C. jejuni*



The arrow heads indicate the direction of the primers

TOPO, as described in section 2.13.8. The presence of the PCR product was confirmed following its release with *Eco*RI from the pCR2.1 back-bone.

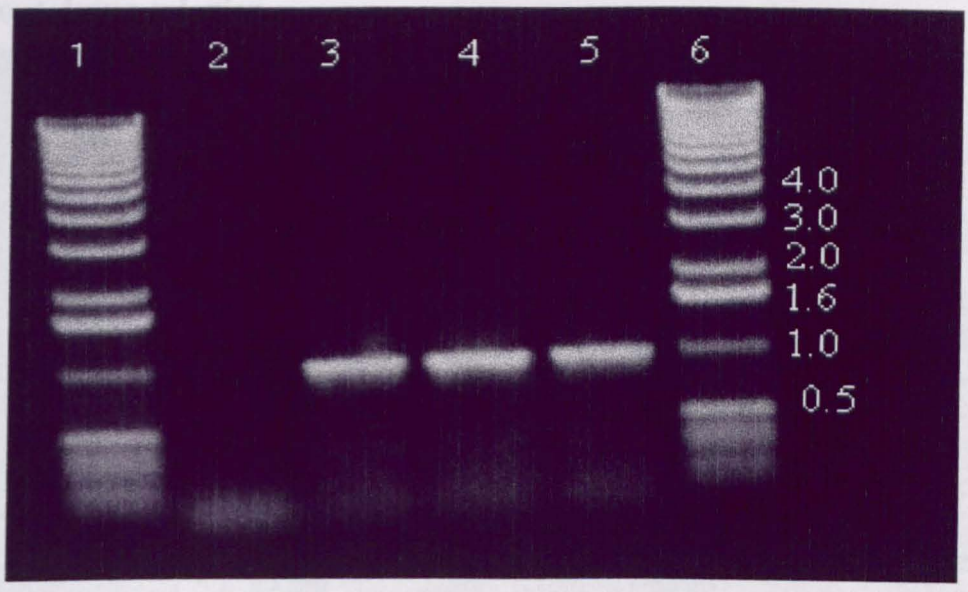
5.3.2.2 Construction of a *htrB*-deficient encoding region in *Campylobacter*

The plasmid pCR2.1, containing the amplified DNA encoding *htrB*, was disrupted by the insertion of the chloramphenicol gene from pAV35 (see Fig. 54). The *hrtB* gene was cut with restriction endonuclease *Cla*I (section 2.13.1), treated with Klenow (section 2.13.3) and purified (section 2.13.2). The Cm^R gene was isolated from plasmid pAV35 using *Pvu*II (section 2.13.1), dephosphorylated (section 2.13.4) and purified (section 2.13.2). The Cm^R gene was ligated into the blunt-ended *Cla*I site of the *htrB* gene (section 2.13.5). The resulting plasmid was transformed in *E. coli* DH5α (section 2.13.6.1) and recovered by selectional plating on LB-agar containing chloramphenicol. The plasmid was isolated as described in section 2.13.9 and returned to *C. jejuni* 81-176 and 11168 by electroporation as described in section 2.13.6.2.

5.3.2.3 Confirmation of the presence of the disrupted *htrB* encoding region

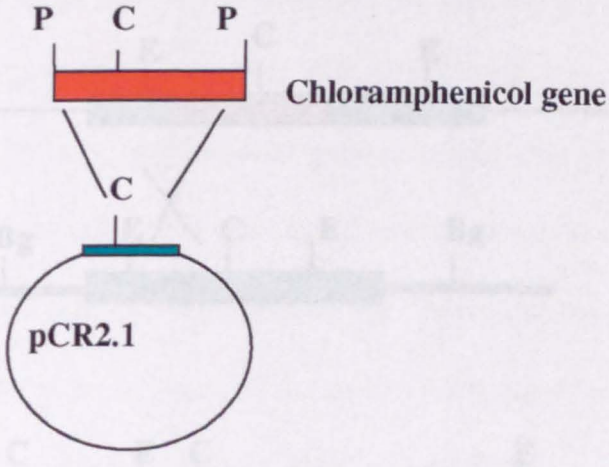
The presence of *htrB*-Cm^R encoding region in the genome of the recovered *C. jejuni* 81-176 colonies following electroporation was determined by probing with fluorescein labelled *htr*B product generated by PCR (section 5.3.2.2). Genomic DNA was isolated (section 2.11.2) from *C. jejuni* 81-176 containing the *htrB*-Cm^R insert and wild-type *C. jejuni* 81-176, digested with restriction endonucleases *Eco*RV, *Cla*I and *Bgl*II (section 2.13.1) and probed for the presence of the *htrB* gene in the genome as described in sections 2.12.1, 2.12.3 and 2.12.4 (see Fig. 55). As a control pCR2.1-TOPO containing the *htrB*-Cm^R insert was digested with the same restriction endonucleases and probed for the presence of the *hrtB* gene. The restriction patterns obtained after hybridization demonstrated the presence of the gene in the wild-type, with one band following digestion with *Bgl*II at approximately 4.3 kb,

Fig 53: Amplification of the *htrB* gene from *C. jejuni* isolates



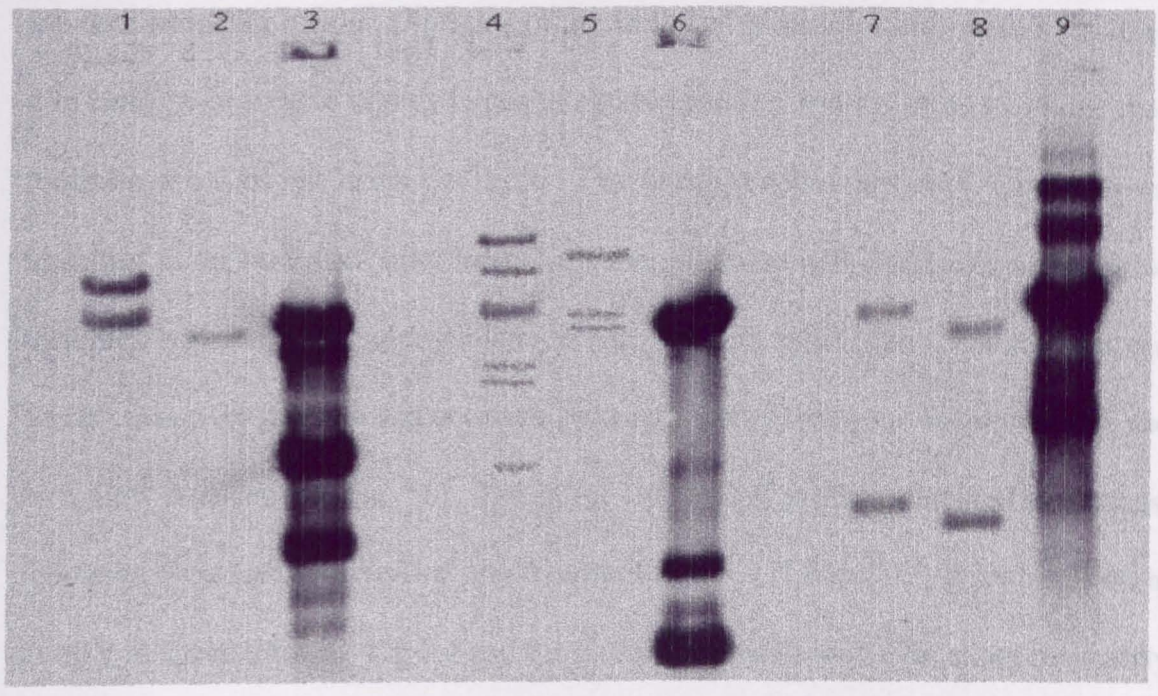
Chromosomal DNA isolated from *C. jejuni* 81116 (Lanes 3), 81-176 (Lane 4) and 11168 was used as templates in PCR reactions using *htrB* specific primers – hREV and hFWD. Template control is indicated in Lane 2 and marker of kb ladder is shown in Lanes 1 and 6.

Fig 54: Schematic of the incorporation of the chloramphenicol resistance gene into the amplified *htrB* gene



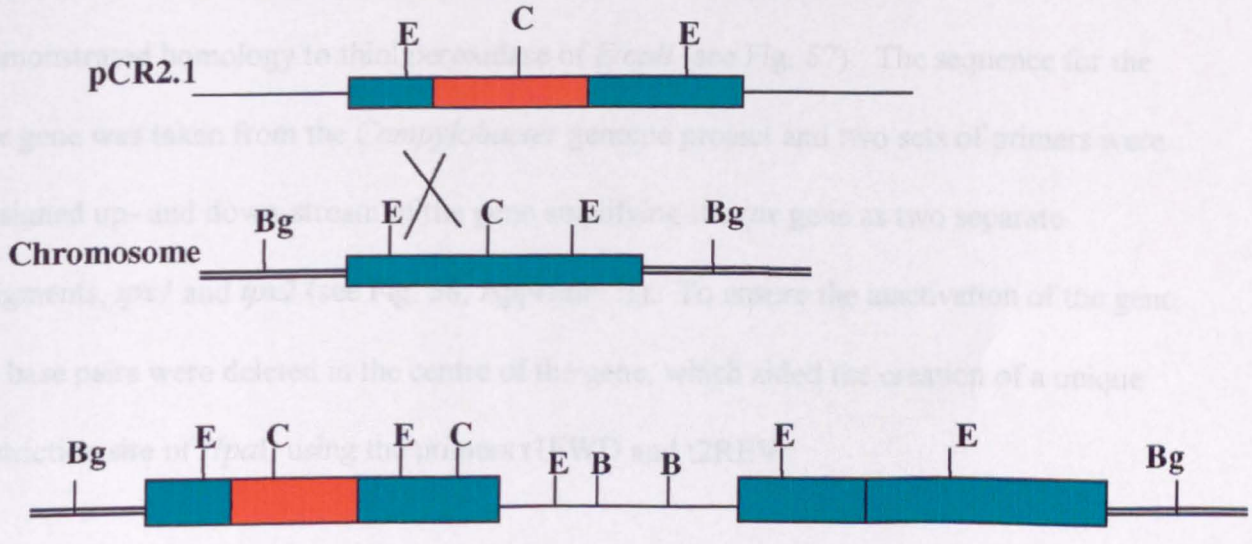
The chloramphenicol gene was isolated from plasmid pAV35 using *Pvu*II. This was ligated into the *Cla*I site within the *htrB* gene in pCR2.1 TOPO. Key: Restriction endonuclease sites are indicated as P, *Pvu*II; C, *Cla*I.

Fig 55: Southern blot analysis of chromosomal DNA of the *C. jejuni* 81-176 *htrB*-Cm^R containing colonies



Key: The chromosomal DNA of *C. jejuni* 81-176 (Lanes 2, 5, 8) and the *htrB*-Cm^R containing colonies (Lanes 1, 4, 7) were digested with *Bgl*II (Lanes 1, 2), *Eco*RV (Lanes 3, 4) and *Cla*I (Lanes 7, 8). The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridization with the 1 kb *htrB* PCR product of *C. jejuni* 81-176 as a DNA probe.

Fig 56: Diagramatic representation of a single cross-over of a gene



The suicide vector was introduced to *C. jejuni* 81-176 by electroporation. The *hrtB*-Cm^R fragment was integrated into the chromosome via a Campbell-like mechanism. Key: E, *Eco*RV; Bg, *Bgl*II, C, *Cla*I

three bands following digestion with *Eco*RV at approximately 4.0, 4.4 and 6.2 kb and two bands following digestion with *Cla*I at approximately 0.6 and 4 kb, due to a *Cla*I site within the *htrB* encoding region. Digestion of the *htrB*-Cm^R insertion colony with *Cla*I identified two bands, which were slightly larger, at approximately 1 and 4.5 kb and was due the presence of a *Cla*I site in the Cm^R gene. Two bands at approximately 4.4 and 5 kb were identified in the *htrB*-Cm^R insertion colony after digestion with *Bgl*II, and six bands were identified with *Eco*RV at approximately 6.5, 6, 4.5, 3.0, 2.8, 1.9 kb. This suggests that a single cross-over reaction had occurred and two copies of the gene were present in the *htrB*-Cm^R colonies (see Fig. 56). The pCR2.1 *htrB*-Cm^R profiles identified four bands following digestion with *Bgl*II at approximately 4.4, 3.9, 1.9 and 0.5, three bands with *Eco*RV at approximately 4.3, 0.4 and 0.1 kb and five bands with *Cla*I at approximately 7, 6.5, 4.5, 3 and 2.8 kb.

5.3.3 Tpx protein

5.3.3.1 Amplification of the *tpx* encoding region in *C. jejuni*

The N-terminal sequence of protein composite no.162 and 163 (see section 4.3.5) demonstrated homology to thiol peroxidase of *E.coli* (see Fig. 57). The sequence for the *tpx* gene was taken from the *Campylobacter* genome project and two sets of primers were designed up- and down-stream of the gene amplifying the *tpx* gene as two separate fragments, *tpx1* and *tpx2* (see Fig. 58; Appendix II). To ensure the inactivation of the gene, 10 base pairs were deleted in the centre of the gene, which aided the creation of a unique restriction site of *Hpa*I, using the primers t1FWD and t2REV.

The PCR reaction described in section 2.13.7, was modified for the amplification of the N-terminal region, by reducing the primer concentration to 50 pM and raising the annealing

Fig. 57: Amino acid alignment of the Tpx protein

Amino acid no.	1											13
162	-	S		T	V	N	F	K	G	N	P	V	K
163	-	G		T	V	N	F	K	G	N	P	V	K
11168	M	S		I	V	N	F	K	G	N	P	V	K
E. coli	M	S	Q	T	V	H	F	Q	G	N	P	V	T
V. cholerae				T	V	T	F	Q	N	N	P	V	S

Amino acid no.	14												26
162	L	K	G	-	-	-	-	-	-	-	-	-	-
163	L	K	-	-	-	-	-	-	-	-	-	-	-
11168	L	K	G	N	S	E	V	G	A	D	A	P	V
E. coli		V	A	N	S	I	P	Q	A	G	S	K	A
V. cholerae		I	S	G	S	F	P	K	V	G	D	R	L

The amino acid sequences for *E. coli* and *V. cholerae* (TagD protein) were taken from Cha *et al.*, (1996). Amino acid sequences for 162 and 163 were taken from Table 12 (section 4.3.5) and sequence for 11168 was taken from the *Campylobacter* genome project.

Fig. 58: Schematic for the amplification of the *tpx* gene of *C. jejuni*

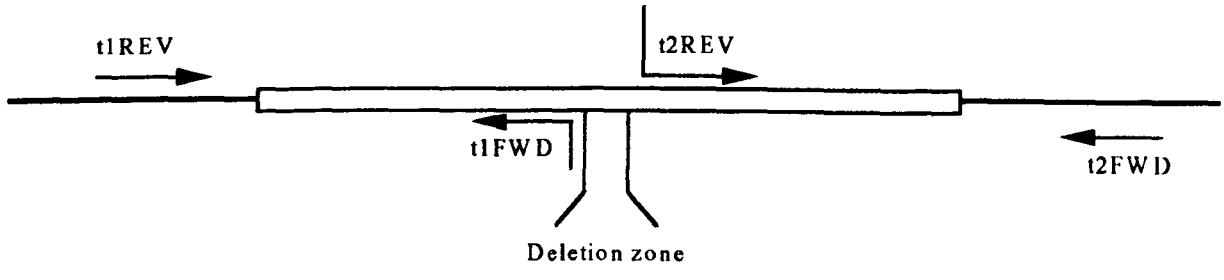
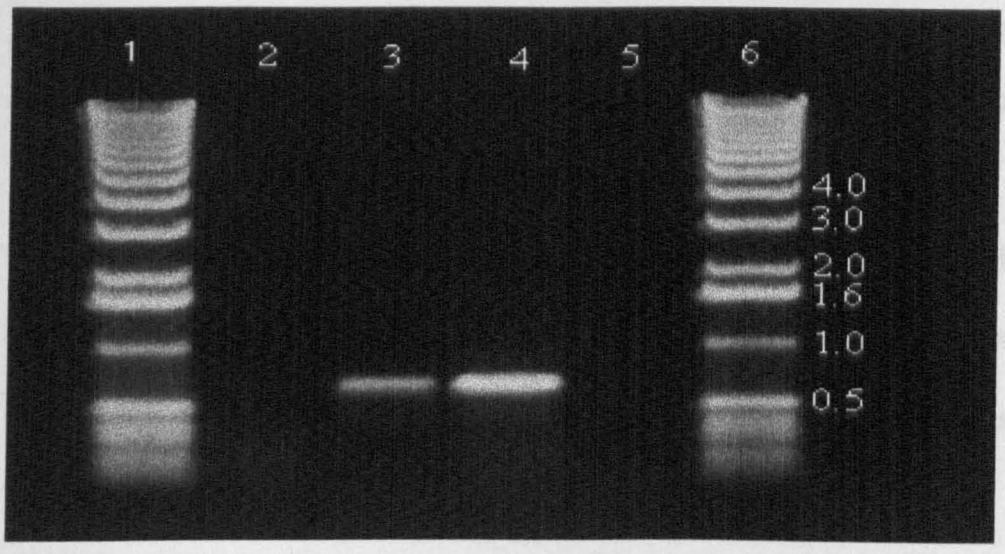
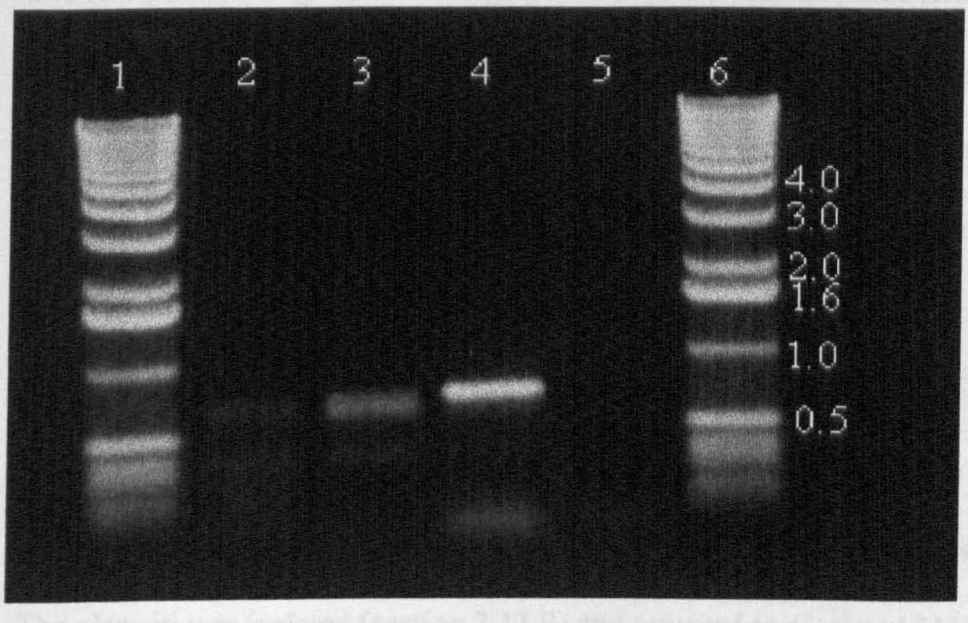


Fig 59: Amplification of *tpx1* fragment from *C. jejuni* isolates



Chromosomal DNA isolated from *C. jejuni* 81116 (Lane 2), 81-176 (Lane 3) and 11168 (Lane 4) was used as templates in the PCR reaction using *tpx*1 specific primers – t1REV and t1FWD. Template control is shown in Lane 5, with Lanes 1 and 6 indicating marker of 1 kb ladder.

Fig 60: Amplification of *tpx2* fragment of *C. jejuni* isolates



Chromosomal DNA isolated from *C. jejuni* 81116 (Lane 2), 81-176 (Lane 3) and 11168 (Lane 4) was used as templates in the PCR reaction using *tpx2* specific primers – t2REV and t2FWD. Template control is shown in Lane 5, with Lanes 1 and 6 indicating marker of 1 kb ladder.

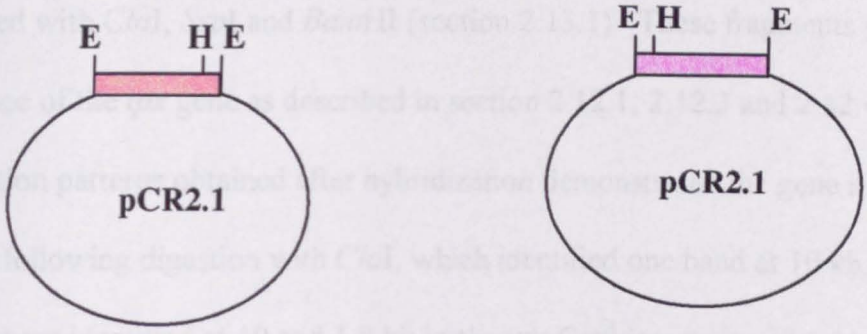
temperature to 53°C. Chromosomal DNA extracted from *C. jejuni* 81116, 11168 and 81-176 was used as templates to amplify the *tpx1* and *tpx2* fragment. An 800 bp fragment was amplified in *C. jejuni* 81-176 and 11168 (see Fig. 59) using primers for the N-terminal region. An 800 bp fragment was generated from *C. jejuni* 81-176 and 11168 using *tpx2* primers corresponding to the C-terminal fragment (see Fig. 60). The *tpx1* and *tpx2* fragments were independantly ligated in plasmid pCR2.1-TOPO, as described in section 2.13.8. To confirm the presence of the PCR product *Eco*RI was used to release the fragment from the backbone of plasmid pCR2.1. The presence of a *Hpa*I site was also confirmed in both clones (section 2.13.1).

5.3.3.2 Construction of the Tpx-deficient encoding region in *C. jejuni*

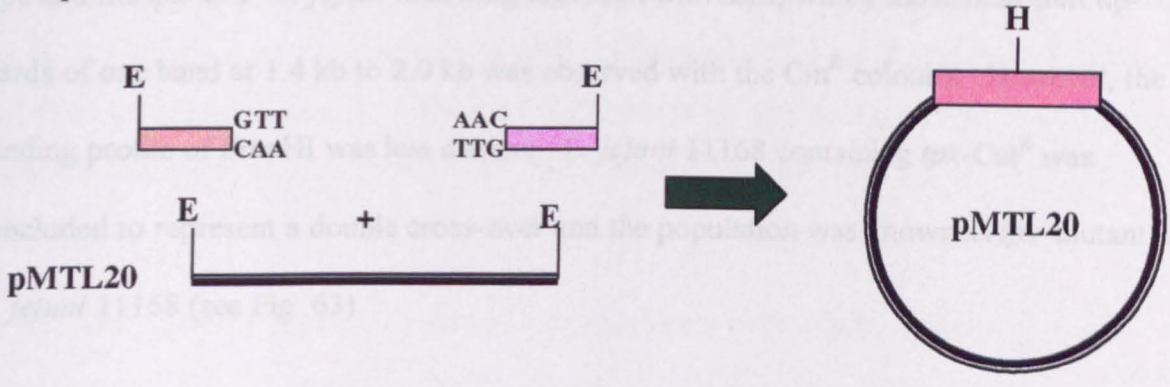
The *tpx* fragments (1 and 2) were released from plasmid pCR2.1-TOPO following digestion with restriction endonucleases *Eco*RI and *Hpa*I, as described in section 2.13.1 (see Fig. 61). The two purified fragments were ligated into the dephosphorylated *Eco*RI site of pMTL20 by a three-way ligation, as described in sections 2.13.2, 2.13.4 and 2.13.5. The *C. jejuni* *tpx* encoding region was disrupted by the insertion of the Cm^R gene from pAV35, into the uniquely created *Hpa*I site. The *tpx* encoding region was digested with *Hpa*I (section 2.13.1) and purified (section 2.13.2). The Cm^R gene was isolated from pAV35 following digestion with *Pvu*II (section 2.13.2), dephosphorylated (section 2.13.4) and purified (section 2.13.2). The Cm^R gene was ligated into the *Hpa*I site of *tpx* as described in section 2.13.5, and the resulting plasmid was transformed into *E. coli* DH5α (section 2.13.6.1). The plasmid was isolated (section 2.13.9) and returned to *C. jejuni* 11168 by electroporation as described in section 2.1.6.2.

Fig. 61: Diagramatic representation of the formation of the *tpx* gene within plasmid pMTL20 and the insertion of the chloramphenicol gene

I. Insertion of amplified *tpx* fragments into pCR2.1 TOPO

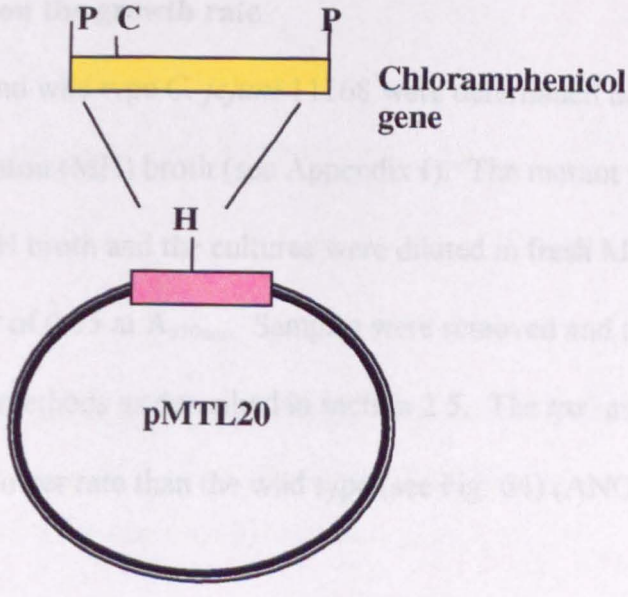


II. The *tpx* fragments were both isolated from plasmid pCR2.1 TOPO following digestion with *Eco*RI and *Hpa*I. The fragments were ligated into the *Eco*RI site of plasmid pMTL20



III. The chloramphenicol gene was inserted into the *Hpa*I site created within *tpx*

Key: E, *EcoR*I; H, *Hpa*I; P, *Pvu*II; C, *Cla*I



5.3.3.3 Confirmation of the presence of the disrupted *tpx* encoding region

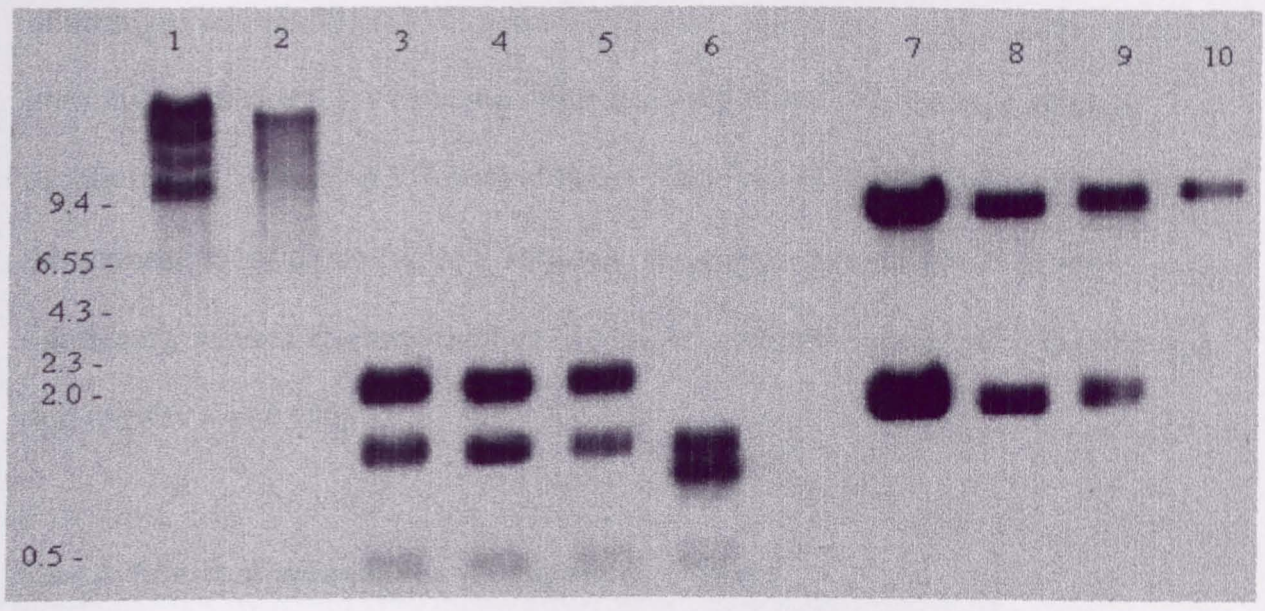
The presence of the *tpx*-Cm^R insert in the genome of *C. jejuni* 11168 was confirmed by probing with a fluorescein labelled *tpx* PCR product (section 5.3.3.1). Genomic DNA isolated from *C. jejuni* 11168 and *C. jejuni* containing *tpx*-Cm^R insert (section 2.11.2) was digested with *Cla*I, *Ssp*I and *Bam*HI (section 2.13.1). These fragments were probed for the presence of the *tpx* gene as described in section 2.12.1, 2.12.3 and 2.12.4 (Fig. 62). The restriction patterns obtained after hybridization demonstrated the gene in wild type *C. jejuni* 11168 following digestion with *Cla*I, which identified one band at 10 kb, whereas two bands were identified at 10 and 1.8 kb in the *tpx*-Cm^R inserts, consistent with the presence of the *Cla*I site present in the inserted Cm^R cassette. Three bands were identified in wild-type and the *tpx*-Cm^R *C. jejuni* following digestion with *Ssp*I, with a substantial shift upwards of one band at 1.4 kb to 2.0 kb was observed with the Cm^R colonies. However, the banding profile of *Bam*HI was less distinct. *C. jejuni* 11168 containing *tpx*-Cm^R was concluded to represent a double cross-over and the population was known as *tpx*$^-$ mutant of *C. jejuni* 11168 (see Fig. 63).

