

Molecular Genetic Characterisation of the
Campylobacter jejuni htrA-regX/Y Locus

A thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Michael James Emery B.Sc. (Hons),
Department of Genetics
University of Leicester

December 1998

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Molecular Genetic Characterisation of the *Campylobacter jejuni* *htrA-regX/Y* Locus

Michael James Emery

Abstract

Campylobacter jejuni is a major cause of gastrointestinal illness throughout the world. The molecular mechanisms underlying *Campylobacter* pathogenesis are poorly understood due to the limited amount of data regarding which virulence determinants are involved. Like other bacteria, *C. jejuni* will have a coordinated pattern of gene regulation in order to adapt to the stresses in the external environment. HtrA is involved in the adaptation to such environmental stresses and has been identified in a variety of organisms, including other bacterial pathogens. In *E. coli*, *htrA* is regulated, in part, by a two-component regulatory system (TCR) consisting of a histidine protein kinase (HPK; CpxA) and a response regulator (RR; CpxR).

Previous work by Henderson (1996), identified a sub-genic fragment (473 bp) with a partial open reading frame (ORF) homologous to *htrA*, but mutation of the *C. jejuni* gene failed to identify a phenotype. The ORF was mapped to a 2.38kb *Bgl*III fragment but only the downstream portion which included 3' *htrA* sequence could be cloned. The 5' end of a RR was identified downstream of *htrA* and showed similarity to members of the OmpR sub-family, including *cpxR* from *E. coli*. The objective of the work described in this thesis was to characterise the 5' region of *htrA*, to determine the level of expression and establish if this expression is regulated by the downstream TCR.

This study focused on both the *htrA* gene and the presence of a putative two-component regulatory system downstream because of the possible involvement of the TCR system in regulating *htrA* in *C. jejuni*. Genetic analysis revealed 5' sequence data of *htrA* including the promoter region and the sequence confirmed the protein as a member of the family of *htrA* heat shock proteases. Analysis of the promoter region failed to indicate the type of regulation which the gene is under but it was established that *htrA* gene is expressed under normal growth conditions, but not in response to temperature.

A two-component system designated, *regX4/regY4* (RR/HPK), was identified downstream of *htrA* and shares basic amino acid sequence features with the orthodox *E. coli* *ompR/envZ* system. Characterisation of the putative *regX4/regY4* system by mutational analysis was difficult as all efforts to create a *regx4* mutant were unsuccessful although a *regY4* mutant was readily obtained allowing preliminary phenotypic analysis to be performed. It remains to be established whether the putative *regX4/regY4* TCR system regulates *htrA* expression in *C. jejuni* although mechanisms are in now in place whereby any possible regulatory relationship can be investigated.

Acknowledgements

Firstly, I would like to thank my supervisor Jules for his friendship, expert supervision, support and encouragement whilst in the lab, and also thank him for reading the “mammoth”. Furthermore, I would like to acknowledge the assistance of many members of the lab, past and present, and thank them for making my time here a more than pleasurable experience; Anne, Karl, Richard, Martin, Ana, Rob, Sue, Jo, Jo, John, Vicky, Wanda, Jane, Mark, Alison, Arjan, Barmy Arnie, cheers for the endless curry and beers, but not the mornings after and Neil, for keeping me topped up with caffeine to avoid me drifting into a coma.

I have been fortunate to have made some excellent friends whilst being at Leicester and they certainly deserve a mention, especially as they are the sole reason why I have retained some sanity whilst being here. In addition to those above, are Tony and Jo, Colin, Rob, Bill (Café Bill’s is always open) and Tracy and Neale; thanks for room when I really needed it. A special thank-you must also go to Sam for her endless support and friendship over the past few years. Cheers everyone.

Finally, I would like to thank my parents and sister for their support and encouragement over the past four and a bit years.

For financial support I acknowledge the University.

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Glossary

The following abbreviations are used in this thesis.

A	Adenine
A (mA)	Ampère (milliAmpère)
aa	Amino acid residue
Amp	Ampicillin
Amp ^R	Resistant to ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
Cm	Chloramphenicol
Cm ^R	Resistant to chloramphenicol
CO ₂	Carbon dioxide
CSA	Campylobacter selective agar base
CTAB	Hexadecyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
EDTA	Disodium ethylenediaminetetraacetate
EtBr	Ethidium Bromide
F	Faradays
g	Angular velocity as multiples of the acceleration due to gravity ($g=9.8 \text{ m.s}^{-2}$)
g	Grams
G	Guanine
h	Hours
HPK	Histidine protein kinase
H ₂ O	Water
IPCR	Inverse polymerase chain reaction
IPCRM	Inverse polymerase chain reaction mutagenesis
J	Joules
K	Kilo (10^3)

kb	Kilobase pairs
kDa	KiloDaltons
Km	Kanamycin
Km ^R	Resistant to kanamycin
L	Litre(s)
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
μ	Micro (10 ⁻⁶)
m	Milli (10 ⁻³)
M	Mole (s)
Mbp	Megabase pairs (10 ⁶ bp)
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
min	Minute(s)
M _r	Molecular Weight
n	Nano (10 ⁻⁹)
OD ₆₀₀	Optical Density at a wave length of 600 nm
ORF	Open reading frame
p	Pico (10 ⁻¹²)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RR	Response regulator
sec(s)	Second(s)
ssDNA	Single stranded DNA
T	Thymine
TCR	Two-component regulatory system
tRNA	Transfer RNA
UV	Ultraviolet
V	Volts
VAIN	Variable atmosphere incubator
v/v	Volume per volume
w/v	Weight per volume

Chapter 1: General introduction

1.1: Background

Members of the genus *Campylobacter* are now recognised as an important cause of acute diarrhoeal disease throughout the world. There are currently 19 species and subspecies, although the exact number is subject to change due to alterations in the classification of new members (Skirrow and Blaser, 1992; Nachamkin *et al.*, 1998). The most important human pathogens within the genus are *Campylobacter jejuni* and *C. coli* which derive their name from the initial association with enteric disease in animals (Jones *et al.*, 1931; Doyle, 1948). *C. jejuni* is responsible for the majority of enteric infections (80-90%) with most of the remainder due to *C. coli* although *C. upsaliensis*, *C. hyointestinalis* and *C. lari* can also infect humans (table 1.1.). *C. jejuni* is recognised as a major cause of food borne gastrointestinal disease throughout many industrialised countries and the increasing frequency and nature of the disease result in *C. jejuni* being of considerable social and economic importance (Tauxe, 1992; ACMSF, 1993) and therefore a matter of public and government concern.

Table 1.1: *Campylobacter* species of clinical importance (adapted from Butzler *et al.*, (1992))

Species	Site isolated	Pathogenicity
<i>Campylobacter jejuni</i>	Faeces	Acute enterocolitis (80-90% of all reported cases of <i>Campylobacter</i> infections)
<i>Campylobacter coli</i>	Faeces	Acute enterocolitis (the second most common causative agent of <i>Campylobacter</i> infections)
<i>Campylobacter lari</i>	Faeces	Acute enterocolitis
<i>Campylobacter upsaliensis</i>	Faeces	Acute enterocolitis
<i>Campylobacter fetus subsp fetus</i>	Blood, various other body fluids	Systemic infection in the immunocompromised patient

Historically, the first reports of enteritis associated with campylobacters were made in the late 19th century in several articles by Ehrlich and other, predominantly German, scientists around at this time (Goossens and Butzler, 1992). Due to their morphological similarity, campylobacters were first placed in the family, *Vibrionaceae*, following their initial description by McFadyean and Stockman (McFadyean and Stockman, 1913). The first example described was probably that of the type species *C. fetus*, (formerly *Vibrio fetus*) which causes foetal and reproductive tract infection and abortion in animals (Mishu *et al.*, 1992). After more extensive research they were assigned their own genus, *Campylobacter* (Sebald and Veron, 1963) due to their different growth requirements, inability to utilise

‘sugars’ and C&G base ratio. (Veron, 1973). Initially, campylobacters were almost exclusively considered as veterinary pathogens (Cover and Blaser, 1989; Mishu *et al.*, 1992). Their importance as human pathogens was only identified about 25 years ago when they were first recognised in the stools of humans with acute enterocolitis. The presence of some *Campylobacter* species as gut inhabitants was suspected previously (Levy, 1946; King, 1957), although went undetected as clinical laboratory techniques at this stage were not suitable for the isolation of the organisms. Furthermore, until the development of selective stool culture techniques in 1972 their importance was not fully appreciated (Dekeyser *et al.*, 1972; Butzler, 1973; Skirrow, 1977).

1.2: *Campylobacter* biology

Campylobacters are slim (1.5-6.0 μm long and 0.2-0.5 μm wide) spirally curved Gram negative rods (Ketley, 1997). It is from these two features that the genus derives its name; from the Greek words “campylo” meaning curved and “bacter” meaning rod (Griffiths and Park, 1990). Under certain adverse environmental conditions or upon ageing, cells may develop a coccoid shape. A great deal of interest has been given to this aspect of *Campylobacter* physiology and its role in transmission as this change has been associated with the transformation from a viable culturable form to a viable but non-culturable (VNC) state (Rollins and Colwell, 1986). *C. jejuni* is non-sporulating although it has been suggested that the VNC form corresponds to a dormant-like stage, enabling bacteria to survive adverse conditions (Ketley, 1995; 1997). The organisms are oxidase positive and possess a polar flagellum at one or both ends of the cell which imparts a high degree of motility on the cell, aided by its spiral morphology and unique “corkscrew-like” motion. Most species are microaerophilic, requiring an O_2 concentration between 3 and 15%, a CO_2 concentration between 3 and 5% and 85% N_2 concentration (Bolton and Coates, 1983), but some are aerotolerant (Griffiths and Park, 1990). There are a wide range of optimal temperatures at which campylobacters grow. For example, *C. jejuni* and *C. coli* are thermotolerant and grow most favourably at 42°C reflecting their adaptation to temperatures found in their normal habitat, the intestine of warm blooded animals and birds. Other species such as *C. fetus* grow at 25°C and 37°C, *C. cinaedi* at 37°C but not at 25°C or 42°C and *C. cryoaerophila* grows at 15°C. Unlike many enteric pathogens, campylobacters are unable to ferment or oxidise

carbohydrates. Their metabolism is of a respiratory nature which utilises amino acids and intermediates from the tricarboxylic acid cycle (Penner, 1988).

1.3: Epidemiology of infection

The epidemiology of *C. jejuni* infection varies throughout the world. In industrialised countries the pattern appears to be more sporadic than epidemic, although common source outbreaks occur. Sporadic cases relate to the consumption of meat, particularly poultry, whereas outbreaks are usually milk or water-borne (Skirrow and Blaser, 1992; Taylor D.N., 1992). The origin of infection is rarely identified, especially for those cases which are sporadic (Ketley, 1995; 1997). For countries in the developed world, campylobacteriosis is a major public health problem (Tauxe, 1992; ACMSF, 1993). Recently in the UK, the incidence of campylobacteriosis has risen dramatically. This could be for a number of reasons; a change in people's eating habits i.e., an increase in the popularity of poultry and the growing trend towards eating in "fast-food" establishments where hygiene standards may be low or where breakdown in control measures has significant consequences (Griffiths and Park, 1990). Furthermore, it may reflect more frequent identification, or reporting of the disease. Despite the increase the number of cases given at any one time it is likely that it will still only be an under-estimate of the total in the general population (Tauxe, 1992).

Despite the increase in *Campylobacter* associated enteritis worldwide, the pattern of infection differs (table 1.2.). In the developed world most cases are sporadic and affect mainly babies less than 4 months old with a second major peak of isolation occurring in young adults between the ages of 20-29 (Skirrow *et al.*, 1987). In adults there is a consistent but as yet unexplained higher incidence of infection in males than in females but after the age of 45 the rate becomes similar (Ketley, 1997). There is also an obvious pattern of seasonal variation in temperate climates as the UK shows the incidence in summer to be twice that of winter after a characteristic rise in late spring (Skirrow and Blaser, 1992). In developing countries on the other hand, *Campylobacter* enteritis is much more common but the clinical characteristics are different. An symptomatic infection predominates and affects young children with no apparent peak in adults. There is no seasonality typical of that seen in industrialised countries and a prevalence of multiple infections complicated with a higher rate of asymptomatic carriage. On the whole, the disease is a lot milder and manifests as a watery, non-inflammatory diarrhoea. The high rate of asymptomatic carriage in developing countries

raises questions about the relative virulence properties of strains from these regions (Blaser *et al.*, 1983b; Black *et al.*, 1988; Taylor D.N., 1992). The view is, that strain differences seen in developing and developed countries might explain the pattern of epidemiology and clinical disease found in these regions. In some cases this may be true. However, in other instances, travellers visiting developing countries still develop *C. jejuni* enteritis with symptoms similar to that seen in developed countries.

Table 1.2: Features of *C. jejuni* infections in developed countries compared with those in developing countries (adapted from Blaser, (1997)).

Feature	Developed countries	Developing countries
Average number of infections/lifetime	0-1	>5
Principal age group affected	Young adults	Children <2 years old
Principal manifestation of illness	Inflammatory diarrhoea	mild, non-inflammatory diarrhoea
Widespread immunity amongst adults	Absent	Present
Principal vehicle	Food	Unknown

Furthermore, in these examples there are no differences in biochemical characteristics between isolates from the two regions, and the serotypes identified are the same. Therefore, it follows that some strains isolated from developing countries may indeed be pathogenic and the differences observed between epidemiology and clinical disease are host specific. The underlying feature that distinguishes the clinical spectrum of disease between developing and developed countries is host immunity in the population from these two areas (Blaser *et al.*, 1983b).

1.4: Transmission of campylobacters to humans

Campylobacters form part of the normal intestinal flora of both wild and domestic animals (Penner, 1988) including animals used in the production of food such as cattle, poultry, and pigs where generally carried asymptotically (Blaser, 1997). As a result, the surface of carcasses can become contaminated by intestinal contents during processing in slaughter houses. Members of the genus have also been identified in members of the animal kingdom as different as flies (Wright, 1983) and turtles (Harvey and Greenwood, 1985). Furthermore, campylobacters can be carried by human pets such as cats and dogs (Skirrow, 1981; Sandstedt *et al.*, 1983) and birds (Skirrow and Benjamin, 1982). Due to the widespread nature of campylobacters throughout the animal kingdom there is a great potential to contaminate both

surface water and soil. This enables transmission to humans by ingestion of contaminated foods of animal origin due to improper handling and/or cooking, consumption of untreated, contaminated water or by direct contact with infected animals, particularly pets. Human to human transmission is rare (Blaser, 1997). The majority of cases of infection due to *Campylobacter* spp. are sporadic and outbreaks are rarely identified. Outbreaks attributed to campylobacters are usually due to the ingestion of unpasteurized milk (Warner, 1986), sewage contamination (Taylor D.N. *et al.*, 1983) or undercooked poultry and other foods (table 1.3.). (Blaser *et al.*, 1983b). Despite this, campylobacteriosis is not considered a consequence of food-poisoning but as a food-borne disease due to the inability of *C. jejuni* to grow below 30°C and therefore multiply on food (Park *et al.*, 1987). It follows that infection depends on the initial gross contamination of the ingested source (Skirrow, 1990). There is geographic variation in transmission characteristics. In developed and developing countries, infection is largely as a result of consumption of contaminated food, most notably, poultry. In the poorest regions, infection is usually the result of contact with domestic animals (Taylor D.N., 1992).

Table 1.3: Principal modes of transmission of *C. jejuni* to humans, by epidemiological setting. (adapted from Blaser, (1997)).

Outbreaks	Sporadic infection
Ingestion of unpasteurised milk	Ingestion of undercooked poultry
Ingestion of contaminated water from a municipal supply	Ingestion of contaminated surface water
Ingestion of undercooked poultry	Ingestion of unpasteurised milk and milk products
	Contact with pets
	Contact with infected persons
	Travel to developing countries

1.5: Clinical features of infection by *Campylobacter* spp.

The clinical consequence of infection is acute abdominal pain often associated with fever and general malaise which progresses to profuse, watery and frequent diarrhoea (Butzler and Skirrow, 1979; Walker *et al.*, 1986). The symptoms are only present in about half of infected patients with the time of onset depending on the incubation period. Usually this ranges between 1 and 7 days although the exact source of infection and therefore exact timing is difficult to establish. In a majority of cases the disease is self-limiting and restricted to a period of 5-8 days, but it occasionally continues longer. In adult volunteer studies with *C. jejuni* to establish the infective dose required to initiate disease, it was discovered that as few as 500 organisms could initiate illness in some volunteers, whereas in others doses of upto 1

$\times 10^9$ were required. Rates of infection were seen to increase with dose but no clear relationship could be shown (Robinson, 1981; Black *et al.*, 1988). In all cases of infection which progress to the diarrhoea stage, stools often contain blood, mucus and faecal leukocytes. Infection can also cause a range of intestinal conditions which vary from changes in the mucosa to colitis, involving loss of mucus, epithelial ulceration, crypt abscesses in the epithelial glands, haemorrhagic lesions and inflammation and oedema of the full thickness of the intestinal wall (Blaser *et al.*, 1980). Although campylobacteriosis can result in severe illness, complications are not common. Reported extra-intestinal complications of the disease include cholecystitis (Darling *et al.*, 1979), Guillain-Barré syndrome (Kaldor and Speed, 1984), erythema nodosum (Galeazzi *et al.*, 1986), appendicitis (Morlet, 1986); meningitis (Goossens *et al.*, 1986), haemolytic-uraemic syndrome (Chamovitz, 1983), reactive arthritis (Butzler *et al.*, 1992) and bacteraemia (Walder, 1982)

1.6: Diagnosis of *Campylobacter* infection

Symptoms present with *Campylobacter* infection can often resemble other types of disease and can vary in severity. For example, a mild infection might resemble viral gastroenteritis and a more severe episode can be indistinguishable from a *Salmonella*, *Shigella* or *Yersinia* induced enteritis (Blaser, 1989). Indeed, severe illness can sometimes be mistaken for appendicitis or ulcerative colitis (Blaser, 1989). Despite this, *C. jejuni* induced enteritis can be diagnosed by isolation of the organism from stool sample culture or occasionally from a different site and by selective filtration or by culturing the organism on selective media (Blaser and Reller, 1981; Kaijser and Megraud, 1992). Selective filtration enables isolation of all *Campylobacter* spp. based on size selection but is of low sensitivity (Kaijser and Megraud, 1992). Culture of the organism on selective media is more sensitive but only identifies a limited number of species, including the most important, *C. jejuni* and *C. coli*. Serology is also, but not routinely, as there is a long delay in waiting for convalescent serum (Blaser, 1989). This technique is used when culture negative cases are seen, in extraintestinal complications such as reactive arthritis (Butzler *et al.*, 1992). A rapid presumptive diagnosis can be made by visualisation of the organism in stools by Gram staining or by darkfield or phase contrast microscopy (Blaser, 1997).

1.7: Treatment of campylobacteriosis

Most infections are fairly mild and self-limiting so therapy usually consists of fluid replacement to prevent dehydration. Antibiotic therapy is not normally required. It is only in those situations where the disease is more severe, such as in the immunocompromised host, or where disease symptoms include bloody diarrhoea or fever, that antibiotics can be administered. The current antibiotic of choice is erythromycin but fluoroquinolones and tetracyclines can be used as alternatives (Blaser, 1997).

1.8: Prevention of *C. jejuni* infection

To prevent *C. jejuni* infection in humans the potential for transmission must be limited at source, for example from broiler chickens (Pearson *et al.*, 1993). To reduce contamination rates and to produce *Campylobacter*-free broiler stocks, there is a necessity to prevent or reduce infection whilst the birds are growing. Furthermore, control measures should concentrate on preventing contamination and cross-contamination of food or utensils used in the preparation. This is particularly important in the handling of raw and cooked meats and the washing of hands and all stages of food processing. Other measures include the purification of water, proper disposal of sewage and the pasteurisation of milk. The need to educate the public is of paramount importance in their adherence to such public health precautions.

In addition to the implementation of effective public health measures, a further possibility to minimise *C. jejuni* infection concerns the use of vaccination to control the disease. For the population of developed countries, education should prevent the majority of *C. jejuni* infections. Vaccination therefore, is not considered a worthwhile option. On the other hand it offers probably the most pragmatic method for effective disease prevention and control for high risk groups, such as young children in developing countries and for international travellers to developing countries (Scott, 1997). There is evidence that protective immunity can develop post-infection given the results from animal model studies (Pavlovskis *et al.*, 1991), experimental infection in humans (Black *et al.*, 1988) and the decline in the illness to infection ratio where *Campylobacter* enteritis is endemic (Taylor D.N., 1992). Therefore, the feasibility to produce a *Campylobacter* vaccine has been investigated.

The development of a vaccine may be complicated as the organism is very diverse and protective epitopes are not clearly defined. Additionally there are the problems associated with the ability of the organism to cause Guillain Barré Syndrome (GBS) and reactive arthritis (RA) post-infection which may cause some problems with the type of vaccine used (Scott, 1997). Nevertheless, several vaccine strategies have been considered. The use of a genetic approach is attractive although somewhat limited until information concerning the pathogenesis of infection is improved. The oral route of administration is the preferred route as this stimulates intestinal immunity, believed to be the most effective against enteric pathogens (McGhee, 1992). One proposal is the use of live attenuated vaccines. Mutants of *C. jejuni* defective in invasion or pili biosynthesis, which are non-virulent in the ferret model are being considered (Yao *et al.*, 1994; Scott, 1997). Together with an additional *recA* mutation, this would prevent reversion to wild type given that *C. jejuni* is a naturally transformable organism (Guerry *et al.*, 1994). Subunit vaccines have been suggested which can either be purified recombinant proteins or expressed in live carrier vaccine strains of *Salmonella* or *Shigella* species (Scott, 1997). Two *Campylobacter* antigens have been considered, flagellin and the protein, PEB1. Flagellin is an immunodominant antigen recognised during infection and may be involved in protection (Martin *et al.*, 1989). PEB1 is also highly immunogenic and conserved amongst *C. jejuni* isolates. It may also function as an adhesin to eukaryotic cells (Pei and Blaser, 1993; Pei *et al.*, 1998). A further target for a subunit vaccine are peritrichous pilus-like appendages whose expression is induced in the presence of several bile salts (Doig *et al.*, 1996b). The final option is to use killed whole cell vaccines which have an advantage for a variety of reasons. They are inexpensive and possess multiple protective antigens which can be important for prevention of *Campylobacter* infection. Furthermore killed whole cell preparations are generally safe for mucosal immunisation (Scott, 1997).

1.9: Pathogenesis of disease

1.9.1: Animal models

The exact pathogenic mechanisms of *Campylobacter* associated enteritis are unclear. Efforts have largely been hampered by the lack of a simple animal model and the inability for some models to mimic closely enough the clinical manifestations of *C. jejuni* pathogenesis. The standard animal models used for other enteric pathogens were found to be negative for *C.*

jejuni (Walker *et al.*, 1986). Therefore, efforts have been undertaken to identify a model which could be used to investigate the organism. To this end, a variety of different animal models have been evaluated. Presently, only humans (Black *et al.*, 1988), young Rhesus monkeys (Russell *et al.*, 1989) and weaning or young ferrets (Bell and Manning, 1990) are known to be naturally susceptible to *C. jejuni*-caused diarrhoeal disease. Nevertheless, other models include, congenitally athymic (nude) mice in a germ free condition (Yrios and Balish, 1986), preconditioned hamsters whose intestinal tract has been flushed with saturated magnesium sulphate (Humphrey *et al.*, 1985), the removable intestinal tie adult rabbit diarrhoea (RITARD) model (Caldwell *et al.*, 1983), oral feeding of *Campylobacter* organisms to adult mice (Blaser *et al.*, 1983a), mice pre-treated with iron (Stanfield *et al.*, 1987) and challenge of infant mice (Abimiku and Dolby, 1987). Furthermore, the chicken can be used as a model for colonisation (Meinersmann *et al.*, 1991). However, none of these animals are seriously affected naturally by infection with *C. jejuni* without prior treatment or surgical procedures (Bell and Manning, 1990). Dogs and cats sometimes develop a severe form of *C. jejuni* induced diarrhoea naturally although not when infected experimentally (Prescott and Karmali, 1978). More recently, use of orally inoculated, colostrum-deprived newborn piglets with *C. jejuni* resulted in illness and pathological lesions similar to those seen in humans (Babakhani *et al.*, 1993). Mice challenged intranasally can also be used to study pathogenesis (Baqar *et al.*, 1996). Given the different variety of animals used and the fact that many require prior treatment before being the subject of analysis it is clear that there is not one simple, single system which surpasses the rest for the study of campylobacteriosis. Each has its advantages and disadvantages and information collected from different sources should be used together to answer questions concerning pathogenesis of the disease.