5.3.4 Identification of the role of Tpx

5.3.4.1 Effects of the *tpx* mutation on the growth rate

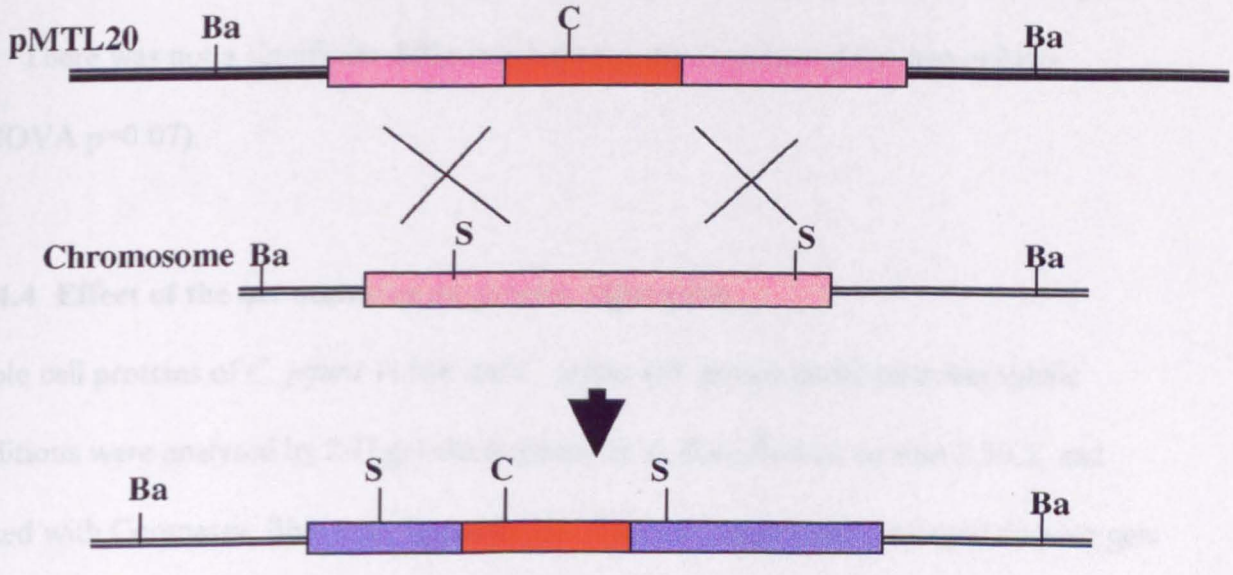
The growth rates of the *tpx*$^-$ mutant and wild type *C. jejuni* 11168 were determined under microaerobic conditions in Muller-Hinton (MH) broth (see Appendix I). The mutant and wild type were grown overnight in MH broth and the cultures were diluted in fresh MH broth to give a starting optical density of 0.05 at A_{650nm}. Samples were removed and the biomass determined by spectrometric methods as described in section 2.5. The *tpx*$^-$ mutant was found to grow at a significantly slower rate than the wild type (see Fig. 64) (ANOVA, $p=0.02$).

Fig 62: Southern blot analysis of chromosomal DNA of *C. jejuni tpx*-Cm^R containing colonies



Chromosomal DNA of *C. jejuni* 11168 (Lanes 2, 6 and 10) and the *tpx*-Cm^R colonies (Lanes 1, 3, 4, 5, 7, 8 and 9) were digested with *Bam*HI (Lanes 1 and 2), *Ssp*I (Lanes 3, 4, 5 and 6) and *Cla*I (Lanes 7, 8, 9 and 10). The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridisation with the 1.6 kb *tpx* PCR product of *C. jejuni* 11168 as a DNA probe.

Fig 63: Diagramatic representation of a double-cross over replacement of a gene



The suicide vector was introduced to *C. jejuni* 11168 by electroporation. The *tpx*-Cm^R fragment was integrated into the chromosome via homologous recombination. Key: Ba, *Bam*HI, C, *Cla*I, S, *Ssp*I.

5.3.4.2 Effect of hydrogen peroxide on the growth rate

The effect of oxidative stress on growth rate was compared using different concentrations of hydrogen peroxide (H_2O_2). *C. jejuni* 11168 and *C. jejeuni tpx*⁻ were grown as described previously in section 5.3.4.1 and were diluted in fresh MH broth containing different concentrations, (0, 0.25, 0.5, 1 mM) of H_2O_2. The *C. jejuni* isolates were both grown in the presence of H_2O_2 (see Fig. 65). However, the growth of the *C. jejuni tpx*⁻ was significantly reduced at a concentration of 0.5 and 1 mM H_2O_2, compared to the wild type cells (ANOVA p=0.006).

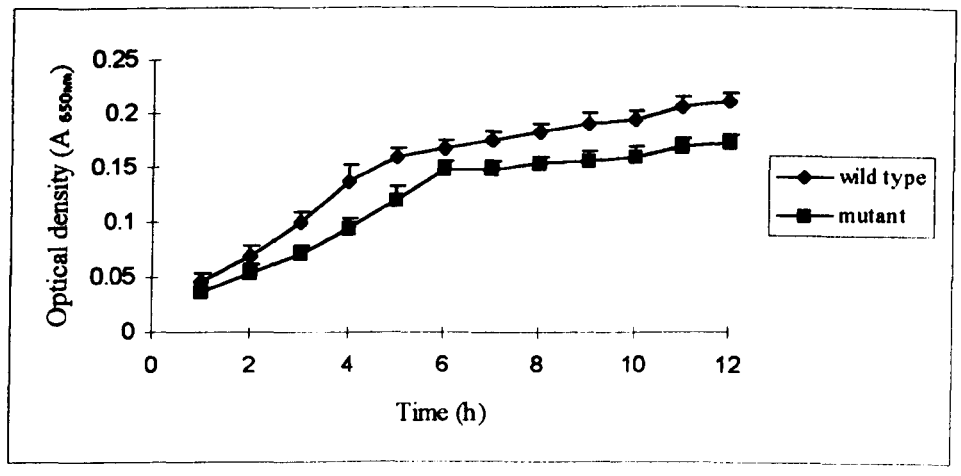
5.3.4.3 Effects of paraquat on the growth of *C. jejuni*

The effects of oxidative stress was assessed using a disk inhibition assay. Sterile disks of filter paper were placed onto the agar plate inoculated with *C. jejuni* 11168 and *tpx*⁻. Volumes (8 µl) of 0.25, 0.5 and 1 mM paraquat were placed onto the disks and the plates were grown overnight in microaerophilic conditions. Growth inhibition was determined by measuring the size of the zones of inhibition surrounding the filter paper disk (see Fig. 66). The size of the clear zone of *C. jejuni* 11168 was larger than that in the lawns of *C. jejuni tpx*⁻. There was not a significant difference between the response of the two isolates (ANOVA p=0.07).

5.3.4.4 Effect of the *tpx* mutation on protein expression

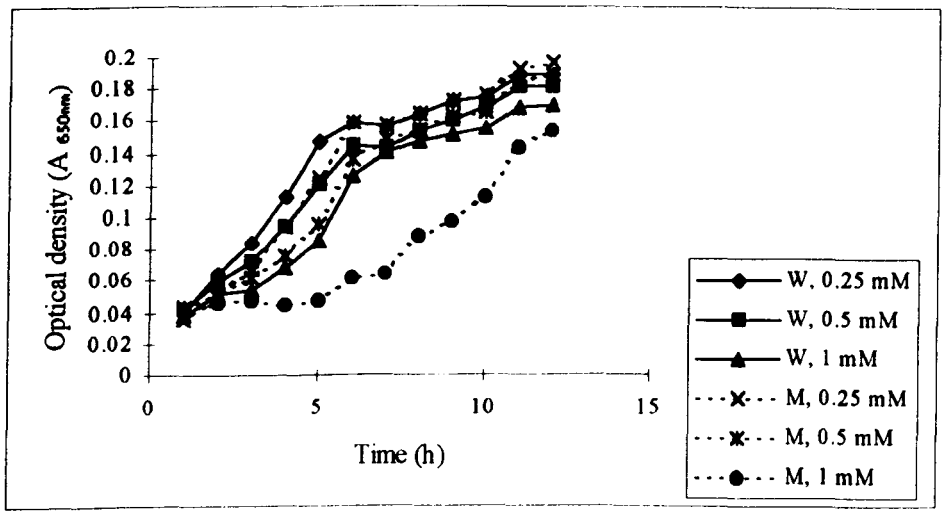
Whole cell proteins of *C. jejuni* 11168 and *C. jejuni tpx*⁻ grown under microaerophilic conditions were analysed by 2-D gel electrophoresis as described on section 2.10.2, and stained with Coomassie Blue (see Appendix II). The BioImage system merged the two gels to produce a normalised composite as described in section 2.10.3. The pI and Mwt were assigned.

Fig. 64: Growth curves of *C. jejuni* 11168 and *C. jejuni tpx*⁻



Note: *C. jejuni* populations were grown in MH broth overnight and used to inoculate fresh MH broth. The OD at $A_{650\,nm}$ was determined at 1 h intervals. Bars represent means ± SE (three separate determinations from three separate experiments). Differences between the strains were found to be significant (p=0.02).

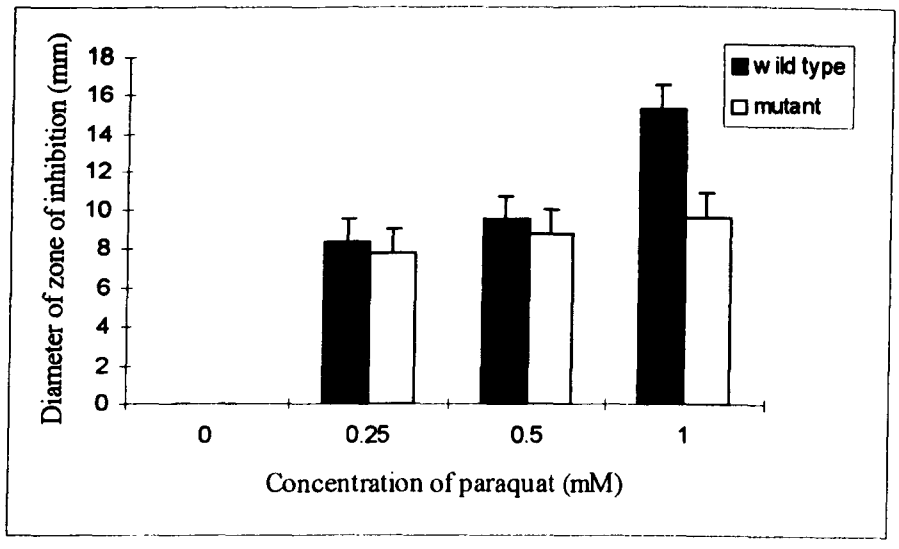
Fig. 65: Effect of hydrogen peroxide on the growth of *C. jejuni* 11168 and *C. jejuni tpx*⁻



Key: W, wild type *C. jejuni* 11168; M, mutant *C. jejuni tpx*⁻.

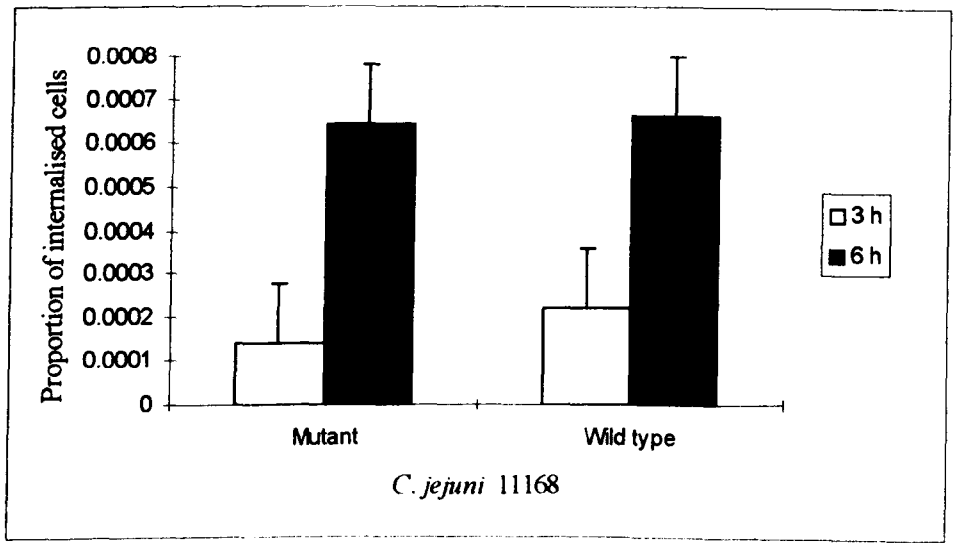
Note: Both *C. jejuni* population were grown in MH broth overnight and used to inoculate fresh MH broth containing 0.25, 0.5, 1 mM concentrations of H_2O_2 and the optical density was determined at 1 h intervals.

Fig. 66: Effect of paraquat on the growth of *C. jejuni* 11168 and *C. jejuni tpx*⁻



C. jejuni 11168 and *C. jejuni tpx*⁻ were grown on MH agar in the presence of 0, 0.25, 0.5, 1 mM concentrations of paraquat. The diameter of the zone of inhibition was measured after incubating in microaerophilic conditions for 48 h.

Fig. 67: The invasiveness of *C. jejuni* 11168 and *C. jejuni tpx*⁻ in Caco-2 cell monolayers cultured on non-permeable supports.



Data represents the proportion of the inoculum internalised by the Caco-2 cells. Bars represent means ± SE (four separate determinations from one separate experiment). Differences between the strains were not found to be significant by ANOVA (p=0.7).

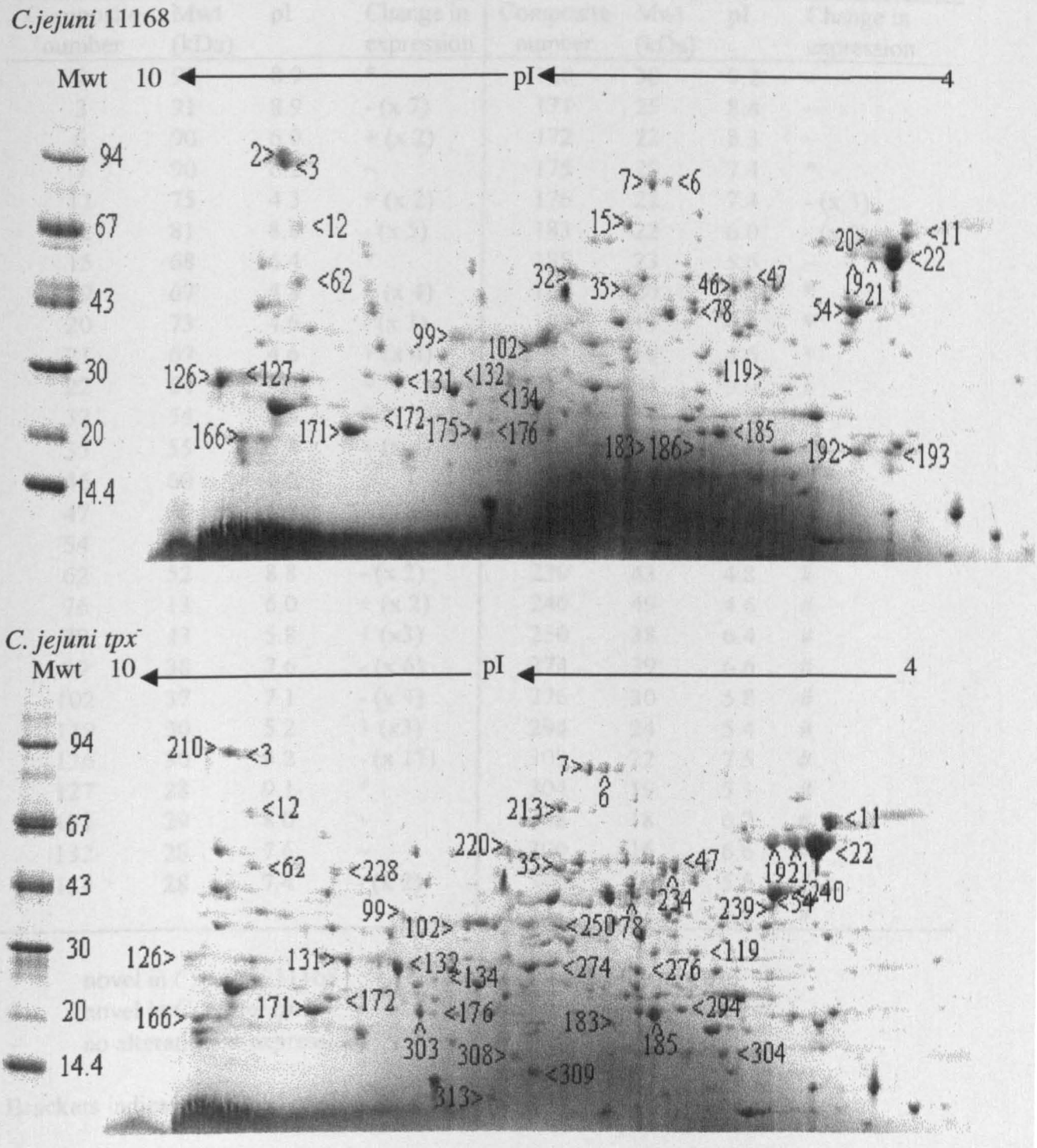
A total of 209 proteins were detected in *C. jejuni* 11168 and 249 proteins in *C. jejuni tpx*⁻ (see Fig. 68). Of these, 143 proteins were matched between the two gels and the remaining proteins - 66 in the wild type and 106 in the mutant were classified as novel. The fainter novel spots with integrated intensities of less than 0.7 were not included in any further analysis as these were not always clearly resolved on the gels. Under these more stringent conditions eight novel proteins were identified in the wild type (composite nos. 2, 15, 32, 127, 175, 186, 192, 193) and 17 novel proteins were identified in the mutant (composite nos. 210, 213, 220, 228, 234, 239, 240, 250, 274, 276, 294, 303, 304, 308, 309, 310, 313) (see Table 18).

The level of expression of the matched proteins, was also affected, following the insertional inactivation of the *tpx* gene. A high proportion of the proteins were either up- or down-regulated, between 2-3 fold (including composite nos. 6, 11, 47, 62, 76, 78, 134, 183), but this was not thought to represent a significant alteration in expression. However, two proteins (composite nos. 21 and 119) were found to be up-regulated between 3-4 fold in and a further two proteins were up-regulated more than 4-fold (composite nos. 19 and 35) in *C. jejuni tpx*. Three proteins (composite nos. 20, 102, 176) were found to be down-regulated between 3-4 fold and four proteins (composite nos. 3, 12, 99, 126) were found to be down-regulated more than 4-fold in *C. jejuni tpx*⁻ compared to *C. jejuni* 11168. The majority of the proteins were found to remain unaltered in their expression (including composite nos. 7, 22, 131, 132, 166, 171, 172, 183).

5.3.4.5 Effect of *tpx* mutation on invasive ability

The ability of the *tpx*⁻ and wild type *C. jejuni* to invade Caco-2 monolayers was investigated as described in section 2.8.2. The proportion of *C. jejuni* located within the Caco-2 monolayer increased over the 6 h time course of the invasion assay for both *C. jejuni*

Fig 68: 2-Dimensional gel electrophoresis of whole cell proteins of *C. jejuni* 11168 and *C. jejuni tpx*⁻ grown under microaerophic conditions



Whole cell proteins were harvested from microaerophilic cultures of both *C. jejuni* populations and focused in the first dimension on IEF strips pH 3 –10. Proteins were stained with Coomassie Blue and the gels were analysed by the BioImage system. Protein composite numbers were assigned. Mwt markers (kDa) and pI are indicated on the gels.

Table 18: Comparison of protein expression of *C. jejuni* 11168 and *C. jejuni tpx*⁻ grown under microaerophilic conditions by 2-D gel electrophoresis

Composite number	Mwt (kDa)	pI	Change in expression	Composite number	Mwt (kDa)	pI	Change in expression
2	92	8.9	*	166	30	9.2	~
3	91	8.9	- (x 7)	171	25	8.4	~
6	90	6.0	+ (x 2)	172	22	8.3	~
7	90	6.2	~	175	20	7.4	*
11	75	4.3	+ (x 2)	176	22	7.4	- (x 3)
12	81	8.8	- (x 5)	183	22	6.0	- (x 2)
15	68	6.4	*	185	23	5.6	~
19	67	4.8	+ (x 4)	186	20	5.8	*
20	73	4.6	- (x 3)	192	19	4.8	*
21	67	4.6	+ (x 4)	193	19	4.6	*
22	64	4.4	~	210	94	9.0	#
32	54	6.8	*	213	76	6.4	#
35	55	6.4	+ (x 4)	220	61	6.8	#
46	60	5.6	~	228	50	8.2	#
47	60	5.5	+ (2)	233	57	5.6	#
54	48	4.8	~	234	57	5.5	#
62	52	8.8	- (x 2)	239	43	4.8	#
76	13	6.0	+ (x 2)	240	49	4.6	#
78	43	5.8	+ (x3)	250	38	6.4	#
99	38	7.6	- (x 6)	274	39	6.6	#
102	37	7.1	- (x 4)	276	30	5.8	#
119	30	5.2	+ (x3)	294	24	5.4	#
126	30	9.2	- (x 17)	303	22	7.5	#
127	28	9.1	*	304	19	5.1	#
131	29	8.0	~	308	18	6.7	#
132	28	7.6	~	309	16	6.6	#
134	28	7.4	- (x 2)	310	14	5.5	#
				313	13	6.9	#

* novel in *C. jejuni* 11168 + up-regulated in *C. jejuni tpx*⁻

\# novel in *C. jejuni tpx*⁻ - down-regulated in *C. jejuni tpx*⁻

~ no alteration in expression

Brackets indicate the level of change in expression.

11168 and the *tpx*⁻ mutant (see Fig. 67). However, the capacity of the *tpx*⁻ to invade the monolayer did not differ from the wild type. The difference between the two cultures was not found to be significant by ANOVA ($p=0.7$).

5.4 Discussion

Pathogenicity is defined as the ability of a bacterium to cause infection (Salyers & Whitt, 1994). Bacteria move through the body and become exposed to numerous environmental cues which may promote the expression of virulence factors. Putative virulence determinants were identified in *Campylobacter* in Chapter 4 and in this chapter some of those proteins were identified at the molecular level. With the many advances in genetic manipulation techniques, genes encoding particular proteins can be identified and insertionally inactivated and returned to the *Campylobacter* genome, allowing the effects of the phenotype to be investigated.

The presence of protein composite no. 278 was found to correlate with an increase in the invasive ability of *Campylobacter*. The role of this protein was investigated at the molecular level using classical molecular techniques. However, it was not possible to identify the role of this protein through this approach.

The failure of the identification of the gene may have arisen for several reasons. The primer was designed to contain only a portion of the identified N-terminal sequence, due to the high number of leucines present in the sequence, which are encoded by six different triplet combination codons. The last nine amino acids were used to create a highly degenerate probe and the probe used, DOT1b, gave the strongest signal in the probing experiments. The chromosomal digests were probed under conditions of low stringency due to the degeneracy of the probe. Under more ideal conditions the stringency should have been raised to demonstrate homology of the particular region to the probe. A region was identified with apparent homology and the resulting clones were sequenced. The generated sequence was transcribed and found to contained a small number of amino acids with homology to the probe, but an open reading frame failed to be identified. However, the

function of the protein was identified through searches of the genome sequence and protein databases, and was found to encode part of the 50S rRNA protein, subunit L9 (see section 4.3.5).

A number of open reading frames were identified with some preceded by a Shine-Dalgarno site, and these translated protein sequences were used to search for homology in the protein data bases. The presence of an alkaline phosphatase-like protein was highlighted in clone 6_{10}A6. This protein functions as an enzyme acting to supply the cell with phosphate from exogenous sources (Nemeyanova *et al.*, 1981). In *E. coli*, the protein is encoded by *phoA* and contains a signal peptide to allow transfer to the periplasm (Michaelis *et al.*, 1983). The presence of an alkaline phosphatase-like enzyme has not previously been reported in *Campylobacter*, though it probably functions similarly to the alkaline phophosphatase of *E. coli*.

Other ORFs were identified in the sequence of 6_{10}A6 with homology to lactate and malate dehydrogenase. The presence of the enzyme lactate dehydrogenase has been suggested previously by Mendz *et al.*, (1997) following the production of lactate from pyruvate. Malate dehydrogenase, an enzyme of the TCA cycle, acts to catalyse the production of oxaloacetate. The presence of malate dehydrogenase has not been previously reported in *Campylobacter*.

Significant homology was identified with a section of the clone 6_{10}A7 to the *fom1* gene of *Streptomyces wedmorensis* and *S. hygroscopicum* (Hidaka *et al.*, 1995). This encodes phosphoenol-pyruvate phosphomutase (FOM1), which is involved in the biosynthesis of the fosfomycin nucleotide binding antibiotic. The presence of such a gene in *Campylobacter* would suggest that it may have the potential to produce fosfomycin. Work by Hidaka *et*

al., (1995) has suggested that this gene may act in self-protection for the bacteria, as fosfomycin is used as an antibiotic in bacterial infections. Fosfomycin was prescribed in an out-break of enterohaemorrhagic *E. coli* in Japan, but under laboratory conditions, it appeared to enhance the release of verotoxins (Yah & Hondo, 1997). Fosfomycin has been identified as an effective antibiotic for the treatment of campylobacteriosis (Gomez-Garces *et al.*, 1995) against a panel of isolates from Spain. The proposed role of a *fomI* gene in *Campylobacter* remains unclear though an interesting report has demonstrated that fosfomycin appears to suppress production of leukotriene B4 from neutrophils which in turn suppresses its expression of IL 8 mRNA (Honda *et al.*, 1998). Given the recent finding of Hickory *et al.*, (1999) that *Campylobacter* spp. stimulates the release of IL 8 from INT407 cells, the presence of a *fomI* gene may act to repress expression of IL 8. This may act to reduce the immune response to infection, causing more severe disease symptoms.

Open reading frames identified in clone 6_{10}G5 also showed significant homology to the FliD protein and FliS protein of *H. pylori*. These proteins were proposed to contribute to the flagella whose structure is composed of a basal unit, hook and filament. The FliD protein has been found to encode a filament cap protein that facilitates the polymerisation of endogenous flagellin at the tip of the filament (Yokoseki *et al.*, 1995). Mutation within *fliD* in *S. typhimurium* prevented growth of the flagella, as flagellin monomers transported through the central channel of the flagellum leaked out without polymerisation (Imada *et al.*, 1998). Interestingly, in *P. aeruginosa* the flagella cap, FliD, was found to have a role in adhesion to mucin (Arora *et al.*, 1998). The *fliD* homologue identified here may have a similar role in *Campylobacter* pathogenesis. FliS has been identified in many organisms including *E. coli* and *S. typhimurium*, and has been found to be essential for the formation of the flagella. Mutations in *fliS* caused impaired flagellin expression and resulted in short flagella, though no function could be assigned to the gene product (Yokoseki *et al.*, 1995).

However, further work found that *fliS* acted to negatively regulate the export of FlgM which acted as an anti-sigma factor, preventing over expression of flagella. Both *fliD*, *fliS* and *fliT* form an operon in *S. typhimurium* and ORFs in *Campylobacter* encoding homologues of *fliD* and *fliS* were found adjacent to one another suggesting a similar operon arrangement (Kutsukake & Ide, 1995).

Following the sequencing of the genome of *C. jejuni* 11168, an alternative molecular approach can be taken to identify the role of genes. An entire gene can be removed from the sequence data and a proposed role assigned by comparison with other known proteins by sequence alignments. However, this does not provide biochemical evidence of the actual role of the protein and genetic manipulation is required to ascertain a possible role. Three genes identified as up-regulated or novel and whose presence correlated with an increased invasive ability were selected to determine their possible role in *Campylobacter* pathogenesis. Using data from the genome sequencing project gene products were amplified by PCR and disrupted through the insertion of a chloramphenicol gene before returning to *Campylobacter* by integration into the genome.

The protein PEB-4 was proposed to have homology to a Gram-positive extracytoplasmic lipoprotein involved in processing export proteins, which was located in the periplasm (Burucoa *et al.*, 1995). PEB-4 was identified in *C. jejuni* 81-176 with a molecular weight of 29 kDa, which was similar to the predicted weight from the gene sequence identified in *C. jejuni* 11168 of 30 kDa. The encoding gene, *peb4A*, was found to possess a signal peptide of 21 amino acids.

On returning the insertionally inactivated *peb4A* gene to *Campylobacter*, no viable colonies were detected. After extensive alteration of conditions of electroporation it was concluded

that the mutation was lethal to the pathogen. The gene was inactivated down-stream of the signal peptide, allowing the first 21 amino acids to be cleaved by a signal peptidase I and hence the truncated protein product would have been able to translocate across the cytoplasmic membrane (Burucoa *et al.*, 1995). However, the lethal effect of this mutation may have been caused by the essential requirement of either the PEB-4 or FdaA proteins. The genes *peb4A* and *fdaA* were proposed form an operon as no consensus sequence for constitutive promoter or terminator sequences was found in the intergenic region (Burucoa *et al.*, 1995). The mutation in the *peb4A* gene may have prevented expression of *fdaA*, which is located further down-stream, demonstrating an effect known as polarity. The gene, *fdaA*, encodes fructose 1, 6-biphosphate aldose in *E. coli* (Alefounder & Perham, 1989) and is involved in glycolytic processes. *Campylobacter* is known not to utilise carbohydrates as energy substrates (Griffith & Park, 1990), but the protein may be involved in the transformation of carbohydrates (Burucoa *et al.*, 1995).

The protein PEB-4 has homology to PrsA of *B. subtilis* (Kontinen *et al.*, 1991) and PrtM of *Lactococcus lactis* (Vos *et al.*, 1989). Work on the role of protein PrsA of *B. subtilis* has demonstrated its involvement in the release and folding of secreted proteins following translocation across cytoplasmic membranes, allowing secretion of exoenzymes (Kontinen & Sarvas, 1993). Mutation of *prsA* showed that it was indispensable for viability. Also in *E. coli*, membrane associated proteins involved in protein export were demonstrated to be essential for viability. Hence, it would seem likely that PEB-4, a membrane associated protein (Kervella *et al.*, 1993), has a role in protein translocation. Interestingly, PrsA has been suggested to act as an extracytoplasmic chaperone to facilitate the correct folding of exoproteins after translocation (Jacobs *et al.*, 1993). Accordingly, PEB-4 may act to provide a similar function in *Campylobacter*.

The presence of the HtrB protein was proposed to aid growth of *E. coli* at high temperature (Karow *et al.*, 1991). The protein homologue predicted in *C. jejuni* 11168 via the genome sequence project has a molecular weight of 35 kDa, similar to the molecular weight of HtrB in *E. coli* of 35.4 kDa. However, this proposed molecular weight did not correspond to that predicted in *C. jejuni* 81116 of 27 kDa (section 4.3.5).

Analysis of the *htrB*-Cm^R containing colonies with restriction endonucleases established that the altered gene had returned to the chromosome via homologous recombination. However, recombination did not appear to be internal to the *htrB* gene. This was demonstrated following treatment with *Bgl*II, as if the deficient gene had integrated into the wild type gene then the single band generated would have been larger in size than that of the wild type, due to the presence of the chloramphenicol gene. However, the probing of the *hrtB*-Cm^R colonies revealed two bands, one a similar size to the wild type and one slightly bigger. The single cross-over reaction occured via a Campbell-like mechanism (Dickinson *et al.*, 1995) whereby the plasmid become integrated, but the bacteria retains a duplicate of the original gene and a gene containing the chloramphenicol gene.

However, insertion appeared to allow transcription of the chloramphenicol gene allowing antibiotic selection. The generation of a single cross-over mutation was unexpected, as 800 bases were amplified either side of the *htrB* gene which should have be sufficient to promote the formation of a *htrB*-deficient mutant. Workers have demonstrated that by increasing the length of the homologous region of DNA returned increased the rate of recombination (Wassenaar *et al.*, 1993). For example, when 567 bases of homology were included homologous recombination was the dominant process in *C. coli*, but reducing the number of bases also reduced the efficiency (Richardson & Park, 1997).