1.9.2: General characteristics of pathogenesis

The absence of a suitable animal model has meant that a lot of questions concerning the molecular mechanisms behind *Campylobacter* disease still remain unanswered. In common with other pathogens, the steps leading upto the clinical presentation of disease and the virulence determinants involved, will be multifactorial in nature. As a food borne pathogen, campylobacters must adapt to variety of situations such as those exhibited by food or water. To this end, they require a number of factors to survive and resist these physiological stresses and to promote successful transmission and infection. The possibility that campylobacters

may undergo a viable non-culturable transition in a stressful environment might be of significance in this case (Rollins and Colwell, 1986; Jones *et al.*, 1991).

Given that *C. jejuni* is a food borne pathogen the organism enters the body via ingestion of contaminated food or water, proceeds to the intestinal tract, multiplies and colonises the intestinal mucus. Experimental and clinical evidence has supported the idea that campylobacters adhere to mucosal surfaces, invade epithelial cells, initiate the inflammatory response and/or produce toxin(s). The resulting damage to epithelial cell function and perturbation of the normal absorptive capacity of the intestine causes the pathological symptoms seen in patients, most notably diarrhoea. *C. jejuni* can progress further and translocate across the intestinal epithelium and penetrate the lamina propria and mesenteric lymph nodes. The result is some of the extraintestinal complications associated with infection. In order to survive the organism must have the ability to resist host defenses. There is an apparent difference in the severity of illness which can be attributed to an individual's susceptibility to the organism and the relative virulence of the infecting strain. Both are important factors in the pathogenesis of *Campylobacter* enteritis (Black *et al.*, 1988).

1.9.2.1: Colonisation

Upon ingestion of food, the organism progresses to the host's intestine *via* the stomach. Once in the stomach it must overcome the hydrochloric acid barrier and proteolytic enzymes in order to progress and to colonise the intestinal mucosa of the distal ileum and colon. The intestinal mucosa is covered with a thick layer of mucus and the association with this mucus and subsequent attachment to the epithelial cells is believed to be the first steps for colonisation. Experimental evidence using a mouse caecal model has shown that *C. jejuni* colonises the intestinal mucosa and associates with intestinal mucus in both the mucus blanket and the mucus-filled crypts (Lee *et al.*, 1986). In support of these findings, and using scanning electron microscopy, *C. jejuni* was found to be present, on, in and below the mucus gel in the lower ileum of infected neonatal mice (Field *et al.*, 1981). The mucus layer is made up of a heterogeneous mixture of proteins, polysaccharides and high molecular weight glycoproteins which results in a highly viscous matrix in which the bacterium can become entrapped. Together with the fact that the mucus layer is awash with antibodies, notably secretory (sIgA), antibacterial agents such as lysozyme or lactoperoxidase and growth inhibitors, including the iron chelator lactoferrin, means that the environment is highly

unfavourable for the bacterium to colonise. In order to traverse the mucus layer to the epithelium and evade this seemingly hostile environment, motility is important. In such a high viscous environment, the spiral morphology of the bacterium is accentuated, motility is less random and the path length of individual cells is increased (Lee *et al.*, 1986; Ferrero and Lee, 1988). The importance of the flagellum in colonisation has been highlighted experimentally in a number of different animal models including mice (Morooka *et al.*, 1985; Newell *et al.*, 1985), rabbits (Caldwell *et al.*, 1983; Pavlovskis *et al.*, 1991) hamsters (Aguero-Rosenfeld *et al.*, 1990) and chicks (Wassenaar *et al.*, 1993b). The experiments have been performed using flagellar mutants which have been either isolated spontaneously (Newell *et al.*, 1985), chemically (Morooka *et al.*, 1985) or by site directed mutagenesis of the flagellar structural components (Wassenaar *et al.*, 1993b). It is therefore believed that the organism's microaerophilic growth requirements, spiral morphology, and high motility in viscous environments imparts an ecological advantage on the organism (Lee *et al.*, 1986; Ferrero and Lee, 1988). In addition to motility, effective colonisation requires chemotaxis. *C. jejuni* has the ability to detect concentration gradients of attractants such as mucin, L-serine and L-fucose and chemorepellants such as several bile acids (Hugdahl *et al.*, 1988). Experimentally, chemically mutagenised, non-chemotactic, fully motile mutants were unable to colonise the neonatal mouse intestinal tract (Takata *et al.*, 1992). Information regarding the molecular mechanisms behind colonisation is limited, although recently one of the regulatory components, *cheY*, has been identified (Marchant, J.E., 1998; Yao *et al.*, 1997). Marchant, (1998) has mutated this gene and discovered a loss of chemotaxis *in vitro*. Further, Yao *et al.*, (1997) demonstrated CheY acted as a repressor by effecting a non-adherent and non-invasive phenotype. Moreover, insertional inactivation of the *cheY* resulted in an increase in adherence and invasion *in vitro*, but the strain was unable to colonise or cause disease in animals (Yao *et al.*, 1997).

1.9.2.2: Adherence

Following colonisation, the bacterium adheres to epithelial cell surfaces to facilitate entry into host cells. From this position, the organism gains some protection against humoral and cellular immune responses (Isberg and Van Nhieu, 1994). The inability to form such an association could result in the bacterium being swept away by the harsh flushing mechanisms of the intestine. As is the case for other intestinal pathogens, the binding of *C. jejuni* to

epithelial or other cells is a prerequisite for disease production. For example, Fauchère *et al.*, (1986) proposed that *C. jejuni* isolated from patients with diarrhoea associated with cultured epithelial cells (HeLa) more frequently than those from patients without diarrhoea or fever.

To identify the mechanisms which contribute bacterial adhesion, efforts have focused largely on the ability of campylobacters to adhere to epithelial-like cell lines *in vitro* (Wooldridge and Ketley, 1997) by using a variety of cell lines; HeLa (Fauchère *et al.*, 1986; Fauchère *et al.*, 1989), INT407 (Yao *et al.*, 1994; Moser and Schroder, 1997), HEp2 (de Melo *et al.*, 1989; Konkel and Joens, 1989; Konkel *et al.*, 1992a) and Caco-2 (Everest *et al.*, 1992; Russell and Blake, 1994). There is no apparent difference in adhesion for either human or animal cell lines (Konkel *et al.*, 1992a).

Konkel and Cieplak, (1992) have identified that new proteins are induced in campylobacters on contact with both viable and non-viable host cells and that some of these new proteins are induced by released host cell components. Furthermore, the use of chloramphenicol to act as a selective inhibitor of bacterial protein synthesis, has shown that metabolically inactive bacteria were able to adhere to host cells and do not require *de novo* protein synthesis.

Adhesion is thought to be multifactorial and many studies have been performed to identify and characterise individual potential *Campylobacter* adhesins which interact with host cell receptors. The point of interaction with the epithelial cell is at the bacterial surface and accordingly, this is where most efforts have focused. Early attention was aimed at the flagellum and its potential role as an adhesin (McSweeney and Walker, 1986). Initial studies using exogenous purified flagella showed no hindrance to adhesion (McSweeney and Walker, 1986). Recent studies indicate that inactivation of flagellin genes has no effect on *C. jejuni* adherence to epithelial cells (Wassenaar *et al.*, 1991), a point later confirmed by other groups (Grant *et al.*, 1993; Moser and Schroder, 1995). Therefore, flagella *per se* may not act as an adhesin but may be essential in the pre-adherence process by providing motility for the bacterium to approach cells (Lee *et al.*, 1986). Indeed, paralysed flagella mutants which have both flagellin components, *flaA* and *flaB* but are non-motile are able to adhere but not initiate invasion (Yao *et al.*, 1994). Further interest has been aimed at the production of aggregative fimbriae in response to the presence of bile salts. The fimbriae are not essential for mediating attachment to intestinal cells *in vitro* but importantly, mutants show a reduced virulence in the ferret model (Doig *et al.*, 1996b). Other possible adhesins include outer membrane proteins (OMPs) and lipopolysaccharide (LPS) (McSweeney and Walker, 1986; Moser and Hellmann,

1989; de Melo and Pechère, 1990). The contribution of LPS to adherence is considered minimal (Moser and Schroder, 1995).

The ability of *C. jejuni* to adhere to epithelial cells is well documented. Of similar importance may be the capacity for the organism to bind to the extracellular matrix. This is a primary means of attachment to tissue surfaces used by a number of bacterial pathogens. Recently, a number of proteins, have been implicated in this process. CadF binds to fibronectin, a large multifunctional glycoprotein of the extracellular matrix (Konkel *et al.*, 1997), the major outer membrane protein (MOMP) and a further 59kDa protein have been found to bind fibronectin as well as the INT407 epithelial cell line (Moser *et al.*, 1997) and lastly a PEB1 (CBF1) mutant showed 50-100 fold less adherence compared to the wild type (Pei *et al.*, 1998). Together, these proteins may play a role in promoting pathogenesis.

1.9.2.3: Invasion

Following colonisation and adhesion, cell invasion and/or toxin production disturbs the normal absorptive capacity of the intestine by damaging epithelial cell function. Only preliminary information regarding the production of toxins and their contribution to pathogenesis has been obtained, whereas epithelial cell invasion is well documented. The process of invasion is considered to be the primary mechanism by which colonic mucosa is damaged, ultimately leading to inflammation and diarrhoea (Ketley, 1995). Knowledge of the invasion process has been derived from studies both *in vitro* and *in vivo*. Information regarding host cell invasion *in vivo* is still rather limited due to the lack of a suitable animal model but nevertheless has been demonstrated experimentally in infected macaque monkeys (Russell *et al.*, 1993), colostrum derived newborn piglets (Babakhani *et al.*, 1993), primary swine intestinal cells (Babakhani and Joens, 1993) and upon immunohistochemical staining and electron microscopy of colonic biopsy material from patients with *Campylobacter* induced colitis (van Spreeuwel *et al.*, 1985).

In contrast, host cell invasion *in vitro* is well established and has been demonstrated by a number of researchers using a variety of cell lines (Fauchère *et al.*, 1986; Konkel and Joens, 1989; de Melo *et al.*, 1989; 1990; Everest *et al.*, 1992). The ability to invade and the degree of invasion appears to be strain dependent (Konkel and Joens, 1989; Everest *et al.*, 1992; Konkel *et al.*, 1992b; Oelschlaeger *et al.*, 1993). Furthermore, clinical isolates tend to be

more invasive (Konkel and Joens, 1989) and continued *in vitro* passaging attenuates invasiveness (Konkel *et al.*, 1990; Babakhani and Joens, 1993). This attenuation may result from the appearance of aflagellate, non-motile mutants (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Yao *et al.*, 1994). Unlike in the process of adhesion, where isolates of *C. jejuni* interact efficiently with cells of both human and non-human origin (Konkel *et al.*, 1992a), invasion of cultured epithelial cells by clinical isolates is more efficient when using cells of human origin (Konkel *et al.*, 1992a).

1.9.2.4: Bacterial factors required for invasion

To date, bacterial factors required for invasion remain unknown although a variety of new bacterial proteins are expressed and other pre-existing proteins enhanced upon invasion of cultured epithelial cells (Konkel and Cieplak, 1992; Konkel *et al.*, 1993). The presence of some of these proteins is believed to be essential. Inhibition of protein synthesis by chloramphenicol precludes internalisation but not attachment (Konkel and Cieplak, 1992; Oelschlaeger *et al.*, 1993) and highlights the requirement for the bacteria to be metabolically active as non-viable organisms (heat-killed or treated with a respiratory inhibitor, sodium azide) are unable to enter INT407 cells (Konkel and Cieplak, 1992).

In competitive inhibition assays, internalisation, but not attachment, was affected by adsorption of an HEp-2 epithelial cell monolayer with bacterial cell lysates. (Konkel and Joens, 1989; Konkel *et al.*, 1990). Pre-treatment of the lysates with proteinase K did not affect invasion although in contrast internalisation was inhibited by treatment of the bacterial cell with sodium *meta* periodate (oxidation). This suggests a role for an intact carbohydrate moiety in the invasion process, and potentially a glycoprotein (Konkel and Joens, 1989). The glycoprotein appears located on the cell surface and the flagellum of invasive strains but only to the flagellum of non-invasive strains suggesting the two share a common epitope. A difference in antigen expression of invasive and non-invasive strains could contribute to the differences observed for internalisation (Klipstein *et al.*, 1985; Konkel *et al.*, 1990).

Another factor required for invasion is motility. Motility *per se* (Szymanski *et al.*, 1995) and expression of the flagellum are considered an essential requirement for invasion. For example, non-motile mutants have a severely reduced ability to penetrate INT407 and Caco-2 cells (Wassenaar *et al.*, 1991; Grant *et al.*, 1993). In addition, successful entry into host cells

is greatly enhanced when the two components of flagellin, FlaA and FlaB are expressed. Mutant bacteria which only express FlaB and not FlaA have an impaired ability to invade compared to the wild type even when centrifuged onto the monolayer (Wassenaar *et al.*, 1994). Furthermore mutants in the gene *pflA* (paralysed flagellin A) in which the flagella are present but non-functional have a reduced ability to enter INT407 cells (Yao *et al.*, 1994).

1.9.2.5: Host factors required for invasion

In contrast to the induction of new bacterial proteins, synthesis of new host proteins is not required for invasion (Konkel and Cieplak, 1992; Oelschlaeger *et al.*, 1993). The mechanism whereby bacterial cells gain entry into the host cell is a matter of conjecture and is not well established. Firstly, it is considered to be an energy dependent process as invasion of *C. jejuni* into HEP-2 cells is inhibited by iodoacetate and dinitrophenol (de Melo *et al.*, 1989). Internalisation of *C. jejuni* shows invagination of the target cell membrane via formation of pseudopodia which envelope the adherent bacteria. In addition, condensed layers of actin like filaments localise at the point of bacterial attachment and below the invaginated plasma membrane (Konkel *et al.*, 1992a). Drugs such as cytochalasin D and B were found to inhibit actin polymerisation and subsequent microfilament formation (Konkel and Joens, 1989; Konkel *et al.*, 1992b;). In contrast, other studies (Oelschlaeger *et al.*, 1993), report a microtubule dependent process without microfilament involvement as drugs which prevent microtubule polymerisation inhibit invasion of some *C. jejuni* strains (Oelschlaeger *et al.*, 1993). The implication that the different uptake pathways, either microfilament or microtubule dependent may vary according to cell line and perhaps the presence and/or abundance of appropriate eukaryotic receptors. In addition, Oelschlaeger *et al.*, who used chemicals to inhibit the formation of clathrin-coated pits and uptake mechanisms, implied that *C. jejuni* enters the host cells via coated pit receptors and from this position, the endosome interacts with the microtubules (Oelschlaeger *et al.*, 1993). This type of pathway would be unusual for an invasive pathogen (Ketley, 1997). Recently, Wooldridge *et al.*, have described a novel mechanism of entry of *C. jejuni* into Caco-2 cells. *C. jejuni* is believed to interact with a receptor located in the caveolae which culminates in a series of signalling events which trigger a signal transduction response across the cell membrane. This is believed to stimulate the small guanosine tri-phosphate-binding protein Rac which mediates the act of membrane ruffling and subsequent phagocytosis (Wooldridge *et al.*, 1996; Ketley, 1997).

1.9.2.6: Epithelial translocation

Translocation is the process whereby viable bacteria pass from the gastrointestinal tract to extra-intestinal sites such as the sub-mucosa, blood and reticulo-endothelial system and promote pathophysiological sequelae such as tissue damage, inflammation and occasional bacteraemia. Translocation was first observed in *Salmonella* (Finlay *et al.*, 1988) but has since been demonstrated for campylobacters both *in vitro* in Caco-2 cells (Everest *et al.*, 1992; Konkel *et al.*, 1992d) and *in vivo* in experimentally challenged chicks (Welkos, 1984) and infant macaque monkeys (Russell *et al.*, 1993). Predominantly, the organism enters the host cell and moves across it exiting via the basolateral surface. Alternatively, the organism may take a paracellular route between tight junctions between cells thereby negating the need to invade at all (Everest *et al.*, 1992; Konkel *et al.*, 1992d; Oelschlaeger *et al.*, 1993). This lends weight to the idea that some non-invasive strains have the ability to translocate despite being classed as non-invasive by other criteria (Konkel *et al.*, 1992d). By taking this paracellular route, and in contrast to the situation for *Salmonella*, there is no resultant loss in tight junction integrity (Finlay *et al.*, 1988). An additional method by which *Campylobacter* gain access to underlying tissues is by way of M-cells which has previously been observed for other enteric pathogens such *Shigella* (Sansonetti, 1991). Entry via M-cells for campylobacters has been observed in rabbits (Walker *et al.*, 1988; 1992) but not in macaque monkeys (Russell *et al.*, 1993b). Translocation requires the bacterium to be metabolically active with bacterial *de novo* protein synthesis although some constitutively expressed components are important in this process (Konkel *et al.*, 1992d). Also important for translocation to occur are motility and the presence of a fully functional flagellum as *flaA*⁻ mutants (*flaA*⁻ *flaB*⁻, *flaA*⁻ *flaB*⁺) are unable to traverse the cell monolayer (Grant *et al.*, 1993).

1.9.2.7: Intracellular residence and survival

Shortly after invasion, *C. jejuni* locates primarily within membrane bound endosomes (de Melo *et al.*, 1989; Konkel *et al.*, 1992b; Russell *et al.*, 1993; Russell and Blake, 1994), there is no lysosomal response and the bacteria preserve their characteristic spiral morphology (Konkel *et al.*, 1992b). As time progresses, about 3 hours post infection, there is a strong lysosomal response, the lysosome surrounds the bacterial containing endosome and the result is lysosome-endosome fusion (de Melo *et al.*, 1989). As a result, the bacteria become coccoid; a stage of the bacterium not considered viable by some researchers (Moran and

Upton, 1987; de Melo *et al.*, 1989). At this point, there is an associated intense phosphatase activity and myelinic structures are present (de Melo *et al.*, 1989). At 6 hours post infection, the numbers of viable bacteria decrease until only a few viable cells remain at 36 hours post-infection (de Melo *et al.*, 1989). Host cell damage has been visualised both *in vitro* (Konkel *et al.*, 1992b) and *in vivo* (Russell *et al.*, 1993) and may in part be due the appearance of the organisms free in the cytoplasm. Typically, cells *in vitro* become vacuolated, rounded and there is a decrease in their viability after 72 hours (Konkel *et al.*, 1992b). The type of cell damage observed *in vivo* in colonic epithelial cells in experimentally challenged monkeys includes cytoplasmic swelling, loss of microvilli, premature apoptosis and exfoliation into the lumen (Russell *et al.*, 1993).

The extent to which bacterial internalisation and cell residence is affected by the host is not well established. Initially it was thought that endosome acidification may play a role in limiting bacterial survival although the use of monensin which inhibits the acidification response did not affect the rate of bacterial killing (Oelschlaeger *et al.*, 1993). In addition intracellular survival may be influenced by reactive oxygen species (Moran and Upton, 1987; Pesci *et al.*, 1994). For example, a superoxide dismutase mutant (*sodB*) has a decreased ability to survive inside INT407 cells compared with the parent (Pesci *et al.*, 1994). Furthermore, an increase in the transition to coccoid form of the bacterium has been observed in the presence of hydrogen peroxide. This effect can be minimised by the addition of active superoxide dismutase *in vitro* (Moran and Upton, 1987). Lastly, isolation and mutation of the catalase gene (*katA*) (hydrogen-peroxide: hydrogen-peroxide oxidoreductase) (Grant and Park, 1995), which does not survive as well as the wild type in response to hydrogen peroxide, suggests that the organism harbours certain defense capabilities to combat oxidative stress.

1.9.2.8: Interaction with professional phagocytes

Inflammation is a common clinical manifestation of *C. jejuni* infection. As a result, professional phagocytes and monocytes infiltrate the intestinal epithelium wherein they act to combat the invading organism (Duffy *et al.*, 1980; Ruiz-Palacios *et al.*, 1981; Black *et al.*, 1988; Russell *et al.*, 1989). The level of phagocytosis and the degree of intracellular killing is reflected in the outcome of infection. The interaction of *C. jejuni* with polymorphonuclear leukocytes (PMNLs) depends on a variety of factors which include specific immune responses and involvement of the complement cascade. In the absence of these two factors, the number

of bacteria which become internalised by PMNLs and the level of killing is strain dependent (Pennie *et al.*, 1986; Autenrieth *et al.*, 1995; Walan *et al.*, 1992). In contrast, antibody and complement opsonised bacteria are readily phagocytosed by PMNLs and efficiently killed by intracellular reactive oxygen intermediates (Walan *et al.*, 1992; Autenrieth *et al.*, 1995). To impose their pathogenic potential, *C. jejuni* must also resist killing by mononuclear phagocytes which are present in the underlying tissues of the lamina propria. *C. jejuni* has been isolated in mononuclear phagocytes from the jejunum, ileum and colon from experimentally challenged chickens (Ruiz-Palacios *et al.*, 1981). Nevertheless, the contribution of the phagocytes to the clearance of *C. jejuni* is not understood. Lastly, internalisation by macrophages has been demonstrated and may be significant for the removal of infecting *C. jejuni* (Kiehlbauch *et al.*, 1985). There is variation in macrophage survival which depends upon the infecting strain and also species of *Campylobacter* used (Kiehlbauch *et al.*, 1985; Banfi *et al.*, 1986; Field *et al.*, 1991). Furthermore, in contrast to phagocytosis by PMNLs, opsonisation by antibody or complement is not required for efficient internalisation by macrophages (Kiehlbauch *et al.*, 1985; Field *et al.*, 1991). The presence of *C. jejuni* in the blood stream may result in bacteraemia unless they become opsonised by antibody and or complement and are subsequently phagocytosed. Most strains appear serum sensitive but bacteraemic strains survive in white blood cells (Wooldridge and Ketley, 1997).

1.9.2.9: Host resistance mechanisms and the immune response

The host employs a number of mechanisms to prevent the bacterium from exerting its pathogenic potential. The first barrier is the acid pH of the stomach (Black *et al.*, 1988). Use of the drug omeprazole suppresses stomach acidity and actually increases the risk of infection (Neal *et al.*, 1996). Additionally, peristaltic motion and harsh flushing mechanisms of the intestine are significant for clearance of the organism as use of anti-motility agents can produce a more severe, possibly even fatal infection (Smith and Blaser, 1985). Furthermore, mucus secretions and secretory IgA limit invasion *in vitro* (McSweeney *et al.*, 1987) and in addition, non-specific inflammatory responses aid clearance of the organism.

Probably the most important component in deciding the outcome of infection is the specific immune response elicited upon interaction with the invading organism. A lack of protective immunity results typically in inflammatory diarrhoea although the extent of the illness depends largely on the virulence of the invading organism. Sometimes, a partial level of

immunity may arise through cross reactivity with antigens from a previous infection. In this instance, the symptoms may only present as a mild form of diarrhoea. The immune response could also be affected by concurrent infection with another pathogen which could stimulate levels of immunity to combat the invading organism (Newell and Nachamkin, 1992). Fully protective immunity can develop upon recurrent challenge with *C. jejuni* and as a result there is an age related decrease in illness due to *C. jejuni*. Moreover, the eventual outcome is usually asymptomatic carriage. This situation is often seen in developing countries. The acquisition of immunity has also been demonstrated in human volunteers (Black *et al.*, 1988). For example, initial challenge with *C. jejuni* results in an inflammatory illness and serum antibody response. Subsequent rechallenge may lead only to colonisation and not to illness. Isolates of *C. jejuni* are generally susceptible to the bactericidal activity of human serum although isolates from extraintestinal infections have a decreased serum sensitivity compared with gastrointestinal isolates (Blaser *et al.*, 1986). At the molecular level the involvement of a specific antibody response has been demonstrated in children from a developing country. Bangladeshi infants have shown specific serum IgA levels to increase linearly with age. This may be indicative of the production of the antibody in the intestine and subsequent immunity of the gut (Blaser *et al.*, 1985). Between 1-4 years old levels of IgG and IgM rise concurrently. Subsequently, there is a drop in the level of IgG production and the level of IgM remains steady. Reduction in the levels of IgG may reflect a level of immunity in the gut sufficient enough to prevent stimulation of IgG (Blaser *et al.*, 1985). The involvement of a specific antibody response has been also been demonstrated following acute infection (Blaser and Duncan, 1984) where the levels of IgG and IgM increase in response to infection and remain elevated for 3-4 weeks before declining to baseline levels. Levels of serum IgA rise rapidly during the first few weeks of infection then fall rapidly (Blaser and Duncan, 1984). Faecal and urine IgA antibodies can also be detected in some patients with infection but appear only detectable during the first few weeks after acute infection (Lane *et al.*, 1987). Furthermore, examination of the antibody levels in both healthy and convalescent sera has revealed a degree of prior exposure to the organism or a level of cross reactivity with another organism (Blaser *et al.*, 1984).

1.10: Putative virulence determinants

1.10.1: Toxin production

For enteric bacteria the production of one or more toxins is an important aetiological agent of diarrhoeal disease. As this is very likely true of *Campylobacter* enteritis, extensive research into the production of toxins by *Campylobacter* has been performed. However, presently, information regarding their production, mechanism of action and importance to the disease process is still in its infancy and therefore remains to be fully established. In trying to determine the contribution of toxins to the disease process a variety of techniques have been used by different researchers. For example, different bacterial species and strains, different culture conditions, tests with animal models, responses of *in vitro* epithelial cell lines, immunological assays and genetic techniques. Unfortunately, a lack of standardisation has lead to confusing data as to whether toxin production can be demonstrated, if so, how many are elicited and finally what relevance they have to pathogenicity.

Faced with the nature of clinical manifestations associated with *Campylobacter* disease, various researchers have postulated that campylobacters elicit similar toxic activities to those of other enterobacteria. For example, the induction of watery diarrhoea might reflect the activity of an enterotoxin matching that of *Vibrio cholerae*. Furthermore, the presence of an inflammatory response might indicate cytotoxic activity similar to infections seen with *Shigella* spp or *Clostridium difficile*. The result is a now a substantial body of evidence to suggest that campylobacters may produce both enterotoxins and cytotoxins. Information regarding these data has been recently reviewed (Wassenaar, 1997) and to date the production of one heat labile enterotoxin (CJT) and potentially five different cytotoxins have been postulated.

The heat labile toxin has a molecular weight between 60-70 kDa and was first described by Ruiz-Palacios *et al.*, (1983) and later confirmed in other reports (Johnson and Lior, 1984; Klipstein and Engert, 1984; Goossens *et al.*, 1985a). CJT is produced by certain strains under appropriate conditions such as exogenous amino acids (Ruiz-Palacios *et al.*, 1983) and iron (McCardell *et al.*, 1986) but has only been partially purified (Daikoku, 1990). Tests on a number of animal models reveal that the enterotoxin stimulates fluid secretion although the results vary according to bacterial strain and animal model used (Ruiz-Palacios *et al.*, 1983; Klipstein and Engert, 1984; McCardell *et al.*, 1984; Saha, 1988; Collins *et al.*, 1992; Everest

et al., 1993). In addition, the enterotoxin causes elongation and rounding of CHO and Y-1 adrenal cells respectively (Ruiz-Palacios *et al.*, 1983; Johnson and Lior, 1984) by altering cytoskeletal components. Crude *C. jejuni* heat-labile enterotoxin causes an increase in cAMP levels in tissue culture cells, binds to GM₁ ganglioside and is neutralised by antibodies against cholera toxin (Ruiz-Palacios *et al.*, 1983; Klipstein and Engert, 1984; Johnson, 1986). In this respect CJT reflects the biological activities of both cholera toxin (CT) and *E. coli* heat-labile toxin (LT). Despite the immunological relatedness and cross reactivity between CJT, CT and LT *in vitro* and *in vivo* their homology at the DNA level as determined by DNA hybridisation studies is poor (Olsvik *et al.*, 1984; Perez-Perez *et al.*, 1992) and is limited to the B subunit of CJT (Klipstein and Engert, 1985; Calva *et al.*, 1989). Degenerate primers designed against conserved regions in both CT and LT to try and amplify the cognate region from *C. jejuni* was unsuccessful (Konkel *et al.*, 1992c). In view of the inability to identify the gene responsible for CJT production and the limited knowledge of its clinical significance, efforts have been made to correlate the presence of CJT with clinical symptoms and seroconversion to CJT⁺. The results of these data are conflicting (Wassenaar, 1997).

In addition to production of CJT, *C. jejuni* has also been reported to produce a number of different cytotoxic factors, although there is still a large amount of confusion concerning the number and specificity. Nevertheless, according to Wassenaar, (1997), *C. jejuni* may produce five different cytotoxins. (Wassenaar, 1997). These are a 70kDa cytotoxin active on HeLa cells CHO cells and other cells but inactive on Vero cells, a cytotoxin active on Vero and HeLa cells, a Shiga-like toxin, a cytolethal distending toxin (CLDT) and a haemolytic cytotoxin.

The 70 kDa cytotoxin is a heat labile , trypsin sensitive agent which appears lethal to various epithelial cell types lines such as HeLa, CHO, Hep-2 and INT407 but not Vero cells and its role in disease is unknown (Wong, 1983; Goossens *et al.*, 1985b; Johnson and Lior, 1986; Daikoku *et al.*, 1989; Pang *et al.*, 1987; Kawaguchi *et al.*, 1990; Mahajan and Rodgers, 1990; Mizuno *et al.*, 1994). It is not neutralised by anti-CT (Guerrant *et al.*, 1987) or anti-Shiga (Johnson and Lior, 1986) or anti *Clostridium difficile* antisera (Mahajan and Rodgers, 1990). The toxin may be associated with the membrane or maybe secreted as the LPS fraction of bacterial lysates exhibited cytotoxic activity (Kawaguchi *et al.*, 1990). Unlike enterotoxin, there was no fluid secretion in 6 or 18-h rabbit ileal loops or suckling mice (Guerrant *et al.*, 1987). A further toxin, distinct from the 70kDa toxin, has been identified (Johnson and Lior, 1984; 1986) and is active against both Vero and HeLa cells (Klipstein and Engert, 1985;

Florin and Antillon, 1992). This cytotoxin cannot be neutralised by anti-*Clostridium* or anti-Vero-antitoxin (Johnson and Lior, 1984; 1986). A third potential toxin is a Shiga-like toxin which shows activity against HeLa cells. Low levels of cell associated cytotoxic factor that is neutralised by anti-Shiga toxin serum have been reported from some strains (Moore *et al.*, 1988). A fourth toxin in *C. jejuni* is CLDT which has been identified from strains causing inflammatory diarrhoea (Johnson and Lior, 1988). The toxin is active on CHO, Vero, HeLa and Hep-2 cells but not Y-1 adrenal cells (Johnson and Lior, 1988) and induces accumulation of cAMP in CHO cells 24h post treatment as opposed to CJT which does so 96h post treatment. Production of CLDT requires expression of three adjacent genes *cdtA*, *cdtB* and *cdtC* which have been cloned and sequenced from *C. jejuni* (Pickett *et al.*, 1996). Analysis of the associated polypeptides of CLDT reveal a unique and unrelated structure compared with other known proteins (Pickett *et al.*, 1996) and is believed to have a novel mode of action (Aragon, 1997; Whitehouse *et al.*, 1998). The toxin causes a rapid irreversible block in the G₂ phase of the cell cycle (Aragon, 1997; Whitehouse, 1998). CLDT is reported to cause a haemorrhagic response in rat ligated intestinal segments *in vivo* but is negative in rabbit ligated ileal segments, in suckling mice and the rabbit skin permeability test. The activity of CLDT activity is neutralised by homologous rabbit antitoxin only and not by anti-CT, anti-Vero and anti *C. difficile* antitoxins (Johnson and Lior, 1988). The role of *C. jejuni* CLDT in disease is unknown but it has been postulated that production of CLDT in close proximity to intestinal cells prevents cell proliferation into functional villous cells, thereby eroding the villous epithelium and upsetting the absorptive function of the epithelium. The final type of toxins thought to be elicited by *C. jejuni* are those which harbour haemolytic activity. McCardell *et al.*, 1986 first identified a cytotoxin which exhibited haemolytic activity and was toxic to CHO cells. The toxin was not neutralised by anti-haemolysin or cytolysin antitoxin of *Vibrio cholerae* (McCardell *et al.*, 1986). More recently, haemolytic activity has been demonstrated by a number of groups. For example haemolysis on blood agar has been demonstrated by ageing bacteria suggesting toxin release upon cell death (Arimi *et al.*, 1990). Both alpha and beta haemolysis have been shown (Misawa *et al.*, 1995) and also non iron-regulated haemolysis by washed bacterial cells (Pickett *et al.*, 1992).

1.10.2: Flagella

The flagellar filament of *Campylobacter* spp., is composed of two flagellin subunits, FlaA and FlaB which are approximately 93% identical to one another. Most heterogeneity between the two amino acid structures lies at the amino and carboxy terminals. The genes which make up the filament, *flaA* and *flaB* are both approximately 1.7kb in size, are contiguous on the chromosome in a head to tail configuration and are separated by a 163bp region. The major differences between the two genes lie in the promoter regions. For example, the two genes are transcribed independently but the *flaA* gene is controlled by a σ^{28} promoter, which is the class of promoter that expresses flagellin genes in *Salmonella*, *Bacillus* and in *E. coli* (Mirel and Chamberlin, 1989; Helmann, 1991). However, the *flaB* gene is controlled by a σ^{54} promoter which is the class of promoter which controls nitrogen-regulated genes in the *Enterobacteriaceae*, and temporally regulated genes in *Caulobacter crescentus* (Sasse-Dwight and Gralla, 1990). Expression of the two genes varies as FlaA is produced in much higher levels than FlaB (Guerry *et al.*, 1991). Furthermore, *flaB* is subject to environmental regulation and is modulated by changes in the level of nitrogen, divalent cations, pH and growth phase (Alm *et al.*, 1993). The regulation of flagellin expression may also be affected by the gene, *flbA* as mutations in this gene are unable to produce flagella and no flagellin is identified in the cytosol (Miller *et al.*, 1993). Genetic analysis of *flaA* and *flaB* by site specific mutations has given an insight into the structure and function of the flagellar filament (Guerry *et al.*, 1991). A *flaA*⁺ *flaB*⁻ mutant has a full length filament and confers motility only slightly less than the wild type and a *flaA*⁻ *flaB*⁺ mutant has a flagellar filament severely truncated in length (~17% of the wild type). Monoclonal antibodies against FlaA and FlaB indicate that the flagellin is composed primarily of FlaA but with minor amounts of FlaB which enhances motility. A *flaA*⁻ *flaB*⁻ mutant is non-motile. The ability to modulate levels of FlaB in response to environmental conditions may have implications to the motility status of the bacterium and in turn, modulation of virulence.

The flagellum is able to undergo phase and antigenic variation (Caldwell *et al.*, 1985; Harris *et al.*, 1987). Antigenic variation permits the bacterium to express flagellins of distinct antigenicities and different apparent molecular masses on SDS-PAGE gels (Harris *et al.*, 1987). The predicted molecular mass of flagellin is ~59 kDa although the aberrant migration on SDS-PAGE gels is thought to be due to post-translational modification (Logan *et al.*, 1989; Alm *et al.*, 1992; Power *et al.*, 1994), particularly, glycosylation of the flagellin subunits (Doig *et al.*, 1996a). Doig *et al.*, (1996a) confirmed flagellins to be glycoproteins as the

flagellin subunits could be oxidised by periodate and react with a sialic acid specific lectin. The molecular basis behind antigenic variation has not been determined although two genes have been identified, *ptmA* and *ptmB* which encode steps involved in the biosynthetic pathway leading upto post-translational modification (Guerry *et al.*, 1996). Mutations in either of these genes results in a loss of reactivity to antisera raised against the initial flagellin component (Guerry *et al.*, 1996)

1.10.2.1: The flagellum and its role in pathogenesis

Several lines of evidence implicate the flagellum as a very important virulence determinant. Firstly, the distinct motility afforded by the flagellum is an absolute requirement for campylobacters to colonise the gastrointestinal tract of humans (Black *et al.*, 1988) and experimentally infected animals (Morooka *et al.*, 1985; Pavlovskis *et al.*, 1991) and cause diarrhoeal disease. For example, aflagellate and flagellated non-motile mutants are unable to colonise or only poorly colonise the intestinal tracts of rabbits (Pavlovskis *et al.*, 1991), suckling mice (Morooka *et al.*, 1985; Newell *et al.*, 1985) and chickens (Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993b). *C. jejuni* maintains its high motility in extremely viscous environments by moving faster and tumbling less (Lee *et al.*, 1986; Ferrero and Lee, 1988) which may offer a significant ecological advantage over other bacteria in colonising the intestine. Potentially, this increases the chances of a successful interaction between the bacterium and the host cell thereby promoting the initial, transient adhesion. In fact, there is evidence of increased attachment of *C. jejuni* to Caco-2 cells in viscous environments (Szymanski *et al.*, 1995). Therefore, this not only identifies a role for the flagellum in motility and colonisation but maybe a second, lesser role in adhesion although there are contrasting reports regarding the involvement of the flagellum in adhesion *per se*. For example, early work by Newell *et al.*, (1985) demonstrated that a flagellate, but non-motile mutant showed an increase in adherence ability to INT407 cells compared with the parent or aflagellate mutants (Newell *et al.*, 1985). Furthermore, removal of the flagellum by shearing reduced bacterial adherence to INT407 cells, whereas potassium cyanide treatment to immobilise the flagellum increased adhesion (McSweegan and Walker, 1986). Also, the addition of purified exogenous flagella to INT407 cells was unable to block adhesion (McSweegan and Walker, 1986). This inability to reduce adhesion may be the result of non-specific, hydrophobic interactions between flagellin bound to epithelial cells and the

Campylobacter cell surface. This results in more bacteria being recruited to the cell surface to subsequently bind (Nuijten *et al.*, 1992). Wassenaar *et al.*, (1991) have shown that non-motile mutants lose their ability to adhere to human intestinal cells *in vitro*, an ability which was partially restored by centrifugation of the bacteria onto the epithelial cell monolayer. Therefore the failure to completely abolish adherence *in vitro* (Newell *et al.*, 1985; McSweeney and Walker, 1986; Wassenaar *et al.*, 1991) suggests that *C. jejuni* possess additional adhesin(s) which enable the interaction with host cells.

The flagellum may contribute significantly to the invasion process. For example non-flagellated (*flaA*⁻, *flaB*⁻) and flagellated but non-motile, paralysed (*flaA*⁺, *flaB*⁺ , *pflA*⁻), mutants are unable to adhere and invade eukaryotic cells (Caco2/INT407) or invade only poorly *in vitro* (Wassenaar *et al.*, 1991; Nuijten *et al.*, 1992; Russell and Blake, 1994; Yao *et al.*, 1994). If the mutant bacteria are centrifuged onto the cell monolayer, the level of invasion rises although does not reach wild type levels. Mutants with only a reduced motility (*flaA*⁺, *flaB*⁻) invade INT407 cells at levels comparable to the wild type. A proposed mechanism of internalisation incorporates one flagellum wrapping around the host cell facilitating internalisation via use of the other (Russell and Blake, 1994). Paralysed mutants, despite the ability to adhere, are therefore prevented from invasion (Yao *et al.*, 1994). Collectively these results identify the need to be fully motile for adhesion and subsequent invasion or alternatively, that the FlaA subunit (or a member of the flagellum that requires the presence of *flaA*) acts as a potential adhesin and is a prerequisite for internalisation (Wassenaar *et al.*, 1991; Nuijten *et al.*, 1992; Russell and Blake, 1994; Yao *et al.*, 1994). This highlights the need for motility in invasion or a co-ordinate regulation of motility and other virulence determinants. Not only does the flagellum have importance in virulence, flagellin is also an immunodominant antigen recognised during infection. It is believed that the development of antibodies to flagellin correlates with the development of protection against disease (Martin *et al.*, 1989).

1.10.3: Lipopolysaccharide (LPS)

Lipopolysaccharides (LPSs) also termed endotoxins are a family of toxic phosphorylated glycolipids that are the major constituents in the outer membrane of most Gram negative bacteria, including *C. jejuni* (Rietschel *et al.*, 1994). They are the major surface antigens (O-antigens) of the bacterium and are essential for the physical integrity of the cell and to

promote efficient functioning of the outer membrane. The general structure of LPS, i.e., that which is common to most bacterial species, consists of polysaccharide or oligosaccharide (OS) covalently linked to a lipid component, lipid A. High relative molecular mass (high- M_r), smooth-form LPS consistent with enterobacteria contains three different domains; the O-antigen, the core oligosaccharide and the Lipid A component. A further type of LPS, low-relative molecular-weight (low- M_r), rough form LPS does not contain an O-specific chain and is produced by enterobacterial mutants that have defects in their LPS biosynthesis genes (Nikaido, 1996). With respect to *C. jejuni*, biochemical characterisation of LPS has been performed by a variety of researchers (Conrad and Galanos, 1990; Moran *et al.*, 1991; Aspinall *et al.*, 1992a; Aspinall *et al.*, 1992b; Aspinall *et al.*, 1993a; Aspinall *et al.*, 1993b; Aspinall *et al.*, 1993c; Aspinall *et al.*, 1993d). A third of *C. jejuni* serotyped strains produce high- M_r LPS (Preston and Penner, 1987) comparable to wild type isolates of enterobacteria. The remaining two-thirds produce low- M_r LPS (Preston and Penner, 1987). Initial investigations compared this form of LPS with that of enterobacterial rough form LPS in which the O-antigen is absent (Logan and Trust, 1984; Mills *et al.*, 1985; Perez-Perez and Blaser, 1985). Further analysis has revealed a closer resemblance with low- M_r LPS of *Neisseria* and *Haemophilus* spp (Aspinall *et al.*, 1993b; Aspinall *et al.*, 1993c; Aspinall *et al.*, 1995a).

As a constituent of the bacterial cell surface, LPS is one of the focal points of interaction with the environment and the also the host cell. This is apparent as LPS molecules harbour binding sites for antibodies and serum factors which are involved in recognition and possible elimination by the host's defence system. Indeed molecular mimicry exists between human ganglioside GM₁ and *C. jejuni* LPS which results in Guillain Barré Syndrome (GBS), furthermore a similar mimicry exists between Gq1b and *C. jejuni* LPS which results in a variant of GBS called Miller Fisher Syndrome (MFS). LPS has a number of endotoxic properties including pyrogenicity and cell toxicity which contribute to the pathogenic potential of the organism (Rietschel *et al.*, 1994). The contribution that LPS makes to the virulence of bacterial infections is further enhanced by variations in saccharide moiety which dictate the efficiency of complement activation, phagocytosis and serum resistance (Liang-Takasaki, 1982). Other virulence attributes can be enhanced by sialylation of LPS, for example, serum resistance of *Neisseria* spp. and *Haemophilus* spp. (Demarco de Hormaeche *et al.*, 1991; Moxon and Maskell, 1992). The contribution of LPS to the virulence of *C. jejuni* is poorly understood.

1.10.4: Aggregative fimbriae

Recent work has demonstrated the production of peritrichous pilus-like appendages when *C. jejuni* was grown in the presence of bile salts (Doig *et al.*, 1996b). The specific bile salts, deoxycholate and chenodeoxycholic acid significantly enhanced pilus production and electron microscopy revealed an aggregative phenotype. Morphologically, the pili were 4-7nm in length and were greater than 1µm in width. The gene(s) encoding the fimbrial subunit has not been found. However a gene which encodes prepilin peptidase (*pspA*) has been identified and is similar to two non-essential *E. coli* proteases; protease IV which is the product of the *sppA* gene and digests cleaved signal peptides (Ichihara, 1986) and SohB, a suppressor of *htrA* (Baird *et al.*, 1991). Site directed mutagenesis of *pspA* results in the loss of pilus production and the non-piliated mutant was still able to adhere to and invade INT407 cells. In addition, the mutant was able to colonise the ferret model of infection but with much reduced symptoms. This is strong evidence to implicate pili in an as yet unknown role in *Campylobacter* virulence (Doig *et al.*, 1996b)

1.10.5: Outer membrane proteins

Several groups have investigated the role of outer membrane proteins (OMPs) in the pathogenesis of disease (Logan and Trust, 1983; de Melo, 1990; Dubreuil *et al.*, 1990; Pei *et al.*, 1991; Fauchère *et al.*, 1992; Kervella *et al.*, 1993; Pei and Blaser, 1993; Burucoa *et al.*, 1995). Most efforts so far have been directed at identifying proteins which bind specifically to eukaryotic cells *in vitro*. De Melo and Pechère (1990) identified four proteins with apparent molecular masses of 28, 32, 36 and 42 kDa that bind to HEp-2 cells and may play a role in *C. jejuni* host cell interactions and ultimately invasion. Fauchère *et al.*, (1992) have described two OMPs of 27 and 29kDa which bind specifically to epithelial cells. The two may co-adhere when binding to epithelial cells but the specific domains involved in adhesion have not been demonstrated. In fact, rabbit sera raised against OMP fractions of between 26-30 kDa precluded adhesion and invasion (Fauchère *et al.*, 1992). Isolation of the major, 27kDa, band (also cell binding factor 1 [CBF1]) blocked adherence of *C. jejuni* to HeLa cells. The other, minor band (also cell binding factor 2 [CBF2]) of 29kDa did not (Kervella *et al.*, 1993). In addition, antibodies raised against CBF1 blocked adherence. The proteins, CBF1 and CBF2 and those identified by de Melo and Pechère, (1990) may represent similar OMPs

to those identified by Pei *et al.* 1990, who identified four major antigenic proteins by acid extraction from *C. jejuni* 81176. The proteins were designated, PEB1 (28kDa), PEB2 (29kDa), PEB3 (30kDa) and PEB4 (31 kDa). *N*-terminal sequence analysis revealed that all four proteins are different although the first 35 amino acids of PEB2 and PEB3 are 51.4% homologous (Pei *et al.*, 1991). PEB2 and PEB3 show little cross reactivity among strains and their functions are unknown. (Pei *et al.*, 1991). On the other hand, PEB-1 is surface exposed and highly conserved amongst strains (Pei *et al.*, 1991; Pei and Blaser, 1993). Analysis of the gene which encodes PEB1 (*peb1A*) suggests that *peb1A* is located within an operon homologous to those for ABC transporters from a variety of bacteria and is the binding component thereof. Furthermore, PEB1 may have a possible role in adherence to tissue culture cells (Pei *et al.*, 1991; Pei and Blaser, 1993) although this binding could be an artefact as PEB1 is not required for short term colonisation of chicks (Meinersmann *et al.*, 1996). In contrast, it has recently been demonstrated that *peb1A* actually enhances *C. jejuni* adherence to and invasion of epithelial cells and intestinal colonisation in a mouse model (Pei *et al.*, 1998). Furthermore, PEB1 is antigenically related and potentially identical to CBF1. The protein, PEB4 shows little cross-reactivity amongst strains and the gene sequence shows a high similarity to an export protein of *Bacillus* species (Burucoa *et al.*, 1995). Given this sequence similarity, PEB4 is considered unlikely to be an adhesin. Furthermore, PEB4 is potentially identical to CBF2 (Kervella *et al.*, 1993). Lastly, the 43kDa major outer membrane protein (MOMP) has been postulated to act as an adhesin (Moser *et al.*, 1997).

1.11: Genetics of *C. jejuni*

C. jejuni has a G+C content of 32-35 mol% and a small genome size of approximately 1.7Mb determined by field inversion gel electrophoresis (Nuijten *et al.*, 1990) and pulse field gel electrophoresis (Chang and Taylor, 1990; Taylor D.E. *et al.*, 1992). The small size of the genome is consistent with the phenotype of *C. jejuni* in its requirement for complex growth media and the inability to metabolise carbohydrates or to degrade complex substrates (Griffiths and Park, 1990; Taylor, D.E., 1992b). The genus *Campylobacter* contains plasmids which may vary in size and copy number (Austen and Trust, 1980; Bradbury *et al.*, 1983; Tenover *et al.*, 1985). Conjugative plasmids from some isolates contain antibiotic resistant genes which can also confer antibiotic resistance in *Escherichia coli* but only a few antibiotic

resistance genes have been identified and cloned, mainly from *C. coli*. For example, chloramphenicol (Wang and Taylor, 1990a), kanamycin (Trieu-Cuot *et al.*, 1985) or tetracycline (Sougakoff *et al.*, 1987) are used for construction of *C. jejuni* vectors and insertional mutants. Presently no transposons have been described for the *Campylobacter* genus but bacteriophages have been described by various groups (Ritchie *et al.*, 1983; Grajewski *et al.*, 1985). Genetic manipulation of campylobacters has proved difficult. "Classical" genetic techniques developed for the analysis of other bacteria such as *E. coli* or *S. typhimurium* either do not work or work only partially in *Campylobacter* species as phylogenetically they are very different. This point is reflected by the comparatively low number of DNA sequences from *C. jejuni* available in GenBank compared with other enteropathogens. Presently there are around 60 *C. jejuni* genes which have been cloned and sequenced. Common problems highlighted by research groups include instability of cloned genes, failure to express the genes of interest and the lack of a system for generalised mutagenesis. (Taylor D.E., 1992b; Tompkins, 1992).