It may be that selection for these colonies may reflect the role of the gene in *C. jejuni*. The colonies were recovered at 37°C, though in *E. coli* the *htrB* gene was found to be essential for growth above 33°C (Karow *et al.*, 1991). Hence, the conditions used to recover *C. jejuni htrB*-Cm^R colonies may have actively selected for those *C. jejuni* colonies which retained a functional *htrB* gene. Incubation of freshly electroporated *C. jejuni* at 30°C did form viable colonies, but these failed to be sub-cultured. These clones had a liquidy appearance and were unable to form distinct colonies, suggesting homologous recombination had occurred and the altered gene had replaced the original copy. The *htrB* gene may have additional roles in *C. jejuni*, apart from its role in permitting growth at high temperatures, which may include involvement in synthesis or maintenance of the cell wall, or both. Such roles for this protein were identified previously in *E. coli* (Karow & Georgopoulis, 1991).

Thiol peroxidase (Tpx) acts to remove oxygen radicals created by oxidative stress and has been identified in a number of organism's, including *E. coli*, *V. cholerae* and *H. pylori* (Wan *et al.*, 1997). The gene putatively thought to encode thiol peroxidase in *C. jejuni* was identified here at the molecular level and its role assessed through creation of a *tpx*-deficient mutant.

The thiol peroxidase (Tpx) protein was previously identified to have a molecular weight of 22 kDa in *E. coli*, which was close to the weight predicted for the Tpx protein of *C. jejuni* from the genome sequencing project. Analysis of the encoding amino acid sequence of the *tpx* gene was compared and found to share homology with many other pathogens (Wan *et al.*, 1997; Zhou *et al.*, 1997). The cloned *tpx* gene of *C. jejuni* 11168 was disrupted by a 10 base pair deletion, created to ensure gene inactivation and the chloramphenicol gene was inserted to allow selection. Genetic analysis of *tpx*-Cm^R colonies confirmed that the gene

had been inserted by homologous recombination into the wild type gene creating a mutagenic effect (Fig. 63).

The growth rate of the *C. jejuni tpx*-deficient mutant (*tpx*$^-$) under microaerophilic conditions was significantly reduced compared to *C. jejuni* 11168. However, the mutant retained the ability to replicate suggesting that the Tpx protein was not an essential protein for cell viability. Application of oxidative stress, through the presence of H_2O_2, caused the differences between the wild-type and the mutant to become more pronounced with a reduction in the growth rate in *C. jejuni tpx*$^-$ at a concentration of 0.5 and 1 mM. In comparison, no discernible effects were observed with the wild type *C. jejuni*, at these concentrations of H_2O_2. This suggests that the *C. jejuni tpx*$^-$ was more sensitive to H_2O_2 than the wild type, with H_2O_2 causing damage to the cell through the generation of oxygen radicals via an iron-catalyzed Fenton reaction (Beyer *et al.*, 1991), Tpx may function in *C. jejuni* to reduce H_2O_2 by breaking down oxygen radicals into water and oxygen, thus preventing cell damage and death.

At a high concentration of paraquat a difference was observed between the zones of inhibition of *C.jejuni tpx*$^-$ and *C. jejuni* 11168. This suggests that the mutant was less sensitive to the presence of the superoxide anions, not affecting viability as observed in the wild type. The unusual response suggests that the functional mechanisms present in *C. jejuni tpx*$^-$, such as catalase and SOD were more effective at removing oxygen radicals in the mutant than the wild type at the higher concentration of paraquat. This was unexpected as the *tpx*$^-$ mutant of *E. coli* on exposure to high level of paraquat formed larger clear zones than the wild type (Cha *et al.*, 1996). Lower levels of superoxide anions did not significantly affect the viability of *tpx*$^-$ when compared to the wild type and the oxidative defence mechanisms appeared to be able to remove the anions present. This suggests that

high levels of superoxide anions, which are normally detoxified in *Campylobacter* by SOD, were able to compensate for the lack of functional Tpx. In addition it would appear that catalase present in *Campylobacter* was able to function effectively to remove these molecules instead of Tpx. This strongly suggests that *Campylobacter* uses different mechanisms to resist oxidative stress created by different sources.

A number of changes were observed in the whole cell protein expression of *C. jejuni tpx*⁻ and *C. jejuni* 11168, whilst grown under microaerophilic conditions as a result of the inactivation of *tpx*. A total of eight and possibly as many as 66 proteins were uniquely identified in the *C. jejuni* 11168 with the up-regulation of 20 proteins. In contrast, in *C. jejuni tpx*⁻ 17 proteins and possibly as many as 106 proteins were identified as unique, with the up-regulation of 19 proteins. The two proteins previously identified through Edman degradation as Tpx (composite nos. 192 and 193; section 4.3.5) were present in *C. jejuni* 11168 but were absent from the proteome of *C. jejuni tpx*⁻. The remaining uniquely identified six proteins, which were detected in *C. jejuni* 11168 but were absent from the mutant may represent proteins that were co-transcribed with *tpx*, or formed a regulon and the absence of a functional *tpx* gene prevented their transcription. At least one or two of the unique proteins identified in *C. jejuni tpx*⁻ may form part of the chloramphenicol gene, but the remaining unique proteins of *C. jejuni tpx*⁻ may be transcribed to functionally replace Tpx. Alternatively, Tpx may have acted to negatively regulate the expression of some genes

The absence of a functional *tpx* gene did not appear to affect the ability of *C. jejuni tpx*⁻ to invade the Caco-2 monolayers over the 6 h period. Both the *C. jejuni* 11168 and *C. jejuni tpx*⁻ were recovered in similar proportions from the monolayer. This was unexpected as previous mutations in another oxygen scavenging molecule, for example *sodB*, was found to

reduce the ability of *C. jejuni* to survive in INT407 monolayers (Pesci *et al.*, 1994) suggesting a role in intracellular survival. Similarly, work by Purdy *et al.*, (1999) demonstrated that the *sodB* mutant of *C. coli* had a reduced potential to colonize the gut of chickens. Peroxidase activity in *Mycobacterium tuberculosis* was also proposed to be an important mechanism for survival in host phagocytes (Manca, *et al.*, 1999). Tpx in *C. jejuni* may act as a defence mechanism at extra-intestinal locations against oxidative burst, generated by PMNs, acting to detoxify the reactive oxygen species.

Alignment of the amino acid sequences of *C. jejuni* Tpx with that of TagD from *V. cholerae*, ToxR regulon from *H. influenzae*, SsaB from *Streptococcus sanguis* amd ScaA from *Streptococcus gordonii* (Cha *et al.*, 1996) highlighted the presence of a conserved cystein residue at position 94 (See Appendix V). Replacement of this residue with serine in *E. coli* resulted in a loss of peroxidase activity suggesting that the protein acted as a nucleophile destroying H_2O_2 and alky peroxides. Tpx was proposed to be located in the periplasmic space of *E. coli*, where it can act to remove exogenous peroxide and may be similarly located in *C. jejuni*. Tpx may act as a defence mechanism against oxidative bursts, created by PMNs, though removal of the functional gene did not alter its ability to survive in monolayers.

In summary, probing of chromosomal DNA with probe DOT1b did not identify the gene encoding protein composite 278. From the clones generated, homology to several interesting genes encoding alkaline phosphatase, *fom1* and flagellar proteins were identified. Mutation of the genes *peb4A* and *htrB* in *C. jejuni* failed to form viable mutants. This suggests that the function of these proteins was essential to the viability of *C. jejuni*. A viable *tpx*⁻ mutant was created, which was shown to act as a defence mechanism against hydrogen peroxide and was not affected by the presence of superoxide anions. Tpx did not

appear to have a role in intracellular survival in Caco-2 cell monolayers. However, the role of Tpx in virulence remains unclear, as it may contribute to survival at extra-cellular locations, and resist oxidative bursts from PMNs. Interestingly, Tpx was identified in *V. cholerae* initially as TagD, a member of the toxin co-regulated pilus gene cluster which was thought to contribute to adhesion (Hughes *et al.*, 1994). Similarly, in *Streptococcus* the *tpx* gene was identified as a member of an operon/gene cluster encoding adhesins (Sampson *et al.*, 1994). Hence *tpx* may function as an adhesin molecule in the initial stage of invasion, or during invasion at extra-intestinal locations.

Chapter 6

General Discussion

Campylobacter remains one of the largest causes of gastroenterititis in the devloped world. How it survives and causes infection has been the aim of numerous reseach groups, though, many aspects of pathogenesis remain unclear (general reviews Ketley, 1995, 1997). The aim of this study was to understand better,

- How *Campylobacter* promotes infection through different invasion mechanisms,
- Surviving exposure to different environmental cues,
- The effect these cues exert on virulence and changes in protein expression
- The identity of some of these proteins at a molecular level.

The panel of *Campylobacter* isolates studied demonstrated significantly different intrinsic abilities to invade Caco-2 cell monolayers whilst grown on solid supports, and these inherent differences may contribute to the wide ranging severity of symptoms of gastroenteritis caused by *Campylobacter*. Isolates which were more invasive (for example, 9519, 10392 and CH1), may produce severe inflammatory diarrhoea and those less invasive isolates (for example 235, 9752 and CH5) may promote milder symptoms, a distinction which was suggested previously by Everest *et al.*, (1992). Recently, work by Hickory *et al.*, (1999) has demonstrated that invasive isolates of *C. jejuni* were associated with a higher level of secretion of IL-8, a potent chemoattractant, in INT407 cells. The highly invasive isolates identified here may similarly induce secretion of IL-8 from Caco-2 cells, and in turn these isolates may promote inflammation in *in vivo* situations and may lead to the accumulation of immune molecules at the site of invasion, causing a more severe disease presentation.

Passaging of selected isolates in Caco-2 cells acted to enhance those isolates, previously identified as invasive, to become more invasive in their nature. This increased invasive ability was correlated with an alteration in the expression of many protein in the passaged isolates CH1P and 799P. Previous workers had demonstrated that *de novo* protein synthesis was essential for internalisation into epithelial cells (Konkel & Cieplak, 1992) and it may be possible that proteins required for invasion, up-take of and intracellular survival of *Campylobacter* may have become expressed consitutively in the passaged isolates in this study. For example, the expression of CiaB which was associated with secretion of proteins in the presence of INT407 cells (Konkel *et al.*, 1999) may have become up-regulated in its expression. Proteins associated with invasion including those involved in motility and chemostaxis were found to alter in expression on passage (see Table 19).

The most noticeable alteration was the expression of flagellin in isolates CH1P and 799P (see Figs. 11 and 12; Tables 3 and 4). Following passage the flagellin subunits shifted becoming more acidic in nature, for example the pI shifts from 4.6 to 4.3. This shift probably reflects post-translational modification of the flagellin which may contribute to antigenic variation of *Campylobacter* flagella. Post-translation modification of *Campylobacter* flagellin has been found to occur by glycosylation, mainly through the addition of sialic acid (Doig *et al.*, 1996; Guerry *et al.*, 1996). Multiple glycoforms of flagellin, similar to those observed here, were found by Doig *et al.*, (1996) to form a charge train following IEF. The gene, *ptmB*, with homology to CMP-N-acetylneuraminic acid synthetase was identified (Doig *et al.*, 1996), which was thought to function solely in the post-translational modification of flagellin. A gene with homology to CMP-N-acetylneuraminic acid synthetase was identified in this study in clone 6_{10}A7 (see section 5.2.5, Table 17), which may have a similar role to *ptmB*. Alternatively, it may be involved

Table 19: Putative identification by position of proteins on 2-D gels following previous identification by N-terminal sequencing: Isolates passaged through Caco-2 cell monolayers

Protein homologue	CH1P	799P	81116P	247P
	Protein composite no.	Protein composite no.	Protein composite no.	Protein composite no.
50 s RNA	N/D	277	256	251
AhpC	192*	298	N/D	205
Aspartate aminotransferase	82	N/D	180	145?
Bacterioferritin	N/D	N/D	N/D	N/D
Catalase	N/D	N/D	N/D	N/D
DnaK	14	35	196	18
Flagellin	27, 29, 30, 252*, 253*	67, 68, 70, 345*, 346*	17, 18, 19, 202*, 204*	43, 45, 46
Fumarate hydratase C	261*	99	31	69
Glutamine synthetase	N/D	N/D	N/D	N/D
GroEL	25	66	14	41
HtrB	176	279	140	223
Methyl accepting receptor	99	357	N/D	113
PEB-1	173	228	138	172
PEB-4	344	225	242	347
Periplasmic protein	225	N/D	N/D	N/D
Protease	38*	133*	76	51
Succinyl coA synthetase	143	231	110	177
Thiol peroxidase	196, 336	301	155	244, 245
Translocation elongation factor	65	182	61	86
Trigger factor	256	357	34	77

Key: The protein composite numbers indicated correspond to the numbers assigned during the analysis by the BioImage system, refer to Tables 3, 4, 5 and 6. N/D, not detected under the environmental cue; *, detected in the non-passaged isolate only.

in the glycosylation of LPS. The changes in flagellin observed here, induced by repeated exposure to, and survival within Caco-2 cell monolayers may represent an additional avoidance mechanism of the organism which may occur *in vivo* acting to prevent subsequent detection of the pathogen by the immune system.

A proposed methyl accepting chemotaxis protein was also found to be up-regulated on passage of the isolates, suggesting a possible role in invasion and survival within the enterocyte monolayers. However, further work would be required to clarify the role of this protein. Other proteins which were up-regulated following intracellular passage included DnaK, trigger factor and PEB-4, suggesting they may contribute to the invasion or survival process. It would appear that HtrB, Tpx and PEB-1 were not enhanced following passage suggesting that they had little, or no direct role in the alteration of invasive or survival ability of passaged isolates. The lack of response by PEB-1 was unexpected as this protein had previously been identified to play a role in internalisation (Pei *et al.*, 1998).

Isolates of *Campylobacter* also demonstrated different abilities to translocate across polarised Caco-2 cell monolayers grown on semi-permeable membranes. Comparison of four contrasting isolates demonstrated that invasiveness did not necessarily correlate with the ability to translocate across the epithelial cells. Hence, although the isolates differed in their invasive ability, they also differed potentially in their mechanism of penetrating the epithelium. This may contribute further to the disease symptoms presented in human cases. Three distinct phenotypes were observed: invasion without translocation, invasion with translocation and translocation without invasion. The ability to translocate paracellularly (translocation without invasion) between the tight junctions, did not induce any noticeable variation in the TEER measurements across the monolayers, or in the morphology of the Caco-2 cells. However, intracellular translocation (invasion with translocation) by

Campylobacter did appear to disrupt both the TEER and the morphology of the Caco-2 cells, suggesting that the process disrupted the monolayer significantly, possibly by a re-arrangement of the cytoskeleton. This work confirms the existence of different invasion phenotypes in different *Campylobacter* isolates (Ketley, 1995) and defines them more clearly.

This study has established that *Campylobacter* differ in their intrinsic behaviour with regard to invasive ability and how the organism can penetrate epithelial cells monolayers. However, the behaviour of a micro-organism both *in vitro* and *in vivo* can also be influenced by environmental factors surrounding the organism (Smith *et al.*, 1990). Encountering different environments often acts to induce phenotypic changes which ensure survival, and has been proposed to act as a cue for the expression of virulence determinants (Mekalanos, 1992). Little work has been carried out on the response of *Campylobacter* to specific environmental cues and the phenotypic changes which subsequently arise. Therefore, in this study, the responses of *C. jejuni* to a range of environmental conditions were examined using continuous culture.

C. jejuni was able to sustain growth in continuous culture under several extremes of culture conditions. Although growth under conditions of iron limitation, extreme oxidative stress and low pH resulted in a lower level of biomass, *C. jejuni* was able to maintain a steady state population with a mean generation time of 8.7 h^{-1}. This was the first demonstration of some of the extremes of environmental conditions which were able to support growth of *Campylobacter*.

C. jejuni changed substantially in morphology, protein expression and virulence (as determined by invasion into Caco-2 cells) in response to each environmental cue. Whilst

grown under iron limitation *C. jejuni* became filamentous in appearance, increasing in length three-fold, a response which was not observed during exposure to any of the other environmental cues. Previous workers had suggested that filamentation occurred in response to a lack of iron, which prevented cell division (Field *et al.*, 1986). Interestingly, trigger factor which has been shown to promote cell division (Griffiths *et al.*, 1995) was found to be substantially down-regulated under iron limitation in this study which further suggests a role for this protein in cell division. In addition, DnaK, which has also been shown to contribute to the process of cell division, was not detected under iron limitation (Bukau & Walker, 1989). Recently Connerton & Connerton (1999) identified a coding region in *C. jejuni* encoding an integral membrane protein which in *E. coli* caused filamentation. This suggests that many proteins may contribute to the filamentation process in *C. jejuni*, some of which may be a result of direct, or indirect interaction of iron molecules with promoter sequences.

Following the changes in protein expression of an iron limited *C. jejuni* culture to a pulse of iron, the up-regulation of different protein components suggested the presence of an iron uptake mechanism. The 75 kDa protein, uniquely identified here in *C. jejuni* 81116 under iron limitation by 1-D SDS PAGE, appeared to respond to the levels of iron present in the medium. A pulse of iron caused the expression of this protein to be reduced until the amount of available iron in the chemostat reached a concentration 5 μM. At this apparently critical concentration the level of intensity of this band rose suggesting an increase in expression. This suggests a possible role as a receptor molecule for iron-bearing ligands. Other iron responsive proteins were also highlighted in different subcellular fractions of *C. jejuni* which may comprise an iron-uptake mechanism similar to that of *E. coli* as described in section 1.13.1 It was surprising that no components of the iron uptake mechanism were identified following N-terminal sequencing of *C. jejuni* grown under iron-limitation. This

may be due to the particular proteins not being present in large enough quantities and not readily detected by the Amido black stain which is used o detect proteins prior to N-terminal sequencing.

Extreme oxidative stress induced by a combination of high DOT and a reduction in carbon source availability induced 50 % of the cells in the chemostat to become coccoid with the two isolates of *C. jejuni* examined. This was not observed under iron limitation or growth at low pH, though a small transient increase in coccoid cells was reported during an iron pulse (Harvey & Leach, 1998). A role for oxygen in coccoid cell formation has been previously suggested (Moran & Upton, 1986; Boucher *et al.*, 1994), but the use of batch cultures prevented the identification of the specific factor(s) involved. Sections of *C. jejuni* examined by EM demonstrated a considerable break-down of ultra-structure in many cells, suggesting that by 48 h the coccal cells represented a degenerate state. However, the phenomenon observed between 24-48 h of a retention of metabolic activity and a recovery in the number of colony forming units, does suggest that a VNC form was induced which may act as a transitionary state during adaptation to oxidative stress in some isolates. The cellular break-down observed at 48 h was most probably due to the oxidative stress exceeding the capacity of the defence mechanisms to remove radical oxygen species from some of the individual cells. These oxygen radicals promoted damage to the lipids, proteins and DNA of the cells (Storz *et al.*, 1990). The release of the intracellular material that was observed in the EM sections was similar to that reported by Moran & Upton (1986).

The responses of batch cultures of *Campylobacter* to acid shock varied, with some isolate more able to survive at the low pH of 3.5 than others. The panel of isolates did not appear to be constitively acid resistant, being only able to survive for short periods of time at low pH values. The lack of resistance to low pH may be related to the low level of CFA present

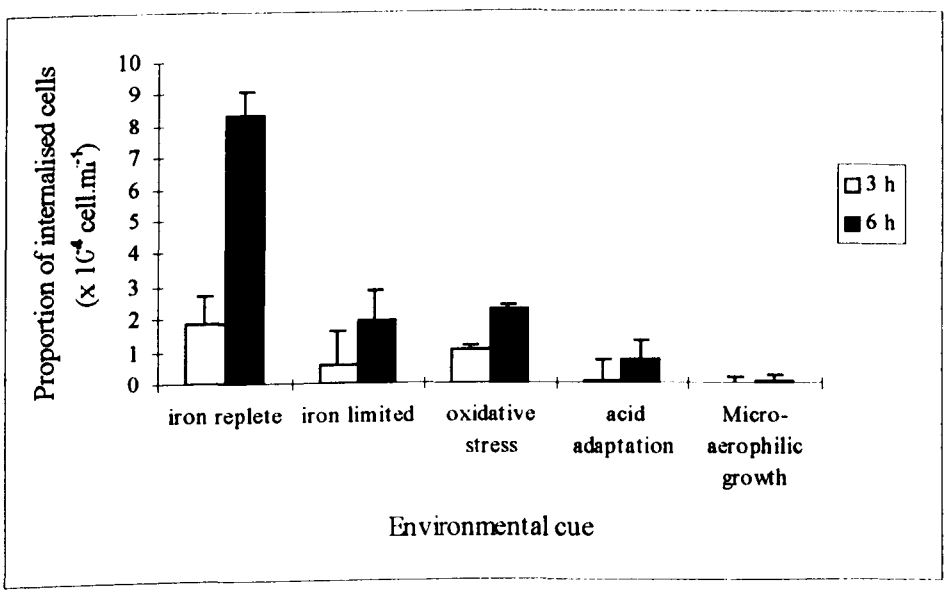
in the cell membrane, as high levels of CFA have been associated with survival to acid shock in *E. coli* (Chang *et al.*, 1999). Growth in continuous culture did enhance survival, but not at a significant level. This may be due to the CFA present in chemostat grown *C. jejuni* which correlated with the CFA present in more resistant stationary phase *E. coli* (Leach *et al.*, 1997). Acid adaptation did enhance survival suggesting the presence of an ATR mechanism similar to that of *S. typhimurium*, which may have involved changes in membrane protein composition or permeability.

The ability of the isolates to resist low pH did not generally correlate well with their invasive ability. Of note, however, were two highly invasive isolates, 9519 and 799, which were more able to with-stand acid shock, than two of the less invasive isolates, 247 and 235, which more acid sensitive. Although the association between acid tolerance and invasion mechanism is not clearly defined, it was notable that, of these particular isolates, 9519 was able to penetrate Caco-2 cell monolayers by mechanisms involving both intracellular invasion and translocation. This suggests that its ability to resist acidic conditions could have promoted its survival during movement through the cells where it may be exposed to phagolysome activity within the cytoplasm under *in vivo* conditions in the human host. The converse may be true of the acid sensitive isolate 235 which may penetrate the epithelium by translocation through tight junctions rather than intracellular invasion. An ability to survive acidic conditions within enterocytes may not therefore be essential for this isolate to invade tissues and cause disease.

Under the conditions of carbon starvation and growth at low pH (pH 5.5) *C. jejuni* remained able to invade Caco-2 cell monolayers and be recovered from within the cells at low levels after 6 h incubation. However, *C. jejuni* grown under oxidative stress, iron limited and iron replete conditions demonstrated a greater invasive potential (see Fig. 69),

especially those grown under iron replete conditions. These differences in invasive ability may be attributed to changes in the phenotype of *Campylobacter* following exposure to the different environments which may occur in *in vivo* situations. The changes in proteome expression of *C. jejuni* under these environmental cues were compared and a number of the affected proteins were identified by Edman degradation. These N-terminal amino acid sequences may provide an explanation of the survival and virulence responses of *Campylobacter* (see Table 20). Four distinct groups of proteins could be discerned amongst those identified from the proteome of *C. jejuni*, including housekeeping proteins, chaperonin proteins, putative virulence determinants and those involved in survival mechanisms.

Fig. 69: Comparison of the effect of different environmental cues on the invasive ability of *C. jejuni* 81116 into Caco-2 cell monolayers



Note: Data was taken from sections 4.2.6, 4.3.6, 4.4.7.

Two enzymes, succinyl coA synthetase and fumarate hydratase C (FumC), the most highly conserved of the TCA cycle, were identified. Two other enyzmes, aspartate aminotransferase and glutamine synthetase, were also identified which were involved in the synthesis of amino acids. Succinyl coA synthetase and aspartate aminotransferase were

Table 20: Putative identification by position of proteins on 2-D gels following previous identification by N-terminal sequencing: Exposure to three different environmental cues.

Protein homologue	Iron replete	Iron limitation	Oxidative stress	Acid adaptation
	Protein composite no.	Protein composite no.	Protein composite no.	Protein composite no.
50 s RNA	138	N/D	277	399
AhpC	125	209	153	243
Aspartate aminotransferase	53	N/D	39	74
Bacterioferritin	221	221	N/D	N/D
Catalase	N/D	177/176/179	N/D	N/D
DnaK	15	N/D	221	14
Flagellin	N/D	N/D	12/13/14	31/32/33
Fumarate hydratase C	22	22	26	46
Glutamine synthetase	37	N/D	N/D	N/D
GroEL	13	13	11	30
htrB	141	N/D	171	396
Methyl accepting receptor	29	N/D	50	89
PEB-1	108	108	125	220
PEB-4	99/101	99	92/261	184
Periplasmic protein	2	N/D	4	6
Protease	18	N/D	62	69
Succinyl coA synthetase	84	N/D	95	188
Thiol peroxidase	149/150	150	162/163	251/255
Translocation elongation factor	46	46	57	103
Trigger factor	37	N/D	N/D	53

Key: The protein composite numbers indicated correspond to the numbers assigned during the analysis by the BioImage system, refer to Tables 8, 10 and 14. N/D, not detected under the environmental cue.

detected under iron replete, oxidative stress and low pH conditions. The up-regulation of these two enzymes may reflect a general increase in energy requirement and protein synthesis of *Campylobacter* under these conditions. Although the regulation of glutamine synthetase was not clearly visible under any environmental condition due to its co-running with trigger factor on the 2-D gels, any up-regulation would have contributed to an increased production of glutamine. FumC was up-regulated under iron limited conditions, oxidative stress and acid adaptation. The up-regulation under iron limitation may reflect this protein's role in providing an alternative enzyme to FumA when iron availability is restricted or when radical oxygen molecules accumulate (Park & Gunsalus, 1995).

The level of expression of translation elongation factor did not alter significantly under any of the environmental conditions, though the functionally related 50S ribosome subunit protein, L9, was up-regulated under all of the environmental conditions applied. The up-regulation of L9 may reflect a general increase in the number of ribosomes and an increased requirement for translation processes to replace damaged proteins.

Chaperones are proposed to act to maintain the correct folding and assembly of proteins including the critical formation of appropriate secondary and tertiary structures in bacterial proteins involved in survival and virulence (Parsons *et al.*, 1997). Two such heat shock proteins were identified here in the proteome of *C. jejuni*, GroEL (HSP 60) and DnaK (HSP 70). These two chaperones interact differently with unfolded polypeptides, with DnaK recognising nascent chains during translation and GroEL recognising secondary structures, facilitating oligomeric protein assembly (Langer *et al.*, 1992). GroEL has been identified previously in *C. jejuni* (Takata *et al.*, 1995; Thies *et al.*, 1999), and shown to play a role in protection from oxidative stress, which was confirmed here in continuous culture experiments with its up-regulation under oxidative stress. DnaK was found previously to be

essential for growth at high temperatures in *E. coli* and also contributed to cell division (Bukau and Walker, 1989). In this study DnaK was up-regulated under all of the environmental conditions suggesting an increased requirement in general protein folding. Additionally, it may reflect a response to stressful conditions which causes increased denaturation of proteins generally within the cell, which DnaK, in conjunction with other proteins such as DnaJ, acts to repair (Gaitanrais *et al.*, 1990).

The protein PEB-4 was previously identified to have homology to an extracytoplasmic export lipoprotein, and possessed a signal peptide suggesting it was transferred to an extracytoplasmic location. The inability to create a viable knock-out mutant of *peb4A* in this study may reflect its vital role in the cell's metabolism, though it may also demonstrate an essential requirement for the down-stream gene, *fdaA*. Further mutational analysis would be required to clarify this, although it does demonstrate the difficulty of creating a knock-out mutation which would solely inactivate *peb4A*. The sequence of the *peb-4a* gene has homology to the gene *prsA* of *B. subtilis* (Kontinen *et al.*, 1991), which acts as an extracellular chaperone. It is possible that *peb4A* may encode a protein which acts in a similar manner to maintain the correct folding of secreted proteins in *C. jejuni*. This protein here was found to be substantially up-regulated by the environmental cues investigated and correlated with an increase in invasive ability, suggesting, perhaps, that it was involved in the secretion of survival or virulence determinants even though it may not act as a virulence determinant *per se*. However, the precise role of the PEB-4 protein in *Campylobacter* remains unclear.

Several of the proteins, whose expression was modulated by the different environmental conditions, have been shown in other studies to be putative virulence determinants of *C. jejuni*. For example, the presence of the flagellin was present in greatest amounts in cells

under oxidative stress and acid adaptation. As oxidative stress also promoted an increase in the invasive ability of *C. jejuni*, it is possible that the up-regulation of flagellin may have promoted invasion and aided internalisation in accordance with the results of other studies (Wassenaar *et al.*, 1991; Yao *et al.*, 1994). The effects of low pH were less clear as flagellin expression in this instance correlated with a reduced invasive ability. The expression of flagellin however, does not imply the production of a fully functional motile organelle. In a previous study (Szymanski *et al.*, 1995), exposure to low pH (pH 5.0) was reported to lead to a loss of motility in *C. jejuni*. The down-regulation of motility (Szymanski *et al.*, 1995) and invasion in this study may reflect an appropriate response with respect to low pH in acidic environments such as the stomach where the expression of invasion determinants may be inappropriate. The importance of fully functional flagella in invasion was also potentially confirmed by comparison of batch and continuous cultures. Continuous cultures were composed predominantly of non-motile cells and were significantly less invasive than batch grown cells.

Chemotaxis has also been proposed to have a role in *Campylobacter* colonising the intestine, following the observation of lack of colonisation by non-chemotactic mutants in a mouse model (Takata *et al.*, 1992). A protein with homology to a methyl accepting chemotaxis protein was identified in the present study as a novel protein under iron replete conditions, where its presence correlated with an increase in invasive ability. This protein was not however, found to be up-regulated under oxidative stress when *C. jejuni* was highly invasive, nor under low pH, when *C. jejuni* was poorly invasive. Up-regulation under iron-replete conditions, which are likely to occur in the gut, may reflect a role for the receptor, in detecting specific chemoattractants present in the intestine. In extra-intestinal locations, where oxidative stress is morely likely to be encountered, the receptor may no longer have a role and may not need to be expressed. Again, under low pH, such as is found in the

stomach where invasion does not occur, expression may also be down-regulated. This demonstrates the extent to which environmental cues may act to regulate the expression of possible virulence determinants. A similar type of chemotaxis receptor was identified in *Campylobacter* (Gonzalez *et al.*, 1998) and was proposed to be involved in the signal transduction processes of the organism. However, mutational analysis found that the receptor did not contribute directly to virulence, but may have a general role in signal tranduction. Further analysis of the protein identified in this study would be required to determine if it had a role in virulence.

PEB-1 was expressed under all of the environmental conditions, though the level of expression varied between the different conditions. This particular protein had previously been associated with invasive ability, and further work had demonstrated that the product of the encoding *peb1a* locus played an important role in epithelial cell interactions and in colonisation of a mouse model (Pei *et al.*, 1998). The protein was up-regulated under iron-replete conditions, when *Campylobacter* demonstrated its greatest invasive ability. However, under oxidative stress, where the expression of PEB-1 was down-regulated, *C. jejuni* remained highly invasive. It was also down-regulated at low pH, but in this instance *Campylobacter* was poorly invasive. Since exposure to iron limitation and oxidative stress conditions are more likely to occur when *Campylobacter* enters extra-intestinal locations, when the protein is consequently likely to be down-regulated in expression, suggesting that PEB-1 may not contribute to more deep seated disease. Instead it would suggest that PEB-1, in a similar manner to that proposed for the methyl-accepting chemotaxis protein, may play a more important role in the initial invasion process.

Proteases have a wide variety of functions including substrate and host cell degradation, and the removal of abnormal and mis-folded proteins and cleavage of signal peptides in the

pathogen (Gottesman, 1999). A protease was identified here, which was unique under iron replete conditions and oxidative stress, and correlated with an increase in invasive ability. Proteases have been identified previously in *C. jejuni*, for example the Clp protease (Griffiths *et al.*, 1995), which was thought to function in the turnover of aberantly folded proteins. However, the true role of the protease identified here remains to be established.

Several bacterial defence mechanisms were identified under the different environmental cues, which guard against oxygen toxicity. Catalase acts to catalyse the dismutation of H_2O_2 and was detected under iron limitation but was not found under any of the other environmental conditions. Although, this protein appeared to be iron-regulated in this study it has been shown not to be under the immediate control of *fur* (van Vliet *et al.*, 1998) but under the control of PerR (van Vliet *et al.*, 1999). The up-regulation of catalase under iron limitation does suggest a role in iron-limited extra-intestinal locations where it may act to minimise the effects, for example, of radical oxygen molecules from oxidative bursts of PMNs.

Alkyl hydroperoxide reductase (AhpC) was expressed under all of the environmental cues. Interestingly, its expression was substantially up-regulated under iron limitation, but did not alter significantly in its expression whilst grown under oxidative stress or low pH. Similar to catalase, AhpC was not observed to be *fur* regulated in *C. jejuni* (van Vliet *et al.*, 1998) but under the control of PerR (van Vliet *et al.*, 1999). The up-regulation of AhpC by *C. jejuni* under iron limitation reported here may reflect a greater need to remove metabolic by-products of oxygen preventing the formation of more damaging endogenous oxygen radicals following their interaction with the scavenged iron molecules. It is possible that when grown under iron replete and low pH conditions the risk from reactive oxygen radicals is reduced. The lack of response of AhpC to oxidative stress was surprising, though

this may reflect an intracellular location in *C. jejuni*. In *E. coli*, AhpC was up-regulated under iron limited and found to be located in the cell matrix (Cha *et al.*, 1996). In this location it was involved in the removal of endogenous oxygen radicals, rather than those which were extracellular, which were created in this study.

A bacterioferritin, an iron storage molecule, was also identified in *C. jejuni* in this study. This was solely identified under iron-limitation where it was uniquely expressed. A similar molecule was identified previously in *C. jejuni* (Wai *et al.*, 1995) and further work demonstrated its role as an iron storage molecule (Wai *et al.*, 1996). The ferritin molecule was thought to sequester free iron away from oxygen present in the cell, reducing the risk of oxygen toxicity created through the Fenton's reaction.

Another member of the antioxidant enzyme family, thiol peroxide (Tpx), was identified here and at the molecular level in *C. jejuni* 11168. Tpx was expressed under iron replete conditions and oxidative stress as an up-regulated doublet protein correlating with an increase in virulence. It was detected in a singlet form under iron limitation, where *C. jejuni* retains some invasive ability, and its expression was not further altered under low pH, when *C. jejuni* was poorly invasive. The presence of a doublet, separated by only a small shift in pI was unexpected. Specific interallelic exchange resulted in an inactivated *tpx* gene within *C. jejuni*. Analysis of the proteome revealed that both proteins had disappeared suggesting it was encoded by a single gene. In addition there was no evidence of a second *tpx* gene in the genome sequence. The doublet arrangement suggests therefore, post-translational modification was occurring either through glycosylation or phosphorylation of the protein.

The *tpx* gene in *C. jejuni* appears to be involved in the reduction of H_2O_2 molecules but not superoxide anions. In *E. coli* Tpx has similarly been shown to reduce H_2O_2 molecules and

was located in the periplasm. The location of Tpx in *C. jejuni* has yet to be clarified, but it may be located in a similar location and function to remove exogenous oxygen radicals preventing damage to the cell membrane, as well as preventing any reactions with iron via the Fenton reaction which would cause further damage.

In addition to the loss of the two Tpx proteins a number of other proteins were lost from the proteome following the disruption of the *tpx* gene, suggesting that several genes were under control of the same promoter that controlled the *tpx* gene. However, no regulon of genes responsive to H_2O_2, similar to that of *oxyR* of *S. typhimurium* and *E. coli*, was detected in the genome of *C. jejuni* (van Vliet *et al.*, 1998). Mutation of *tpx* was not found to attenuate the invasive ability of *C. jejuni* suggesting that it did not have a role in internalisation, or in intracellular survival in enterocytes. Although, the ability of Tpx to provide protection from oxidative bursts in extra-intestinal locations remains unknown, it may carry out this role due to the presence of the conserved cystein residue at position 94.

A periplasmic protein of 19 kDa was up-regulated under iron replete, oxidative stress and low pH conditions. van Vliet *et al.*, (1998) identified this protein to be iron regulated, and further work has demonstrated that the protein is not immunogenic (Janvier *et al.*, 1998). However, the role and identity of this protein remains unclear.

The protein with homology to the high temperature requirement B (HtrB) protein of *E. coli* was detected under iron replete, oxidative stress and low pH, which correlated in two instances with an increased invasive ability. However, initial attempts at homologous recombination resulted in the inactivated gene becoming integrated into the genome by a Campbell-like mechanism (Dickinson *et al.*, 1995) whereby two copies of the DNA were present including the functional wild-type gene. Further attempts at homologous

recombination of the inactivated gene into the genome failed to form viable colones at 37°C, suggesting it may be essential for growth at high temperatures. In *E. coli*, inactivation of the *htrB* gene was found to allow growth only at 30°C. However, further work demonstrated a role in cell wall maintenance in *E. coli*, which may also account for the lack of viable colonies which were recovered from *C. jejuni*

This study has demonstrated that the genotypic make-up and the environment that *Campylobacter* finds itself in contributes to its ability to persist and cause infection. Invasion of the Caco-2 cell line was shown to vary between isolates and occurred through three possible mechanisms; invasion, invasion with translocation and translocation without invasion. Repeated exposure to the enterocyte cell monolayer enhance the invasive ability of the isolates and was correlated to changes in protein expression, possibly through constitutive expression of adhesion molecules, protein secretion systems or virulence determinants.

The use of continuous culture allowed controlled growth of *C. jejuni* in a number of defined environmental conditions. Exposure to these different environmental cues was found to affect protein expression and the pathogen's ability to invade the Caco-2 cell line. Indeed, exposure to iron replete conditions was found to substantially enhance invasive ability when compare to the response induced when grown in the remaining environmental cues of oxidative stress, iron limitation and low pH.

Protein expression was responsive to environmental cues, being able to adapt probably via signal transduction mechanisms. Alternatively, expression may be altered by a more direct interaction using, for example, the Fur protein which has been identified in *C. jejuni* (Chan

et al., 1995; Wooldridge *et al.*, 1994). Other regulators may exist in *C. jejuni* which respond to changing levels of oxygen or hydrogen ions.

The expression of some proteins was correlated with an increased invasive ability and a proportion were identified by Edman degradation. Three of these proteins were identified further at a molecular level and gene-knock-out studies demonstrated that the proteins PEB-4 and HtrB were essential for viability. Tpx was identified to be important during exposure to H_2O_2, though it did not appear to act as a virulence determinant. This protein may act as an adhesin, functioning in the initial stages of invasion or at extra-intestinal sites. Interestingly analysis of protein expression of the *tpx* deficient mutant revealed the expression of other proteins suggesting that *tpx* or other members of the operon were able to regulated protein expression.

In summary, *Campylobacter*, though a fastidious organism, was able to survive in extreme environments and retain the potential to invade through changes in protein expression. The range of disease symptoms presented in clinical cases may not only be influenced by strain and host variation, but also on the environment that the isolate has been exposed to before and during invasion and the mechanism of penetrating the epithelium.

To futher expand how different invasion phenotypes in *Campylobacter* can translocate across and invade into epithelial cell monolayers, fluorescent stains specific to the cytoskeleton, for example F-actin, Zonala Occludin proteins located in tight junctions and protein receptors could be used. In addition inhibitors of eukaryotic cell signalling and re-organisation, such as cytochalsin B could be used to determine the role of the eukaryotic cell in deciding the route taken through the epithelium.

Many of the proteins identified by Edman degradation were present in doublet forms, separated by only a small shift in pI and possessed the same amino acid sequences, for example PEB-4, Tpx and catalase. To identify the post-transloational modification on these proteins, mass spectrometric techniques would be used to determine if the proteins were glycosylated, contained siliac acid or phosphate groups.

The N-terminal sequences of further proteins whose expression correlated with an increase in virulence could be determined. The selected proteins could be from cultures that were oxidatively stress or grown under iron replete conditions. Alternatively, the novel proteins expressed by *C. jejuni* CH1P and *C. jejuni* 799P, whose invasive ability was enhanced could be identified. These proteins may represent novel survival and virulence determinants.

The response of *C. jejuni* to low pH conditions revealed a complex story. To determine the role of the bacterial membrane in protecting *C. jejuni* from low pH the phospholipid composition of the membrane could be analysed by Fast-atom bombardment mass spectrometry. In addition the exposure of a *C. jejuni fur* mutant to low pH would identify if *fur* played a role in the response.

To determine their role in pathogenesis gene knock-out studies could be performed on selected genes. Previously identified N-terminal sequences from the iron-replete gels could be identified further. Of particular interest would be the proteins with homology to the methyl accepting protein that may have a role in chemotaxis and the protease that may have a role in protein-turnover or in the cleavage of leader sequences.

The function of the genes' *peb4a* and *htr*B in *Campylobacter* was not identified from the gene knock-out studies carried out in this study. To determine the role of these proteins, the gene could be over- or under-expressed. This can be achieved through cloning the PCR product into, for example pBAD TOPO (Invitrogen, Netherlands) and *E. coli* is transformed with the resulting plasmid. The level of expression can be controlled through the *araBAD* promoter in the presence of varying concentration of arabinose, with protein expression monitored by visualisation on SDS PAGE.

Additionally, through the use of lux technology, the luxAB promoter could be inserted into the identified novel and up-regulated genes to determine the conditions which induce expression. This may provide an indication as to where the gene is expressed during infection of hosts.

The effect of the *tpx* mutation on *C. jejuni* could be assessed further. Some of the proteins characterized as up-regulated or novel could be identified by N-terminal sequencing. Site specific substitution on the cystein residue at position 94 could be carried out to determin if this residue is responsible for the peroxidase activity. The role of *tpx* as a possible adhesin could be investigated using Caco-2 cells and macrophage cell lines.

Acar, J. F. & Goldstein, F. W. (1997). Trends in bacterial resistance to fluoroquinolones. *Clin Infect Dis* **24** (Suppl 1), S67-73.

Adak, G. K., Cowden, J. M., Nicholas, S. & Evan, H. S. (1995). The Public Health Laboratory Service national case-control study of primary indigenous sporadic cases of campylobacter infections. *Epidemiol Infect* **115**, 15-22.

Adamski, F. M., Atkins, J. F. & Gesteland, R. F. (1996). Ribosomal protein L9 interactions with 23S rRNA: the use of a translational bypass assay to study the effect of amino acid substitutes. *J Mol Biol* **261**, 357-371.

Alefounder, P. R. & Perham, R. N. (1989). Identification, molecular cloning and sequence analysis of a gene cluster encoding the Class II fructose 1, 6-biphosphate aldolase, 3-phosphoglycerate kinase and a putative second glyceraldehyde 3-phosphaate dehydrogenase of *Escherichia coli*. *Mol Microbiol* **3**, 723-732.

Alex, L. A. & Simon, M. I. (1994). Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. *Trend Genet* **10**, 133-138.

Alon, U., Surette, M. G., Barkai, N. & Leibler, S. (1999). Robustness in bacterial chemostxis. *Nature* **397**, 168-171.

Allos, B. M. (1997). Association between *Campylobacter* infection and Guillain-Barré syndrome. *J Infect Dis* **176** (Suppl 2), S125-128.

Allos, B. M. & Blaser M. J. (1995). *Campylobacter jejuni* and the expanding spectrum of related infections. *Clin Infect Dis* **20**, 1092-1101.

Alm, R. A., Guerry, P. & Trust, T. J. (1993). The *Campylobacter* σ^{54} *flaB* flagellin promoter is subject to environmental regulation. *J Bacteriol* **175**, 4448-4455.

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, is responsible for mucin adhesion. *Infect Immun* **66**, 100-1007.

Bagg, A. & Neilands, J.B. (1987). Ferric uptake regulation protein acts as a repressor, employing iron (III) as a co-factor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry* **26**, 5471-5477.

Baig, B. H., Wachsmuth, I. K. & Morris, G. K. (1986). Utilization of exogenous siderophores by *Campylobacter* species. *J Clin Microbiol* **23**, 431-433.

Baja, V., Lucas, R. L., Hwang, S. & Lee, C. A. (1996). Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental regulatory factors is mediated by control of *hilA* expression. *Mol Microbiol* **22**, 703-714.

Bearaowski, E., Jose, P., Davies, H., Stephenson, M. & Webster, D. (1989). Interaction of *Campylobacter* species with antibody, complement and phagocytes. *Gut* **30**, 906-911.

Bearson, S., Bearson, B. & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**, 173-180.

Bearson, S., Benjamin, W. H. Jr., Swords, W. E. & Foster, J. W. (1996). Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. *J Bacteriol* **178**, 2572-2579.

Bell, J. A. & Manning, D. D. (1990). A domestic ferret model of immunity to *Campylobacter jejuni*-induced enteric disease. *Infect Immun* **58**, 1848-1852.

Belli, W. A. & Marquis, R. E. (1991). Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. *Appl Environ Microbiol* **57**, 1134-1138.

Benaissa, M., Babin, P., Quellard, N., Pezennec, L., Cenatiempo, Y. & Fauchère, J. L. (1996). Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect Immun* **64**, 2331-2335.

Benjamin, M. M. & Datta, A. R. (1995). Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **61**, 1669-1672.

Beumer, R. R., de Vries, J. & Rombouts, F. M. (1992). *Campylobacter jejuni* non-culturable coccoid cells. *Int J Food Microbiol* **15**, 153-163.

Beyer, W., Imlay, J. & Fridovich, I. (1991). Superoxide dismutases. *Prog Nucleic Acid Res Mol Biol* **40**, 221-253.

Black, R. E., Levine, M. M., Clements, M. L., Hughes, T. P. & Blaser, M. J. (1988). Experimental *Campylobacter jejuni* infections in humans. *J Infect Dis* **157**, 472-479.

Boucher, S. N., Slater, E. R., Chamberlain, A. H. L. & Adams, M. R. (1994). Production and viability of coccoid forms of *Campylobacter jejuni*. *J Appl Bacteriol* **77**, 303-307.

Brás, A. M., Chatterjee, S., Wren, B. W., Newell, D. G. & Ketley, J. M. (1999). A novel *Campylobacter jejuni* two-component regulatory system inportant for temperature-dependent growth and colonisation. *J Bacteriol* **181**, 3298-3302.

Brown, J. L., Ross, T., McMeekin, T. A. & Nichols, P. D. (1997). Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. *Int J Food Microbiol* **37**, 163-173.

Braun, V. (1995). Energy-coupled transport and signal transduction through the Gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol Rev* **16**, 295-307.

Bukau, B. & Walker, G. G. (1989). Cellular defects caused by deletion of the *Escherichia coli dnaK* gene indicated roles for heat shock protein in normal metabolism. *J Bacteriol* **171**, 2337-2346

Burucoa, C., Fremaux, C., Pei, Z., Tummuru, M., Blaser, M. J., Cenatiempo, Y. & Fauchère, J. L. (1995). Nucleotide sequence and characterization of *peb4A* encoding an antigenic protein in *Campylobacter jejuni*. *Res Microbiol* **146**, 467-476.

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T. M., Marsh, P. D., Keevil, C. W. & Leach, S. A. (1998). Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. *Appl Environ Microbiol* **64**, 733-741.

Caldwell, M. B., Walker, R. I., Stewart, S. D. & Rogers, J. E. (1983). Simple adult rabbit model for *Campylobacter jejuni* enteritis. *Infect Immun* **42**, 1176-1182.

Camili, A., Beattie, D. T. & Mekalanos, J. J. (1994). Use of genetic recombination as a reporter of gene expression. *Proc Natl Acad Sci USA* **91**, 2634-2638.

Canil, C., Rosenshine, I., Ruchkowski, S., Donnenberg, S., Kaper, J. B. & Finlay, B. B. (1993). Enteropathogenic *Escherichia coli* decreases the transepithelial electrical resistance of polarised epithelial monolayers. *Infect Immun* **61**, 2755-2762.

Cawthraw, S. A., Wassenaar, T. M., Ayling, R. & Newell, D. G. (1996). Increased colonisation potential of *Campylobacter jejuni* strain 81116 after passage through chickens and its implication on the rate of transmission within flocks. *Epidemiol Infect* **117**, 213-215.

Chambers, S. P., Prior, S. E., Barstow, D. A. & Minton, N. P. (1988). The pMTL *nic*⁻ cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**, 139-149.

Cha, M-K., Kim, H-K. & Kim I-H. (1995). Thioredoxin-linked "Thiol peroxidase" from the periplasmic space of *Escherichia coli*. *J Biol Chem* **48**, 28635-28641.

Cha, M-K., Kim, H-K. & Kim I-H. (1996). Mutation and mutagenesis of thiol peroxidase of *Escherichia coli* and a new type of thiol peroxidase family. *J Bacteriol* **178**, 5610-5614.

Chan, V. L., Louie, H. & Bigham, H. L. (1995). Cloning and transcription regulation of the ferric uptake regulatory gene of *Campylobacter jejuni*. *Infect Immun* **50**, 941-943.

Chang, Y-Y. & Cronon, J. E. (1999). Membrane cyclopropanol fatty acid content is a major factor in aicd resitance in *Escherichia coli*. *Mol Microbiol* **33**, 249-259.

Christman, M. F., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). Positive control of a regulon for defences against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**, 753-762.

Connerton, P. L. & Connerton, I. F. (1999). Identification of a gene encoding an immuno-reactive membrane protein from *Campylobacter jejuni*. *Lett Appl Microbiol* **28**, 233-237.

Conte, M. P., Longhi, C., Polidoro, M., Petone, G., Buonfiglio, V., Di Santo, S., Papi, E., Seganti, L., Visca, P. & Valenti, P. (1996). Iron availability affects entry of *Listeria monocytogenes* into the enterocyte cell line Caco-2. *Infect Immun* **64**, 3925-3929.

Crosa, J. H. (1997). Signal transduction and transcriptional and post-transriptional control of iron-regulated genes in bacteria. *Microbiol Mol Biol Rev* **61**, 319-336.

Czuprynski, C. J. & Balish, E. (1981). Pathogenesis of *Listeria monocytogenes* for gnotobiotic rats. *Infect Immun* **32**, 323-331.

Davies, M. J., Coote, P. J. & O'Byrne, P. (1996). Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth phase-dependent acid resistance. *Microbiology* **142**, 2975-2982.

DeMelo, M. A., Gabbiani, G. & Pechère, J-C. (1989). Cellular events and intracellular survival of *Campylobacter jejuni* during infection of Hep-2 cells. *Infect Immun* **58**, 1749-1756.

Demple, B. (1991). Regulation of bacterial oxidative stress genes. *Ann Rev Genet* **25**, 315-337.

Demple, B. (1996). Redox signalling and gene control in the *Escherichia coli soxRS* oxidative stress regulon - a review. *Gene* **179**, 53-57.

Deptartment of Health (1997). Chapter 7, Food Safety pp 218-225. In, *On the state of the Public Health 1997.* The Stationary Office, London.

Dickinson, J. H., Grant, K. A. & Park, S. F. (1995). Targeted and random mutagenesis of the *Campylobacter coli* chromosome with integrational plasmid vectors. *Curr Microbiol* **31**, 92-96.

DiRita, V. J. & Mekalanos, J. J. (1989). Genetic regulation of bacterial virulence. *Ann Rev Genet* **23**, 455-482.

DiRita, V. J. (1992). Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. *Mol Microbiol* **6**, 451-458.

Doig, P., Kinsella, N., Guerry, P. & Trust, T. J. (1996a). Characterisation of a post-translational modification of *Campylobacter* flagellin - identification of a sero-specific glycosyl moiety. *Mol Microbiol* **19**, 379-387.

Doig, P., Yao, R. J., Burr, D. H., Guerry, P. & Trust, T. J. (1996b). An environmentally regulated pilus-like appendage involved in *Campylobacter* pathogenesis. *Mol Microbiol* **20**, 885-894.

Dorman, C. J. (1991). DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. *Infect Immun* **59**, 745-749.

Doyle, A. & Griffiths, J. B. (1998). Chapter 2 pp57-61. *In Cell and tissue culture: Laboratory procedures in Biotechnology*. John Wiley & Sons, Ltd.

Emery, M. J., Henderson, J. & Ketley, J. M. (1997). Identification of a 2-component regulatory system down-stream of the *C. jejuni htrA* gene. In, *Campylobacter, Helicobacter and related organsims; Proceedings of the 9th International Workshop held in Cape Town, South Africa.* Edited by A. J. Lastovica, D. G. Newell & E. E. Lastovica. New York, Plenium Press.

Engle, M. J., Goetz, G. S. & Apers, D. H. (1998). Caco-2 cells express a combination of coloncyte and enterocyte phenotypes. *J Cell Physiol* **174**, 362-369.

Ernst, R. K., Dombroski, D. M. & Merrick, J. M. (1990). Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. *Infect Immun* **58**, 2014-2016.

Evans, D. J., Evans, D. G., Lampert, H. C. & Nakano, H. (1995a). Identification of four new prokaryotic bacterioferritins, from *Helicobacter pylori, Anabaena variabilis, Bacillus subtilis* and *Treponema pallidum*, by analysis of gene sequences. *Gene* **153**, 123-127.

Evans, D. J., Evans, D. G., Takemura, T., Nakano, H., Lampert, H. C., Graham, D. Y., Granger, D. N. & Kvietys, P. R. (1995). Characterisation of a *Helicobacter pylori* neutrophil-activating protein. *Infect Immun* **63**, 2213-2220.

Everest, P. H., Goossens, H., Butzler, J. P., Lloyd, D., Knutton, S., Ketley, J. M. & Williams, P. H. (1992). Differentiated Caco-2 cells as a model for enteric invasion by *Campylobacter jejuni* and *Campylobacter coli*. *J Med Microbiol* **37**, 319-325.

Everest, P. H., Goossens, H., Sibbon, P., Lloyd, D., Knutton, S., Leece, R., Ketley, J. M. & Williams, P. H. (1993). Pathological changes in the rabbit ileal loop model caused by *Campylobacter jejuni* from human colitis. *J Med Microbiol* **38**, 316-321.

Farr, S. B. & Kogoma, T. (1991). Oxidative stress response in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Rev* **55**, 561-585.

Fauchère, J-L, Rosenau, A., Veron, M., Moyen, E. N., Richard, S. & Pfister, A. (1986). Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human faeces. *Infect Immun* **54**, 283-287.

Ferrero, R. L. & Lee, A. (1988). Motility of *Camplobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. *J Gen Microbiol* **134**, 53-59.

Field, L. H., Headley, V. L., Payne, S. M. & Berry, L. J. (1986). Influence of iron in growth, morphology, outer membrane protein composition, and synthesis of siderophores in *Campylobacter jejuni*. *Infect Immun* **54**, 126-132.

Field, L. H., Underwood, J. L., Payne, S. M. & Berry, L. J. (1993). Characteristics of an avirulent *Campylobacter jejuni* strain and its virulence enhanced variants. *J Med Microbiol* **38**, 293-300.

Finlay, B. B. & Falkow, S. (1990). *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. *J Infect Dis* **162**, 1096-1106.

Finlay, B. B. & Falkow, S. (1997). Common themes in microbial pathogenicity revisited. *Microbiol Mol Rev* **61**, 136-169.

Firehammer, B. D. & Myers, L. L. (1981). *Campylobacter fetus* subsp. *jejuni*: its possible significance in enteric disease of calves and lambs. *Am J Vet Res* **42**, 918-922.

Fitzgeorge, R. B., Baskerville, A. & Lander, K. P. (1981). Experimental infection of Rhesus monkeys with a human strain of *Campylobacter jejuni*. *J Hyg* **86**, 343-351.

Flahaut, S., Laplace, J.-M., Frère, J. & Auffray, Y. (1998). The oxidative stress response in *Enterococcus faecalis*: relationship between H_2O_2 tolerance and H_2O_2 stress proteins. *Lett Appl Microbiol* **26**, 259-264.

Foster, J. W. (1991). *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. *J Bacteriol* **173**, 6896-6902.

Foster, J. W. (1993). The acid tolerance reponse of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. *J Bacteriol* **175**, 1981-1987.

Foster, J. W. (1999). When protons attack: microbial strategies of acid adaption. *Curr Opin Microbiol* **2**, 170-174.

Foster, J. W. & Hall, H. K. (1990). Adaptive acidification tolerance response of *Salmonella typhimurium*. *J Bacteriol* **172**, 771-778.

Foster, J. W. & Hall, H. K. (1991). Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. *J Bacteriol* **173**, 5129-5135.

Foster, J. W. & Hall, H. K. (1992). Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. *J Bacteriol* **174**, 4317-4323.

Gardel, C. L. & Mekalanos, J. J. (1994). Regulation of cholera toxin by temperature, pH and osmolarity. *Methods Enzymol* **235**, 517-526.

Geilhausen, B., Schutt-Gerowitt, H., Aleksic. S., Koenen, R., Mauff, G. & Pulverer, G. (1996). *Campylobacter* and *Salmonella* contaminating fresh chicken meat. *Zentrabl Bakteriol* **284**, 241-245.

Gilbert, P. (1985). The theory and relevance of continuous culture. *J Antimicrob Chemotherapy* **15**, Suppl A, 1-6.

Gomez-Garces, J. L., Cogollos, A. & Alos, J. L. (1995). Susceptibilities of fluoroquinolone-resistant strains of *Campylobacter jejuni* to 11 oral antimicrobial agents. *Antimicrob Agents Chemother* **39**, 542-544.

Gonzalez, I., Richardson, P. T., Collins, M. D. & Park, S. F. (1998). Identification of a gene encoding a methyl-accepting chemotaxis-like protein from *Campylobacter coli* and its use in a molecular typing scheme for campylobacters. *J Appl Microbiol* **85**, 317-326.

Goodson, M. & Rowbury, R. J. (1989). Habituation to normally lethal acidity by prior growth at a sublethal acid pH value. *Lett Appl Microbiol* **8**, 77-79.

Goosen, H., van den Abbele, R., Butlzler, J. P. & Williams, P. (1989). Study of iron uptake on *Campylobacter fetus* and *Campylobacter jejuni*. In *Campylobacter V, Proc of Fifth Internaltional Workshop on Campylobacter Infections*. Edited by G. M. Ruiz-Palcios, Dept Infectious Disease, Insttituto Nacional de la Nutricion, Mexico.

Gorden, J. & Small, P. L. C. (1993). Acid resistance in enteric bacteria. *Infect Immun* **61**, 364-367.

Gort, A. S. & Imlay, J. A. (1998). Balance between endogenous superoxide stress and antioxidant defences. *J Bacteriol* **180**, 1402-1410.

Gottesman, S. (1999). Regulation by proteolysis: developmental switches. *Curr Opin Microbiol* **2**, 142-147.

Grant, C. C. R., Konkel, M. E., Cieplak, W., Jr & Tompkins, L. S. (1993). Role of flagella in adherence, internalisation, and translocation of *Campylobacter jejuni* in nonpolarised and polarised epithelial cell cultures. *Infect Immun* **61**, 1764-1771.

Grant, K. A. & Park, S. F. (1995). Molecular characterisation of *katA* from *Campylobacter jejuni* and generation of a catalse-deficient mutant of *Campylobacter coli* by interspecific allelic exchange. *Microbiology* **141**, 1369-1376.

Greenberg, J. T. & Demple, B. (1989). A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J Bacteriol* **171**, 3933-3939.

Greenberg, J. T., Monach, P., Chou, J. H., Josephy, P. D. & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci USA* **87**, 6181-6185.

Griffiths, E. (1989). The iron uptake systems of pathogenic bacteria, pp 69-137. In, *Iron & Infection: Molecular, Physiological and Clinical aspects*. Edited by J. J. Bullen, E. Griffiths. John Wiley & Sons, New York.

Griffiths, P. L. (1993). Morphological changes of *Campylobacter jejuni* growing in liquid culture. *Lett Appl Microbiol* **17**, 152-155.

Griffiths, P. L. & Park, R. W. A. (1990). Campylobacters associated with human diarrhoel disease. *J Appl Bacteriol* **69**, 281-301.

Griffiths, P. L., Park, R. W. A. & Connerton, I. F. (1995). The gene for *Campylobacter* trigger factor: evidence for multiple transcription start sites and protein products. *Microbiol* **141**, 1359-1367.

Guerry, P., Doig, P., Alm, R. A., Burr, D. H., Kinsella, N. & Trust, T. J. (1996). Identification and characterisation of genes required for post-translational modification of *Campylobacter coli* VC167 flagellin. *Mol Microbiol* **19**, 369-378.

Guerry, P., Perez-Casal, J., Yao, R., McVeigh, A. & Trust, T. J. (1997). A genetic locus involved in iron utilisation unique to some *Campylobacter* strains. *J Bacteriol* **179**, 3997-4002.

Guest, J. R., Miles, J. S., Roberts, R. E. & Woods, S. A. (1985). The fumerase genes of *Escherichia coli*: location of the *fumB* gene and discovery of a new gene (*fumC*). *J Gen Microbiol* **131**, 2971-2984.

Guthrie, B. & Wickner, W. (1990). Trigger factor depletion or overproduction causes defective cell division but does not block protein export. *J Bacteriol* **172**, 5555-5562.

Harris, L. A., Logan, S. M., Guerry, P. & Trust, T. J. (1987). Antigenic variation of *Campylobacter* flagella. *J Bacteriol* **169**, 5056-5071.

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

Harvey, P., Battle, T. & Leach, A. (1999). Different invasion phenotypes of *Campylobacter* isolates in Caco-2 cell monolayers. *J Med Microbiol* **48**, 461-470.

Hassett, D. J., Howell, M. L., Ochsner, U. A., Vasil, M. L., Johnson, Z. & Dean, G. E. (1997). An operon containing *fumC* and *sodA* encoding fumerase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa*: *fur* mutants produce elevated alginate levels. *J Bacteriol* **179**, 1452-1459.

Hazeleger, W. C., Janse, J. D., Koenraad, F. J., Beumer, R. R., Rombouts, F. M. & Abee, T. (1995). Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*. *Appl Environ Microbiol* **61**, 2713-2719.