The creation of genomic libraries in either high copy number cosmids or medium copy number cosmids typifies the problems encountered (Labigne-Roussel *et al.*, 1992). For example cloning *C. jejuni* genes with large inserts in *E. coli* can result in the collapse of insert DNA. Without selection, *E. coli* recipients transfected with cosmids containing large *Campylobacter* inserts can delete the foreign DNA leaving only cosmids that contain little or no insert DNA (Lee *et al.*, 1985) suggesting that some *Campylobacter* sequences are unstable in *E. coli*. It is thought that the high A-T content of *C. jejuni* compared with that of *E. coli* could lead to instability problems due to the presence of promoter-like sequences and thus may lead to collapsing inserts. Long regions of A-T rich DNA that are also rich in static bends may also serve as upstream activators of promoters (Morrison., 1990). These strong promoters are only stable in vectors in which efficient terminator signals protect plasmid-control elements from excessive transcription (Brosius, 1984). In addition, different patterns of DNA methylation between *E. coli* and *Campylobacter* may result in an initial clone instability (Labigne-Roussel *et al.*, 1987; Taylor D. E., 1992b). Some problems with instability are overcome by providing a selective pressure to maintain the *Campylobacter* gene in *E. coli* (Lee *et al.*, 1985). To reduce further problems of clone instability the use of small inserts or bacteriophage vectors is preferred (Ketley, 1995).

Provided the target gene has been cloned successfully there may be problems with a lack of expression of certain *C. jejuni* genes in *E. coli*. This may be due to unusual promoter

sequences that are not recognised or are only recognised poorly by *E. coli* transcriptional machinery and the failure of *E. coli* to process some gene products due to the lack of accessory gene products (Taylor D. E., 1992b). Furthermore, the limit of expression of some *Campylobacter* genes can be attributed to different codon usage (Wang and Taylor, 1990b; Taylor D.E., 1992c). Despite these problems there are examples of genes which have been successfully cloned and identified as a result of their expression in *E. coli*. These are mainly housekeeping genes as they tend to be highly conserved across species boundaries and encode similar functions in many bacterial genera, for example, *glyA* (Chan *et al.*, 1988) and partial complementation of the iron responsive global regulator *fur* (Wooldridge *et al.*, 1994). Expression of more specialised, non-housekeeping genes could be more problematic as they are not so highly conserved and there may be problems associated with the host's inability to carry out post-translational modifications. (Taylor D.E., 1992a; 1992b). Some *C. jejuni* genes can impart a phenotypic change on the *E. coli* host when expressed which has enabled them to be cloned and identified, for example, the catalase gene *katA* (Grant and Park, 1995) confers a catalase-positive phenotype on catalase-negative *E. coli* and additionally, *sodB* (Pesci *et al.*, 1994) is enzymatically active in *E. coli*. Some genes have been identified in *E. coli* by an antibody screening method. The flagellin genes were identified in this way (Nuijten *et al.*, 1989) and *C. jejuni* ferritin (*cft*) was cloned using information derived from the N-terminal amino acid sequence of the purified protein (Wai *et al.*, 1996). An alternative method for cloning several *C. jejuni* genes uses the polymerase chain reaction with degenerate oligonucleotide primers (PCRDOP) (Wren *et al.*, 1992). The technique uses PCR with degenerate oligonucleotide primers which are designed to anneal against conserved regions within families of proteins. The amplified product from the gene of interest can be used subsequently as a probe against a genomic libraries to identify more of the gene. The PCRDOP method is unfortunately restricted to the isolation of genes which encode known proteins with highly conserved domains and therefore genes which are not novel.

Despite the apparent difficulties, progress has been made in genetic manipulation of these organisms. Shuttle vectors have been developed which are capable of replicating in both *E. coli* and *C. jejuni*, for example pIL550 (Labigne-Roussel *et al.*, 1987), the pUOA series of vectors (Wang and Taylor, 1990a; 1990b), the pRY series (Yao *et al.*, 1993) and the promoter probe vector pSP73 (Purdy and Park, 1993).

The identification and characterisation of more *C. jejuni* genes has been hindered largely by the lack of a system for random insertional mutagenesis (Taylor D.E., 1992b) Transposons of

both Gram positive and Gram negative origin have been tested in *C. jejuni* and in addition a “hybrid transposon” has been constructed which contains the *C. coli apha-3* kanamycin gene, and a *C. jejuni* promoter driven transposase (Ketley, 1995). None of these transposons were found to transpose in a *C. jejuni* background. Nevertheless, *TnphoA* was able to mutate cloned *C. jejuni* DNA in an *E. coli* background to enable identification of genes encoding secreted protein products and therefore putative virulence determinants (Ketley, 1995). A method of shuttle transposon mutagenesis using mini-Tn:Km, has been developed to generate mutations in *Campylobacter* DNA within an *E. coli* host (Labigne-Roussel *et al.*, 1992). The disrupted gene of interest is introduced into *Campylobacter* by homologous recombination. The technique has also been used successfully in *Helicobacter pylori* (Haas *et al.*, 1993). A method of semi-random insertional mutagenesis has been developed by Yao *et al.*, (1994) who identified a gene required for motility (*pflA*). The approach was successfully adapted from work previously applied to *Haemophilus influenzae* (Sharetsky *et al.*, 1991) and involves introduction of a resistance gene into restricted chromosomal DNA fragments which are then circularised. The method takes advantage of the fact that some strains of *C. jejuni* are naturally transformable and the circularised DNA is introduced into the chromosome to enable homologous recombination between identical sequences. A particular drawback of this method is the availability of suitable restriction sites in the genes of interest.

The absence of a suitable transposon mutagenesis system for *C. jejuni* has also meant that other indirect methods for mutagenesis have been developed. Defined insertional mutants are constructed by cloning the gene of interest into a suitable vector and introducing an antibiotic resistance cassette into the target gene by utilising a unique restriction site or one created by inverse PCR (IPCR) mutagenesis (Wren *et al.*, 1994). With either technique, the mutant construct is introduced into *Campylobacter* to allow for homologous recombination by a double crossover event to obtain a chromosomal mutant. Gene disruption and displacement was first used to inactivate 16S rRNA (Labigne-Roussel *et al.*, 1988). The length of flanking sequence either side of the point of insertion of the antibiotic resistance cassette is an important factor in dictating whether homologous recombination and genetic exchange is possible (Wassenaar *et al.*, 1993a; Ketley, 1995). At present, as little as 200-400 bp of flanking DNA has proved sufficient for recombination to occur (Wassenaar *et al.*, 1993a; Ketley, 1995) although the efficiency of exchange may be low. To increase the efficiency of recombination by increasing the amount of flanking DNA, the plasmid based IPCRM technique has been modified for use on circularised chromosomal loops. The loops can then

either be cloned for further analysis and characterisation or directly used to mutate the target gene in *C. jejuni*

In order to assess gene function the ability to transfer DNA efficiently back into *C. jejuni* cells is important. The efficiency can vary remarkably between strains (Wassenaar *et al.*, 1993a) and most strains are transformed more effectively with homologous DNA than heterologous DNA due to restriction and modification. Currently there are three methods for the introduction of recombinant DNA into *C. jejuni*, electrotransformation, natural transformation, and conjugation. Electrotransformation, (Miller *et al.*, 1988) has been used to transform both suicide and shuttle vectors successfully (Wassenaar *et al.*, 1991; Wooldridge *et al.*, 1994) although the efficiency can vary markedly with the strain. Natural transformation is a characteristic displayed by most *Campylobacter* strains (Wang and Taylor, 1990b) and is common amongst Gram positive bacteria. Only a few Gram negative bacteria are considered naturally competent for example, *Neisseria gonorrhoeae* (Goodman and Scoocca, 1988) and *Haemophilus spp.*, (Danner *et al.*, 1980) and take up DNA by binding to a small specific DNA sequence (Smith *et al.*, 1995). It is thought that *Campylobacter* DNA may also contain a specific sequence necessary for binding and uptake into *Campylobacter* cells. Natural transformation has been used to construct defined and random mutants in *Campylobacter* (Yao *et al.*, 1994; Dickinson *et al.*, 1995). Conjugation has also been used as a delivery system for both shuttle vectors (Labigne-Roussel *et al.*, 1987; Yao *et al.*, 1993) and suicide vectors (Labigne-Roussel *et al.*, 1988) using mobilisation of the plasmid from *E. coli* to *C. jejuni*. As the variety of techniques available to identify genes encoding virulence determinants in *C. jejuni* is very limited new methodologies are sought to circumvent this problem.

1.12: Aims.

The molecular mechanisms surrounding *Campylobacter* pathogenesis are poorly understood due to the limited amount of data regarding which virulence determinants are involved. This is partly due to technical problems associated with identifying potentially important genes and their subsequent characterisation. Nevertheless, the PCR-DOP technique, described above, has been used extensively in this laboratory, with some success, to identify potential candidates in the virulence process. Several putative members of the two component regulator family in *C. jejuni* (Ketley, 1995) have been identified, most notably *cheY* (Marchant J. E., 1998) and *racR*

(Brás, 1996). Of particular relevance to this study was use of the technique to identify the *htrA* locus of *C. jejuni* (Henderson, 1996).

HtrA is proposed to be a member of the serine protease family and thus possibly involved in the stress response of *C. jejuni*. The adaptation of *C. jejuni* to a stressful environment is likely to be important for continued survival and virulence of the organism. PCR-DOP methodology identified a small sub-genic fragment of *htrA* which was subsequently used to mutate the chromosomal *htrA* locus but analysis of the mutant did not reveal a phenotype. Nevertheless, the PCR-DOP fragment enabled flanking regions of the gene to be identified using IPCR methodology to allow further characterisation of the gene and its surrounding loci. Taking these findings, the aim of the study was to further investigate *htrA* due its potentially important role in the stress response (chapters 4 and 5) and to examine regulatory genes downstream of *htrA* with a view to their characterisation (chapters 7 and 8). The information could be used to gain a better understanding of the role of *htrA* in the pathogenesis of *C. jejuni*.

2: General materials and methods

2.1: Bacterial strains and growth conditions

2.1.1: *Campylobacter jejuni* strains

81116 (NCTC 11828) (Palmer *et al.*, 1983); **480** (NCTC 12744) (King *et al.*, 1991)

Campylobacter strains were grown on *Campylobacter* Blood Free Selective Agar Base (CSA; Oxoid, Unipath UK) or Mueller Hinton Agar (MHA; Oxoid, Unipath) plates in a Variable Atmosphere Incubator (VAIN; Don Whitley Scientific Ltd, UK) containing 6% O₂, 6% CO₂, and 82% N₂ at either 37°C or 42°C. Liquid cultures in MH broth (MHB) (Oxoid) were shaken at approximately 200 rpm on a G2 Gyrotory Shaker (New Brunswick Scientific Co, Inc., Edison, NJ, USA). Strains were incubated in Mueller Hinton Broth (MHB) (Oxoid, Unipath UK) at either 37°C or 42°C.

2.1.2: *Escherichia coli* K-12 strain derivatives

DH5 α F': (F'*endA1 hsdR17 (r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ (lacIZYA-argF) U169 deoR (ϕ 80dlac Δ (lacZ)M15)*) (Hanahan, 1983).

MC4100: (F'*araD139 Δ (argF-lac)U169 rpsL 150 (Str^r) relA1 flb5301 deoC1 ptsF25 rbsR*) (Silhavy *et al.*, 1984).

All *E. coli* strains were grown in Luria Bertani broth (LB), or on Luria Agar (LA) with the desired antibiotic supplements and incubated at the desired temperature. Liquid cultures were shaken at approximately 240 rpm on a G10 Gyrotory Shaker (New Brunswick Scientific Co, Inc., Edison, NJ, USA).

2.2: General plasmids used in this study (appendices 2 and 3)

pUC19: (Yanisch-Perron *et al.*, 1985)

pBluescript II SK⁻: (Short *et al.*, 1988).

pTNS#A: pBluescript derivative containing a segment of *C. jejuni* 81116 *flaA* gene interrupted by the *C. coli* kanamycin resistance gene (Trieu-Cuot *et al.*, 1985). pTNS#A was isolated by Wassenaar *et al.*, 1991 (Wassenaar *et al.*, 1991).

pJMK30: 1.5 kb *Campylobacter coli* (*apha-3*) Km^R gene in pUC19 with copy of polylinker on both sides (van Vliet *et al.*, 1998)

pAV35: pBluescript SK⁻ derivative containing a Cm^R gene cassette, bracketed by six restriction sites (van Vliet *et al.*, 1998). The primers used in construction were RAA17 and RAA18 (Yao *et al.*, 1993). The chloramphenicol cassette is inserted into the *Bam*HI cassette of the original vector

pAV123: pUC19 derivative containing promoterless chloramphenicol and kanamycin cassette.

pMW10: LacZ transcriptional assay vector (Wösten *et al.*, 1998).

p23E5: promoter of *C. jejuni metK* inserted into *Bam*HI of pMW10 (Wösten *et al.*, 1998)

2.3: Storage of bacterial strains.

Bacterial strains were incubated overnight on LA, CSA or MHA plates containing the appropriate antibiotic selection. The cells were harvested from the plate and resuspended in 0.5ml of LB or MHB broth within a 1.25ml cryotube. A volume of 0.5ml 50% glycerol was then added to the cell suspension, mixed thoroughly and then stored at -80°C.

2.4: Media and preparation of antibiotics

2.4.1: Media

All reagents used in media were of analytical grade, supplied by Fisons Scientific Equipment, Loughborough, UK., unless otherwise stated.

Luria-Bertani broth (LB) (Roth, 1970) was prepared by adding 10g of bacto-tryptone (Difco), 5g of bacto-yeast extract (Difco) and 10g of NaCl to 0.95L of deionised water. The

pH was adjusted to 7.0 with 5N NaOH. The volume was adjusted to 1L and the medium was autoclaved at 121°C for 15 mins.

Luria-Bertani agar (LA) prepared as above but with 15g/L of grade A bacteriological agar (BBL) added.

Mueller Hinton agar (MHA) was prepared by dissolving 15.2g of Mueller Hinton Agar base (Oxoid, Unipath UK) in 0.4L of deionised water and then autoclaved at 121°C for 15 mins.

Mueller Hinton broth (MHB) was prepared by dissolving 21g of Mueller Hinton broth powder (Oxoid, Unipath UK) in 1L of deionised water and was autoclaved at 121°C for 15 mins.

***Campylobacter* blood-free selective agar (CSA)** was prepared by dissolving 22.75g of *Campylobacter* blood-free selective agar base (Oxoid, Unipath UK) in 0.5L of deionised water and then autoclaved at 121°C for 15 mins.

SOB medium was prepared by dissolving 20g/L of bacto-tryptone (Difco), 5g/L of yeast extract (Oxoid, Unipath UK), and by adding 0.01M NaCl, 0.005M KCl, 0.01M MgCl₂ and 0.01M MgSO₄. The final volume was adjusted with deionised water. The medium was autoclaved at 121°C for 15 mins and aliquoted into 20ml Sterilin containers to be stored at -20 °C until required.

SOC medium was prepared by adding 0.02M glucose (filter sterilised) to sterilised SOB medium

X-gal. Where appropriate the media also contained 20µg/ml 5-bromo-4-chloro-3-indoyl- β-D-galactoside (X-gal) as the majority of cloning experiments utilised the vector pUC19 or pBluescript SK-.

2.4.2: Antibiotic supplements

All antibiotics were purchased from Sigma. Prior to use, all antibiotic stock solutions were filter sterilised (table 2.1.).

Table 2.1: Antibiotics and their respective concentrations.

	Stock solution concentration	Storage	Final concentration in media
Ampicillin	100 mg/ml in H ₂ O	4°C	100 µg/ml
Kanamycin	50 mg/ml in H ₂ O	4°C	50 µg/ml
Chloramphenicol	20 mg/ml in 95% Ethanol	4°C	20 µg/ml

2.5: Miscellaneous buffers and solutions

All reagents used in solutions were of analytical grade, supplied by Fisons Scientific Equipment, Loughborough, UK unless otherwise stated.

10 x Phosphate buffered saline (PBS): 1.37M NaCl, 0.27M KCl, 0.015M KH₂PO₄ and 0.08M Na₂HPO₄. After adjusting the pH of the buffer to pH7.4 with 2N HCl, the final volume was adjusted with distilled water. The buffer was autoclaved at 121°C for 15 mins.

0.5M EDTA was prepared by dissolving 0.5M of EDTA (disodium diaminoethane tetra acetate) in distilled water. 5M NaOH was added to pH8.0 to dissolve the EDTA. The final volume was adjusted with distilled water. The solution was autoclaved at 121°C for 15 mins.

Tris-HCl buffers: 1M Tris was dissolved in distilled water to give a volume of approximately 60% the final volume required. Concentrated HCl was added to give a pH just above the desired values of 6.8, 7.2, 7.5, 8.0 and 9.5. The buffer was allowed to equilibrate overnight with mixing before final adjustments were made to the pH. The final volume of buffer was adjusted with distilled water and autoclaved at 121°C for 15 mins.

TAE buffer: 0.04M Tris [hydroxymethyl] aminomethane (Tris), 0.001M EDTA. After adjusting the pH of the buffer to pH7.8 with glacial acetic acid, the final volume was adjusted with distilled water. The buffer was autoclaved at 121°C for 15 mins.

TE Buffer: 0.01M Tris-HCl, 0.001M EDTA, pH8.0. The buffer was autoclaved at 121°C for 15 mins.

CTAB/NaCl solution: This solution was prepared by dissolving 0.7M of NaCl in distilled water, followed by the slow addition of 10% (w/v) of CTAB (hexadecyltrimethyl ammonium

bromide; Sigma), whilst heating and stirring. The final volume was adjusted by the addition of distilled water. The solution was not autoclaved.

IPTG (isopropylthio- β -D-galactoside) was prepared by dissolving 200mg/ml IPTG in distilled water. The solution was filter sterilised and dispensed in small aliquots which were then stored at -20°C.

Phenol/chloroform was prepared by mixing 200ml of liquefied phenol and 200ml of chloroform. 0.4g 8-hydroxyquinoline (Sigma) were added as an antioxidant. The pH was equilibrated to 7.5 by extracting twice with 150ml of 1M Tris pH7.5, followed by one extraction with 150ml of 0.1M Tris pH7.5 and finally 150ml of 0.01M Tris pH7.5. The pH was checked with pH-sensitive indicator papers (BDH). The phenol/chloroform mix was stored in a shatter-proof bottle at 4°C in the dark under 0.01M Tris-HCl pH7.5.

Chloroform/isoamyl alcohol 24:1 was prepared by mixing 240ml of chloroform with 10ml of isoamyl alcohol.

Proteinase K (Sigma) was prepared by dissolving 20mg/ml proteinase K in distilled water at a concentration of 20mg/ml and stored in small single-use aliquots at -20°C.

RNAse A (Sigma) was prepared by dissolving 10mg/ml RNAse A in distilled water to a final concentration of 10mg/ml and aliquoted into 1.5ml tubes. The samples were boiled for 15 mins to inactivate DNases and stored at -20°C.

3M sodium acetate pH5.2 was prepared by adding 3M sodium acetate to distilled water. The pH was adjusted to pH 5.2 with glacial acetic acid and the final volume adjustments were made with distilled water. The solution was autoclaved at 121°C for 15 mins.

10% (w/v) SDS was prepared by adding sodium lauryl sulphate to distilled water and heating until dissolved. The solution was not autoclaved.

X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) was prepared by dissolving 20mg/ml X-gal in dimethylformamide. The solution was stored at -20°C in the dark to prevent damage by light.

20 x SSC: 3M NaCl, 0.3M $C_6H_5Na_3O_7 \cdot 2H_2O$ (*tri*-sodium citrate) (pH7.0)

STE: 0.1M NaCl, 10mM Tris (pH 8.0) and 1mM EDTA. The solution was autoclaved.

***Campylobacter* electroporation buffer (CEB):** 0.272M sucrose and 15% (v/v) glycerol). The solution was autoclaved.

2.6: Preparation of plasmid DNA from *E. coli* DH5 α F'/MC4100 host cells

Several protocols were used for the preparation of plasmid DNA depending upon the yield and quality of the DNA required.

2.6.1: Small-scale plasmid DNA preparation (mini-prep)

Small-scale plasmid DNA preparation (3-5ug) was used to analyse recombinants after initial isolation. This protocol was modified from the plasmid preparation procedure described by Birnboim and Doly, (1979). 1.5ml of culture was centrifuged at 11600g for 10 mins and the supernatant was discarded. The pellet was resuspended in 100 μ l of solution P1 (100ug/ml RNaseA, 50 mM Tris/HCl, 10 mM EDTA) prior to the addition of 200 μ l of solution P2 (200 mM NaOH, 1% SDS). The microcentrifuge tube containing the cells was then gently mixed by inversion and incubated at 37°C for 5 mins. A further 150 μ l of pre-chilled solution P3 (3M potassium acetate, pH 4.8) was added and the preparation was gently mixed by inversion and incubated on ice for 10 mins. The preparation was then centrifuged at 11600g for 10 mins and the supernatant was removed. The supernatant was then extracted with phenol/chloroform (previously equilibrated with 100 mM Tris pH7.2) and the upper aqueous layer was retained. The aqueous layer was then extracted with a 24:1 mixture of chloroform:iso-amyl-alcohol and once again retained. A 1/10th volume of 3M sodium acetate pH 5.2 was added to the supernatant and gently mixed by inversion. A further 3 volumes of absolute ethanol were then added to the preparation and stored at -20°C for 15 mins to promote DNA precipitation. The contents of the microcentrifuge tube were then centrifuged at 11600g at room temperature for 15 mins prior to the removal of the supernatant. The pellet containing plasmid DNA was then washed in 70% ethanol and dried in a 37°C heating block for 10 mins. The pellet was then resuspended in 50ul of 1 x TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0). All plasmid DNA preparations were stored at -20°C.

2.6.2: Small scale plasmid preparation (Qiagen)

To prepare larger plasmid yields (up to 20µg) Qiaprep Spin plasmid kits (Qiagen, UK) were used. High quality plasmid DNA was required for DNA sequencing and Southern blot preparations. The protocol was followed according to the manufacturers instructions. 1-5ml of overnight culture was pelleted and resuspended in 250µl of buffer P1 prior to the addition of 250µl of buffer P2. The solutions were mixed gently by inversion. A further 350µl of pre-chilled buffer N3 (supplied by Qiagen) was added and mixed by inversion and incubated on ice for 5 mins. The cell debris was pelleted by centrifugation for 10 mins at 11600g and the clear supernatant was recovered. The supernatant was transferred into a Qiaprep-spin column which was placed within a disposable 2ml centrifuge tube. The column/microcentrifuge tube was centrifuged for 1 min at 11600g and the elution was removed and discarded. The column was then washed by application of 0.5ml buffer PB (supplied by Qiagen) and centrifuged for 1 min and the drain flow-through was again removed. The column was washed a second time with 0.75ml of buffer PE (supplied by Qiagen) and centrifuged at 11600g for 1 min. The elution was removed and any residual ethanol which remained from the PE wash step was eliminated by a further spin at 11600g for 1 min. Plasmid DNA was eluted from the column by the application of 100µl of 1 x TE or H₂O to the column immediately before a final spin at 11600g for 1 min.

2.6.3: Large-scale plasmid preparation (Qiagen)

For high quality plasmids yields of up to 100µg, Qiagen Tip-100 columns were used. 100ml of an overnight culture was harvested in two 50ml tubes by centrifugation at 3020g for 20 mins at 4°C. The supernatants were removed and both pellets were resuspended in a total of 4ml of buffer P1. A volume of 4ml buffer P2 was then added, mixed by inversion and incubated at 37°C for 5 mins. A volume of 4ml buffer P3 was then added and also mixed by inversion. The preparation was then centrifuged at 3020g for 30 min at 4°C. The clear supernatant was then removed from the centrifuge tube and added to a Qiagen Tip-100 column which had been pre-equilibrated with 3ml of buffer QBT (50 mM NaCl, 50 mM MOPS, 15% ETOH, 0.15% Triton X-100 pH7.0). The column was then washed twice with 10ml of buffer QC (1.0M NaCl, 50 mM MOPS, 15% ETOH pH7.0) and allowed to empty by

gravity flow. The plasmid DNA was eluted with 5ml of buffer QF (1.25M NaCl, 50 mM MOPS, 15% ETOH pH8.2) into a plastic universal containing 3.5ml of isopropanol. The DNA/isopropanol mixture was then gently mixed and split into 6 equal 1.4ml portions. Each tube was incubated on ice for 10 mins to promote DNA precipitation and then centrifuged for 30 min at 11600g at room temperature. The pellets were then washed with 70% ethanol, dried under vacuum and resuspended in 40µl 1 x TE.

2.7: Preparation of *C. jejuni* genomic DNA

2.7.1: Small-scale preparation of *C. jejuni* DNA

Campylobacter cells were spread onto a fresh CSA or MHA plate to make a “swab” plate which was incubated overnight in a VAIN at 37°C. Cells were recovered from the agar plate and resuspended in 1.5ml MHB. The cells were pelleted by spinning at 11600g for 10 mins at 4°C. The supernatant was removed and the cells were resuspended in 0.5ml STE in a 1.5ml microcentrifuge tube. 50µl of 10% SDS were then added and mixed with the resuspended cells. A further 5µl of 20 mg/ml proteinase K and 2.5 µl of 10mg/ml RNase A was then added to the cells prior to incubation for 10 mins at 37°C. After this incubation step, 75µl of 5M NaCl was added and gently mixed with the cell suspension. A volume of 63µl of 10% CTAB/0.7M NaCl was added and mixed gently but thoroughly and the solution was incubated for 20 mins at 65°C. The solution was then extracted with an equal volume of chloroform/isoamyl-alcohol, centrifuged and the supernatant transferred to a new 1.5ml microcentrifuge tube. Genomic DNA was then precipitated by the addition of 0.7 volumes isopropanol, centrifuged at 11600g for 10 mins, washed in 1ml of 70% ethanol and dried. The DNA was gently resuspended in 500µl 1 x TE.

2.7.2: Large scale preparation of *C. jejuni* DNA

For large scale preparation of *Campylobacter* the process was essentially the same. 4 swab plates were made and the cells were harvested in 12ml of MHB, pelleted by spinning at 3020g for 20 mins at 4°C and resuspended in 9ml of PBS. 2ml of 10% SDS was added and mixed with the resuspended cells and a further 100µl of 20 mg/ml proteinase K was then added to

the cells prior to incubation for 1 h at 37°C. After this incubation step, 1.8ml of 5M NaCl was added and gently mixed with the cell suspension. A volume of 1.5ml 10% CTAB/0.7M NaCl was added, mixed and incubated for 20 mins at 65°C. The solution was then extracted with an equal volume of chloroform/isoamyl-alcohol. Genomic DNA was then precipitated by the addition of 0.7 volumes isopropanol, recovered using a glass rod, washed in 1ml of 70% ethanol and dried in air for 5 mins. The DNA was gently resuspended in the minimum volume of 1 x TE (typically 4ml) and incubated at 37°C overnight to ensure that the genomic DNA had completely redissolved in the TE.

2.8: Analysis of DNA by agarose gel electrophoresis

DNA fragments were separated by electrophoresis on horizontal agarose gels. Horizontal agarose gel electrophoresis tanks, well combs and casting trays were manufactured to a standard design by the Leicester University School of Biological Sciences workshop. The gel was prepared by dissolving agarose (SeaKem, FMC bioproducts,UK) in 1 x TAE buffer to a final concentration of 1.0%-2.0% (w/v) depending on the size range to be separated. EtBr was added to the molten agarose to a final concentration of 0.5 μ g/ml. The gel was cast on the perspex tray with a comb inserted in one end to a depth of 0.5-0.75cm. The solidified gels were submerged in electrophoresis tanks containing 1 x TAE buffer. Prior to loading, 2 μ l of 5 x TAE sample buffer (5 x TAE buffer, 15% (v/v) glycerol, 0.3% (w/v) orange G (Sigma)) were added to 0.01ml of DNA sample. The samples were loaded into the wells created by removal of the comb. Molecular weight markers (250 ng) were used and consisted of commercially prepared λ DNA restricted with *Hind*III (Gibco-BRL; 23130bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp, 2027 bp, 564bp and 125bp) and ϕ X174 DNA restricted with *Hae*III (Gibco-BRL; 1353bp, 1078bp, 872bp, 603bp, 310bp, 281bp, 271bp, 234bp, 194bp, 18bp, 72bp). The gels were run at a constant voltage of 11V/cm. The gels were electrophoresed until the orange dye had travelled through approximately 80% of the gel. The DNA was visualised by placing the gel on an ultraviolet (UV) transilluminator (UV Products Ltd) and exposing it to UV light (290nm). DNA bands were photographed using a Polaroid MP4 land camera.

2.9: Purification of DNA

2.9.1: Polyallomer wool gel extraction

DNA fragments were initially separated on an agarose gel. Small blocks of agarose containing the fragment of interest were excised using a scalpel and transferred into 0.5ml microcentrifuge tubes which was pierced in the base with a syringe needle and contained a few strands of polyallomer wool (Interpret™; obtained from a local tropical fish supplier). The 0.5ml tube was placed inside a 1.5ml tube whose cap had been removed and centrifuged at room temperature for 5 mins at 11600g. The liquid collected in the 1.5ml tube was transferred to a fresh tube, and the tubes containing the polyallomer wool and agarose were centrifuged twice for 5 mins. The liquid fractions collected after the three centrifugations were mixed together. Following a phenol/chloroform extraction, the DNA was ethanol precipitated washed in 70% ethanol and dried for 10 mins in a waterbath. The pellet was resuspended in distilled water or TE.

2.9.2: Phenol/chloroform:chloroform isoamyl alcohol extraction

A more traditional method for the removal of proteins from nucleic acid solutions was to extract the DNA with phenol:chloroform and then with a 24:1 chloroform:iso-amyl alcohol mixture (Sambrook *et al.*, 1989). Purification of DNA using this protocol was frequently used throughout this study, particularly after restriction modification of DNA. Solutions containing the DNA to be purified were adjusted to 400µl by the addition of H₂O. Tris-saturated phenol was prepared as described in (Sambrook *et al.*, 1989). An equal volume of phenol:chloroform was then added to the DNA solution and mixed by vortex until an emulsion formed. DNA of high molecular weight was mixed gently by inversion. The mixture was centrifuged at 11600g for 5 mins and the upper aqueous layer was transferred to a fresh tube. An equal volume of chloroform:iso-amyl alcohol (24:1) was added to the solution and mixed. The mixture was then centrifuged at 11600g for 5 mins and the upper aqueous layer was transferred to a fresh tube and ethanol precipitated.

2.9.3: Ethanol precipitation of DNA

To increase DNA concentration in solution or to remove an excess of salt or to purify it, DNA was precipitated by adding 1/10th volume of 3M sodium acetate pH5.2 and 2.5 volumes of 100% ethanol to the sample. If a small DNA fragment was to be precipitated, 1µl of 10mg/ml tRNA (type A from *E. coli*, Sigma) was also added to the tube as a co-precipitant. The sample was incubated for 20 mins on ice and subsequently centrifuged at 11600g in a microcentrifuge. The supernatant was discarded and the pellet was gently washed in 1ml of 70% (v/v) ethanol. The supernatant was removed after 5 mins centrifugation and the DNA was either air dried or dried in a 37°C heating block or water bath. The resulting dried sample was resuspended in an appropriate volume of either TE (pH 8.0) or dH₂O and stored at -20°C.

2.9.4: Purification of PCR amplified DNA

The Qiaquick PCR purification kit (Qiagen) was used to purify double stranded PCR products away from primers, nucleotides and polymerase. Initially 5 volumes of Buffer PB were added to 1 volume of PCR reaction and mixed gently. A Qiaquick spin column was placed into a 2ml collection tube and the sample was added to the column. The column was then centrifuged at 11600g for 1 min and the flow-through fraction was discarded. The sample was then washed by the application of 0.75ml of Buffer PE to the column. The column was centrifuged at 11600g for 1 min and the flow-through fraction was discarded. Residual Buffer PE was removed by an additional spin at 11600g for 1 min. The column was then transferred to a clean microcentrifuge tube and the DNA was eluted from the column by the application of 50µl of TE (pH 8.0) or H₂O. The column was then centrifuged at 11600g for 1 min and the flow-through fraction was retained. DNA amplified from PCR reactions using this protocol was suitable for subsequent restriction modification, ligation and PCR amplification.

2.10: Determination of DNA concentration in solution

The concentration of DNA in solution was determined by diluting 0.01ml of the sample to be tested in 0.99ml of water and transferring it into a 1ml quartz cuvette. The absorbance was measured at 260 and 280nm, using a Pharmacia Ultraspec III spectrophotometer. The

concentration was calculated given 1.0 A₂₆₀ unit equals 50µg/ml of double stranded DNA or 40µg/ml of single stranded DNA. The ratio of A₂₆₀/A₂₈₀ was also determined in order to assess the purity of the DNA; a ratio of 1.5-1.9 was considered acceptable.

2.11: Enzyme modification of genomic and plasmid DNA.

2.11.1: Restriction endonuclease digestion

Restriction endonucleases were provided by Gibco-BRL at a concentration of 10 units/µl and the corresponding restriction buffers were provided at a 10 x normal concentration. For most restriction enzyme reactions, 2-5 units of enzyme were added to 0.2-0.5µg of DNA sample in a 20µl reaction containing 1 x concentration of the respective enzyme buffer. The digestion conditions applied were those suggested by the manufacturer for each endonuclease and usually consisted of a 4h incubation at 37°C. In cases where larger amounts of DNA were digested, all the components of the reaction were scaled up accordingly and the incubation time extended to overnight. The restriction reaction was terminated by adding 2µl of 5 x TAE sample buffer and the digest was loaded onto an agarose gel for analysis. In instances where the DNA was to be sub-cloned, the reaction was terminated by inactivating the restriction endonuclease. Inactivation conditions consisted of heating the sample at 65°C for 10 mins or, when the endonuclease was heat-resistant, by extraction with phenol/chloroform and chloroform/iso-amyl alcohol as described above.

2.11.2: Dephosphorylation

Dephosphorylation of restricted plasmid DNA with 5' compatible ends was performed using calf intestinal alkaline phosphatase (CIP, Pharmacia). The DNA was resuspended in H₂O and 10 x CIP buffer (0.01M ZnCl₂, 0.01M MgCl₂, 0.1M Tris-HCl; pH8.3 Pharmacia) to give a final volume of 20µl. CIP was diluted 20-fold in reaction buffer and 0.1unit CIP was added to the digested DNA and incubated for 30 mins at 37°C. After dephosphorylation the reaction was terminated by the addition of 0.5µl of 0.5M EDTA and 80µl of distilled water. CIP was inactivated by phenol/chloroform extraction and the DNA was ethanol precipitated.

2.11.3: Ligation of genomic and plasmid DNA

Ligation reactions were carried out using T4 DNA ligase at 5U/ μ l (Gibco-BRL) and 5 x ligation buffer (50 mM Tris HCl pH7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT 5% polyethylene glycol-8000; supplied by Gibco-BRL). In a typical ligation reaction the molar concentration of insert was 2-3 times that of the vector. In 10 μ l reactions, ligations were typically incubated overnight at 16°C, although intramolecular ligation reactions were incubated for 3hrs at room temperature. In some experiments the vector DNA was dephosphorylated to prevent the intramolecular ligation of the vector DNA.

2.12: Transformation by electroporation

2.12.1: Electrotransformation of plasmid DNA into *E. coli* DH5 α and MC4100 host strains

2.12.1.1: Cell preparation

E. coli cells were transformed by an electroporation protocol as described by Dower *et al*, (1988). Briefly, an overnight culture of cells was diluted 1:100 in 50ml of LB broth and incubated at 37°C until the culture had reached an optical density (OD₆₀₀) of 0.5-0.7. The culture was then cooled on ice to prevent further growth and subsequently centrifuged at 3020g for 15 mins at 4°C. The supernatant was discarded and the cells were gently resuspended in 20ml of ice cold dH₂O. The cells were washed five times in 20ml of ice cold dH₂O. After the final wash the cells were centrifuged at 3020g for 5 mins and resuspended in 500 μ l of dH₂O.

2.12.1.2: Transformation

Prior to electroporation, the respective DNA sample was ethanol precipitated to remove excess salt and resuspended in 10 μ l of distilled water. In a 0.5ml tube, 40 μ l of electrocompetent cells were mixed with 5 μ l of DNA and transferred into an ice-cold electroporation cuvette (BioRad) with a 0.5mm electrode gap. The cuvette was placed in the BioRad Gene Pulser cuvette holder. Electroporation was performed with an electrical discharge of 1.5kV, a capacitance of 25 μ F and with a resistance load of 1000 Ω . After electroporation, the time

constant shown on the Gene Pulser unit was noted; values below 18ms indicated a trace contamination with salts, which would be expected to result in a reduction in the efficiency of transformation. Immediately after electroporation, the cuvette contents were resuspended in 1ml of SOC medium in a glass test tube and incubated with shaking for 1hr at 30 or 37°C. The culture was transferred into a 1.5ml tube and centrifuged briefly at room temperature, at 13000g. The cell pellet was resuspended in 0.1ml of the recovery medium (SOC). Serial dilutions were prepared and plated out on LA containing the appropriate antibiotic selection for transformants.

2.12.2: Electrotransformation of plasmid DNA into *C. jejuni* strains 81116 and 480

2.12.2.1: Cell preparation

C. jejuni 81116 or 480 cells from a frozen stock kept at -80°C were plated onto MHA or CSA and incubated overnight at 42°C or 37°C, respectively, in a VAIN with a microaerophilic atmosphere. The strain was passed 2-3 times onto fresh plates and finally used to prepare electrocompetent cells. Bacterial cells were harvested from four plates showing fully confluent growth and resuspended in 10ml of ice-cold *Campylobacter* electroporation buffer (CEB). The cell suspension was transferred into a sterile 50ml centrifuge tube and centrifuged for 30 min at 4°C, 3200g in a Heraeus Megafuge 1R. The supernatant was discarded and the cells washed twice in 10ml of ice-cold CEB. The bacterial cells were finally resuspended in 0.3ml of ice-cold CEB and used immediately.

2.12.2.2: Transformation

In a 0.5ml tube, 50µl of electrocompetent cells were mixed with 5µl DNA and stored on ice for 10 mins. The mix was transferred into an ice-cold electroporation cuvette (BioRad) with a 0.5mm electrode gap. The cuvette was placed in the BioRad Gene Pulser cuvette holder. Electroporation was performed with an electrical discharge of 2.5kV, a capacitance of 25µF and with a resistance load of 200Ω. After electroporation, the time constant typically varied between 4.3 and 4.6ms. Immediately after electroporation, the cuvette contents were resuspended in a 0.1ml of SOC medium or MHB and plated onto MHA or CSA and incubated overnight at 42°C or 37°C, in a microaerophilic atmosphere. In addition, the percentage of cell killing due to electroporation was assessed by electroporating cells with no DNA and

plating out dilutions onto MHA or CSA. The number of electroporated cells was compared to a non-electroporated control. The plates were incubated overnight at 42°C or 37°C, in a microaerophilic atmosphere.

After a period of recovery, typically 5hr-overnight the cells were washed off the plates with SOC medium or MHB and centrifuged for 5 mins at room temperature, 3200g in a Heraeus Megafuge 1R. The bacterial pellet was resuspended in the same medium and plated onto MHA or CSA containing the appropriate antibiotic selection for transformants. The plates were incubated at 42°C or 37°C, in a microaerophilic atmosphere for up to a week.

2.13: DNA amplification using the Polymerase Chain Reaction.

2.13.1: Amplification of purified plasmid and chromosomal DNA

Amplification of DNA using the polymerase chain reaction (PCR) was performed within 200 or 500µl centrifuge tubes in a automatic thermal DNA cycler (Hybaid Omnigene). The standard PCR reaction mixture (10µl) consisted of 50 mM KCl, 10 mM Tris HCl pH9.0, 0.1% Triton X-100, 0.2 mM dNTPs, 1pmol of each primer, 3 units of *Taq* polymerase (BioTaq, supplied by Advanced Biotechnologies, UK) and an appropriate concentration of template DNA (typically 2-20ng). The reaction mixture was made up to the required volume by the addition of sterile distilled water. Mineral oil (RNase, DNase and protease free, Sigma) was added to each centrifuge tube to prevent evaporation of the PCR reaction mixture during thermal cycling. PCR cycling conditions were adapted from Sambrook *et al.*, (1989) and are given in table 2.2.

Table 2.2: General PCR conditions.

	Seg no.	Temp.(°C)	Time.(mins:secs)	Cycle (No.)
Stage 1	1	95	01:00	35
		A	01:00	
		72	B	

Temperature A, typically 50°C or 55°C, and time B were adjusted to individual PCR requirements. Cycling conditions for individual PCR reactions are given in each results section separately.

2.13.2: Amplification of DNA from bacterial colonies

Colony PCR was used to screen a large number of transformants to check whether a cloning experiment had worked. The method is based on that described by Güsson and Clarkson, (Güsson and Clarkson, 1989). For the colony PCR, template was prepared by resuspending the bacterial colony in 100µl of distilled water. The cell suspension was boiled for 5 mins to lyse the bacterial cells and centrifuged for 5 mins at room temperature, 13000g in a microcentrifuge. The supernatant was transferred to a clean, 500µl tube, and 1-2µl was used as template DNA.

2.14: Determination of DNA sequence by automated sequencing

DNA was prepared for sequencing using the ABI Prism Dye terminator cycle sequencing ready reaction kit (Applied Biosystems International, USA). All reagents were supplied with the kit. For each reaction 200ng of DNA was mixed with 3.2pmol of primer and 8µl of the automated sequencing reaction mix (1.58µM A-DyeDeoxy, 94.74µM T-DyeDeoxy, 0.42µM C-DyeDeoxy, 47.37µM G-DyeDeoxy, 78.95µM dITP, 15.79µM dATP, 15.79µM dCTP, 15.79µM dTTP, 168.42 mM Tris-HCl (pH 9.0), 4.21 mM (NH₄)SO₄, 42.10 mM MgCl₂, 0.42units/µl AmpliTaq DNA Polymerase, supplied by Applied Biosystems International) in a 20µl volume. The reaction mixtures were then overlaid with 20µl of mineral oil and placed into a thermal cycler (Hybaid Omnigene). Template purity is of extreme importance for automated sequence analysis. Fresh plasmid DNA to be sequenced was therefore prepared using the Qiagen small-scale plasmid purification kit to optimise template quality. The PCR program for automatic cycle sequencing is given in table 2.3.

Table 2.3: PCR conditions for automated sequencing.

	Seg no.	Temp.(°C)	Time.(mins:secs)	Cycle (No.)
Stage 1	1	96	00.30	25
	2	50	00:15	
	3	60	04:00	

Once the PCR reaction was complete, the sequencing reaction mixture was removed and a 1/10th volume of 3M sodium acetate (pH 5.2) was added and mixed prior to the addition of 2-3 volumes of absolute ethanol. This was incubated on ice for 20 mins to promote DNA precipitation. The pellet of DNA was then formed by centrifugation at 11600g for 15 mins and gently washed twice in 70% ethanol. Excess 70% ethanol was removed and the pellet air dried. The dry DNA pellet was given to the Protein and Nucleic Acids Chemistry Laboratory for sequence determination using the ABI 337 automated DNA sequencer. The DNA sequence data was returned to the university computer network and analysed using the Wisconsin Package, (Version 8, September 1994) via the Genetics Computer Group.

2.15: Analysis of genomic or plasmid DNA by Southern hybridisation

2.15.1: Transfer of DNA to a nitrocellulose membrane

The detection of specific sequences separated by agarose gel electrophoresis was performed using protocols based upon those described by Southern, 1975. High purity genomic or plasmid DNA was prepared using the protocols described above and digested with the appropriate restriction enzyme(s). For Southern analysis, DNA 5-10 μ g of genomic DNA and 100-200ng of plasmid DNA was loaded into each well of the agarose gel. Once the electrophoresis was complete the gel was photographed and the gel washes commenced. If the target DNA was more than 15 kb in length then the gel was washed twice in depurinating solution (0.2N HCl). Otherwise the depurinating step was omitted and the gel was washed twice in denaturing solution for 10 mins, twice in neutralising solution for 10 mins and finally in 2 x SSC for 10 mins, all at room temperature. DNA was transferred from the agarose gel to a nitrocellulose membrane by capillary transfer for a minimum of 2hrs. The DNA was fixed to the membrane by exposure to UV radiation using a calibrated UV transilluminator. Detection of target DNA bound to the nylon membrane was accomplished using a Non-radioactive random prime system. Both protocols were used during this study and are described below.

2.15.2: Non-radioactive Southern hybridisation (Gene Images Random Prime Alkaline Phosphatase System™ (Amersham Life Science))

2.15.2.1: Labelling

DNA to be labelled as the probe for the Southern hybridisation was first purified by one of the methods described above. The probe labelling reaction mixture consisted of 10µl of nucleotide mix, 5µl of primers, 50ng denatured DNA and 4 units of Klenow enzyme. The probe labelling reaction mixture was made up to 50µl total volume by the addition of H₂O. The mixture was prepared as follows; a minimum of 50ng probe DNA was denatured by boiling for 5 mins in a water bath. The primers and nucleotide mix were added immediately after boiling and the DNA was placed on ice to cool. Once cool the enzyme solution (4units of Klenow) was added to the mixture and gently mixed prior to incubated for 1 h at 37°C. The labelled probe was denatured by boiling for 5 mins before being added to the pre-hybridisation solution.

2.15.2.2: Detection (CDPstar™ system (Amersham Life Science)).

The membrane was washed in 5 x SSC prior to pre-hybridisation whilst the hybridisation buffer (5 x SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulphate, 0.5% (w/v) blocking agent) was preheated to 60°C. The required volume of hybridisation buffer (typically 50-100ml) was added to the membrane and incubated for at least 30 mins at 65°C with constant, gentle agitation. The boiled probe was then added to the pre-hybridisation buffer containing the membrane which was then incubated with constant gentle agitation overnight at 65°C. Stringency wash buffer 1 (0.1% (w/v) SDS, 1 x SSC) and stringency wash buffer 2 (0.1% (w/v) SDS, 0.5 x SSC) were prepared and pre-warmed to 65°C. After the hybridisation was complete, the membrane was transferred into 250ml stringency wash buffer 1 and incubated for 15 mins at 65°C. This wash step was repeated. The membrane was then transferred to 250ml stringency wash buffer 2 and incubated for 15 mins at 65°C. This wash step was also repeated. Following the hybridisation washes the membrane was briefly rinsed in an excess of diluent buffer (100 mM Tris-HCl, 300 mM NaCl, pH adjusted to 7.0). The membrane was then incubated in 5ml of liquid blocking agent made up to 50ml in diluent for 1 hr at room temperature. The membrane was briefly washed in excess diluent buffer prior to the addition of Anti-Fluorescein Alkaline Phosphatase (Anti-Fi AP) conjugate diluted 5,000-fold in

freshly-prepared 0.5% (w/v) BSA in diluent buffer. The membrane was incubated with gentle agitation for a further 60 min at room temperature. Unbound conjugate was removed by washing the membrane three times in an excess volume of 0.3% (v/v) Tween-20 for 10 mins at room temperature. Excess diluent buffer was first drained from the membranes which were then placed DNA side up onto a fresh sheet of Saran Wrap. Detection reagent was sprayed evenly over the surface of the membrane and incubated for 5 mins at room temperature. Excess detection reagent was removed and the membrane was placed between two fresh pieces of Saran Wrap without the formation of air pockets. The membrane was then placed DNA side up in a film cassette together with a single piece of autoradiography film. The film was exposed at either room temperature or 37°C for up to 2hr, after which the first film was developed. The time scale to develop any subsequent films was altered accordingly.

2.16: Methods for the manipulation of proteins

2.16.1: Preparation of fractionated proteins of *Campylobacter*

Bacterial strains were grown overnight on MHA in a microaerophilic environment at 37 or 42°C. The cells were harvested with MHB, chilled on ice and their OD₆₀₀ determined using a Pharmacia Ultraspec III spectrophotometer. Bacterial cells (normally 3-4 swab plates) were harvested by 10 mins centrifugation at 4°C, 3200g in a Heraeus Megafuge 1R washed once with 1 x PBS and then resuspended in 1ml of TES (10mM Tris-Cl pH 7.5, 10mM EDTA, 25% (w/v) sucrose, filter sterilised). The cells were then pelleted by centrifugation for 10 mins at 11600g at room temperature. The cells were resuspended in 1ml of TES and subsequently centrifuged. The bacterial cells were resuspended in a volume of ice cold dH₂O according to this formula:

$$(a) \quad \text{Volume dH}_2\text{O required } (\mu\text{l}) = [\text{initial volume of cells } (\mu\text{l}) \times \text{OD}_{600}] / 30$$

The resuspension was incubated on ice for 10 mins and centrifuged for 5 mins at room temperature, 13000g using a Sigma 113 centrifuge. The supernatant is the periplasmic fraction and the pellet is the spheroplast fraction. The spheroplast fraction was resuspended in an equal volume of 10mM Tris-Cl pH7.5 as in (a). The cells were disrupted on ice by sonicating with 4-5 pulses of 15secs each with a 15sec interval, using a Soniprep sonicator (MSE) with a 0.3cm diameter probe. Non-disrupted organisms were removed by

centrifugation at 13000g in a micro-centrifuge at 4°C for 15 mins. The membrane fraction was pelleted by centrifugation (Beckman TL-100 ultracentrifuge) at 50 000 rpm, for 10 mins at 20°C. The cytoplasmic fraction is contained within the supernatant and the pellet contains the crude membrane fraction. The pellet was resuspended in 1/6 of the volume calculated in (a) in solubilisation buffer (10mM Tris-Cl pH, 7.5., 7mM EDTA, 0.6% (w/v) sarcosyl) and incubated for 30 mins at 37°C to aid solubilisation. The outer membranes were pelleted by centrifugation (Beckman TL-100 ultracentrifuge) at 50 000 rpm for 10 mins at 20°C; the supernatant contained the inner membrane fraction which was retained and the pellet contained the outer membrane fraction. The outer membrane fraction was washed once with solubilisation buffer and subsequently ultracentrifuged (Beckman TL-100). The pellet was washed twice more in 1 x PBS each time followed by centrifugation. The outer membrane fraction was finally resuspended in 1/8 of the volume of calculated in (a). To samples to be electrophoresed, an equal volume of 2 x SDS-PAGE sample buffer (0.25M Tris-HCl, pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, 0.1% (w/v) bromophenol blue) was added and the suspension was incubated at 95°C for 5 mins, the tube was vortexed and briefly centrifuged at 11600 g in a microcentrifuge. All protein extracts were stored at -20°C.