Hickory, T. E., Bagor, S., Bougeois, A. L., Ewing, C. P. & Guerry, P. (1999). *Campylobacter jejuni* stimulated secretion of interleukin-8 by INT407 cells. *Infect Immun* **67**, 88-93.

Hidaka, J., Goda, M., Kuzuyama. T., Takei, N., Nidaka, M. & Seto, H. (1995). Cloning and nucleotide sequences of fosfomycin biosynthetic genes of *Streptomyces wedmorensis*. *Mol Gen Genet* **249**, 274-280.

Hidalgo, I. J., Raub, T. J. & Borchardt, R. T. (1989). Characterisation of the human colonic carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterol* **96**, 736-749.

Hodgson, A. E., McBride, B. W., Hudson, M. J., Hall, G. & Leach, S. A. (1998). Experimental *Campylobacter* infection and diarrhoea in immunodeficient mice. *J Med Microbiol* **47**, 799-809.

Honda, J., Okubo, Y., Kusaba, M., Kumagai, M., Saruwatari, N. & Oimzumi, K. (1998). Fosfomycin suppress the production of IL-8 from monocytes via the suppression of neutrophil function. *Immunopharmacology* **39**, 149-155.

Horwitz, M. A. (1987). Characterisation of avirulent mutant *Legionella pneumophilia* that survive but do not multiply within human monocytes. *J Exp Med* **166**, 1310-1328.

Hossain, A., Stwart-Tull, D. E. S. & Freer, J. H. (1993). Heat-labile heat stable haemolysis of *C. jejuni*. *FEMS Immunol Med Microbiol* **6**, 335 -340.

Hu, W. N., Band, R. N. & Kopachik, W. J. (1991). Virulence related protein synthesis in *Naegleria fowleri*. *Infect Immun* **59**, 4278-4282.

Hugdahl, M. B., Beery, J. T & Doyle, M. P. (1988). Chemotactic behaviour of *Campylobacter jejuni*. *Infect Immun* **56**, 1560-1566.

Hughes, K. J., Everiss, K. S., Harkey, C. W. & Peterson, K. M. (1994). Identification of *Vibrio cholera* ToxR-activated gene (*tagD*) that is physically linked to the toxin-coregulated pilus (*tcp*) gene cluster. *Gene* **148**, 97-100.

Hughes, R. A. C. & Rees, J. H. (1997). Clinical and epidemiologic features of Guillain-Barré Syndrome. *J Infect Dis* **176** (Suppl 2), S92-98.

Imada, K., Vonderviszt, F., Furukaun, Y., Oosawa, K. & Namba, K. (1998). Assembly characteristics of flagellar cap protein HAP2 of *Salmonella*: decamer and pentamer in the pH sensitive equilibrium. *J Mol Biol* **277**, 883-888.

Imlay, J. A. & Fridovich, I. (1992). Exogenous quinones directly inhibit the respiratory NADH dehydrogenase in *Escherichia coli*. *Arch Biochem Biophys* **296**, 337-346.

Jacobs, M., Anderson, J. B., Kontinen, V. & Sarvas, M. (1993). *Bacillus subtilis* PrsA is required *in vivo* as an extracytoplsmic chaperone for secretion of active enzymes synthesised either with or without pro-sequences. *Mol Microbiol* **8**, 957-966.

James, B. W., Machline, W. S., Fitzgeorge, R. B., Dennis, P. J. & Keevil, C. W. (1995). Influences of iron-limited continuous culture on physiology and virulence of *Legionella pneumophilia*. *Infect Immun* **63**, 4224-4230.

Janvier, B., Constantinidou, C., Aucher, P., Marshall, Z. V., Penn, C. W. & Fauchere, J. L. (1998). Characterisation and gene sequencing of a 19 kDa periplasmic protein of *C. jejuni* and *C. coli*. *Res Microbiol* **149**, 95-107.

Jayaraman, G. C., Penders, J. E. & Burne, R. A. (1997). Transcriptional analysis of the *Streptococcus mutans hrcA, grpE and dnaK* genes and regulation of expression in response to heat shock and environmental acidification. *Mol Microbiol* **25**, 329-341.

Johnson, W. M. & Lior, H. (1988). A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microbiol Path* **4**, 115-126.

Jones, B. D. & Falkow, S. (1994). Identification and characterisation of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalisation. *Infect Immun* **62**, 3745-3752.

Jones, D., M, Sutcliffe, E. M., Rios, R., Fox, A. J. & Curry, A. (1993). *Campylobacter jejuni* adapts to aerobic metabolism in the environment. *J Med Microbiol* **38**, 145-150.

Jones, D. M, Sutcliffe, E. M. & Curry, A. (1991). Recovery of viable but non-culturable, *Campylobacter jejuni*. *J Gen Microbiol* **137**, 2477-2482.

Jordan, K. N., Oxford, L. & O'Byrne, C. D. (1999). Survival at low pH stress by *Escherichia coli* O157:H7: Correlation between alterations in the cell envelope and increased acid tolerance. *Appl Environ Microbiol* 65, 3048-3055.

Karita, M. & Blaser, M. J. (1998). Acid tolerance response in *Helicobacter pylori* and difference between *cagA*$^{+}$ and *cagA*$^{-}$ strains. *J Inf Dis* **178**, 213-219.

Karlyshev, A. V., Henderson, J., Ketley, J. M. & Wren, B. W. (1999). Procedure for the investigation of Bacterial Genomes: Random Shot-Gun Cloning, Sample Seqencing and Mutagenesis of *Campylobacter jejuni*. *BioTechniques* **26**, 50-56.

Karow, M. & Georgopoulos, C. (1991). Sequencing, mutational analysis and transcriptional regulation of the *Escherichia coli hrtB* gene. *Mol Microbiol* **5**, 2285-2292.

Karow, M., Fayet, O., Cegielska, A., Ziegelhoffer, T. & Georgopoulous, C. (1991). Isolation and characterisation of the *Escherichia coli htrB* gene, whose product is essential for bacteria viability above 33°C in rich media. *J Bacteriol* **173**, 741-750.

Kernéis, S., Bogdonova, A., Kraehenbuhl, J. P. & Prinault, E. (1997). Conversion by Peyer's patch lymophocytes of human enterocytes into M cells that transport bacteria. *Science* **277**, 949-952.

Kervella, M., Pagès, J-M., Pei, Z., Grollier, G., Blaser, M. J. & Fauchère, J-L. (1993). Isolation and characterisation of two *Campylobacter* glycine-extracted proteins that bind to HeLa cell membrane. *Infect Immun* **61**, 3440-3448.

Ketley. J. M. (1995). Virulence of *Campylobacter* species: a molecular genetic approach. *J Med Microb* **42**, 312-327.

Ketley, J. M. (1997). Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**, 5-21.

Kiehlbauch, J. A., Albach, R. A., Baum, L. L. & Chang, K-P. (1985). Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. *Infect Immun* **48**, 446-451.

Kim H-K., Kim, S-J., Lee, J-W., Lee, J-W., Cha M-K. & Kim, I-H. (1996). Identification of a promoter in the 5'-flanking region of the *E. coli* thioredoxin-linker thiol peroxidase gene: evidence for the existence of oxygen-regulated transcriptional regulatory protein. *Biochem Biophy Res Comm* **221**, 641-646.

King, E. O. (1957). Human infection with *Vibrio fetus* and a closely related vibrio. *J Infect Dis* **101**, 119-128.

Kinsella, N., Guerry, P., Cooney, J. & Trust, T. J. (1997). The *flgE* gene of *Campylobacter coli* is under the control of the alternative sigma factor σ^{54}. *J Bacteriol* **179**, 4647-4653.

Klebba, P. E., McIntosh, M. A. & Neilands, J. B. (1982). Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli. J Bacteriol* **149**, 880-888.

Klebba, P. E., Rutz, J. M., Liu, J. & Murphy, C. K. (1993). Mechanism of TonB catalyzed iron transport through the enteric bacterial cell envelope. *J Bioenerg Biomembr* **25**, 603-611.

Klose, K.E. & Mekalanos, J. J. (1997). Simultaneous prevention of glutamine synthesis and high-affinity transport attentuates *Salmonella typhimurium* virulence. *Infect Immun* **65**, 587-596.

Konkel, M. E. & Joens, L. A. (1989). Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. *Infect Immun* **57**, 2984-2990.

Konkel, M. E. & Cieplak, W. Jr. (1992). Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalisation. *Infect Immun* **60**, 4945-4949.

Konkel, M. E., Corwin, M. D., Joens, L. A. & Cieplak, W., Jr. (1992). Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells. *J Med Microbiol* **37**, 30-37.

Konkel, M. E., Hayes, S. F., Joens, L. A. & Cieplak, W., Jr. (1992a). Characteristics of the internalisation and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microbiol Pathog* **13**, 357-370.

Konkel. M. E., Mead, D. J., Hayes, S. F. & Cieplak, W., Jr. (1992b). Translocation of *Campylobacter jejuni* across human polarised epithelial monolayer cultures. *J Infect Dis* **166**, 308-315.

Konkel, M. E., Garvis, S. G., Tipton, S. L., Anderson, D. E. Jr. & Cieplak, W. Jr. (1997). Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni. Mol Microbiol* **24**, 953-963.

Konkel, M. E., Kim, B. J., Rivera-Amill, V. & Garvis, S. G. (1999). Bacterial secreted proteins are acquired for the internalisation of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol* **32**, 691-701.

Kontinen, V. P., Saris, P. M. & Sarvas, M. (1991). A gene (*prsA*) of *Bacillus subtilis* involved in a novel, late stage of protein export. *Mol Microbiol* **5**, 1273-1283.

Kontinen, V. P. & Sarvas, M. (1993). The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high level secretion. *Mol Microbiol* **8**, 727-737.

Kusters, J. G., Gerritis, M. M., van Strijp, J. A. G. & Vandenbroucke-Grauls, C. M. J. E. (1997). Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. *Infect Immun* **65**, 3672-3679.

Kutsukake, K. & Ide, N. (1995). Transcriptional analysis of the *flgK* and *fliD* operones of *Salmonella typhimurium* which encode flagellar hook-associated proteins. *Mol Gen Genet* **247**, 2785-281.

Labigne-Roussel, A., Harel, J. & Tompkins, L. (1987). Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. *J Bacteriol* **169**, 5320-5323.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K. & Hartl, F. U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356**, 683-689.

Lawlor, K. M., Daskaleros, P. A., Robinson, R. E. & Payne, S. M. (1987). Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. *Infect Immun* **55**, 594-599.

Leach, S. A. (1997). Growth, survival and pathogenicity of enteric campylobacters. *Rev Med Microbiol* **8**, 113-124.

Leach, S., Harvey, P. & Wait, R. (1997). Changes with growth rate in the membrane lipid composition of and amino acid utilization by continuous cultures of *Campylobacter jejuni*. *J Appl Microbiol* **82**, 631-640.

Leclerc, G. J., Tartera, C. & Metcalf, E. S. (1998). Environmental regulation of *Salmonella typhimurium* invasion-defective mutants. *Infect Immun* **66**, 682-691.

Lee, C. A. & Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci USA* **87**, 4304-4308.

Lee, I. S., Lin, J., Hall, H. K., Bradley, B. & Foster, J. W. (1995). The stationary-phase sigma factor (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. *Mol Microbiol* **17**, 155-167.

Lewthwaite, J., Skinner, A. & Henderson, B. (1998). Are molecular chaperones microbial virulence factors? *Trend Microbiol* **6**, 426-428.

Litwin, C. M., Boyko, A. S. & Calderwood S. B. (1992). Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. *J Bacteriol* **174**, 1897-1907.

Litwin, C. M. & Calderwood, S. B. (1993). Role of iron in regulation of virulence genes. *Clin Microbio Rev* **6**, 137-149.

Litwin, C. M. & Calderwood, S. B. (1994). Analysis of the complexity of gene regulation by *fur* in *Vibrio cholerae*. *J Bacteriol* **176**, 240-248.

Madara, J. L. (1997). The chameleon within: improving antigen delivery [comment]. *Science* **277**, 910-911.

Manca, C., Paul, S., Barry, C. E., Freedman, V. H. & Kaplan, G. (1999). *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes *in vitro*. *Infect Immun* **67**, 74-79.

Manning, G., Cawthraw, S. A., Fearnley, C. & Newell, D. G. (1997). PEB3: A potential role in the colonisation of chickens by *C. jejuni*. In, *Campylobacter, Helicobacter and related organsims; Proceedings of the 9th International Workshop held in Cape Town, South Africa*. Edited by Lastovica, A. J., Newell, D. G. & Lastovica, E. E. Plenium Press.

Marchant, J. E., Henderson, J., Wren, B. W. & Ketley, J. M. (1997). Role of the *cheY* gene in the chemostaxis of *Campylobacter jejuni*. In, *Campylobacter, Helicobacter and related organsims; Proceedings of the 9th International Workshop held in Cape Town, South Africa*. Edited by Lastovica, A. J., Newell, D. G. & Lastovica, E. E. Plenium Press.

Matsudaria, P. (1987). Sequence from picomolar quantities of proteins electroblotted onto polyvinylindene difluoride membranes. *J Biol Chem* **262**, 10035-10038.

Medema, G. J., Schets, F. M., van de Giessenn, A. W. & Havelaar, A. H. (1992). Lack of colonisation of 1 day old chicks by viable, non-culturable *Campylobacter jejuni*. *J Appl Bacteriol* **72**, 512-516.

Mekalanos, J. J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol* **174**, 1-7.

Mendz, G. L., Ball, G. E. & Meek, D. J. (1997). Pyruvate metabolism in *Campylobacter* spp. *Biochim Biophys Acata* **1334**, 291-302.

Michaelis, S., Inouye, H., Oliver, D. & Beckwith, J. (1983). Mutations that alter the signal sequence of alkaline phosphotase in *E. coli*. *J Bacteriol* **154**, 366-374.

Mickelsen, P. A. & Sparling, P. F. (1981). Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. *Infect Immun* **33**, 555-564.

Miller, J. F., Mekalanos, J. J. & Falkow, S. (1989). Co-ordinate regulation and signal transduction in the control of bacterial virulence. *Science* **243**, 916-922.

Miller, J. F., Dower, W. J. & Tompkins, L. S. (1988). High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. *Proc Natl Acad Sci USA* **85**, 856-860.

Miller, S., Pesci, E. C. & Pickett, C. L. (1993). A *Campylobacter jejuni* homolog of the LcrD/FlbF family of proteins is necessary for flagella biogensis. *Infect Immun* **61**, 2930-2936.

Mishu, B. & Blaser, M. J. (1997). Role of infection due to *Campylobacter jejuni* in the initiation of Guillain-Barré syndrome. *Clin Infect Dis* **17**, 104-108.

Moran, A. P. & Upton, M. E. (1986). A comparative study of the rod and coccoid forms of *Campylobacter jejuni* ATCC 29428. *J Appl Bacteriol* **60**, 103-110.

Moran, A. P. & Upton, M. E. (1987a). Effect of medium supplements, illumination and superoxide distmutase on the production of coccoid forms of *Campylobacter jejuni* ATCC 29428. *J Appl Bacteriol* **62**, 43-51.

Moran, A. P. & Upton, M. E. (1987b). Factors affecting production of coccoid forms by *Campylobacter jejuni* on solid media during incubaction. *J Appl Bacteriol* **62**, 527-537.

Moser, I., Schroeder, W. & Salnikow, J. (1997). *Campylobacter jejuni* major outer membrane protein and a 59 kDa protein are involved in binding to fibronectin and INT 407 cell membrane. *FEMS Microbiol Lett* **157**, 233-238.

Mullis, K. & Faloona, F. (1987). Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Meth Enzymol* **55**, 335-350.

Nachamkin, I., Yang, X-H. & Stern, N. J. (1993). Role of *Campylobacter jejuni* flagella as colonisation factors for three-day-old chicks: analysis with flagellar mutants. *Appl Environ Microbiol* **59**, 1269-1273.

Neilands, J. B. (1981). Microbial iron compounds. *Ann Rev Biochem* **50**, 715-731.

Nesmeyanova, M. A., Motlockh, O. B., Kolot, M. N. & Kulaev, I. S. (1981). Multiple forms of alkaline phophatase from *E. coli* with repressed and depressed biosynthesis of the enzyme. *J Bacteriol* **146**, 453-457.

Newell, D. G., McBride, H. & Pearson, A. D. (1984). The identification of outer membrane proteins and flagella of *Campylobacter jejuni*. *J Gen Microbiol* **130**, 1201-1208.

Nickenon, C. A. & Curtis, R. (1997). Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. *Infect Immun* **65**, 1814-1823.

Nierhaus, K. H. (1996). Protein synthesis. An elongation factor turn-on. [News, comment]. *Nature* **379**, 491-492.

O'Driscoll, B., Gahan, C. G. M. & Hill, C. (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid tolerant mutant which demonstrated increased virulence. *Appl Environ Microbiol* **62**, 1693-1698.

Oelschaeger, T. A., Guerry, P. & Kopecko, D. J. (1993). Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci USA* **90**, 6884-6888.

Oliver, J. D., Nilsson, L. & Kjelleberg, S. (1991). Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. *Appl Environ Microbiol* **57**, 2640-2644.

Otto, B. R., Verweij-van Vught, A. M. J. J. & MacLaren, D. M. (1992). Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit Rev Microbiol* **18**, 217-235.

Park, K. R., Giard, J-C., Eom, J. H. Bearson, S. & Foster, J. W. (1998). Cyclic AMP receptor protein and TyrR are required for the acid pH and anaerobic induction of *hgaB* and *aniC* in *Salmonella typhimurium*. *J Bacteriol* **181**, 689-694.

Park, S. F. & Richardson, P. T. (1995). Molecular characterisation of a *Campylobacter jejuni* lipoprotein with homology to periplasmic-siderophore-binding protein. *J Bacteriol* **177**, 2259-2264.

Park, S. J. & Gunsalus, R. P. (1995). Oxygen, iron, carbon and superoxide control of the fumarase *fumA* and *fumC* genes of *Escherichia coli*: role of the *araA*, *fnr*, and *soxR* gene products. *J Bacteriol* **177**, 6255-6262.

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M-A., Rutherford, K. M., van Vliet, A. H. M., Whitehead, S. & Barrell, B. G. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665-668.

Parsons, L. M., Limberger, R. J. & Shayegani, M. (1997). Alterations in levels of DnaK and GroEL result in diminished survival and adherence of stressed *Haemophilius ducreyi*. *Infect Immun* **65**, 2413-2419.

Peabody, R. G., Ryan, M. J. & Wall, P. G. (1997). Outbreaks of *Campylobacter* infection: rare events for a common pathogen. *Comm Dis Report* 7, R33-37.

Pederson, K. J. & Pierson, D. E. (1995). Ail expression in *Yersinia enterocolitica* is affected by oxygen tension. *Infect Immun* **63**, 4199-4201.

Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X-Z., Kopecko, D. J., Bourgeois, A. L., Fauchère, J-C. & Blaser, M. J. (1998). Mutation in the *peb1a* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonisation of mice. *Infect Immun* **66**, 938-943.

Pei, Z. & Blaser, M. J. (1993). PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in Gram-negative nutrient transport systems. *J Biol Chem* **268**, 18717-18725.

Pei, Z., Ellison, R. T. & Blaser, M. J. (1991). Identification, purification, and characterisation of major antigenic proteins of *Campylobacter jejuni*. *J Biol Chem* **266**, 16363-16369.

Perraud, A-L., Weiss, V. & Gross, R. (1999). Signalling pathways in two-component phosphorelay systems. *Trend Microbiol* 7, 115-120.

Pesci, E. C., Cottle, D. L. & Pickett, C. L. (1994). Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infect Immun* **62**, 2687-2694.

Pickett, C. L., Auffenberg, T., Pesci, E. C., Sheen, V. L. & Jusuf, S. S. D. (1992). Iron acquisition and hemolysin production by *Campylobacter jejuni*. *Infect Immun* **60**, 3872-3877.

Pickett, C. L., Pesci, E. C., Cottle, D. L., Russell, G., Erdem, A. N. & Zeytin, H. (1996). Prevalance of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* spp. *cdtB* genes. *Infect Immun* **64**, 2070-2078.

Piddock, L. J. (1995). Quinolone resistance and *Campylobacter* spp. *J Antimicrob Chemothero* **36**, 891-898.

Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151-156.

Polotsky, Y., Dragunsky, E. & Ehavkin, T. (1994). Morphologic evaluation of the pathogenesis of bacterial enteric infections. *Crit Rev Microbiol* **20**, 161-208.

Prescott, J. F. & Karmali, M. A. (1978). Attempts to transmit campylobacter enteritis to dogs and cats. *Can Med Assoc* **119**, 1001.

Purdy, D., Cawthraw, S., Dickenson, J. H., Newell, D. G. & Park, S. F. (1999). Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonisation. *Appl Environ Microbiol* **65**, 2540-2546.

Purdy, D. & Park, S. F. (1994). Cloning, nucleotide sequence and characterization of a gene encoding superoxide dismutase from *Campylobacter jejuni* and *Campylobacter coli*. *J Gen Microbiol* **140**, 1203-1208.

Raja, N., Goodson, M., Smith, D. G. & Rowbury, R. J. (1991). Decreased DNA damage by acid and increased repair of acid-damaged DNA in acid-habituated *Escherichia coli*. *J Appl Bacteriol* **70**, 507-511.

Rathman, M., Sjaastad, M. D. & Falkow, S. (1996). Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages *Infect Immun* **64**, 2765-2773

Ratnaker, P., Rao, S. P. & Catanzaro, A. (1996). Isolation and characterisation of a 70 kDa protein from *Mycobacterium avium*. *Microb Pathog* **21**, 471-486.

Richardson, P. T. & Park, S. F. (1995). Enterochelin acquisition in *Campylobacter coli*: characterisation of components of a binding protein-dependent transport sytem. *Microbiol* **141**, 3181-3191.

Richardson, P. T. & Park, S. F. (1997). Integration of heterologous plasmid DNA into multiple sites on the genome of *Campylobacter coli* following natural transformations. *J Bacteriol* **179**, 1809-1812.

Rodriguez, G. G., Phippa, D., Ishiguro, K. & Ridway, H. F. (1992). Use of a fluorescent redox probe for a direct visualisation of actively respiring bacteria. *Appl Environ Microbiol* **58**, 1801-1808.

Roggenkamp, A., Bittner, T., Leitritz, L., Sing, A. & Heesemann, J. (1997). Contribution of the Mn-cofactored superoxide dimutase (SodA) to the virulence of *Yersinia enterocolitica* serotype 08. *Infect Immun* **65**, 4705-4710.

Rollins, D. M. & Colwell, R. R. (1986). Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl Environ Microbiol* **52**, 531-538.

Roof, M. B., Kramer, T. T., Roth, J. A. & Minion, F. C. (1992). Characterisation of a *Salmonella choleraesuis* isolate after repeated neutrophil exposure. *Am J Vet Res* **53**, 1328-1332.

Rotimi, V. O., Egwari, L. & Akande, B. (1990). Acidity and intestinal bacteria: an *in vitro* assessment of the bactericidal activity of hydrochloric acid on intestinal pathogens. *Afr J Med med Sci* **19**, 275-280.

Rowbury, R. J. (1995). An assessment of the environmental factors influencing acid tolerance and sensitivity in *Escherichia coli*, *Salmonella* spp. and other enterobacteria. *Lett Appl Microbiol* **20**, 333-337.

Rowbury, R. J. (1997). Regulatory components, including integration host factor, CycB and H-NS, that influence pH responses in *Escherichia coli*. *Lett Appl Microbiol* **24**, 319-328.

Rowbury, R. J. & Goodson, M. (1993). PhoE porin of *Escherichia coli* and phosphate reversal of acid damage and killing and acid induction of the *cadA* gene product. *J Appl Bacteriol* **74**, 652-661.

Ruiz-Palacios, G. M., Torres, J., Torres, N. I., Escamilla, E., Ruiz-Palacios, B. R. & Tamayo, T. (1983). Cholera-like enterotoxin produced by *Campylobacter jejuni*: characterisation and clinical significance. *Lancet* **2**, 250-253.

Russell, R. G., Blaser, M. J., Sarmiento, J. L. & Fox, J. (1989). Experimental *C. jejuni* infection in *Macaca nemestrina*. *Infect Immun* **57**, 1438-1444.

Russell, R. G., O'Donnoghue, M., Blake, D. C., Jr, Zulty, J & SeTolla, L. J. (1993). Early colonic damage and invasion of *Campylobacter jejuni* in experimentally challenged infant *Macaca mulatta*. *J Infect Dis* **168**, 210-215.

Russell, R. G. & Blake, D.C., Jr. (1994). Cell association and invasion of Caco-2 cells by *Campylobacter jejuni*. *Infect Immun* **62**, 3773-3779.

Sails, A. D., Bolton, F. J., Fox, A. J., Wareing, D. R. & Greenway, D. L. (1998). A reverse transcriptase polymerase chanin reaction assay for the detection of thermophilic *Campylobacter* spp. *Mol Cell Probes* **12**, 317-322.

Salyers, A. A. & Whitt, D. D. (1994). Chapters 3 and 4, pp 30-62. In, *Bacterial Pathogenesis: a molecular approach*. American Society for Microbiology, Washington.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: A laboratory manual Vol I, II & III. Cold Harvour Laboratory Press.

Sampson, J. S., O'Connor, S. P., Stinson, A. R., Tharpe, J. A. & Russell, H. (1994). Cloning and nuclerotide sequence analysis of *psaA*, the *Streptococcus pneumoniae* gene encoding a 37 kDa protein homologous to the previously reported *Streptococcus* spp. adhesins. *Infect Immun* **62**, 319-324.

Schwartz, D., Konforti, N., Perry, R., Goossens, H., Butzler, J. P., Williams, P. & Goldhar, J. (1994). Iron-regulated proteins in outer membranes of *Campylobacter jejuni* diarrhoea isolates and immune response to the proteins in patients. *Zbl Bakt* 280, 338-347.

Sebald, M. & Véron. M. (1963). Teneur en bases de L'ADN et classification de vibrions. *Ann de l'Inst Pasteur* **105**, 897-910.

Skirrow, M. B. (1977). *Campylobacter* enteritis: a "new" disease. *Br Med J* **2**, 9-11.

Skirrow, M. B. (1990). *Campylobacter*. *Lancet* **336**, 921-923.

Skirrow, M. B. & Benjamin, J. (1980). "1001" Campylobacters: cultural characteristics of intestinal campylobacters from man and animals. *J Hyg Lond* **85**, 427-442.

Skirrow, M. B. & Blaser, M. J. (1992). Clinical and epidemiological considerations. In *Campylobacter jejuni: Current Status and Future Trends*, pp. 3-8. Edited by I. Nachamkin, M. J. Blaser & L. S. Tompkins. Washington, DC: American Society for Microbiology.

Simmonson, C., Brener, D. & DeVoe, I. W. (1982). Expression of a high-affinity mechanism for acquisition of transferrin iron by *Neisseria menigitidis*. *Infect Immun* **36**, 107-113.

Silversmith, R. E. & Bourret, R. B. (1999). Throwing the switch in bacterial chemotaxis. *Trend Microbiol* 7, 16-21.

Skroupski, K. & Taylor, R. K. (1997). Control of the ToxR virulence regulon in *Vibrio chloerae* by environmental stimuli. *Mol Microbiol* **25**, 1003-1009.

Smith, H. (1990). Pathogenicity and the microbe *in vivo*. *J Gen Microbiol* **136**, 377-383.

van Spreeuwel, P., Duursma, G. C., Meijer, C. J. L., Bax, R., Rosekrans, P. C. M. & Lindeman, J. (1985). *Campylobacter* colitis: histological, immunohistochemical and ultrastructure finding. *Gut* **26**, 945-951.

Stanbury, P. F. & Whitaker, A. (1989). Chapter 2, Microbial growth kinetics pp 11-25. In, *Principles of fermentation technology*. Pergamon Press, Oxford. UK.

Steele, T. W., Sangster, N. & Lanser, J. A. (1985). DNA related and biochemical features of *Campylobacter* spp. isolated in Central and Southern Austrialia. *J Clin Microbiol* **22**, 71-74.

Stern, N. J., Jones, D. M., Wesley, I. V. & Rollins, D. M. (1994). Colonization of chicks by non-culutrable *Campylobacter* spp. *Lett Appl Microbiol* **18**, 333-336.

Stoeber, J. A. & Payne, S. M. (1988). Iron regulated hemolysin production and utilisation of heme and hemolysin by *Vibrio cholerae*. *Infect Immun* **56**, 2891-2895.

Stoller, G., Tradler, T., Rucknagel, K. P., Ralfeld, J-C. & Fischer, G. (1996). An 11.8 kDa proteolytic fragment of the *E. coli* trigger factor represents the domain carrying the peptidly-prolyl cis/trans isomerase activity. *FEBS Lett* **384**, 117-122.

Storz, G., Tartaglia, L. A., Farr, S. B. & Ames, B. N. (1990). Bacterial defences against oxidative stress. *Trend Genet* **6**, 363-368.

Stuart, J., Sufi, F., McMulty, C. & Park, P. (1997). Outbreak of *Campylobacter* enteritis in a boarding school associated with bird pecked bottle tops. *Comm Dis Report* 7, R38-40.

Szymanski, C. M., King, M., Haardt, M. & Armstrong, G. D. (1995). *Campylobacter jejuni* motility and invasion of Caco-2 cells. *Infect Immun* **63**, 4295-4300.

Takata, T., Wai, S. N., Takade, A., Sawae, Y., Ono, J. & Amoka, K. (1995). The purification of a GroEL-like stress protein from aerobically adapted *Campylobacter jejuni*. *Microbiol Immunol* **39**, 639-645.

Takeuchi, A. (1967). Electron microscopy studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *Am J Pathol* **50**, 109-136.

Taylor, D. E. (1992). Genetics of *Campylobacter* and *Helicobacter*. *Ann Rev Microbiol* **46**, 35-64.

Thies, F. L., Weishaupt, A., Karch, H., Hartung, H-P. & Giegerich. G. (1999). Cloning, sequencing and molecular analysis of the *Campylobacter jejuni groESL* biscistronic operon. *Microbiology* **145**, 89-98.

Unden, G. & Schirawski, J. (1997). The oxygen-responsive transcriptional regulator FNR of *Escherichia coli*: the search for signals and reactions. *Mol Microbiol* **25**, 205-210.

van Vliet, A, H. M., Wooldridge, K. G. & Ketley, J. M. (1998). Iron-responsive gene regulation in a *Campylobacter jejuni fur* mutant. *J Bacteriol* **180**, 5391-5298.

van Vliet, A. H. M., Baillion, M-L, A., Penn, C. W. & Ketley, J. M. (1999). *Campylobacter jejuni* contains two Fur homologs: characterisation of iron-responsive regulation of peroxide stress defence genes by the PerR repressor. *J Bacteriol* **181**, 6371-6376.

Véscovi, E. G., Soncini. F. C. & Groisman, E. A. (1996). Mg^{2+} as an extracellular signal environmental regulation of *Salmonella* virulence. *Cell* **84**, 165-174.

Vos, P., van Asseldorik, M. M., van Jeveren, F., Siezen, R., Simons, G, de-Vos, W. N. (1989). A maturation protein is essential for production of active forms of *Lactococcus lactis* SKII serine proteinase located in or secreted from cell envelope. *J Bacteriol* **171**, 2795-2802.