2.16.2: Quantitation of proteins using Bradford's colorimetric method.

The amount of protein in samples was quantified using the Bradford method (Ausubel *et al.*, 1992). A BSA (bovine serum albumin) 1mg/ml stock solution was prepared in PBS and diluted to specific final concentrations. 20 μ l from each BSA solution of known concentration or 2 μ l from each *C. jejuni* protein sample were added to 1ml of Bradford's reagent (containing 0.1g of Coomassie brilliant blue R250 (Sigma) dissolved in 100ml of 85% phosphoric acid and 850ml of distilled water and filtered in a vacuum filter unit through a Whatman filter No.1) and their A_{595nm} determined using a Pharmacia Ultraspec III spectrophotometer. The absorbances from the BSA solutions were used to plot a standard curve (A_{595nm} versus concentration) to determine the protein concentration.

2.16.3: One-dimensional electrophoresis of proteins

Fractionated cellular proteins were analysed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), (Laemmli, 1970). SDS-PAGE was performed either using Mini-Protean II (BioRad) apparatus producing "mini-gels" with dimensions of 7.2 x 10.2cm or a large Protean II apparatus producing "maxi-gels" with dimensions of 16 x 16cm. All gels

were of 0.75mm thickness. The stacking gels were of 5% (v/v) and the separating gels of 11% (v/v) final concentration (table 2.4.) using 30%:0.8% (w/v) acrylamide mix (Protogel, supplied by National Diagnostics; 30%:0.8% acrylamide:bis-acrylamide). Electrophoresis was performed using SDS-PAGE running buffer: 0.025M tris, 0.192M glycine, 0.19% (w/v) SDS, pH8.3-8.6. "Mini-gels" were electrophoresed at a constant current of 15mA. "Maxi-gels" were electrophoresed for 5h at 25mA (constant) or overnight at a voltage of 40V (constant).

Prior to electrophoresis, non-radiolabelled protein samples and protein molecular weight standards, Pharmacia Biotech, (LMW Electrophoresis Calibration Kit Phosphorylase b; 94 kDa, Bovine Serum Albumin; 67 kDa, Ovalbumin; 43 kDa, Carbonic Anhydrase; 30 kDa, Soybean Trypsin Inhibitor; 20.1 kDa, α -Lactalbumin; 14.4 kDa) were boiled for 3 mins and loaded on the gel. 10 μ l of the stainable molecular weight standards were loaded onto "mini-gels" (or 15 μ l on "maxi-gels"). Electrophoresis was interrupted when the dye front entered the bottom tank buffer. The gels were stained for at least 5h (preferably overnight) in Coomassie brilliant blue staining solution (containing 0.25g of Coomassie brilliant blue R250 (Sigma) dissolved in 45ml of methanol, 45ml of distilled water and 10ml of glacial acetic acid and gently rocked. The gels were destained in 40% (v/v) methanol, 10% (v/v) glacial acetic acid solution until the background became clear and then were rehydrated in distilled water, photographed and dried using a BioRad Gelair drier programmed to dry at 80°C.

Table 2.4: Method for the preparation of SDS-polyacrylamide gels.

Solutions	"Mini-gel"		"Maxi-gel"	
	5%	11%	5%	11%
	<u>Stacking gel</u>	<u>Separating gel</u>	<u>Stacking gel</u>	<u>Separating gel</u>
2 x Buffer A (0.75M Tris-HCl, 0.2% (w/v) SDS, pH8.8)		2.7ml		13.5ml
2 x Buffer B (0.25M Tris-HCl, 0.2% (w/v) SDS, pH6.8),	2ml		10ml	
Distilled water	1.3ml	0.56ml	6.5ml	2.8ml
30% Acrylamide mix (30:0.8 acrylamide : bis-acrylamide)	0.7ml	2ml	3.5ml	10ml
10mg/ml APS (ammonium persulphate; Sigma)	0.2ml	0.19ml	0.5ml	0.95ml
TEMED (N,N,N',N'-tetramethylethylenediamine; Sigma)	0.016ml	0.015ml	0.04ml	0.075ml

2.17: Miscellaneous experimental procedures

2.17.1: Analysis of c-myc expression using an enzyme linked immunosorbent assay (ELISA)

2.17.1.1: Preparation of bacterial samples

Bacterial samples were grown overnight on MHA swab plates with appropriate selection in a microaerophilic atmosphere (VAIN, Don Whitley Scientific) at either 37 or 42°C. The cells were harvested, pelleted and resuspended in 500µl of coating buffer (0.1M Na₂HCO₃, pH 9.6), together with protease inhibitor, PMSF to a final concentration of 1mM. The cells were then sonicated on low power, amplitude 1 (MSE Sonicator), until the suspension became clear. The total protein concentration for each sample was calculated using a standard curve obtained by using a Bradford assay.

2.17.1.2: Application of ELISA

Total protein samples at their respective amounts (µg) were made up to 100µl with coating buffer and aliquoted onto the 96 microwell ELISA plate (Immulon-1, Dynatech). The plate was incubated at room temperature for 1h and subsequently washed three times with Tris-buffered saline (TBS; pH7.2) containing 0.05% Tween 20 (polyoxyethylene [20] sorbitan monolaurate). Non-specific binding sites were blocked using TBS containing 2% skimmed milk powder for 1h at room temperature, 100µl per well. The plate was washed 3 times with TBS Tween, as above. The primary c-myc antibody (9E10) was diluted 1:200 in 100% foetal calf serum and 50µl was added per well. The dilution of primary c-myc antibody (Genosys, Cambridge, UK) to use was obtained from a standard curve against a positive control human c-myc peptide (OP-11-3059, Genosys, Cambridge, UK). The antibody was left to incubate for 1h before washing again three times with TBS Tween, as above. 50µl of the second antibody, a rabbit anti-mouse IgG antibody directly linked to horse-radish peroxidase diluted to 1:1000 was added and left to incubate for 1h at room temperature. Once again, the plate was washed three times with TBS Tween. The colour was developed in reactive wells using 0.75mgml⁻¹ of the soluble chromogen *o*-phenyldiamine in 0.5M formic acid with 0.006% hydrogen peroxide as a substrate, 100µl per well. The reaction was stopped after 5 mins incubation using 100µl 0.1M H₂SO₄. The colour development was measured using an ELISA plate reader (BIO-RAD, model 3550) set to 490nm. The negative control for each experiment was 100µl of

coating buffer, no antigen. The positive control for each experiment was human c-myc peptide, 80ng in 100µl of coating buffer.

2.17.2: Chloramphenicol Assays

2.17.2.1: Single colony growth experiment

Bacterial samples were grown overnight at 37°C on a Mueller-Hinton swab plate with appropriate selection. The cells were harvested in sterile PBS and resuspended to an OD₆₀₀ of 0.1. 10 fold serial dilutions were made from each sample using a 96 microwell plate (Nunc) and 10 µl of each dilution (10⁻¹, through 10⁻⁶) for each bacterial sample was spotted onto MHA plates. Each Mueller-Hinton plate contained one of the following amounts of chloramphenicol; 0, 2, 5, 10 or 20µgml⁻¹. The cells were incubated overnight at either 37 or 42°C and were examined for growth of single colonies after ~24 hrs growth.

2.17.2.2: Chloramphenicol disc sensitivity assay

Bacterial samples were grown overnight on MHA swab plates with appropriate selection and the cells were harvested in sterile PBS to calculate the OD₆₀₀. 25mls of MHA was poured into sterile Petri dishes and allowed to set. Subsequently, to 5ml of molten MHA maintained at 42°C, each bacterial sample was added to a final OD₆₀₀ of 0.4. Before the bacterial agar sample was allowed to solidify, it was poured on top of the pre-made MHA plates and left to set. In order to test the chloramphenicol sensitivity of the bacterial samples, a chloramphenicol sensitivity disc (30µg) was placed in the centre of each plate and the plates were left overnight at either 37 or 42°C. The presence or absence of a halo was examined the following morning. The size of the halo gave an indication of the sensitivity of each bacterial sample.

2.17.3: LacZ assay using β -galactosidase activity as a measure of promoter activity

2.17.3.1: Preparation of *C. jejuni* 480 cells

C. jejuni 480 host cells, with the respective promoter cloned within plasmid pMW10 were grown overnight on MHA swab plates at 37, or 42°C. The following morning the cells were subsequently harvested and resuspended in sterile PBS at a “calculated” OD₆₀₀ value of 0.4. Before starting the assay the “actual” OD₆₀₀ value was determined, which typically varied between 0.37 and 0.45. The samples were maintained on ice.

2.17.3.2: β -galactosidase assay

To 50 μ l of cell sample, 450 μ l of Z-Buffer (0.06M Na₂HPO₄·7H₂O, 0.04M NaH₂PO₄·H₂O, 0.01M KCl, 0.001M MgSO₄·7H₂O and 0.05m β -Mercaptoethanol), 8 μ l of 0.1% SDS and 15 μ l of chloroform were added and vortexed together for 30 secs. Each sample mixture was placed in a 28°C waterbath and allowed to equilibrate for 5 mins. At this point, 100 μ l of *O*-nitrophenyl- β -galactopyranoside (ONPG, 4mgml⁻¹) was added, mixed thoroughly and the solution was incubated at 28°C for a set time for the yellow colour to develop, typically between 7 and 12 mins. The activity of β -galactosidase in *C. jejuni* was measured by the conversion of *O*-nitrophenyl- β -galactopyranoside to nitrophenol as described by Miller, (1972). The reaction was terminated by addition of 250 μ l of 1M Na₂CO₃ and the colour development was measured at two different optical densities, OD₄₂₀ and OD₅₅₀ using a spectrophotometer (Pharmacia LKB, Ultraspec III). Miller units were calculated using the following formula.

$$\frac{1000 \times (OD_{420} - (1.75 \times OD_{550}))}{OD_{600} \times 0.1 \times t \text{ (s)}}$$

2.18: Bioinformatics

2.18.1: Homology searches and sequence analysis tools

<http://www2.ebi.ac.uk/clustalw/>

http://www.toulouse.inra.fr/prodom/doc/blast_form.html

<http://www.ncbi.nlm.nih.gov/BLAST>

<http://www.ncbi.nlm.nih.gov/gorf/bl2.html>

<http://expasy.hcuge.ch/sprot/prosite.html>

2.18.2: Signal peptide prediction

<http://www.cbs.dtu.dk/services/SignalP>

2.18.3: Kyte and Doolittle hydropathy and protein secondary structure predictions

<http://expasy.hcuge.ch/cgi-bin/protscale.pl>

2.18.4: Prediction of protein transmembrane regions.

http://www.ch.embnet.org/software/TMPRED_form.html

Chapter 3: The heat shock response:

3.1: Introduction

The heat-shock response is a universal physiological phenomenon that occurs in both eukaryotic and prokaryotic cells. It is a highly conserved biological response that allows the cell to deal with adverse conditions. In prokaryotes, the products of many heat shock genes act as molecular chaperones or cellular proteases enabling the cell to process partially folded proteins in the cytoplasm targeting them for secretion, proteolysis or refolding (Goff, 1985; Ang, 1991; Gething, 1992). The result is a protective response under stressful conditions (Gross, 1996). Many of the heat shock genes are constitutively expressed and have an important role in house-keeping functions in addition to maintaining the integrity of the cell in unfavourable conditions. In order to facilitate this dual response the organism requires a highly co-ordinated pattern of gene expression (Herendeen, 1979; Yamamori, 1982).

3.2: General characteristics of the *E. coli* heat shock response

The best characterised heat shock response is that elicited in *E. coli*. Within the normal growth range, the variation in rate of synthesis of most proteins exhibits very little change. But, after an upshift in temperature there is a group of about 20 proteins that undergo a transient increase (10-20 fold) in their rate of synthesis. Conversely, a downshift in temperature sees an immediate decrease in the level of synthesis and an eventual low temperature steady state level (Lemaux, 1978; Neidhart, 1987). This group of proteins comprises the *E. coli* heat shock proteins (HSPs) whose expression is regulated at the transcriptional level (Yamamori, 1980; Cowing, 1985) in response to heat, ethanol, viral infection and misfolded proteins (reviewed in Neidhart, 1987).

To adapt to environmental conditions and direct an appropriate response the highly regulated pattern of gene expression is controlled by the σ -subunit of the RNA polymerase holoenzyme which initiates transcription at relevant promoter sites (Burgess, 1969; deHaseth, 1988). With respect to housekeeping genes, transcription is mediated during exponential growth by the primary σ -factor, σ^{70} or *RpoD*, which is essential for growth under normal temperature conditions (Helman and Chamberlin, 1988). Alternative σ -factors control the transcription of certain sets of genes whose products are required for specific functions, e.g. sporulation,

nitrogen fixation or flagella synthesis (Gross, 1992). Transcription of most of these genes are under the influence of changes in environmental stimuli. There are two different heat shock responses in *E. coli* which are triggered differently depending on the cellular localisation of aberrant protein production. Misfolded cytoplasmic proteins induce the classical heat-shock regulon which is transcribed by the alternative σ -factor, σ^{32} , a product of *rpoH* (*htpR* or *hin*). Accumulation of abnormal proteins in the cell envelope induces a second alternative σ -factor, σ^E (σ^{24}) which implies there is inducible transduction machinery in the inner membrane (Grossman, 1984; Erickson, 1987; Erickson and Gross, 1989; Wang, 1989).

3.3: The σ^{32} regulon

The *rpoH* gene of *E. coli* reveals strong homology to *rpoD* (Landick, 1984; Yura, 1984) and was the first alternative sigma-factor to be described in *E. coli* (Grossman, 1984). The *rpoH* gene encodes a 32 kDa sigma-factor, σ^{32} , whose function directs core RNA polymerase to promoters different from those whose transcription is directed by σ^{70} (Cowing, 1985). This allows regulation by *rpoH* to be distinctly different from that of most other cellular proteins.

3.3.1: Role of the σ^{32} regulon

The overall contribution of the σ^{32} regulon has been determined by the effect of mutations in *rpoH* on cell viability. Cells which lack σ^{32} demonstrate that members of the regulon are required for growth throughout the normal growth range in addition to high temperatures. For example, inactivation of *rpoH* renders the cell viable below 20°C albeit with major growth defects (Zhou, 1988). The requirement for σ^{32} at normal temperatures can be attributed to the fact that chaperones of the regulon promote normal folding of most cellular proteins *in vivo* as *rpoH* strains show extensive aggregation of newly synthesised proteins (Gragerov, 1992). Furthermore, HSPs of the regulon also promote proteolysis, as *rpoH* strains are defective in degradation of abnormal peptides and other specific proteins (Baker, 1984; Goff *et al.*, 1984). In addition, some chaperones contribute to the proteolytic processes as mutant strains appear defective in this process (Keller, 1988; Straus, 1988). It has been demonstrated that some σ^{32} HSPs are required at all temperatures, but a greater concentration of HSPs is necessary as the temperature increases (Yamamori, 1982).

3.3.2: Members of the σ^{32} regulon

Components of the σ^{32} regulon constitute a very diverse array of heat shock proteases and chaperones which control the various protein interactions within the cell. The molecular chaperones include DnaK, DnaJ and GrpE which function as one chaperone group and GroEL and GroES which function as another. The two systems have different modes of action (reviewed in Georgopolous, 1993). Together, these chaperones play a significant role in cellular protein folding reactions i.e., folding of nascent chains, maintenance of proteins destined to be secreted in a translocation-competent form and refolding of proteins after thermal damage (Kusukawa, 1989; Gragerov, 1992; Nelson, 1992; Wild, 1992; Hendrick, 1993; Horwich, 1993; Schroder, 1993; Ziemienowicz, 1993). Furthermore, these proteins have a role in general proteolysis (Keller, 1988; Straus, 1988). Other chaperones include CbpA, which is a functionally similar homologue of DnaJ (Ueguchi, 1994), HtpG, which is potentially involved in protein translocation (Ueguchi, 1992) and IbpA and IbpB which are associated with inclusion bodies and perform chaperone functions (Jakob, 1993). In addition to chaperones, there is an array of proteases. For example, Lon (La), is an ATP dependent serine protease which plays an important role in general protein degradation by removing abnormal proteins from the cell (Chung, 1981). Lon probably works in conjunction with DnaK, DnaJ and GrpE to degrade abnormal proteins (Keller, 1988; Straus, 1988). In addition, the Clp family of two component proteases (ClpP, ClpA, ClpX and ClpB), which are heat inducible proteases, degrade a variety of specific proteins and play a secondary role in degrading abnormal proteins (Katayama-Fujimura, 1987; Hwang, 1987; 1988; Kitagawa, 1991; Squires and Squires, 1992). Finally, HflB, is the only essential protease currently known in *E. coli* (Herman *et al.*, 1993).

3.3.3: Regulation of σ^{32}

Temperature affects expression of σ^{32} but how HSPs are influenced accordingly depends upon how translation, stability and activity of σ^{32} is altered by a distinct set regulatory mechanisms (Gross, 1996). Synthesis of σ^{32} is not affected at the transcriptional level but moreover at the translational level by RNA secondary structure and an increase in temperature is reflected by an increase in translation (Straus *et al.*, 1989). Thermal regulation is controlled by specific *cis*-acting regions of *rpoH* RNA which in turn affect synthesis of σ^{32} (Kamath-Loeb and

Gross, 1991; Nagai *et al.*, 1991). In addition, specific *trans*-acting elements affect σ^{32} synthesis, including the DnaJ-DnaK-GrpE chaperone group (Straus *et al.*, 1990). Not only do HSPs affect translation of σ^{32} they are important in regulating its stability. The HflB protease, described previously, is thought to be the major protease responsible for degrading σ^{32} (Herman *et al.*, 1995) with further degradation facilitated by the DnaK-DnaJ-GrpE chaperone group (Tilly *et al.*, 1989; Straus *et al.*, 1990). The particular role played by these chaperones in promoting degradation is not well established.

The best characterised feature of σ^{32} is its activity and how the molecule is controlled. Loss of activity occurs most drastically upon a decrease in temperature and when HSPs are in excess (Straus *et al.*, 1989; 1990). Nevertheless, this is a reversible phenomenon as extraction of inactivated σ^{32} from the cell results in the molecule regaining activity (Straus *et al.*, 1989). Importantly, DnaK, DnaJ and GrpE are involved in this process as mutations in either of these components render the cell defective in inactivation (*cit* in Straus *et al.*, 1989). The mechanism is believed to be a reversible interaction between HSPs and σ^{32} which precludes formation and activity of the σ^{32} containing holoenzyme. Ultimately this results in a decrease in transcriptional activity (Straus *et al.*, 1989).

It is clear that the interaction between chaperones, proteases and σ^{32} constitutes a very complex process in which the mechanisms are not well established. This is also true for the signals which govern expression of the σ^{32} regulon. The present understanding is that the signal transduction pathway which controls translation induction is different from that which affects the stability of σ^{32} . For example, an increase in temperature, ethanol and induction of unfolded cytoplasmic proteins all stabilise σ^{32} , although translational induction is only stimulated by temperature and ethanol (Straus *et al.*, 1987; Wild *et al.*, 1993; Kanemori *et al.*, 1994). Furthermore, the HSPs, DnaK, DnaJ and GrpE are involved in the stabilisation of σ^{32} but not in translational induction (Straus *et al.*, 1990). In contrast, secondary structure of RNA is involved in translational induction but not stability of σ^{32} (Nagai *et al.*, 1991). DnaK, DnaJ and GrpE, are all required for inactivation, stabilisation and translational repression. Together these findings indicate that HSPs play a regulatory role in sensing a stimulus although there is no solid evidence for this conclusion (Gross, 1996).

3.4: The σ^E (σ^{24}) regulon.

3.4.1: Activation, regulation and the role of the σ^E regulon.

The *rpoH* gene is transcribed from four different promoters, three of which are initiated under normal growth conditions and require RNA polymerase complexed with σ^{70} . The fourth promoter, *rpoHp3* is not recognised by σ^{70} and shows a different pattern of expression (Erickson, 1987). Therefore, *E. coli* must have a further heat inducible alternative sigma-factor in addition to that of σ^{32} . This has been designated σ^E (σ^{24}) (Erickson and Gross, 1989; Wang, 1989). This alternative sigma factor transcribes *rpoH* from *rpoHp3* at lethal temperatures (Erickson, 1987). σ^E is the product of the *rpoE* gene and cells which lack *rpoE* are viable at 30, 42 and 43°C although at the higher temperatures the bacteria form colonies with greatly reduced efficiency. Moreover, upon exposure to lethal temperatures the mutant dies more rapidly than the parent strain. (Hiratsu *et al.*, 1995; Raina *et al.*, 1995; Rouviere *et al.*, 1995). Similar to the situation for σ^{32} , activity of σ^E is induced by heat and ethanol (Erickson, 1987; Neidhart, 1987; Erickson and Gross, 1989; De Las Penas *et al.*, 1997a). In contrast to the control of cytoplasmic stress, σ^E responds to extracytoplasmic stress, specifically in the periplasm (Mecsas *et al.*, 1993; Rouviere *et al.*, 1995; De Las Penas *et al.*, 1997a). Transcription of *rpoE* was initially thought to be induced exclusively by misfolded outer membrane proteins (OMPs) (Mecsas *et al.*, 1993) and many environmental changes affect processing of OMPs. These include temperature, osmolarity, dessication and starvation. Therefore, σ^E activity was believed to be affected by changing OMP levels and mutations which cause the proteins to fold inappropriately (Mecsas *et al.*, 1993). Indeed now, σ^E is known to respond to the accumulation of any exported protein that is unstable or misfolded (Missiakas *et al.*, 1995; Raina *et al.*, 1995). For example, porins, which are components of the outer membrane which undergo a complex series of folding and oligomerisation steps prior to their insertion into the outer membrane (Pugsley, 1993). A disruption in this pathway can lead to a build up in folding intermediates that are believed to be sensed by the σ^E pathway (Mecsas *et al.*, 1993; Rouviere and Gross, 1996). Similarly, when periplasmic folding catalysts are absent, for example, Dsb proteins (protein disulphide isomerases), SurA and FkpA (peptidyl prolyl isomerases) and Skp (involved in OMP folding reactions), an increase in activity of σ^E results (Missiakas *et al.*, 1996; Chen and Henning, 1996).

Genetic and biochemical evidence has provided additional information about how regulation of σ^E is mediated. The activity of σ^E is under the control of three genes, *rseA*, *B* and *C* which are located immediately downstream of *rpoE*. Topological analysis has revealed that RseA is an inner membrane protein whose *N*-terminal domain is located in the cytoplasm. Purified cytoplasmic domain binds to and inhibits σ^E -directed transcription *in vitro*. Moreover, deletion of RseA results in a 25 fold induction of σ^E and overexpression of RseA decreases repression of σ^E activity. These findings suggest RseA acts as an anti-sigma factor. RseB is a periplasmic protein and co-purifies with the periplasmic domain of RseA indicating that it exerts a negative activity on $E\sigma^E$ through RseA. Additional evidence for the negative effect on $E\sigma^E$ is provided in part by the knowledge that deleted *rseB* causes a slight induction in σ^E . RseC is localised to the inner membrane and deletion of the gene has no effect on $E\sigma^E$ activity under steady state conditions. In conditions when σ^E should be induced, deletion of RseB and/or C shows wild type induction of σ^E activity. This implies that a number of $E\sigma^E$ regulating pathways are present or that RseA can function alone in both sensing and transmitting information (De Las Penas *et al.*, 1997b; Missiakas *et al.*, 1997a).

3.4.2: Members of the σ^E regulon.

In response to a number of different inducers, RNA polymerase containing σ^E mediates transcription of at least ten different genes which function to maintain envelope integrity under stress conditions (Raina *et al.*, 1995; Rouviere *et al.*, 1995). To date only four have been identified (Erickson, 1987; Lipinska *et al.*, 1988; Erickson and Gross, 1989; Wang, 1989; Raina *et al.*, 1995; Rouviere *et al.*, 1995; Danese and Silhavy, 1997a;). These are *rpoH*, which encodes σ^{32} and functions to alleviate cytoplasmic stress, *fpkA* which encodes a periplasmic peptidyl prolyl isomerase, *rpoE* itself which supports the idea that *rpoE* is autoregulated and *degP* (*htrA*), a periplasmic protease, and the focus of the next part of the discussion.

3.5: HtrA

3.5.1: Introduction

Most information regarding HtrA has been gathered from *E. coli*. A number of studies have verified important characteristics of the protein. For example, HtrA (DegP) is a 48kDa periplasmic serine protease induced by heat shock and is indispensable for survival at temperatures above 42°C (Lipinska *et al.*, 1989; Strauch *et al.*, 1989). Like many other heat shock proteins, homologues of HtrA have been discovered in a wide variety of organisms, including eubacteria, cyanobacteria, yeast and humans (Zumbrunn and Trueb, 1996; Ponting, 1997). Some bacterial species have more than one example of HtrA (Bass *et al.*, 1996; Glazebrook *et al.*, 1996). Nevertheless, HtrA is not a prerequisite for independent life as the protein is absent from some bacterial species e.g. *Mycoplasma genitalium* and *Methanococcus janaschii* (Pallen and Wren, 1997). Nucleotide sequence analysis of members of the HtrA family of heat shock proteins has identified putative proteolytic active sites which suggest they all function as serine proteases (Ponting, 1997), although there appears to be one exception, the *htrA* homologue from *Rickettsia* (reviewed in Bass *et al.*, 1996). The physiological role of the *E. coli* HtrA serine protease and at least some other HtrA homologues is to degrade aberrant proteins arising in the periplasmic space under stress conditions (Neidhart, 1987). Mutations in the *htrA* gene are pleiotropic and may affect, heat, oxidative or osmotic stress responses as well as virulence of a number of organisms such as *E. coli* (Lipinska *et al.*, 1990), *Brucella abortus* (Roop *et al.*, 1994; Tatum *et al.*, 1994; Elzer *et al.*, 1994; 1996), *Brucella melitensis* (Phillips *et al.*, 1995), *Salmonella typhimurium* (Johnson *et al.*, 1990; 1991; Baumler *et al.*, 1994) and *Yersinia enterocolitica* (Li *et al.*, 1996). Interestingly, an *htrA* mutant of *Helicobacter pylori* has no obvious phenotype (Kleanthous, 1994). Given the relative importance of the molecule to the physiology of the cell a great deal of interest has surrounded its structural and functional properties and the complex series of signal transduction pathways which determine how *htrA* is regulated. Additionally, the fact that *htrA* mutants of a number of bacterial pathogens show a reduced virulence in animal models has raised the possibility of their use as potential live vaccine candidates (Chatfield *et al.*, 1992; Li *et al.*, 1996). The HtrA family of serine proteases has been recently reviewed by Pallen and Wren, (1997).

3.5.2: Structural and functional relationships of *E. coli htrA*

3.5.2.1: Primary, amino acid structure

E. coli HtrA is synthesised as a 51 kDa unstable precursor protein and is processed by removal of an amino acid leader peptide (Lipinska *et al.*, 1988; 1989; 1990). Removal of this signal peptide occurs at the signal peptidase I cleavage site, 26 amino acid residues from the *N*-terminus. Following this cleavage site there are 50 amino acids of unknown function and then a 30 residue proline/serine/glutamine rich region which has the characteristics of a flexible segment linking domain called a Q-linker (Pallen and Wren, 1997; Wooton and Drummond, 1989). Experiments using the mature 48kDa protein have demonstrated ATP-independent endopeptidase activity and the ability to degrade β -casein (Cavard *et al.*, 1989). To date, the only identified natural substrate for HtrA is the acylated precursor form of the colicin A lysis protein (Cavard *et al.*, 1989; Cavard, 1995). Proteolytic activity of HtrA is inhibited by diisopropyl fluorophosphate, a specific inhibitor of serine proteases (Cavard *et al.*, 1989; Lipinska *et al.*, 1990). The HtrA protein contains the amino acid sequence GNSGGAL, starting at position 208 of the mature protein (Brenner, 1988; Lipinska *et al.*, 1990). This sequence is similar to the consensus sequence GNSGGPL, surrounding the active serine residue of many trypsin-like serine proteases (Brenner, 1988; Ponting, 1997). Together with the serine residue, histidine and aspartate residues within the trypsin-like serine proteases constitute a catalytic triad essential for their proteolytic activity (Brenner, 1988; Ponting, 1997). The importance of two of these putative catalytic residues, Ser²¹⁰ and His¹⁰⁵ for *E. coli* HtrA been demonstrated. Mutation and exchange for alternative amino acids totally eliminates protease activity without a detectable change in secondary structure (Skorko-Glonek *et al.*, 1995b). The amino acid sequence around the active site serine resembles that of trypsin-like serine proteases, although that which surrounds His¹⁰⁵ (Brenner, 1988) bears no resemblance to those found around active histidines of trypsin-like serine proteases (Skorko-Glonek *et al.*, 1995b). Nevertheless, as His¹⁰⁵ is the only one present in HtrA (Brenner, 1988) it is conceivable that this histidine is the element of the catalytic triad present in most serine proteases (Skorko-Glonek *et al.*, 1995b). There are many aspartates conserved in HtrA proteins (Brenner, 1988; Skorko-Glonek *et al.*, 1995b) but none of them are surrounded by sequences found around known active site aspartates in other molecules. This makes it difficult to determine which one is the important element of the catalytic triad (Skorko-Glonek *et al.*, 1995b). Following the serine protease active site, the *C*-terminus contains two PDZ

domains (Fanning and Anderson, 1996; Ponting, 1997). PDZ domains, also termed DHR or GLGF domains, are named after the three eukaryotic proteins (post synaptic density protein, disc large and zo-1 protein) where they were first identified. PDZ domains are 80-100 amino acid repeats which interact with C-terminal tetrapeptides (X-Ser/Thr-X-Val-COO) of ion channels and/or receptors (Ponting, 1997). Formerly, PDZ domains were identified mainly in eukaryotes such as mammals, flies and worms although recently their identification has widened to other phyla such as higher plants, yeast and subsequently bacteria. The functional significance of these domains is discussed below.

3.5.2.2: Secondary, tertiary and quaternary structure

The major secondary structural element of HtrA is β -sheet (Skorko-Glonek *et al.*, 1995b). The serine protease domain at the N-terminus interacts to form a double antiparallel β -barrel, and the PDZ domain in the C-terminus manifests into a β -sandwich structure. Alterations to the 3D structure of the molecule are effected by temperature (Skorko-Glonek *et al.*, 1995b; Fanning and Anderson, 1996) which in turn affects the proteolytic activity of the molecule (Skorko-Glonek *et al.*, 1995b). Individually folded HtrA protein molecules bind together to form oligomeric complexes, possibly dodecamers (Waller and Sauer, 1996; Kolmar *et al.*, 1996). The importance of these structures is not well established although it is postulated that oligomerisation may facilitate allosteric interaction between subunits. Together, with the presence of multiple cleavage sites centralised into one enzyme unit, the processivity of the molecule may be enhanced. It is proposed that oligomerisation favours formation of a ring like structure with an inner channel harbouring the active sites (Kolmar *et al.*, 1996).

3.5.2.3: HtrA PDZ domains and substrate recognition

PDZ domains in eukaryotes bind to either C-terminal tetrapeptides, other internal sequences or to other PDZ domains and are thus are an integral part in mediating a number of protein-protein interactions (Fanning and Anderson, 1996; Ponting, 1997). Moreover, they may have a role in assembling proteins into large membrane associated complexes which can initiate signal transduction. The significance of PDZ domains in HtrA is not well established although it has been postulated that they contribute to the assembly of individual HtrA molecules into oligomeric complexes, or alternatively, facilitate the recognition of target

proteins. An “anemone” model (Pallen and Wren, 1997) which is an extension of the model proposed by Kolmar *et al.*, 1996 (an oligomeric ring with an active site inner channel), implicates PDZ domains as fundamental to these processes (Pallen and Wren, 1997). It is believed that projections from PDZ domains (tentacles) bind to unfolded target proteins and transfer them to the inner channel. The inner channel is lined with multiple active sites where the target protein is degraded (or refolded). For this to occur, the transfer stage must be accompanied by a conformational change within the multi-subunit complex. To facilitate this process, the protein targeted for degradation by HtrA must possess a C- terminal or internal recognition sequence which can be recognised and bind to the PDZ domains, be present in an unfolded conformation displaying its recognition sequence enabling it to fit into the active site channel and finally contain cleavage sites recognised by active site domains (Ponting, 1997). HtrA is thought to recognise specific targets by binding to a short sequence either within the protein or at the C- terminus. Presentation of the internal sequence or short C-terminal sequence to the PDZ domain would occur when the periplasmic protein becomes denatured, ultimately targeting it for destruction (Pallen and Wren, 1997). Specific recognition of the protein as a target for the protease is different to that of the cleavage site within a target and it has been established that these independent processes are carried out by individual domains in the HtrA protease, the PDZ domain and the catalytic triad (Kolmar *et al.*, 1996; Pallen and Wren, 1997). It has been demonstrated how PDZ domains act but not how the catalytic triad determines the cleavage specificity of HtrA (Kolmar *et al.*, 1996).

3.5.2.4: Location and the role of HtrA in the cell

In contrast to early reports, (Swamy and Goldberg, 1982), HtrA has now been found located on the periplasmic side of the inner membrane (Skorko-Glonek *et al.*, 1997). The main physiological role of HtrA within the cell, identified by mutational analysis of the *htrA* gene, is to degrade abnormal periplasmic proteins (Strauch and Beckwith, 1988; Strauch *et al.*, 1989). There is a link between the inability of null mutants to degrade abnormal periplasmic proteins and thermal sensitivity stemming from the fact that a non-proteolytic, correctly folded HtrA variant, when supplied in *trans* is unable to relieve temperature stress of an HtrA⁻ mutant (Skorko-Glonek *et al.*, 1995a; 1995b). HtrA is able to stabilise, unstable envelope-associated proteins but not cytosolic proteins (Strauch and Beckwith, 1988). This point is supported by the fact that mutations in *htrA* prevent degradation of certain fusion proteins and

mutant proteins located in the periplasm and inner membrane but not in the cytosol. Further direct evidence for HtrA's role in degrading denatured proteins is given by experiments with thermally aggregated endogenous proteins, labelled as the S-fraction, and mutants defective in HtrA (Laskowska *et al.*, 1996). Addition of exogenous purified HtrA protease participated in the removal of aggregated proteins of the S-fraction. During the time course of the experiment a third of the S-fraction was converted to TCA soluble products. As noted, the S-fraction contains a mixture of different aggregated material (Laskowska *et al.*, 1996), but previously, the only recognised normal substrate for *E. coli* HtrA was the acylated precursor form of colicin A lysis protein (Cavard, 1995). Recently, in *Haemophilus influenzae* unprocessed HMW1, a non-pilus adhesin which mediates attachment to human epithelial cells was found to be degraded in part by the *H. influenzae* HtrA protease (Geme and Grass, 1998).

3.5.2.5: Suppressors of HtrA

Null mutants in *htrA* show a temperature sensitive phenotype (Lipinska *et al.*, 1990). Suppression of this phenotype can be afforded in two different ways, either by a second site mutation (Strauch and Beckwith, 1988) or suppression by a particular determinant in multicopy (Baird and Georgopoulos, 1990; Baird *et al.*, 1991; Tatum *et al.*, 1994; Waller and Sauer, 1996). The proteolytic defect removed by extragenic suppressors could involve, i) altering the specificity or increasing the production of another protease that can now adopt the function of the *htrA* gene product, ii) elimination of the production of the toxic protein(s) which accumulate in the absence of the HtrA protein or iii) alteration of the membrane to allow leakage of the toxic protein(s). The latter example is especially true of the accompanying mutation causing lipoprotein deficiency, i.e., a mutation in the *lpp* gene which prevents a build up of abnormal periplasmic proteins by effecting their release from the periplasm into the surrounding medium (Strauch and Beckwith, 1988). Suppression by multicopy expression has been documented using *B. abortus* HtrA to complement for the lack of the corresponding *E. coli* copy of the protein (Tatum *et al.*, 1994). Other examples include an HtrA homologue in *E. coli*, HhoA (see below) and overexpression of SohB (Baird *et al.*, 1991) and SohA (Baird and Georgopoulos, 1990). SohB might have a protease function in the cell envelope of *E. coli*. It bears homology with the inner membrane enzyme protease IV of *E. coli* which digests cleaved signal peptides. In this example, there is partial complementation for the missing HtrA function (Baird *et al.*, 1991). Additional suppression is provided by

SohA a protein involved in export from the cytoplasm, although the mechanism behind the suppression is not understood (Baird and Georgopoulos, 1990).

3.5.3: Regulation of HtrA

HtrA is under the influence of a complex series of signal transduction pathways (Pallen and Wren, 1997) (figure 3.1.). Stresses that perturb the bacterial envelope in *E. coli* increase the synthesis of the periplasmic HtrA protease. Consistent with this observation is that HtrA is not regulated at the transcriptional level by the classical heat shock regulon involving σ^{32} but a second heat shock σ -factor, σ^E (Lipinska *et al.*, 1988). As discussed earlier, σ^E controls extracytoplasmic stress response in *E. coli* whose activity is induced not only by heat and ethanol but also uniquely, disruption of protein folding in the periplasm (Mecenas *et al.*, 1993; Rouviere *et al.*, 1995; Missiakas and Raina, 1997a). σ^E controls transcription of at least 10 genes, one of which is *htrA*. How increased transcription of *htrA* upon protein misfolding in the periplasm is signalled to the σ^E regulon has been addressed genetically by constructing a transcriptional fusion between the *htrA* promoter and the *lacZ* gene and searching for *trans*-acting mutations which affected the expression of LacZ (Raina *et al.*, 1995). Many mutations mapped to the *rpoE* gene but others mapped to the Cpx operon (Danese *et al.*, 1995) and implicated two new genes *prpA* and *prpB* (Missiakas and Raina, 1997b) (see below). These observations indicated that *htrA* transcription is also regulated by a second system further to that of the σ^E regulon.

3.5.3.1: The Cpx signal transduction pathway

The Cpx signal transduction pathway is prominent in regulating protein trafficking factors in the bacterial envelope. The *cpx* locus consists of a two component inner membrane sensor (CpxA) and a cognate response regulator (CpxR). CpxA encodes a protein with homology to the classical histidine kinases of the two component systems (Weber and Silverman, 1988) such as EnvZ or CheA, reviewed in (Parkinson, 1993) (see chapter 6). Mutations in CpxA decrease but do not abolish transcription of *htrA* as there is still σ^E -dependent transcription. CpxR encodes a protein with homology to classical DNA binding regulatory proteins, such as OmpR (Dong *et al.*, 1993). A recently identified third member, CpxP, is a pH regulated

periplasmic protein that aids combating extracytoplasmic protein mediated toxicity (Dance and Silhavy, 1998). Cpx mediated suppression of extracytoplasmic stress is in part due to production of HtrA.

Stresses to the bacterial envelope activate the Cpx pathway, but the signals can be distinct from those which activate the σ^S regulon implying that the two systems overlap but are not identical.

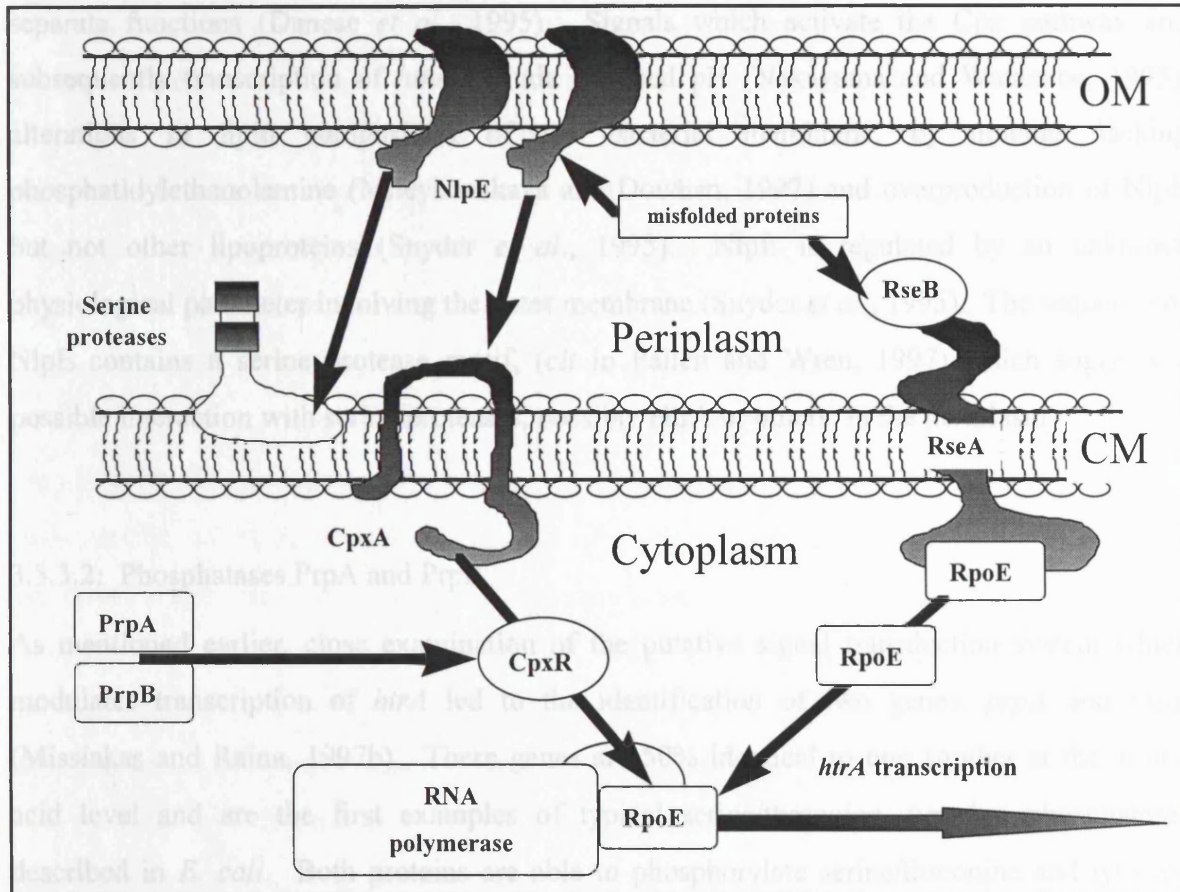


Figure 3.1: Regulatory network leading to transcription of *htrA*. NlpE is regulated by an unknown physiological parameter involving the outer membrane and harbours a serine protease motif suggesting that it interacts with serine proteases (HtrA or others). NlpE senses misfolded proteins and activates CpxA by unknown mechanisms. CpxA phosphorylates CpxR which activates transcription of *htrA*. Prp phosphatases fine tune regulation of Cpx system. Misfolded proteins in the periplasm activate *rpoE* which is modulated by RseA and RseB. RseA normally sequesters RpoE and releases RpoE when RseB senses misfolded proteins by unknown mechanism. RpoE activates transcription of *htrA*. CpxR, PrpA, PrpB and RpoE interact with other signal transduction systems and network with other cellular processes. OM: Outer Membrane; CM: Cytoplasmic Membrane. (Figure adapted from Pallen and Wren, (1997)).

periplasmic protein that aids combating extracytoplasmic protein mediated toxicity (Danese and Silhavy, 1998). Cpx mediated suppression of extracytoplasmic stress is in part due to production of HtrA.

Stresses to the bacterial envelope activate the Cpx pathway, but the signals can be distinct from those which activate the σ^E regulon implying that the two systems overlap but carry out separate functions (Danese *et al.*, 1995). Signals which activate the Cpx pathway and subsequently transcription of *htrA* include elevated pH (Nakayama and Watanabe, 1995), alterations in lipid composition of the bacterial membrane, by mutants lacking phosphatidylethanolamine (Mileykovskaya and Dowhan, 1997) and overproduction of NlpE but not other lipoproteins (Snyder *et al.*, 1995). NlpE is regulated by an unknown physiological parameter involving the outer membrane (Snyder *et al.*, 1995). The sequence of NlpE contains a serine protease motif, (*cit* in Pallen and Wren, 1997) which suggests a possible interaction with serine proteases, possibly HtrA or others, in the periplasm.

3.5.3.2: Phosphatases PrpA and PrpB

As mentioned earlier, close examination of the putative signal transduction system which modulates transcription of *htrA* led to the identification of two genes, *prpA* and *prpB* (Missiakas and Raina, 1997b). These genes are 50% identical to one another at the amino acid level and are the first examples of typical serine/threonine, tyrosine phosphatases described in *E. coli*. Both proteins are able to phosphorylate serine/threonine and tyrosine residues *in vitro* (Missiakas and Raina, 1997b). The interaction with CpxA/R *in vivo* suggests, however, that they may also function as aspartate and histidine phosphatases (Missiakas and Raina, 1997b). PrpA and PrpB behave as positive modulators of CpxA/R-dependent *htrA* transcription and consequently, mutations in either gene decrease the basal level of transcription. Consistent with these findings when present in multicopy *prpA* and *prpB* can induce *htrA* transcription even in the absence of misfolded proteins (Missiakas and Raina, 1997b).

3.5.4: Homologues of HtrA

Homologues of a particular gene or protein can be classified into two main areas, paralogues and orthologues,

3.5.4.1: Paralogues

Paralogues are homologous genes which are present in the same genome and are thought to have arisen from a common ancestor through gene duplication (Pallen and Wren, 1997). There are two paralogues of *E.coli htrA*, *htrA* homologue A (*hhoA* or *degQ*) and *htrA* homologue B (*hhoB* or *degS*) (Bass *et al.*, 1996; Waller and Sauer, 1996). At the DNA level, *hhoA* and *hhoB* are contiguous on the chromosome and transcribed in the same direction. The *htrA* gene is induced at elevated temperatures by the σ^E containing RNA polymerase but nothing resembling σ^E like promoter sequences are apparent for either *hhoA* or *hhoB* (Erickson and Gross, 1989; Bass *et al.*, 1996; Waller and Sauer, 1996). HhoA and HhoB encode proteins of 455 and 355 amino acids respectively. Both have an N-terminus characteristic of standard signal peptides (Pugsley, 1993) including basic N domains, hydrophobic cores and signal peptidase cleavage sites. HhoA has a 27 residue signal peptide sequence which suggests a periplasmic origin (Waller and Sauer, 1996). The cellular location of HhoB is not known as the signal sequence is not removed from His tagged purified protein, the significance of which is unclear (Waller and Sauer, 1996). HhoA is 58% and HhoB is 35% identical to HtrA and there is 30% identity with each other (Waller and Sauer, 1996). HhoA possesses two PDZ domains as is the case for HtrA whereas HhoB has only one (Ponting, 1997). Despite their similarities at the protein level, deletion mutants in either *hhoA* or *hhoB* do not confer a thermosensitive phenotype on the cell, characteristic of *htrA* mutants (Bass *et al.*, 1996; Waller and Sauer, 1996). Purified HhoA has serine protease activities and is able to complement the temperature sensitive phenotype of *htrA* null mutants when present in multicopy, supporting its function as a serine protease *in vivo* (Bass *et al.*, 1996; Waller and Sauer, 1996). The two proteins HtrA and HhoA are homologous endoproteases found in the periplasm and are very similar in size, 474 compared to 455 amino acids respectively (Bass *et al.*, 1996; Waller and Sauer, 1996; Kolmar *et al.*, 1996). They have very similar substrate specificities and cleave either substrates which aggregate following heat shock or other stress conditions and/or newly secreted proteins prior to folding and disulphide bond formation (Kolmar *et al.*, 1996). Cross-linking studies on HhoA have determined that the protein forms dodecamers in solution indicative of many other periplasmic proteases, especially HtrA

(Kolmar *et al.*, 1996). HhoA is inhibited by diisopropyl fluorophosphate and mutagenesis experiments of the catalytic triad components support its function as a serine protease similar to HtrA (Bass *et al.*, 1996; Waller and Sauer, 1996). Null mutants of *hhoA* have no obvious growth defect. In contrast, null mutants of *hhoB* show a small colony phenotype, which can be rescued by plasmids expressing *hhoB* but not *hhoA* or *htrA* (Bass *et al.*, 1996; Waller and Sauer, 1996). In conclusion HhoB appears to function differently from either HhoA or HtrA (Bass *et al.*, 1996; Waller and Sauer, 1996).