Wai, S. N., Takata, T., Takade, A., Hamasaki, N. & Amako, K. (1995). Purification of ferritin from *Campylobacter jejuni*. *Arch Microbiol* **164**, 1-6.

Wai, S. N., Nakayama, K., Umene, K., Moriya, T. & Amako, K. (1996). Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. *Mol Microbiol* **20**, 1127-1134.

Walan, A., Dahlgren, C., Kihlström, E., Stendahl, D. & Lock, R. (1992). Phagocyte killing of *Campylobacter jejuni* in relation to oxidative activation. *APMIS* **100**, 424-430.

Wallis, M. R. (1994). The pathogenesis of *Campylobacter jejuni*. *Br J Biomed Sci* **51**, 57-64.

Willis, W. L. & Murray, C. (1997). *Campylobacter jejuni* seasonal recovery: observations of retail market broilers. *Poult Sci* **76**, 314-317.

Walker, R. I., Caldwell, M. B., Lee, E. C., Guerry, P., Trust, T. J. & Ruiz-Palacois, G. M. (1986). Pathophysiology of *Campylobacter* enteritis. *Microbiol Rev* **50**, 81-94.

Walker, R. I., Schmauder-Chock, E. A., Parker, J. L. & Burr, D. (1988). Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. *Can J Microbiol* **34**, 1142-1147.

Wan, X, Y., Zhou, Y., Yan, Z. Y., Wang, H. L., Hou, Y. D. & Jin, D. Y. (1997). Scavengase p20: a novel family of bacterial antioxidant enzymes. *FEBS Lett* **407**, 32-36.

Wassenaar, T. M., Bleumink-Pluym, N. M. C. & van der Zeijst, B. A. M. (1991). Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. *EMBO J* **10**, 2055-2061.

Wassenaar, T. M., Fry, B. N. & van der Zeijst, B. A. M. (1993). Genetic transformation of *Campylobacter*: evaluation of natural transformation and electro-transfer. *Gene* **132**, 131-135.

Wassenaar, T. M., Engelskirchen, M., Park, S. & Lastovica, A. (1997). Differential uptake and killing of *Campylobacter jejuni* by human peripheral monocytes/macrophages. *Med Microbiol Immunol* **186**, 139-144.

Wassenaar, T. M. (1997). Toxin production by *Campylobacter* spp. *Clin Microbiol Rev* **10**, 466-476.

Weinberg, E. D. (1990). Cellular iron metabolism in health and disease. *Drug Metab Health Dis* **22**, 531.

Which Magazine. (1996). How safe is chicken? October, 8-9.

Whitehouse, C. A., Balbo, P. B., Pesci, E. C., Cottle, D. L., Mirabito, P. M. & Pickett, C. L. (1998). *Campylobacter jejuni* cytolethal distending toxin causes a G_2-phase cell cycle block. *Infect Immun* **66**, 1934-1940.

Willis, W. L. & Murray, C. (1997). *C. jejuni* seasonal recovery observations of retail market broilers. *Poult Sci* **76**, 314-317.

Wood, A. C., Oldfield, N. J., O'Swyer, C. A. & Ketley, J. K. (1999). Cloning, mutation and distribution of a putative lipopolysaccaride biosynthesis locus in *Campylobacter jejuni*. *Microbiology* **145**, 379-388.

Wooldridge, K. G., Williams, P. H. & Ketley, J. M. (1994). Iron-responsive genetic-regulation in *Campylobacter jejuni* - cloning and characterization of a *fur* homolog. *J Bacteriol* **176**, 5852-5856.

Wooldridge, K. G., Williams, P. H. & Ketley, J. M. (1996). Host signal transduction and endocytosis of *Campylobacter jejuni*. *Microb Pathog* **21**, 299-305.

Yao, R., Burr, D., Doig, P., Trust, T. J., Niu, H. Y. & Guerry, P. (1993). Construction of a new *Campylobacter* cloning vector and a new mutational *cat* cassette. *Gene* **130**, 127-130.

Yao, R. J., Burr, D. H., Doig, P., Trust, T. J., Niu, H. Y. & Guerry, P. (1994). Isolatation of motile and nonmotile insertional mutants of *Campylobacter jejuni* - the role of motility in adherance and invasion of eukaryotic cells. *Mol Microbiol* **14**, 883-893.

Yao, R., Burr, D. & Guerry, P. (1997). CheY mediated modulation of *Campylobacter jejuni* virulence. *Mol Microbiol* **23**, 1021-1031.

Yoh, M. & Honda, T. (1997). The stimulating effect of fosfomycin an antibiotic in common use in Japan on the production/release of Verotoxin-1 from enterohaemorrhagic *Escherichia coli* O157:H7 *in vitro*. *FEBS Immunol Med Microbiol* **19**, 57-64.

Yokoseki, T., Kutsakake, K., Ohnishi, K. & Iino, T. (1995). Functional analysis of the flagellar genes in the *fliD* operon of *Salmonella typhimurium*. *Microbiology* **141**, 1715-1722.

Yrios, J. W. & Balish, E. (1986). Pathogenesis of *Campylobacter* spp. in athymic and euthymic germfree mice. *Infect Immun* **53**, 384-392.

Zhou, Y., Wan, X-Y., Wang, H-L., Yan, Z-Y., Hou, Y-D. & Jin D-Y. (1997). Bacterial scavengase p20 is structurally and functionally related to peroxiredoxins. *Biochem Biophy Res Com* **233**, 848-852.

Appendix I: Media for bacterial growth

ACES buffered chemically defined (ABCD) medium

Component	Quantity
ACES	10 000 $mg.l^{-1}$
KH_2PO_4	220 $mg.l^{-1}$
Na_2SO_4	150 $mg.l^{-1}$
$CaCl_2.2H_2O$	0.56 $mg.l^{-1}$
$MgSO_4.7H_20$	214 $mg.l^{-1}$
NH_4VO_3	1.17 $mg.l^{-1}$
$ZnSO_4.7H_2O$	28.75 $mg.l^{-1}$
$CoCl_2.6H_2O$	0.48 $mg.l^{-1}$
$CuSO_4.5H_2O$	0.03 $mg.l^{-1}$
$MnCl_2.4H_20$	0.02 $mg.l^{-1}$
$Na_2MoO_4.2H_2O$	1.21 $mg.l^{-1}$
$NiSO_4.6H_20$	0.53 $mg.l^{-1}$
Alanine, Arginine, Asparagine, Aspartic acid, Glutamine, Glycine, Glutamic acid, Histidine, Isoleucine, Leucine, Lysine, Methioine, Phenylalanine, Proline, Threonine, Tryptophan, Valine	100 $mg.l^{-1}$ of each amino acid
Serine	2000 $mg.l^{-1}$
Sodium pyruvate	1000 $mg.l^{-1}$
Potassium α-ketoglurate	1000 $mg.l^{-1}$
Inositol	2 $mg.l^{-1}$
Thiamine HCl	2 $mg.l^{-1}$
Calcium pantothenate	2 $mg.l^{-1}$
Nicotinamide	1 $mg.l^{-1}$
Biotin	0.1 $mg.l^{-1}$
Thiotic acid	0.1 $mg.l^{-1}$
Coenzyme A	0.1 $mg.l^{-1}$
Cysteine HCl	500 $mg.l^{-1}$
Glutathione	500 $mg.l^{-1}$
Tyrosine	50 $mg.l^{-1}$
$FeSO_4.7H_2O$	40 $mg.l^{-1}$
Haemin	2 $mg.l^{-1}$

Medium was filter sterilised (0.45 and 0.22 μm filters, Sartorius) and prepared in 20 l volumes

Complex medium - Cpmod4

Component	Amount
Bactotryptone (Difco)	10 $g.l^{-1}$
Protease peptone (Oxoid)	10 $g.l^{-1}$
Glucose	1 $g.l^{-1}$
Yeast extract (Difco)	2 $g.l^{-1}$
NaCl	5 $g.l^{-1}$
Sodium pyruvate	1.1 $g.l^{-1}$

Medium was sterilzed by autoclaving (15 lb in^{-2} for 30 min) in 20 l volumes.

LB-Broth

Component	Quantity
Bactotryptone	10 $g.l^{-1}$
Bacto-yeast extract	5 $g.l^{-1}$
Sodium chloride	10 $g.l^{-1}$

pH adjusted to pH 7.0 - 7.2 with sodium hydroxide.
Sterilised by autoclaving 121°C, 15 lb in^{-2}

LB-Agar
Prepared as described above with the addition of Bacto-agar (15 $g.l^{-1}$)

Mueller-Hinton
Obtained from Oxoid, Basingstoke

Broth
21 $g.l^{-1}$ Mueller-Hinton broth in distilled water
Sterilised by autoclaving 121°C, 15 lb in^{-2}

Agar plates
38 $g.l^{-1}$ Mueller-Hinton agar in distilled water
Sterilised by autoclaving 121°C, 15 lb in^{-2}

Appendix II: Buffers

Buffers used in gel electrophoresis

Concentrated sample buffer for 1-D gel electrophoresis

Component	Quantity
Tris-HCl, pH 7.9	0.05 M
Sodium lauryl sulphate (SDS)	10 $g.l^{-1}$
Bromophenol blue	1 $g.l^{-1}$

2-D gel electrophoresis

Rehydration Solution for IEF Strips

Component	Quantity
Urea	8M
Triton X-100	0.5 % (v/v)
Pharmalyte 3-10 (Pharmacia Biotech)	0.5 % (v/v)
DTT	0.013 M
IPG 3-10 Buffer (Pharmacia Biotech)	0.5 % (v/v)

Bromophenol blue added to give colour. Made up to 25 ml with sterile water

Lysis Solution A

Component	Quantity
Urea	9 M
Triton X-100	2 % (v/v)
DTT	0.13 M
Pharmalyte 3-10	2 % (v/v)
4,2-amino-ethyl benzene sufonyl fluoride	0.8 mM

Bromophenol Blue added to give colour.
Made up to 25 ml with sterile water. Stored for one month at -20°C.

Lysis Solution B

Component	Quantity
Urea	9 M
DTT	65 mM
Pharmalyte 3-10	2 % (v/v)
Triton X-100	0.52 % (v/v)

Made up to 25 ml with sterile water. Stored for one month at -20°C.

Equilibration Solution for IEF Strips	
Component	Quantity
Tris-HCl, pH 6.8	0.5 M
Urea	6 M
Glycerol	30 % (v/v)
SDS	12.5 $g.l^{-1}$

Allow 20 ml for one incubaction

Electroblotting Buffer (Matsudaira, 1987)

Component	Quantity
CAPS pH 11.0	12.5 % (v/v)
Methanol	12.5 % (v/v)

Protein Staining Techniques

Stain	Component	Quantity
Coomassie Blue	Coomassie Blue R250	1.16 $g.l^{-1}$
	Absolute alcohol	25 % (v/v)
	Acetic acid	8 % (v/v)
Amido Black	Amido Black	0.1 % (v/v)
	Acetic acid	1 % (v/v)
	Methanol	40 % (v/v)

Recombinant DNA techniques

Extraction of DNA (Pitcher *et al.*, 1989)

GES Reagent	
Component	Quantity
Guanidium thiocyanate	60 g
EDTA, pH 8.0	0.5 M
Deionised water	20 ml
N-lauroyl sarcosine	10 % (w/v)

Buffer was prepared by mixing guanidium thiocyanate, 0.5 M EDTA, pH 8.0 (20 ml) and deionised water (20 ml) and heating to 65°C. The solution was cooled and 5 ml of 10 % (v/v) Sarkosyl was added. The volume was made up to 100 ml with deionised water and filter sterilsed (0.45 µm filter; Satorius) and stored at room temperature.

TE Buffer	
Component	Quantity
Tris-HCl	10 mM
EDTA, pH 8.0	1 mM

TAE Buffer (1x)

Component	Quantity
Tris-acetate, pH7.6	40 mM
EDTA (sodium)	1 mM

Neutralisation Buffer

Component	Quantity
Tris-HCl pH 7.2	0.5 M
Sodium chloride	1.5 M
EDTA	1 mM

Used in the final incubation step of preparing the agarose gel for Southern Blotting

20x SSC

Component	Quantity
Na_3 citrate	0.3 M
Sodium chloride	3 M

Non-radioactive probe detection

Fluorescein labelled probe

Buffer	Component	Quantity
Hybridization buffer	5x SSC	
	Hybridization buffer component (Amersham, UK)	0.1 % (w/v)
	SDS	0.02 % (w/v)
	Liquid block (Amersham, UK)	20- fold dilution
Buffer 1	Sodium chloride	0.15 M
	Tris base	0.1 M
Buffer 2	Sodium chloride	0.4 M
	Tris base	0.1 M

The pH of Buffer 1 and 2 was altered to pH 7.5 using 2 M HCl.

Peroxidase labelled probe

Buffer	Component	Quantity
Hybridization Buffer II	Gold hybridization buffer (Amersham)	0.125ml.cm^2
	Sodium chloride	0.5 M
	Blocking agent (Amersham)	5 % (w/v)
Primary wash buffer	Urea	6 M
	SDS	0.4 % (w/v)
	20x SSC	use as 1-5x SSC
Secondary wash buffer	2x SSC	

The Hybridization buffer was prepared by mixing for 1 h, following by heating for 0.5-1 h at 42°C.

Electroporation buffer

Component	Quantity
Sucrose	272 mM
Glycerol	15 % (v/v)

Appendix III: Primers

Primers designed to sequence the clones A6, A7 and G5 (see section 5.2.5)

Clone	Primer direction	Primer
A6	reverse	5'-GGT AAA TAC ATC GTC GCA ACA -3'
	forward	5'-AAC AAG TTG AAC GCT GGA TTA-3'
	reverse	5'-TCA ATG CCC GCT ATC ATC-3'
	forward	5'-GGG GTA AGT ATT TTG GTA-3'

Clone	Primer direction	Primer
A7	reverse	5'-AGT TTT CCC ACC CGT ATC AGC A-3'
	forward	5'-GTA TTG CTT GAT GGC GAT GAG-3'
	reverse	5'-TCT GCG ATT GCT TCT TAT-3'
	forward	5'-ATC AGC CAT AAC TCA AAG T-3'

Clone	Primer direction	Primer
G5	reverse	5'-ATT CGC CAT TGT ARC ATA-3'
	forward	5'-TTT GGT TCT GGG GTT TTA-3'
	reverse	5'-TTT AAA GTT AAG CCA AAA-3'
	forward	5'-GCG CAG GGT CAG TAT CAA-3'

Primers designed to amplify specific genes using the Polymerase chain reaction (PCR)

(see section 5.3.1.1, 5.3.2.1 and 5.3.3.1)

Name	Sequence
PEB-4 rev	5'-GTCGCACGAACTTTGATTGCAGG-3'
PEB-4 fwd	5'-GCGCGACAGCTAAAGCATCAAG-3'
htrB rev	5'-GCGATTGTCCTTTAGAGTTGC-3'
htrB fwd	5'-CTTGCTTTTTTAGAATCCACC-3'
tpx1 rev	5'-GATCTTAGCATTAAAATAACC-3'
tpx1 fwd	5'-ACAGGTTAACGCTATGGGAAGATTTTGCAGT-3'
tpx2 rev	5'-ACAGGTTAACCATACTTACTACAATCACTTC-3'
tpx2 fwd	5'-GGACTTTATGAAGATATGGC-3'

Appendix IV: Recombinant DNA techniques

Calculations for probe labelling

T_m of an oligonucleotide can be calculated according to the Wallace rule:

$$T_m = (4 \times \text{number of G+C bases}) + (2 \times \text{number of A+T bases})$$

Calculation of oligonuceotide concentration

$$E = (A \times 15200) + (G \times 12010) + (C \times 7050) + (T \times 8400)$$

where A, G, C and T represent the number of times each base occurs in the oligonucleotide sequence and E is the molar extinction coefficient (the absorbance of a 1 molar solution).

Ligation reaction

Tube	Plasmid (1 µl)	Insert (1 µl)	Ligase (1 µl)	Ligase Buffer (1 µl)	Water (1µl)	Total (1 µl)
1*	1	-	-	-	9	10
2	1	-	-	-	9	10
3	1	-	1	1	7	10
4	-	1	1	1	7	10
5	1	1	1	1	6	10
6	1	2	1	1	5	10
7	1	4	1	1	3	10
8	1	6	1	1	1	10

1*, the plasmid added to this tube was uncut, acting as a control to ensure the transformation reaction worked effectively; 2, the plasmid in this instance was cut with a restriction endonuclease and treated with alkaline phosphatase, acting as a control to ensure that the plasmid had been cut; 3, the cut plasmid treated with AP was incubated with ligate to endure that the AP treated had worked and that the plasmid did not re-ligate itself; 4, the insert of *C. jejuni* DNA was used as a control; 5-8 were used as experimental reactions with an increasing proportion of *C. jejuni* insert.

Appendix V: Sequence data

Alignment of alkaline phosphatase protein

Alignment of FOM1 protein

Alignment of FliS protein

Alignment of FliD protein

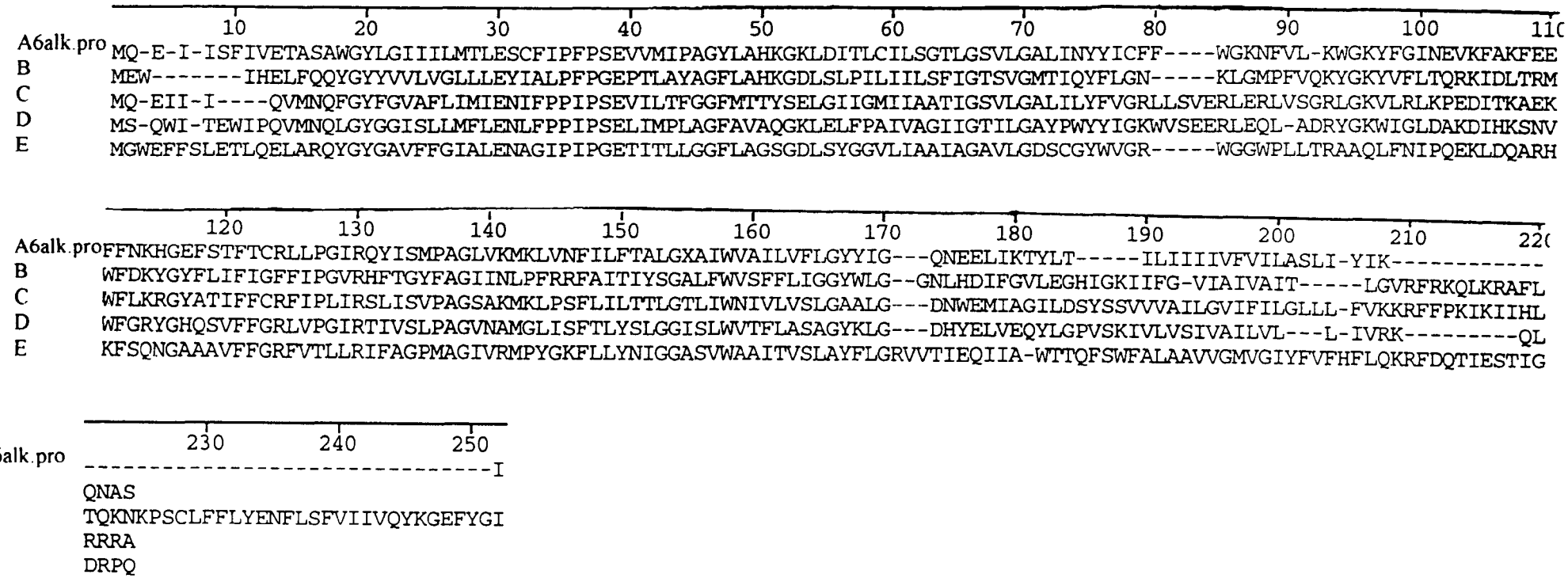
Amino acid alignment of the thiol peroxidase family

Sequence data of Clone A6

Sequence data of Clone A7

Sequence data of Clone G5

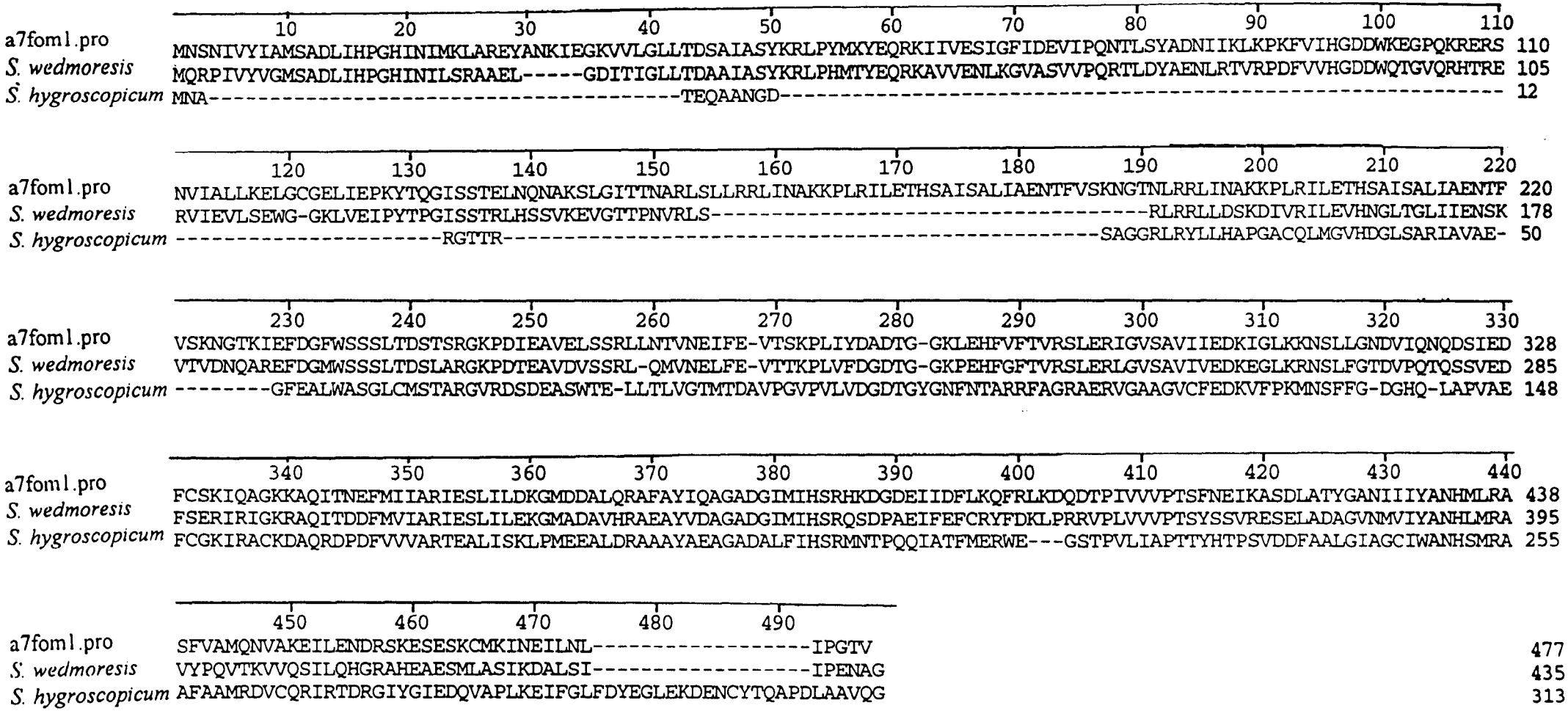
Amino acid alignment of the protein alkaline phosphotase



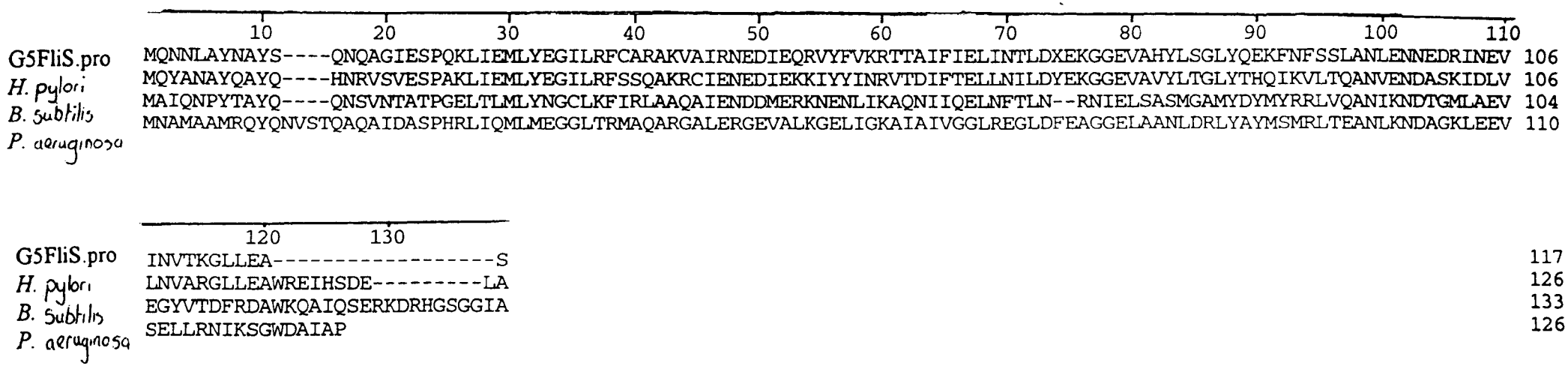
Key: A6alk.pro, sequence from clone A6; B, *Bacillius cereus*, C, *Lactobacillis lactis*;

D, *Synechocystis* alkaline phosphatase, E, *Synechocystis* DedA

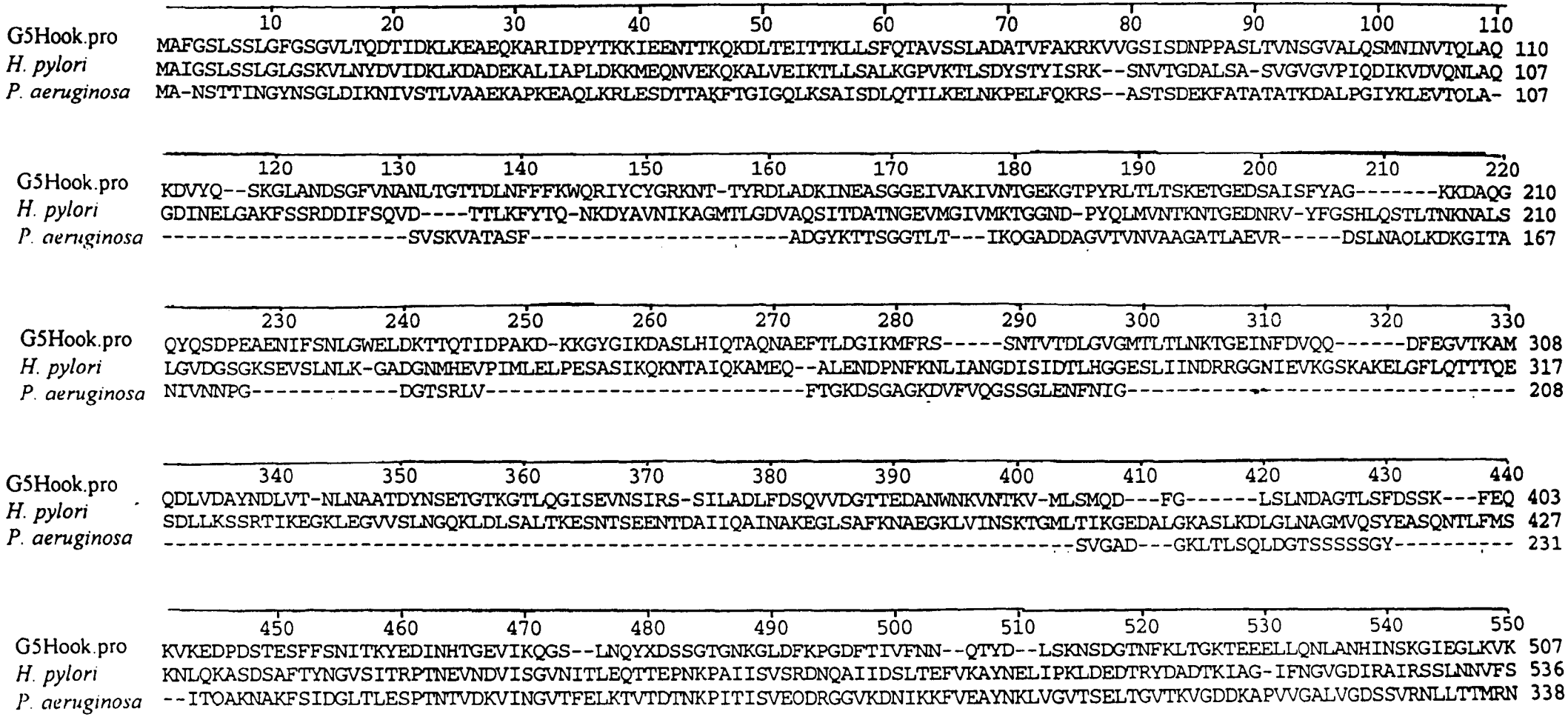
Amino acid alignment of the protein FomI, encoding phosphoenolypyruvate phosphormutase

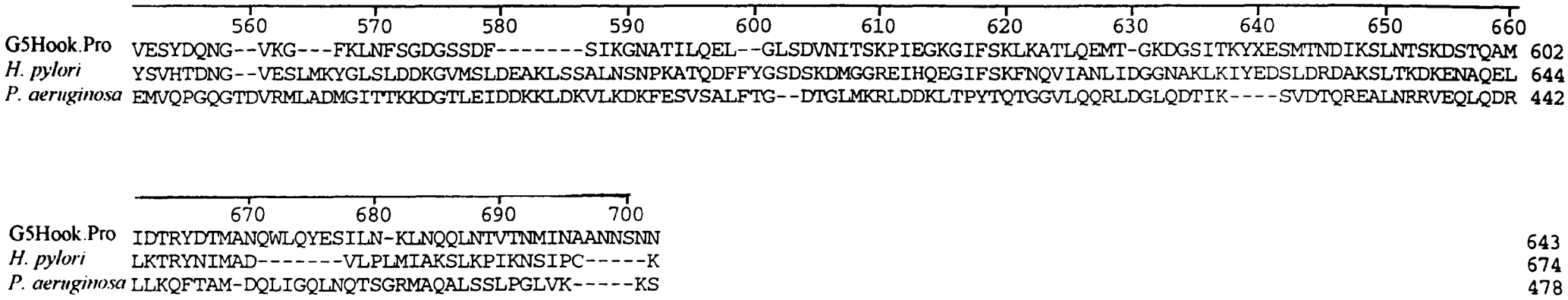


Amino acid alignment of the protein FliS

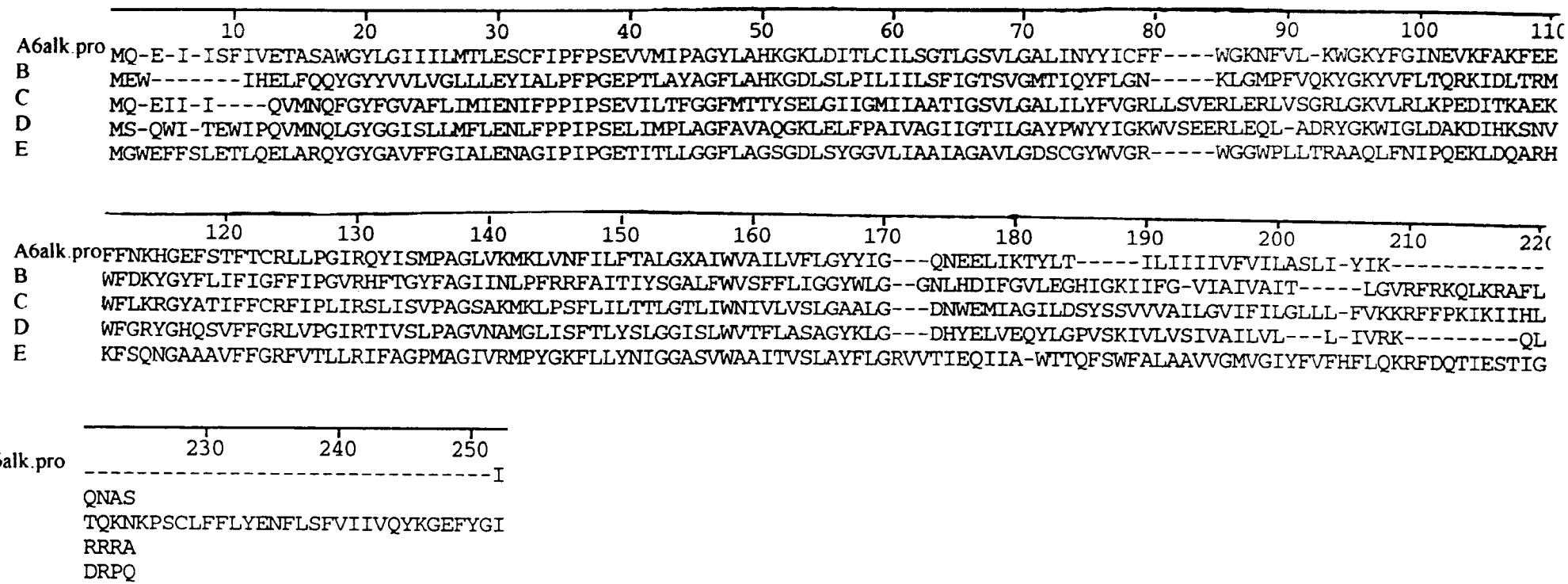


Amino acid alignment of the protein FliD




560 570 580 590 600 610 620 630 640 650 660
G5Hook.Pro VESYDQNG--VKG---FKLNFSGDGSSDF-------SIKGNATILQEL--GLSDVNITSKPIEGKGIFSKLKATLQEMT-GKDGSITKYXESMTNDIKSLNTSKDSTQAM 602
H. pylori YSVHTDNG--VESLMKYGLSLDDKGVMSLDEAKLSSALNSNPKATQDFFYGSDSKDMGGREIHQEGIFSKFNQVIANLIDGGNAKLKIYEDSLDRDAKSLTKDKENAQEL 644
P. aeruginosa EMVQPGQGTDVRMLADMGITTKKDGTLEIDDKKLDKVLKDKFESVSALFTG--DTGLMKRLDDKLTPYTQTGGVLQQRLDGLQDTIK----SVDTQREALNRRVEQLQDR 442
670 680 690 700
G5Hook.Pro IDTRYDTMANQWLQYESILN-KLNQQLNTVTNMINAANNSNN 643
H. pylori LKTRYNIMAD-------VLPLMIAKSLKPIKNSIPC-----K 674
P. aeruginosa LLKQFTAM-DQLIGQLNQTSGRMAQALSSLPGLVK-----KS 478

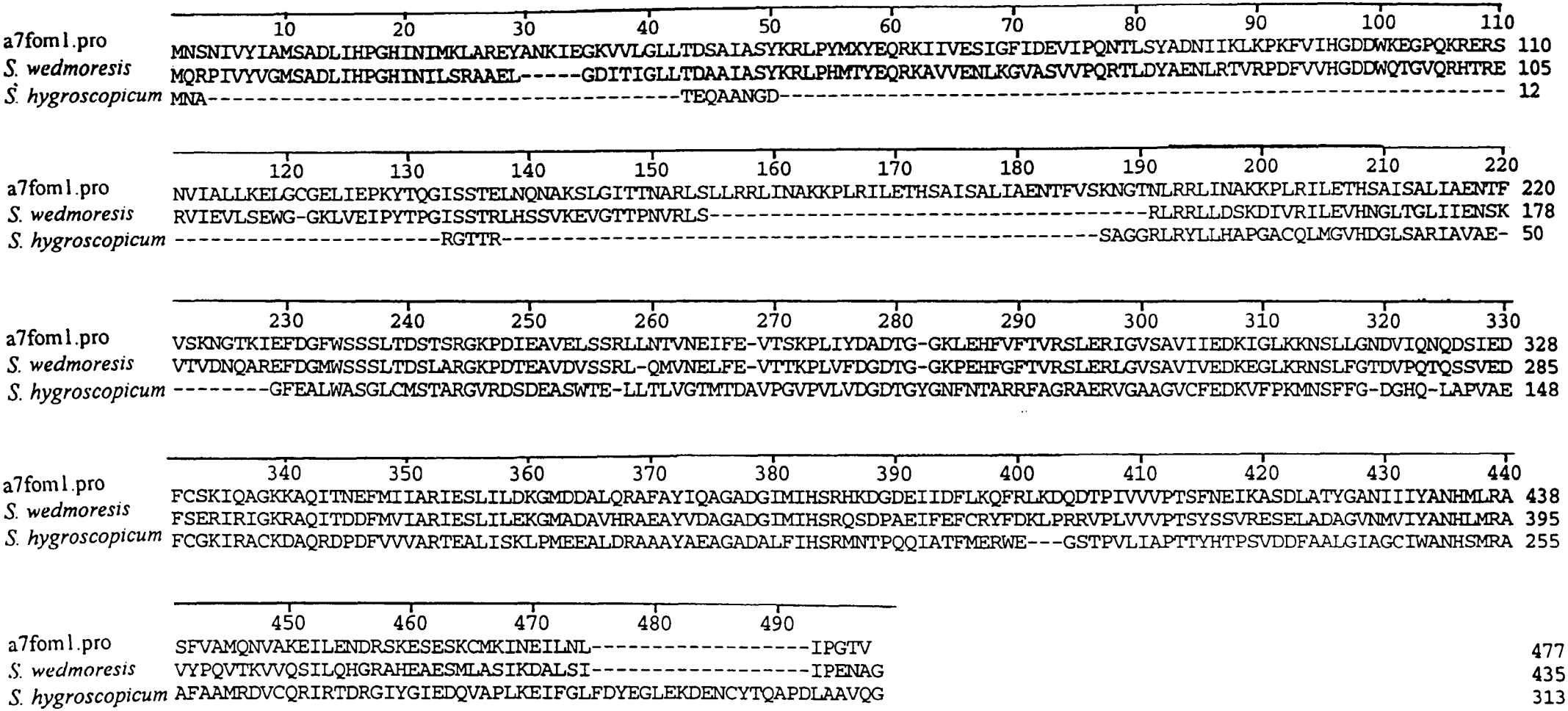
Amino acid alignment of the protein alkaline phosphotase



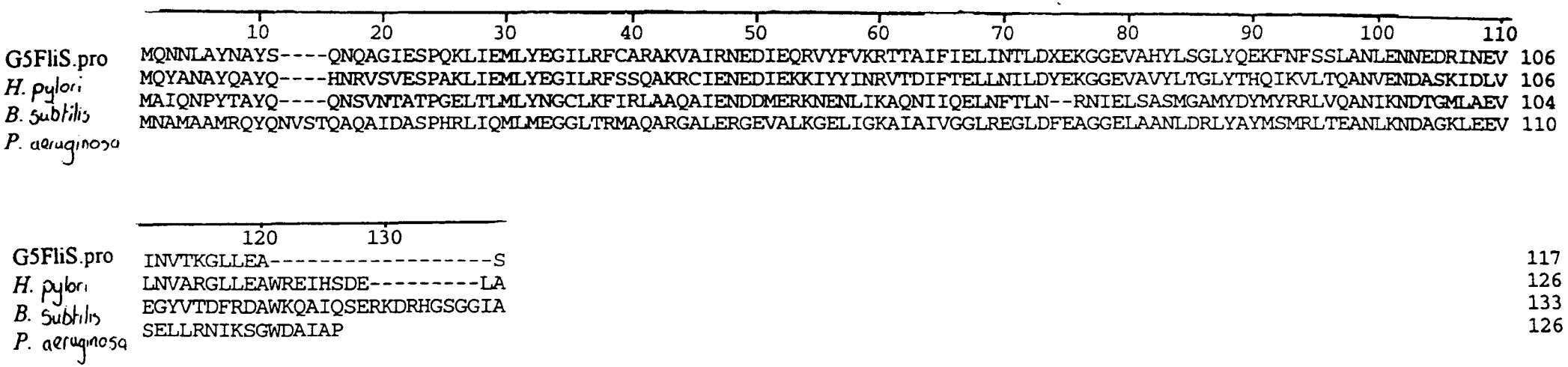
Key: A6alk.pro, sequence from clone A6; B, *Bacillius cereus*, C, *Lactobacillis lactis*;

D, *Synechocystis* alkaline phosphatase, E, *Synechocystis* DedA

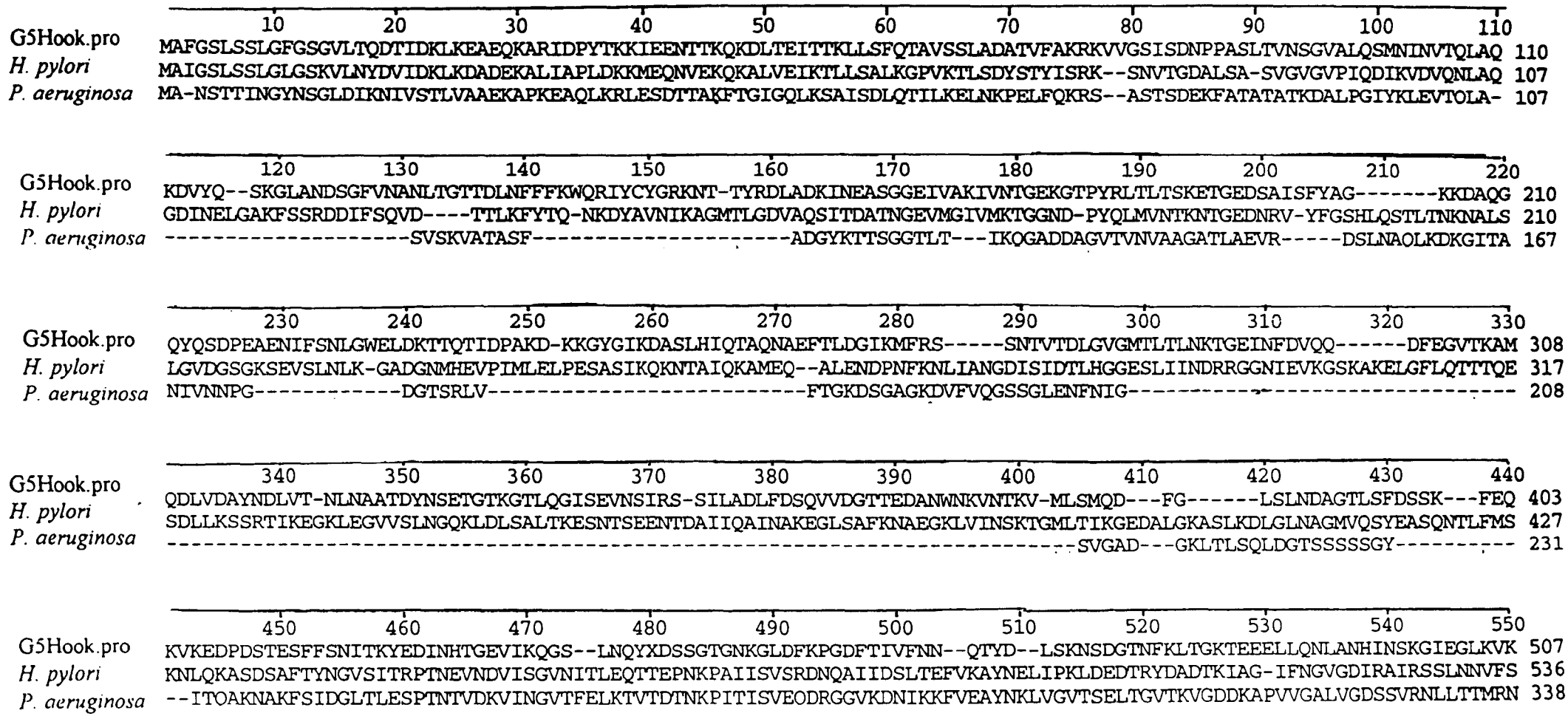
Amino acid alignment of the protein FomI, encoding phosphoenolypyruvate phosphormutase

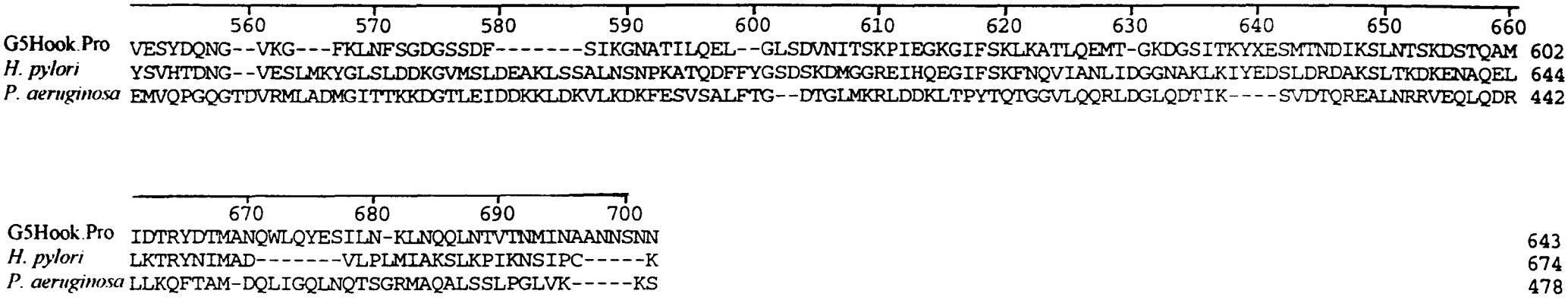


Amino acid alignment of the protein FliS



Amino acid alignment of the protein FliD



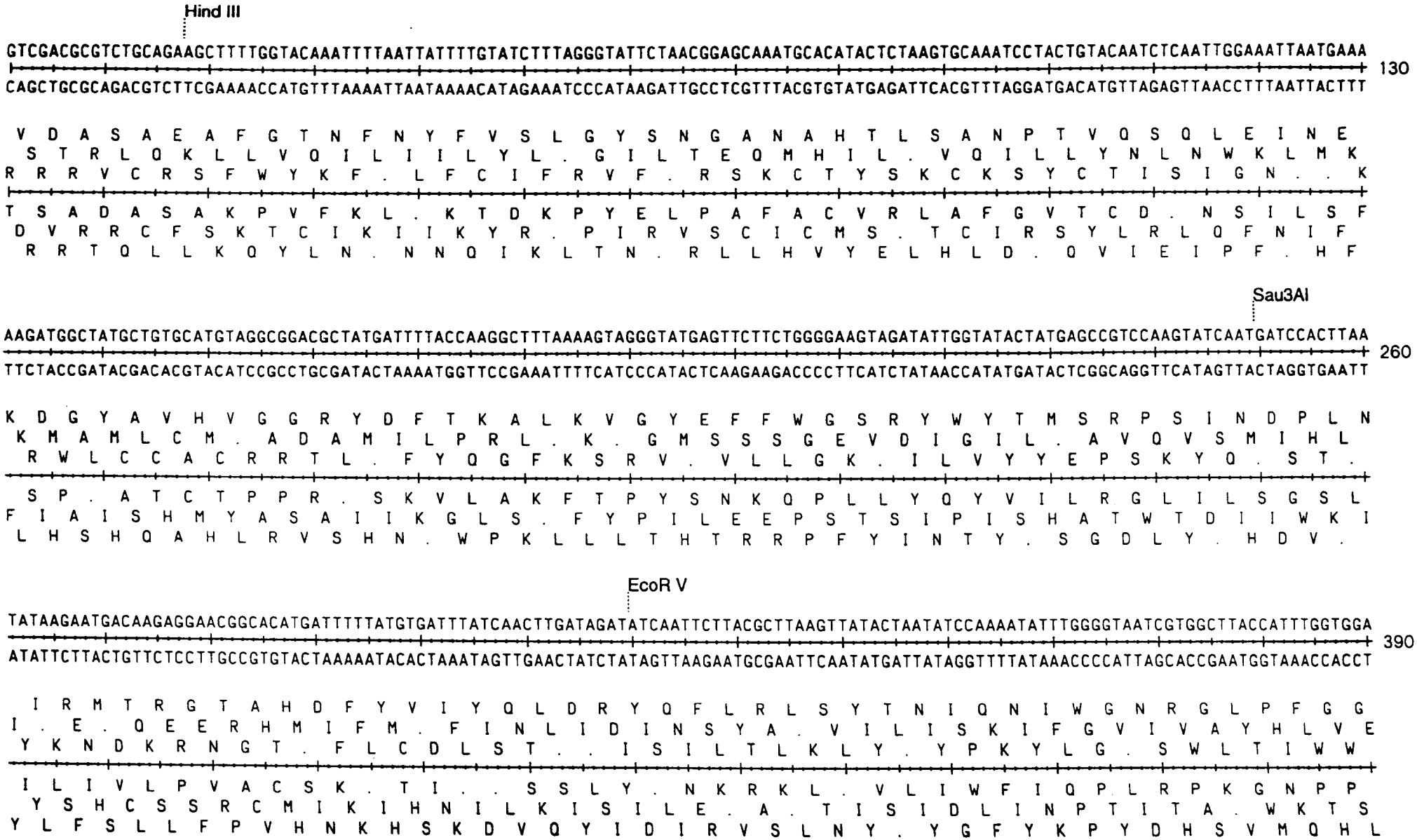

560 570 580 590 600 610 620 630 640 650 660
G5Hook.Pro VESYDQNG--VKG---FKLNFSGDGSSDF-------SIKGNATILQEL--GLSDVNITSKPIEGKGIFSKLKATLQEMT-GKDGSITKYXESMTNDIKSLNTSKDSTQAM 602
H. pylori YSVHTDNG--VESLMKYGLSLDDKGVMSLDEAKLSSALNSNPKATQDFFYGSDSKDMGGREIHQEGIFSKFNQVIANLIDGGNAKLKIYEDSLDRDAKSLTKDKENAQEL 644
P. aeruginosa EMVQPGQGTDVRMLADMGITTKKDGTLEIDDKKLDKVLKDKFESVSALFTG--DTGLMKRLDDKLTPYTQTGGVLQQRLDGLQDTIK----SVDTQREALNRRVEQLQDR 442
670 680 690 700
G5Hook.Pro IDTRYDTMANQWLQYESILN-KLNQQLNTVTNMINAANNSNN 643
H. pylori LKTRYNIMAD-------VLPLMIAKSLKPIKNSIPC-----K 674
P. aeruginosa LLKQFTAM-DQLIGQLNQTSGRMAQALSSLPGLVK-----KS 478

Amino acid alignment of the thiol peroxidase family

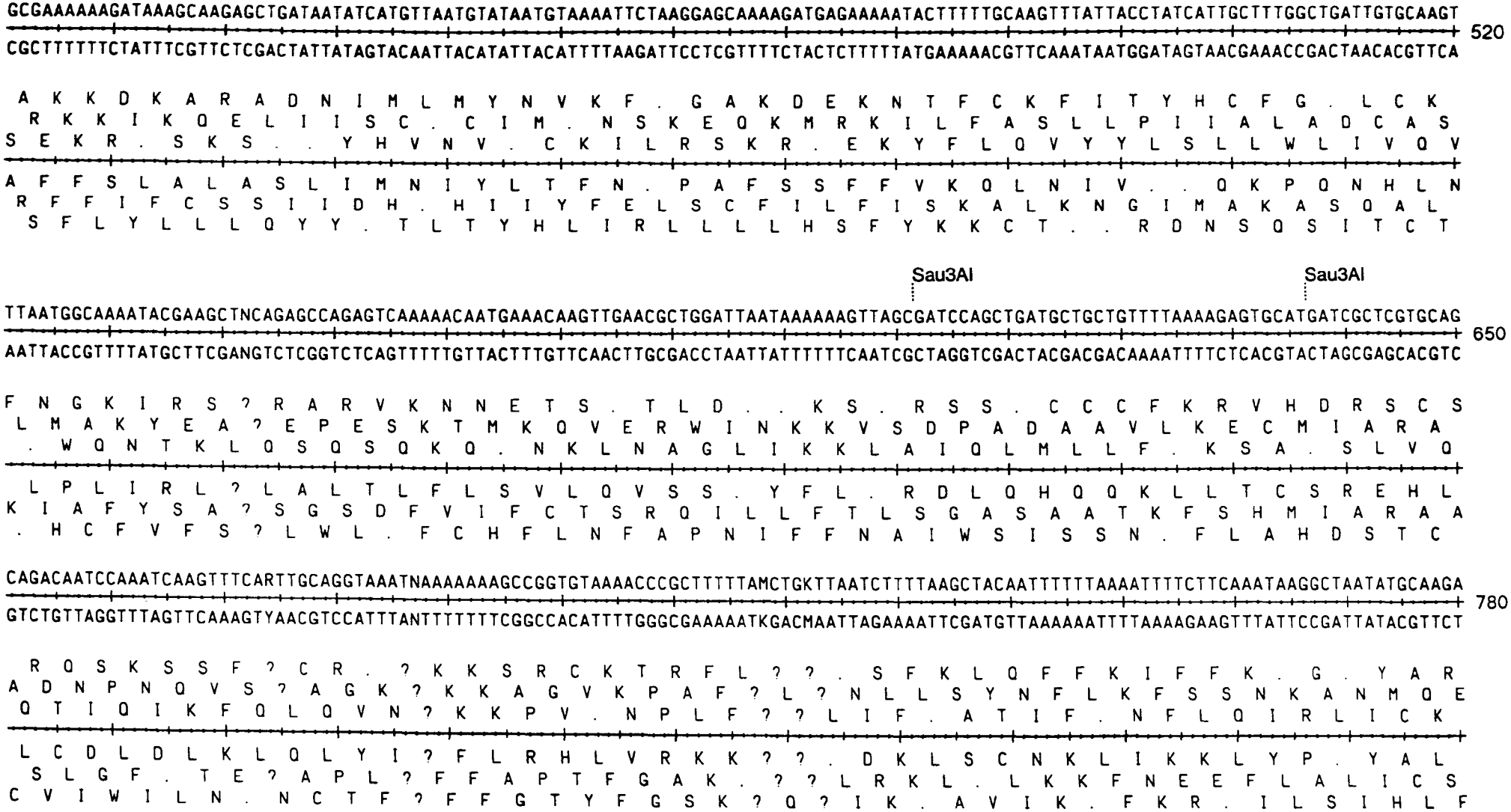
```
          1                                                  50
ScaA      ~~~TTFLGNP VTFTGKQLQV GDTAHDFSLT ATDLSKKTLA DFAGKKKVLS
SsaB      ~~~TTFLGNP VTFTGKQLQV GDTAHDFSLT ATDLSKKTLA DFAGKKKVLS
FimA      ~~~ATFLGNP VTFTGSQLQV GEIAHDFSLI TPALEKKSLA DFAGKKKVLS
Etpx      SQTVHFQGNP VTVANSIPQA GSKAQTFTLV AKDLSDVTLG QFAGKRKVLN
ToxR      ~MTVTLAGNP IEVGGHFPQV GEIVENFILV GNDLADVALN DFASKRKVLN
TagD      ~MTVTFQNNP VSISGSFPKV GDRLPSFTLC GADLNDLNNE DFKGKKIVMS
Ctpx      MSIVNFKGNP VKLKGNSVEV GADAPKVNLK AKDLSVIEIG AAG.KTQIIL
               - -++ -- --    - +     - +  - - +-- -  --  +- -

          51                                                 100
ScaA      IIPSIDTGVC STQTRRFNQE LSDLDNTVVI TVSVDLPFAQ GKWCAAEGIE
SsaB      IIPSIDTGVC STQTRRFNQE LSDLDNTVVI TVSVDLPFAQ GKWCAAEGIE
FimA      IIPSIDTGIC SMQTRHFNKT LSDLEDTVVL TVSVDLPFAQ GKWCAAEGLD
Etpx      IFPSIDTGVC AASVRKFNQL ATEIDNTVVL CISADLPFAQ SRFCGAEGLN
ToxR      IFPSIDTGVC ATSVRKFNQQ AAKLSNTIVL CISADLPFAQ ARFCGAEGIE
TagD      IFPSIDTPVC SKSVKVLQNA LMTRSDTVLL CVSADLPFAM SRFCTEHAVA
Ctpx      SVPSLDTPVC ATEAREFNKK VASYNGAEVI VVSMDLPFAM GRFCSTEGIE
          --++-++--+     - --         ----   + +++++-    +  --

          101                                                150
ScaA      NAVMLSDYFD HSFGRDYAVL INEW...HLL ARAVLVLDEN NTVTYAEYVD
SsaB      NAVMLSDYFD HSFGRDYAVL INEW...HLL ARAVLVLDEN NTVTYAEYVD
FimA      NAIMLSDYYD HSFGKAYGLL INEW...HLL ARAVLVLDAD NKITYVEYLD
Etpx      NVITLSTFRN AEFLQAYGVA IADGPLKGLA ARAVVVIDEN DNVIFSQLVD
ToxR      NAKTVSTFRN HALHSQLGVD IQTGPLAGLT SRAVIVLDEQ NNVLHSQLVE
TagD      NVTNASFFRE PAFTERFGVN LNEGALRGLA ARAVIVADEF GVITHSELVN
Ctpx      NLSVASDFVA KEFGEKYGVL INEGALEGLL ARAVFVIKE. GKVAYKELVN
          +    + -     -          - - - -+  -+++ +       -    --

          151                        177
ScaA      NINTEPDYDA AIAAVKNL~~ ~~~~~~~
SsaB      NINTEPDYDA AIAAVKSL~~ ~~~~~~~
FimA      NINSEPNYDA AIEAVKVLG~ ~~~~~~~
Etpx      EITTEPDYEA ALAVLKA~~~ ~~~~~~~
ToxR      EIKEEPNYEA ALAVLA~~~~ ~~~~~~~
TagD      EITNEPDYDR ILMSL~~~~~ ~~~~~~~
Ctpx      EITEMPDIAK LDAFFGGSSC CGGCGCH
          -+   ++-
```

The Cys-94, which is highly conserved is shown in red. Symbols: +, position is perfectly conserved; -, position is well conserved. Abbreviations: ScaA, SsaB, FimA, adhesion proteins from *S. sanguis*, *S. gordonii* and *S. parasanguis*, respectively; Etpx, *E. coli* thiol peroxidase; ToxR, regulon from *H. influenza*; TagD, TagD protein from *V. cholerae*, Ctpx, *C. jejuni* thiol peroxidase taken from the genome sequence.

DNA sequence of clone A6



DNA sequence of clone A6



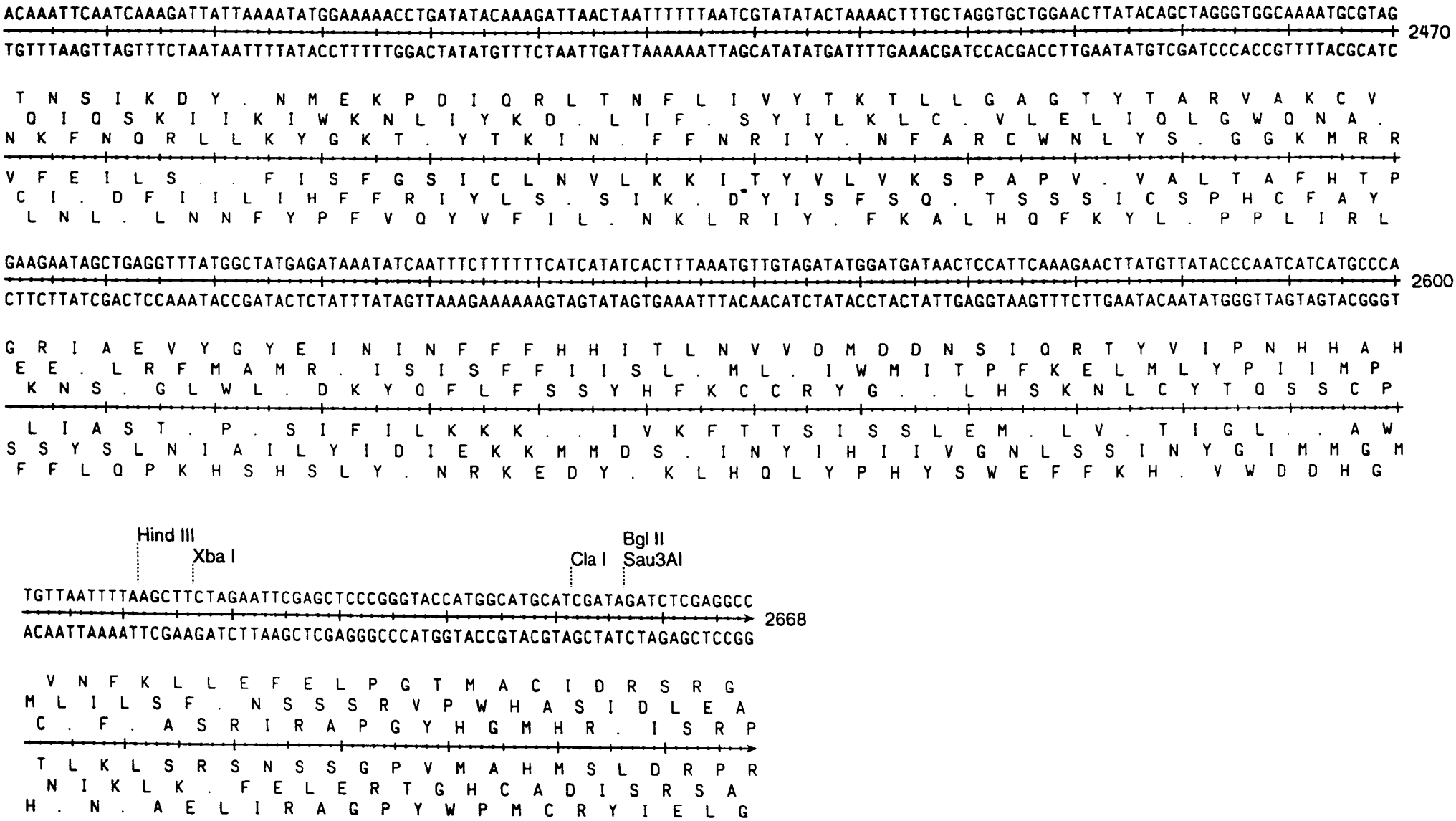
DNA sequence of clone A6

DNA sequence of clone A6

DNA sequence of clone A6

DNA sequence of clone A6

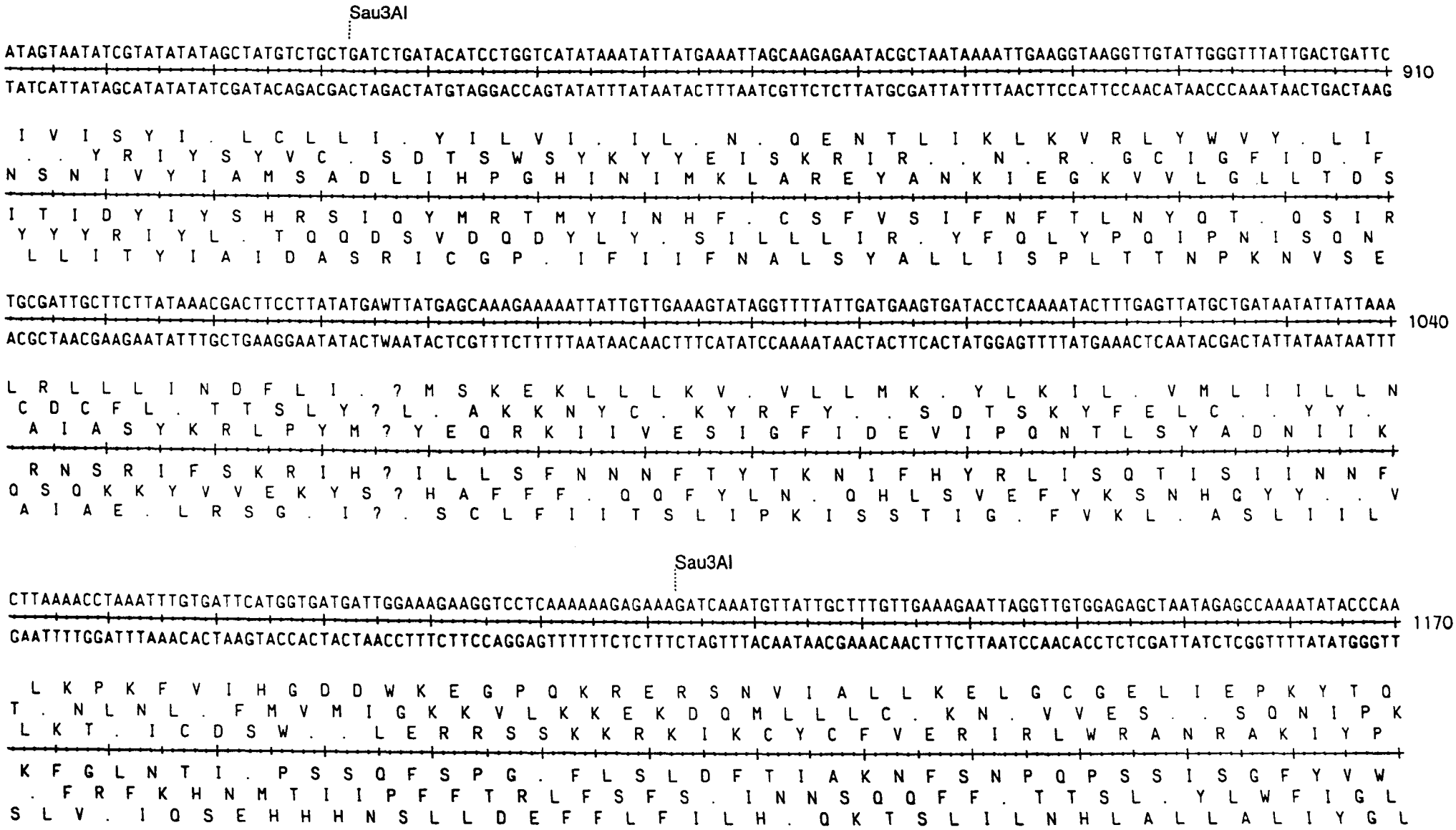
DNA sequence of clone A6



DNA sequence of clone A7

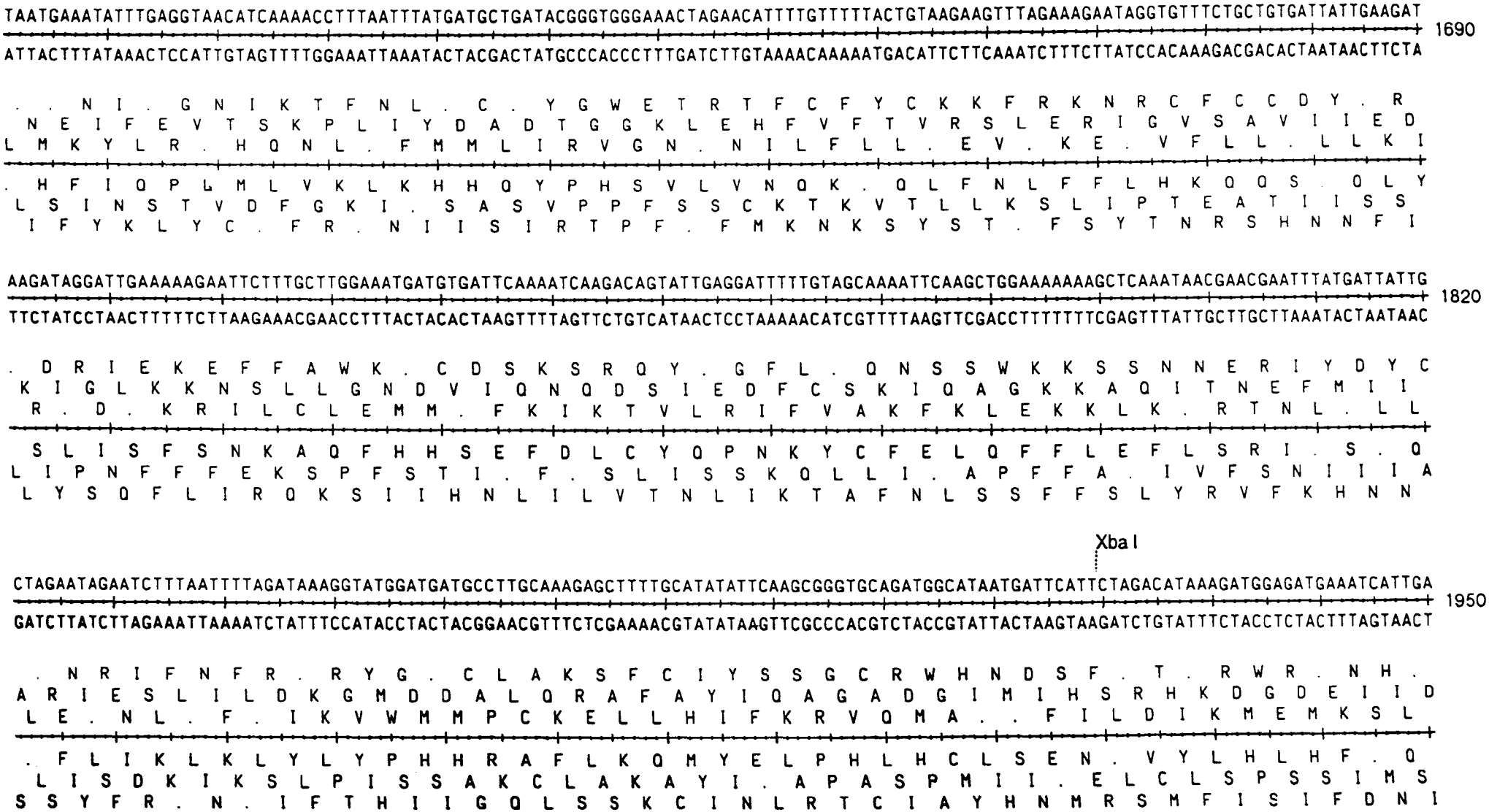
DNA sequence of clone A7

DNA sequence of clone A7

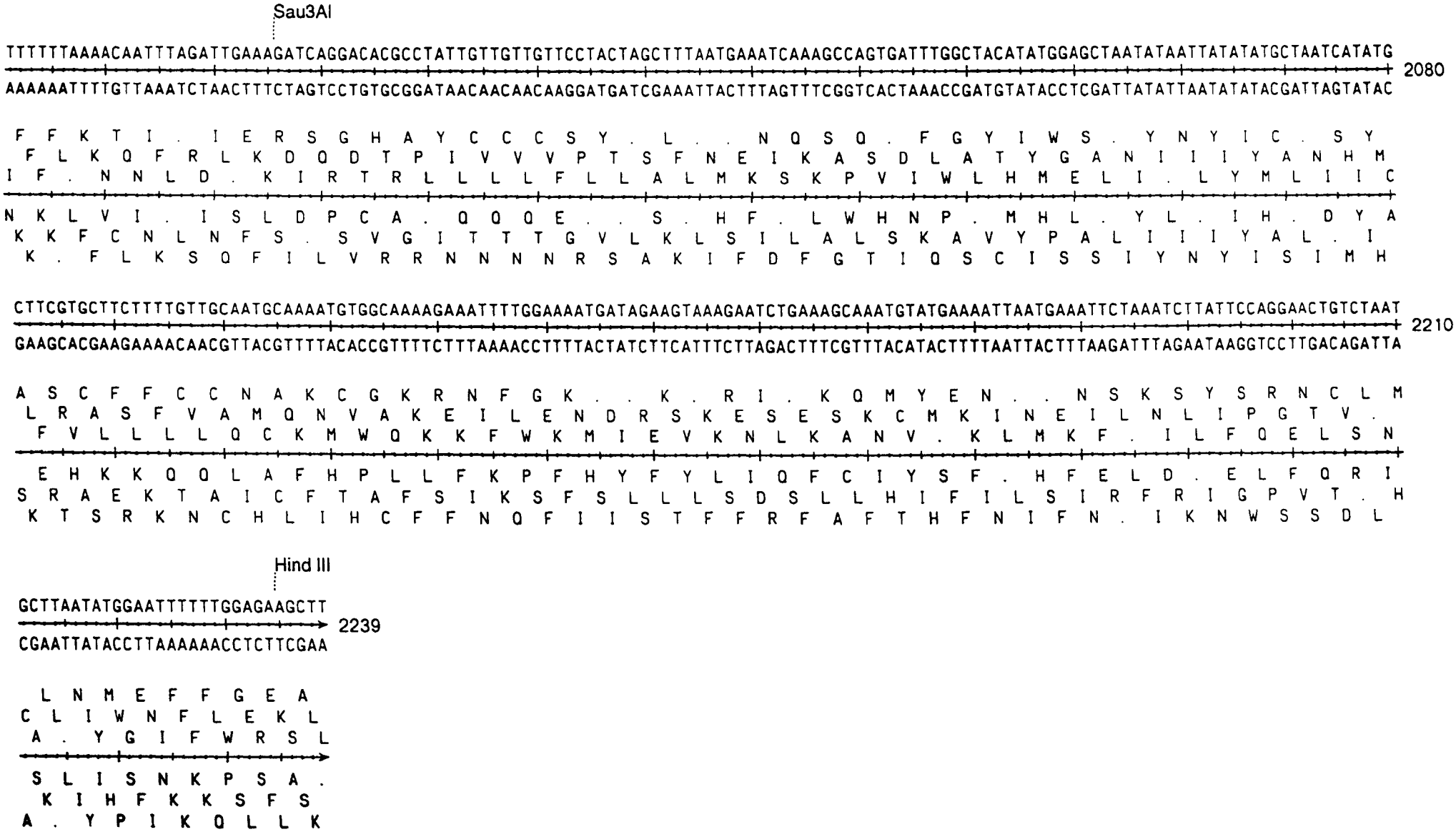


DNA sequence of clone A7

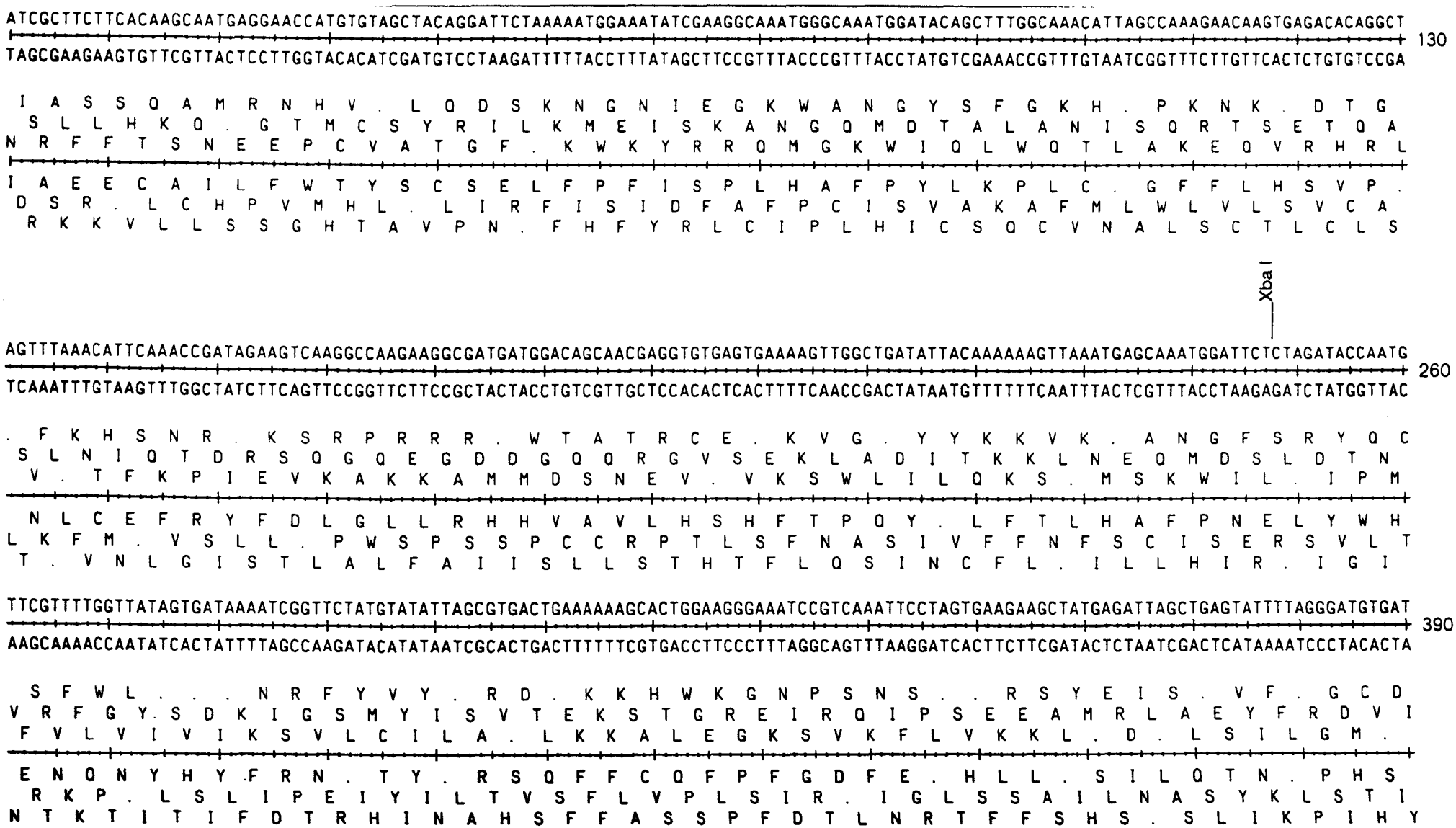
DNA sequence of clone A7



DNA sequence of clone A7

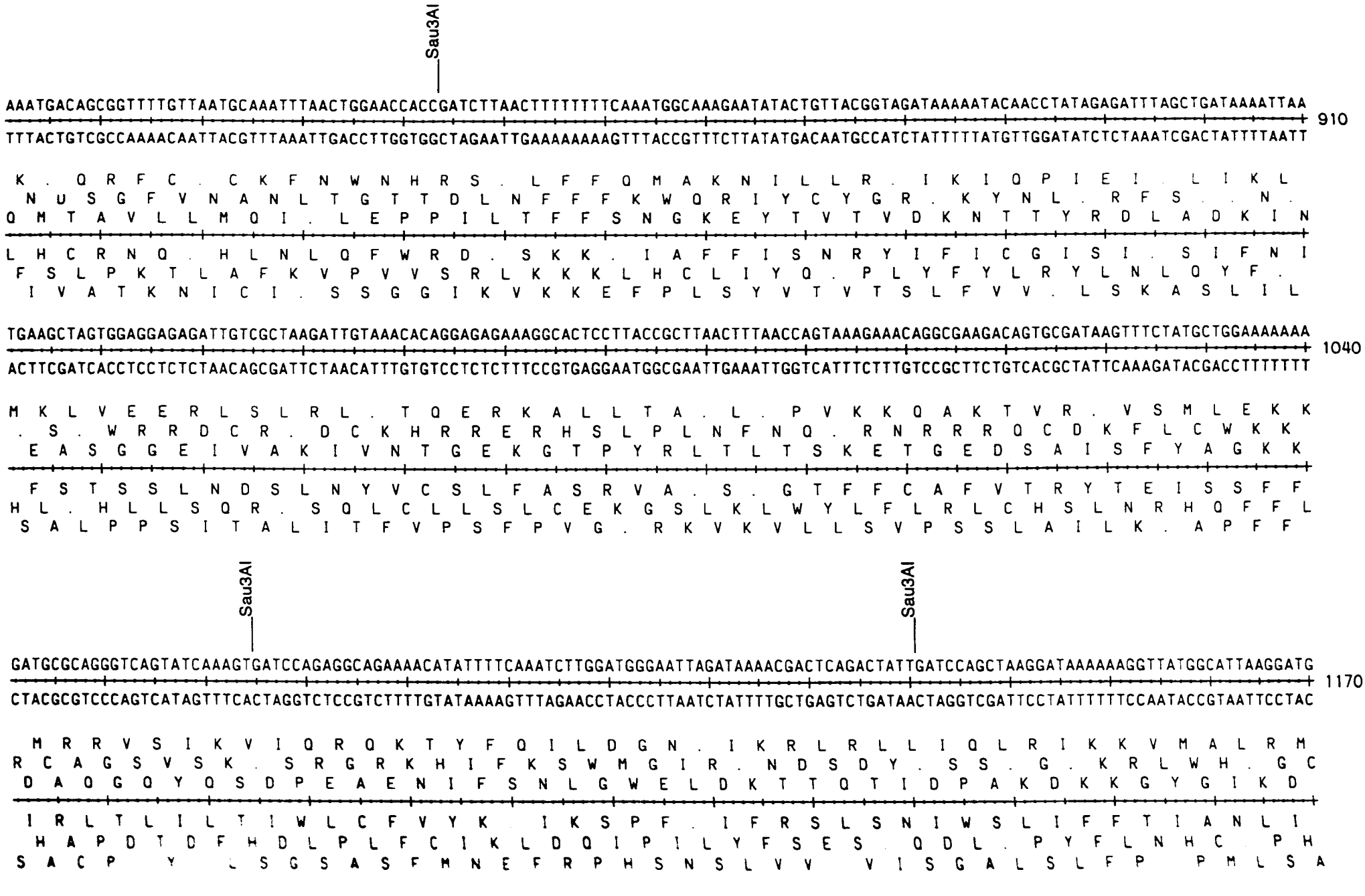


DNA sequence of clone G5

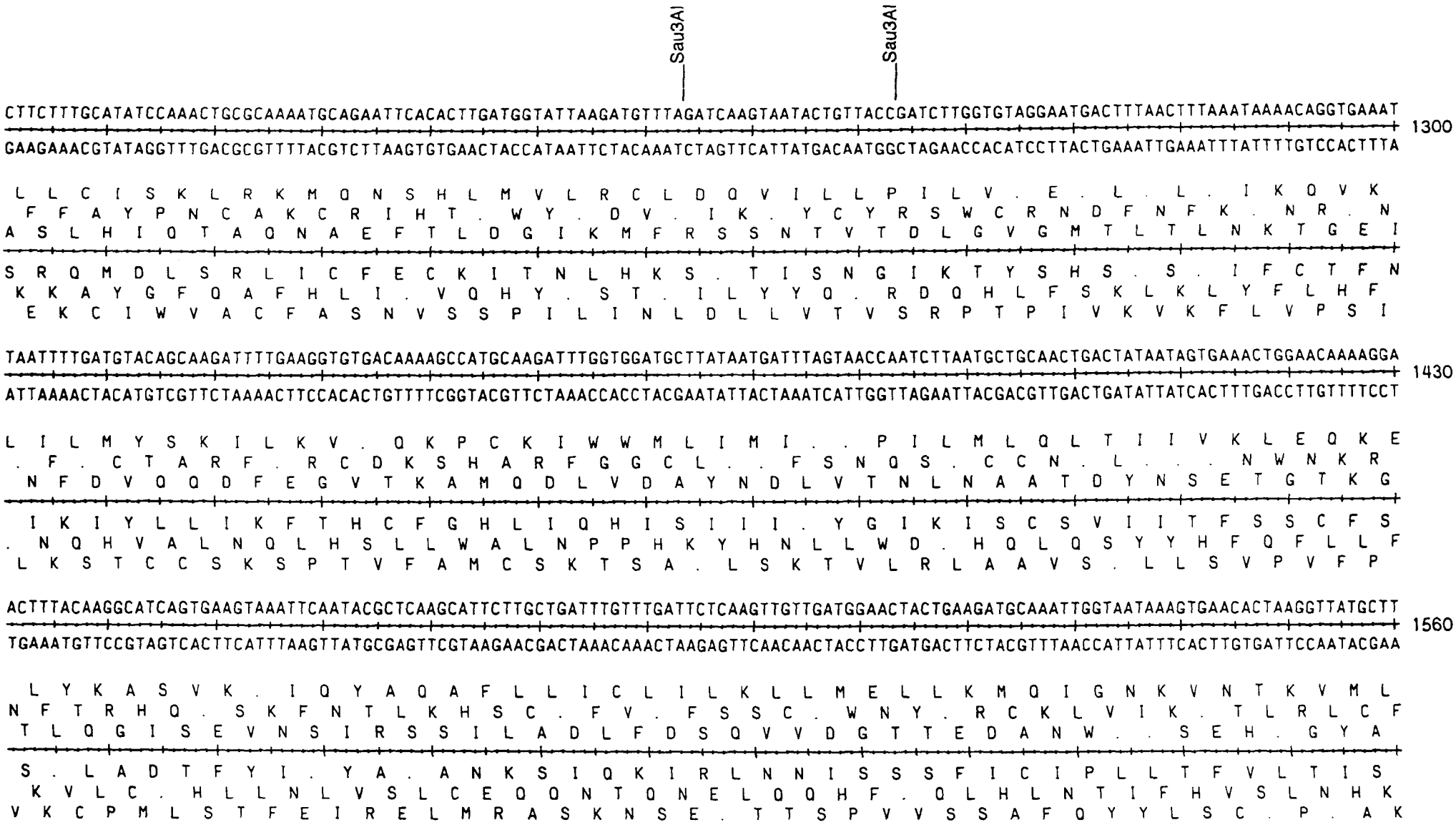


DNA sequence of clone G5

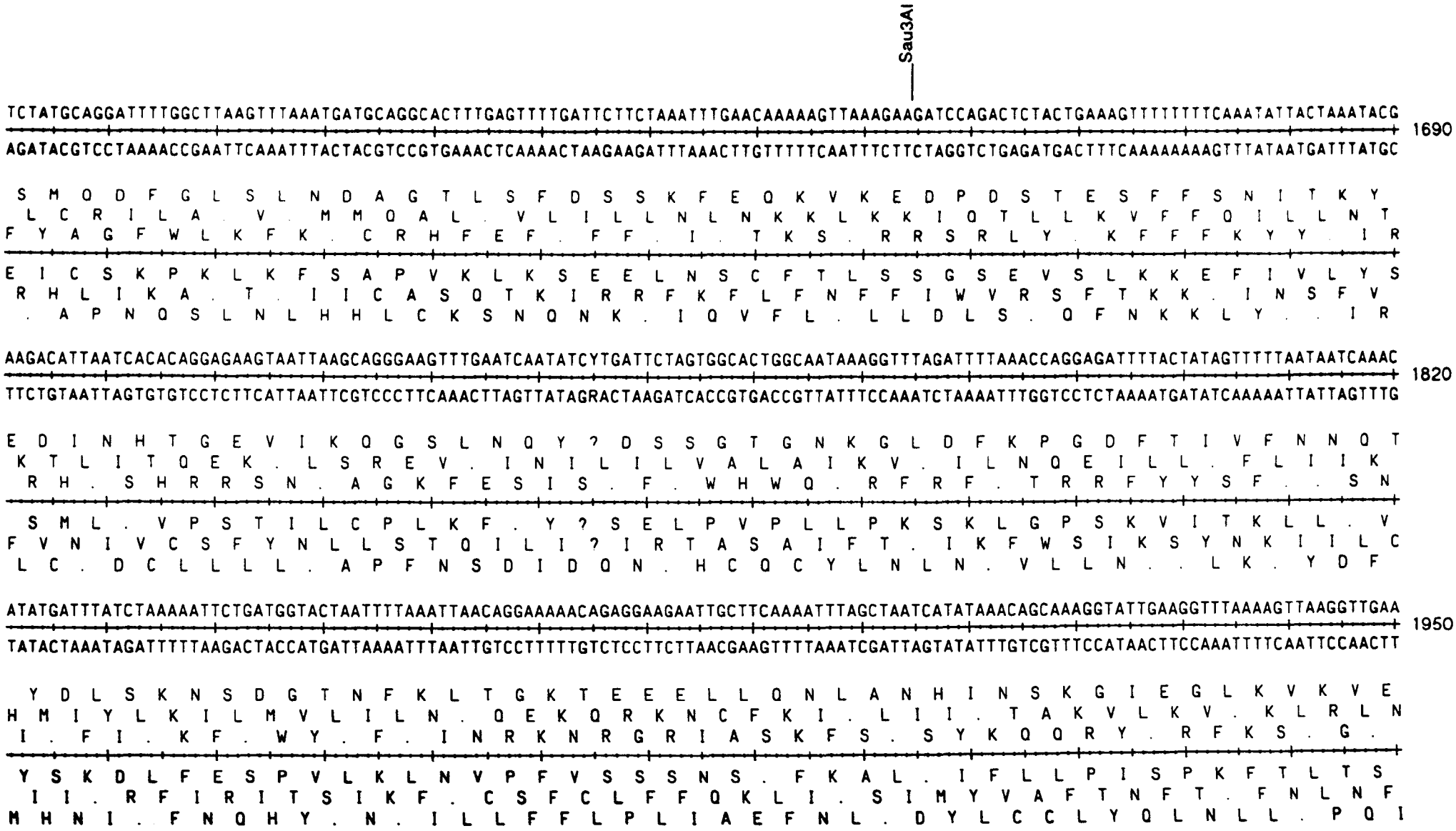
DNA sequence of clone G5



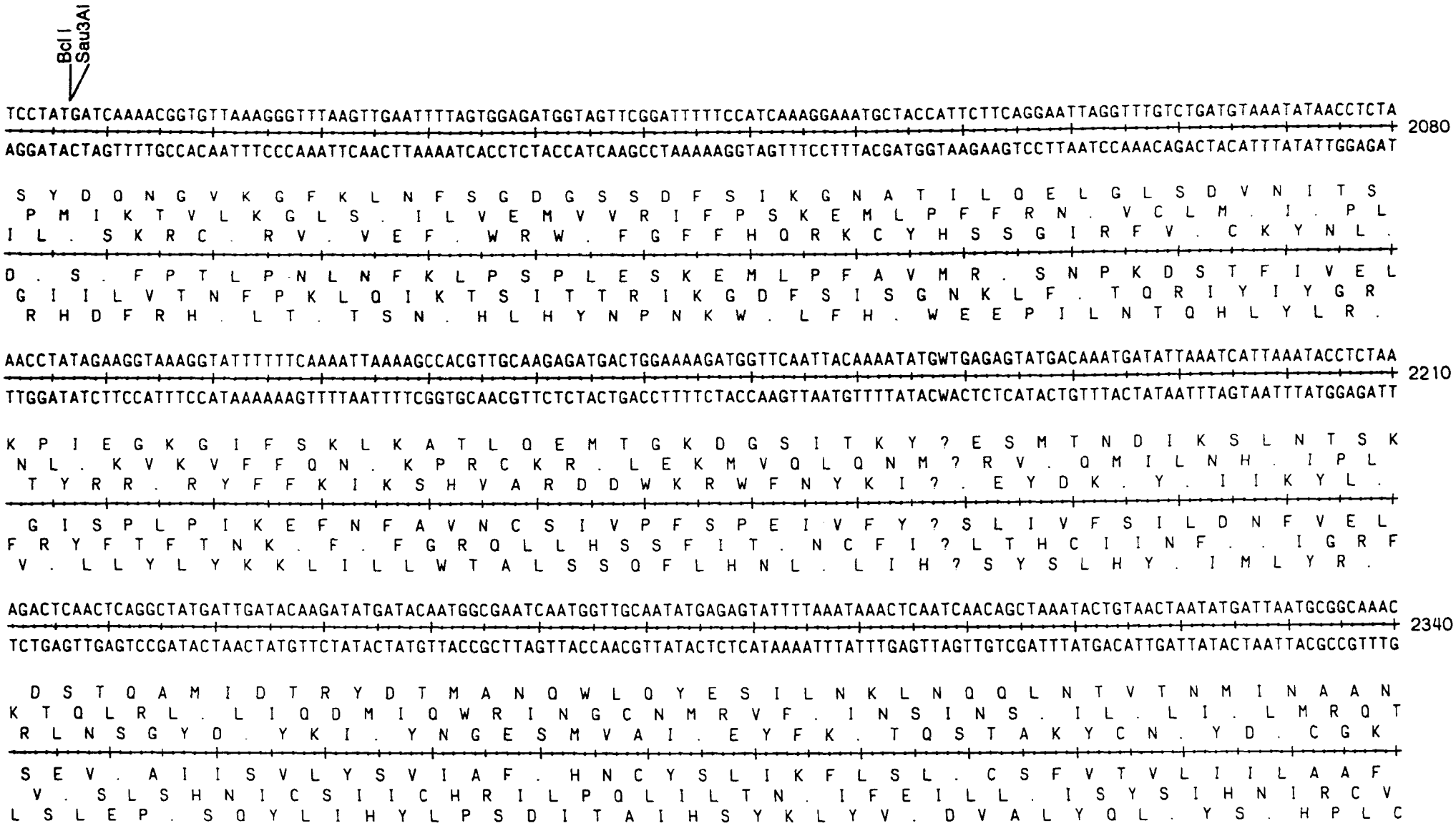
DNA sequence of clone G5



DNA sequence of clone G5



DNA sequence of clone G5



DNA sequence of clone G5

DNA sequence of clone G5