3.5.4.2: Orthologues

HtrA homologues have been identified in a variety of different organisms including eubacteria with high and low G-C content in their genome, cyanobacteria, humans and yeast (table 3.1.). All of these examples possess PDZ domains at the C-terminus (Pallen and Wren, 1997). Moreover, HtrA homologues from Gram positive bacteria and cyanobacteria, like HhoB from *E. coli* and *H. influenzae* have just one PDZ domain but all other examples contain two (*cit* in Pallen and Wren, 1997). The human protein L56 possesses domains homologous to HtrA preceded by an insulin-like growth factor binding domain and a serpin domain (Zumbrunn and Trueb, 1996). The yeast hypothetical protein N1987 consists of two tandem HtrA-like repeats each complete with two PDZ domains (Ponting, 1997). Some of the bacterial examples highlighted above have multiple HtrA-like examples. The presence of HtrA, HhoA and HhoB in *E. coli* has been discussed already but a similar situation is also true of *Haemophilus influenzae*, *Brucella abortus* and *Pseudomonas aeruginosa* (table 3.1.) suggesting that more family members remain to be identified in other bacteria. To date, all of the bacterial examples contain all three active site residues of the catalytic triad but with one exception, *Rickettsia*, which intriguingly has all three components missing. Why this should be the case is unclear. Some proteases can also act as chaperones, particularly those of the cytoplasm and this may also be true of HtrA (Bass *et al.*, 1996; Suzuki *et al.*, 1997). The hypothesis is that the HtrA family of proteins bind periplasmic proteins that have been secreted or those denatured under stressful conditions. At this point they can either release the bound protein toward a folding pathway in chaperone-like mode or degrade it using its serine protease activity (Bass *et al.*, 1996; Suzuki *et al.*, 1997).

Table 3.1: HtrA and related proteins from different prokaryotic and eukaryotic examples

Species	UID(s)	Protein name	Length (aa)	Phenotype	Comments
<i>Azotobacter vinelandii</i>	1345104	MucD	473	NT	in <i>algU/mucABCD operon</i>
<i>Bacillus subtilis</i>	1064809, 1731164	YyxA	400	NT	hypothetical protein
<i>Bartonella henselae</i>	1708339, 310329	HtrA	503	NT	PCR target, antigenic
<i>Brucella abortus</i>	144118, 497157	HtrA	500 or 513	ts (+) ox (-), att (+/-) mac (+)	antigenic, can substitute for <i>E. coli</i> HtrA
<i>Brucella abortus</i>	497155	HtrA-like	474	att (+/-)	not antigenic
<i>Campylobacter jejuni</i>	881375, 1109748	HtrA	466	ts (-) ox (-)	no obvious phenotype
<i>Escherichia coli</i>	41761, 78483, 146414, 473819, 547687, 628706, 1552739, 1786356	HtrA, DegP, Protease Do	491 (474)	ts (+) deg (-)	inner membrane serine protease active in periplasm
<i>Escherichia coli</i>	558912, 606173, 729710, 984379, 1587317	HhoA, DegQ	455	ts (-) osm (-)	serine protease, Prc suppressor
<i>Escherichia coli</i>	558913, 606174, 729711, 984380, 1587318, 1789630	HhoB, DegS	355	ts (-) osm (-) small colonies	gene adjacent to <i>hhoA</i>
<i>Haemophilus influenzae</i>	1074213, 1170273, 1573965	HtrH, HhoB	340	NT	hypothetical protein
<i>Haemophilus influenzae</i>	1075129, 1170411, 1574189	HtrA	466, 463	NT	protective antigen
<i>Helicobacter pylori</i>	Kleanthous, PhD thesis, London	HtrA	462	ts (-) ox (-)	antigenic
<i>Homo sapiens</i>	1513059, 1621244	Serine protease with IGF binding motif	480	NT	putative serine protease with IGF binding and serpin domains, absent in transformed cells

Chapter 3: The heat shock response

Species	UID(s).	Protein name	Length (aa)	Phenotype	Comments
<i>Mycobacterium leprae</i>	699150	HtrA	533	NT	hypothetical protein
<i>Mycobacterium tuberculosis</i>	505551,629197	34kDa antigen	361	NT	antigenic
<i>Pseudomonas aeruginosa</i>	1184675,	AlgW	389	ts (+) ox (+)	regulates alginate production no (leader peptide?)
<i>Pseudomonas aeruginosa</i>	1184684, 1220194	MucD	474	ts (++) ox (+)	regulates alginate production
<i>Rhizobium meliloti</i>	951170, 1586653	RmDEGP	484	ts (+/-) able to induce nodules	
<i>Rickettsia tsutsugamushi</i>	473503, '1220497,	47kDa protein	466	NT	hypothetical protein
<i>Rickettsia typhi</i>	1220499, 1220501	47kDa protein	466	NT	
<i>Saccharomyces cerevisiae</i>	1730771	N1897, YNM3_YEA ST		NT	contains two tandem HtrA-like repeats
<i>Salmonella typhimurium</i>	1183950	HtrA	475	ts (-) ox (+) att (+) mac (+)	
<i>Synechocystis sp</i>	1651868	HhoA	394	NT	hypothetical protein
<i>Synechocystis sp</i>	1652463	HtrA	452	NT	hypothetical protein
<i>Synechocystis sp</i>	1653090	HhoB	416	NT	hypothetical protein
<i>Yersinia enterocolitica</i>	1419351, 1526428	HtrA, GsrA	464 or 478	ts (+), ox (+) osm (+) att (+) mac (+)	

Key to mutant phenotypes: thermosensitive, (ts); sensitive to oxidative stress, (ox); sensitive to osmotic stress, (osm); attenuated in animal model, (att); decreased survival in macrophages, (mac); inability to degrade abnormally folded periplasmic proteins, (deg); and not tested, (NT), (-) indicates the mutant does not show phenotype; (+/-) indicates an equivocal result; (+) indicates that the mutant shows the phenotype; (++) indicates the mutant shows a marked phenotype. UIDs are the accession codes for subsequent database retrieval. Variations in length for the same protein are usually the results of frameshifts at the C-terminus. Table adapted from Pallen and Wren, (1997).

3.5.5: Phenotypes of HtrA mutants

Null mutations in the *htrA* gene have been constructed in several bacterial species and they exhibit a range of overlapping phenotypes (table 3.1.). These include thermosensitivity, susceptibility to oxidative stress and osmotic stress *in vitro*; *in vivo*, various bacterial pathogens such as *S. typhimurium* (Johnson *et al.*, 1991; Chatfield *et al.*, 1992; Baumler *et al.*, 1994), *B. abortus* (Roop *et al.*, 1994; Tatum *et al.*, 1994; Elzer *et al.*, 1994; 1996) and *Yersinia enterocolitica* (Wren *et al.*, 1995; Li *et al.*, 1996; Yamamoto *et al.*, 1996; 1997) have altered survival capacity in macrophages and animal models. The variation in phenotypes observed could be for a number of reasons. HtrA homologues may function differently *per se* or it could reflect a difference in the regulation of gene expression or other properties linked to the physiology of the organism. Furthermore the strain in which the mutant has been generated may carry a secondary gene or mutation which complements some of the functions of HtrA. Lastly it may be a consequence of the different environmental stresses encountered by the bacterium during infection or its free living state.

3.5.6: HtrA and virulence

A number of bacteria in which *htrA* homologues have been identified are pathogens and have a significant part of their life-cycle in an intracellular environment. In most cases, the presence of an *htrA* homologue has been implicated in their survival within this hostile environment. These include *S. typhimurium*, *B. abortus* and *Y. enterocolitica*

3.5.6.1: *Salmonella*

The HtrA homologue in *Salmonella* was identified by the use of selective transposon mutagenesis to look for avirulent mutants of a pathogenic strain of *S. typhimurium*. The transposon mutants of *htrA* are avirulent as a consequence of their impaired ability to survive and/or replicate in host tissues (Johnson *et al.*, 1991; Baumler *et al.*, 1994). They have a decreased survival capacity within macrophages which is a prerequisite for virulence (Fields, 1989). They are not temperature sensitive like *E. coli* (Lipinska *et al.*, 1990) or *B. abortus* (Elzer *et al.*, 1994; Tatum *et al.*, 1994) but are sensitive to oxidative stress elicited by redox cycling reagents such as menadione and hydrogen peroxide (Johnson *et al.*, 1990). This

would account for their decreased survival in macrophages which exhibit oxidative killing mechanisms. *Salmonella htrA* mutants also have decreased ability to survive within mice. The LD₅₀ rises 10 000 fold following intravenous injection and moreover, the *htrA* mutant was unable to kill mice after oral inoculation with the bacteria being quickly removed from the spleen and liver (Chatfield *et al.*, 1992). Nevertheless, challenging the mice with the *htrA* mutant offered protection against subsequent rechallenge with a virulent strain (Chatfield *et al.*, 1992). These findings have raised the possibility that mutant *Salmonella* strains have potential as vaccine candidates (Chatfield *et al.*, 1992). Mutations in *htrA* (Chatfield *et al.*, 1992) with or without accompanying mutations in genes encoding regulators (*cya/crp* and *ompR*) (Curtiss, 1987; Dorman, 1989) or purine metabolism enzymes (*purA* and *purE*) (McFarland, 1987; O'Callaghan, 1988) have shown promise as typhoid vaccines (Levine *et al.*, 1996; Tacket *et al.*, 1997) or as systems to deliver heterologous antigens to the immune system, e.g. Herpes simplex virus and tetanus toxin (Chabalgoity *et al.*, 1996).

3.5.6.2: *Brucella*

Screening of genomic libraries with anti-*Brucella* antisera identified an immunoreactive antigen which had homology to *E. coli* HtrA (Roop *et al.*, 1994; Tatum *et al.*, 1994). Construction of a *B. abortus* isogenic *htrA* mutant established that the bacterium has a temperature sensitive phenotype and an increased sensitivity to oxidative killing mechanisms in a disc sensitivity assay compared with the parent (Elzer *et al.*, 1994; Roop, 1994; Tatum *et al.*, 1994). Compared with virulent *B. abortus*, the isogenic *htrA* mutants show a decreased resistance to killing by cultured murine neutrophils and macrophages and a significant attenuation during the early stages of infection in the BALB/c mouse model. Observations on bacterial recovery from the spleen and liver of infected mice showed that the level of mutants was initially lower but 60 days post-infection they had increased to levels greater than the wild type (Elzer *et al.*, 1994; Tatum *et al.*, 1994). The same pattern of early attenuation followed by recovery in the BALB/c mouse model was also apparent in *htrA* mutants constructed from a strain of *B. melitensis* (Phillips *et al.*, 1995) and a *B. abortus* vaccine strain (Robertson *et al.*, 1996).

3.5.6.3: *Yersinia*

A homologue of *E. coli htrA* has been isolated independently in *Yersinia enterocolitica* by two groups (Li *et al.*, 1996; Yamamoto, 1996; 1997) and has been designated *htrA* and *gsrA*, (global stress response) respectively. The *Y. enterocolitica gsrA* gene was identified as being essential for protecting cells under both extracellular environmental stress and intracellular stress in macrophages due to phagocytosis. Yamamoto *et al.*, (1996; 1997), showed that an isogenic mutant had a temperature sensitive phenotype, increased susceptibility to hydrogen peroxide and the superoxide anion O_2^- increased susceptibility to high salt concentrations and an increased inability to survive within macrophages. Li *et al.*, (1996) identified the *htrA* homologue using PCR with degenerate oligonucleotide primers (Wren *et al.*, 1992). The gene was subsequently cloned, sequenced and mutated and the resulting isogenic mutant was avirulent in a murine yersiniosis model. Subsequent re-challenge with the parent demonstrated that partial protection to the mouse had been afforded by the mutant. The apparent attenuation of a *Y. enterocolitica htrA (gsrA)* mutant for mice raises the possibility of its use as a vaccine candidate (Li *et al.*, 1996).

The reasons why *htrA* mutants are attenuated are not well established. The proteolytic activity of the molecule may impede host defenses or *htrA* mutants are simply less viable and killed more easily (Bass *et al.*, 1996; Pallen and Wren, 1997). The finding that *htrA* mutants are more susceptible to heat and especially oxidative stress suggests the latter is true as the action of the host's oxidative killing mechanisms are efficient in rendering the invading strain avirulent. A suggested reason for this attenuation is that host bactericidal mechanisms probably increase the amount of denatured proteins in the periplasm to a level where the bacterium is unable to cope. Moreover, an absence of HtrA to combat this lethal effect results in derangement of envelope function. More extravagant hypotheses implicate a role for HtrA to interact with exported virulence factors (Bass *et al.*, 1996; Pallen and Wren, 1997). An accumulation of misfolded proteins directly interferes with oligomerisation and export of such virulence factors. A combination of HtrA's putative chaperone/protein processing and PDZ interactions might function to fold and/or export certain virulence factors. Without HtrA, this mechanism is prevented (Fanning and Anderson, 1996; Pallen and Wren, 1997; Ponting, 1997).

Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region

4.1: Introduction and summary of previous work

The *htrA* gene was found to be conserved in a number of different bacterial species including, *E. coli* (Lipinska *et al.*, 1988) *S. typhimurium* (Johnson *et al.*, 1991) *H. pylori* (Kleanthous, 1994) and *B. abortus* (Elzer *et al.*, 1994; Roop *et al.*, 1994; Tatum *et al.*, 1994). These data facilitated the application of PCR-DOP methodology to investigate whether *C. jejuni* also possessed an *htrA* homologue (Wren *et al.*, 1992; Henderson, 1996). For this purpose, degenerate oligonucleotide primers were designed against sequence data from *E. coli* and *S. typhimurium* to enable PCR amplification on *C. jejuni* template DNA. The result yielded a 473bp product which encoded a fragment of the *htrA* gene, the origin of which was confirmed by Southern hybridisation and PCR. Examination of the open reading frame highlighted that the sub-genic fragment of the proposed *C. jejuni htrA* homologue shared extensive homology with other members of the HtrA protein family.

As the PCR-DOP fragment shared sequence similarity with other *htrA* homologues it was necessary to determine whether there was also functional homology with those organisms in which *htrA* had been identified. As mentioned previously, *htrA* mutants have a range of overlapping phenotypes, including thermal sensitivity in *E. coli*, oxidative stress sensitivity in *S. typhimurium* and both temperature sensitivity and oxidative stress sensitivity in *Brucella abortus*. Intriguingly, an *H. pylori htrA* mutant does not have thermal or oxidative stress sensitivity. A chromosomal *htrA* mutant was constructed in *C. jejuni* with a view to identifying a phenotype. Phenotypic analysis of the mutant compared to the parent strain identified that there was no significant difference in growth profile at 37°C, response to starvation, survival characteristics at 37, 42 and 46°C, sensitivity to oxidative stress, total and periplasmic protein profiles, ability to either associate with or survive within Caco-2 cells and colonisation potential in the avian gut.

The failure to identify a phenotype together with the fact that only a sub-genic PCR-DOP fragment had been identified, prompted efforts to identify the complete open reading frame of the protein and also the putative promoter region upstream. Initially, it was unclear as to whether the PCR-DOP fragment was part of a true *htrA* gene and whether this was the reason for the lack of a phenotype, particularly with regards to the response to both thermal and oxidative stress. Identification of the complete open reading frame would facilitate further

analysis of the amino acid homology between the proposed *C. jejuni* HtrA and other HtrA homologues with a view to confirming its authenticity. Furthermore, identification of the putative promoter region might reveal information about whether the gene is actually expressed and how it is regulated in *C. jejuni*. The regulation of *htrA* in *E. coli* and *S. typhimurium* is dependent upon σ^E which is encoded by *rpoE*. These bacteria have σ^E or σ^E -like promoter regions upstream of the *htrA* gene. On the other hand, sequence analysis of the *H. pylori* genome has failed to identify σ^E gene homologue and therefore a lack of σ^E or σ^E -like promoter region for the *htrA* gene.

Previous attempts to isolate the complete *htrA* gene from *C. jejuni* were unsuccessful. A *C. jejuni* strain F132 *Sau3A1* lambda-Zap library was constructed and screened by hybridisation with the *htrA* PCR-DOP fragment but provided only 70bp of additional *htrA* sequence (Kiernan *et al.*, 1993). This indicated that parts of the *htrA* gene might be unstable in standard *E. coli* hosts. In an attempt to overcome these cloning difficulties, inverse PCR (IPCR), was utilised to isolate the flanking regions of the *C. jejuni* *htrA* gene. The resultant amplicon was 2.38kb which was comparable to the size predicted by Southern hybridisation. Sub-cloning experiments using both high and low copy vectors only isolated the 3' end of the 2.38kb fragment (~1.34kb, designated pJH16) which contained the 3' end of *C. jejuni* *htrA* and the 5' end of a response regulator, *regX4* (figure 4.1.). The inability to obtain 5' sequence of *htrA* (~1.04kb) suggests that this region contains sequence that is unstable and cannot be maintained in *E. coli*. Although only 3' sequence of *htrA* was obtained, analysis of the sequence gave further support that the proposed *htrA* locus was indeed an *htrA* homologue. Clearly, identification of the complete open reading frame and promoter region was necessary to confirm this point. Moreover, sequence analysis of the promoter region may reveal information concerning the regulation of *htrA* expression in *C. jejuni* and allow expression of the gene to be monitored. In pursuit of these objectives, two parallel approaches were applied. The first involved construction of a complete *Sau3AI* library of the upstream, ~1.04kb, region as it was believed that this might limit any detrimental effect on the host cell that cloning of the whole fragment might have previously had. The second approach involved direct sequence analysis of the 2.38 kb fragment containing *htrA* that could be amplified from chromosomal loops.

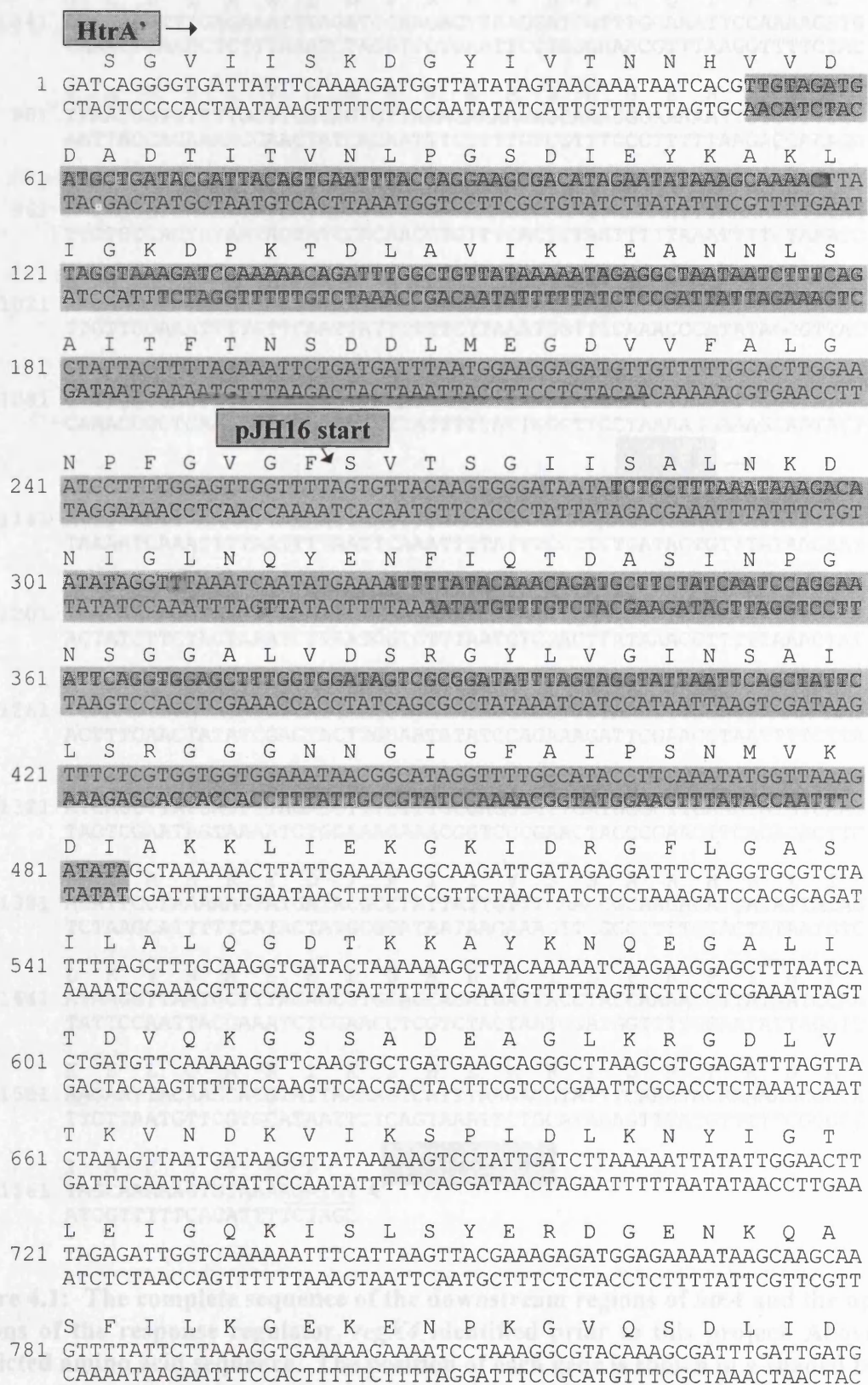


Figure 4.1 The complete sequence of the downstream region of the *htrA* gene. The sequence is shown in the 5' to 3' direction. The sequence is highlighted by a shaded area indicating the predicted start and end of the *htrA* gene. The sequence 3' to the shaded area denotes the sequence identified from the AZAP library. The start and end of sequence contained within this part is given. The arrow denotes the stop codon of *htrA*. The 3' restriction *Bgl*I site is underlined.

Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region.

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      G L S L R N L D P R L K D R L Q I P K D
841  GTTTGAGTTTGAGAAATTTAGATCCAAGACTTAAGGATCGTTTGCAAATTCACAAAGATG
      CAAACTCAAACCTTTTAAATCTAGGTTCTGAATTCCTAGCAAACGTTTAAGGTTTTCTAC

      V N G V L V D S V K E K S K G K N S G F
901  TTAATGGTGTGTTTGGTTGATAGTGTTAAAGAAAAGAGCAAAGGGAAAAATTCTGGTTTCC
      AATTACCACAAAACCAACTATCACAATTTCTTTTCTCGTTTCCCTTTTAAAGACCAAAGG

      Q E G D I I I G V G Q S E I K N L K D L
961  AAGAGGGTGATATTATCATAGGTGTTGGACAAAGTGAAATTAATAATTTTAAAGATTAG
      TTCTCCCACTATAATAGTATCCACAACCTGTTTCACTTTAATTTTAAATTTTCTAAATC

      E Q A L K Q V N K K E F T K V W V Y R N
1021 AACAAGCTTTAAACAAGTTAATAAAAAAGAATTTACCAAAGTTTGGGTATATCGCAATG
      TTGTTTCGAAATTTTGTTCATTATTTTTTCTTAAATGGTTTCAAACCCATATAGCGTTAC

      G L A T L L V L K #
1081 GTTTGGCGACTTTGCTTGTGCTTAAATAAAAAATAACGGAAGGATTTTCCTTTTCGTTATAA
      CAAACCGCTGAAACGAACACGAATTTATTTTTATTGCCTTCCTAAAAGGAAAGCAATATT

      RegX4 →
      M T N I L
1141 ATTTTAGTTTAAAATTAAAATTAAGTTTAAAATAAAGGAAAACATGACAAATATTCTTA
      TAAAATCAAATTTTAAATTTTAAATTTTCAATTTTATTTCTTTTGATACTGTTTATAAGAA

      M I E D D L E L A E I T A E Y L E K F D
1201 TGATAGAAGATGATTTAGAATTAGCAGAAATTACAGCTGAATATTGGAATAATTTGATA
      ACTATCTTCTACTAAATCTTAATCGTCTTTAATGTCGACTTATAAACCTTTTAAACTAT

      M K V D I A H E P Y I G L S K L A L K E
1261 TGAAAGTTGATATAGCTCATGAACCTTATATAGGTCTTTCTAAGCTTGCATTAAGAAT
      ACTTTCAACTATATCGAGTACTTGAATATATCCAGAAAGATTGCAACGTAATTTTCTTA

      Y Q L I I L D L S L P G L D G L E V C E
1321 ATCAGCTTATCATTTTAGACCTTTCTTTGCCAGGGCTTGATGGGCTTGAAGTGTGTGAAG
      TAGTCGAATAGTAAATCTGGAAGAAACGGTCCCGAACTACCCGAACCTTCACACACTTC

      E I R K K Y D T P I I V S S A R H D I T
1381 AGATTTCGTAAAAGTATGATACGCCTATTATTGTTTCAAGCGCAAGACATGATATTACAG
      TCTAAGCATTTTTCATACTATGCGGATAATAACAAAGTTTCGCGTTCTGTACTATAATGTC

      D K V N A L E L G A D D Y L P K P Y N P
1441 ATAAGGTAAATGCTTTAGAGCTTGGAGCAGATGATTACCTACCAAACCTTATAATCCAA
      TATTCCAATTACGAAATCTCGAACCTCGTCTACTAATGGATGGTTTGGAAATATTAGGTT

      K E L Q A R I K S H L R R I S N T K S A
1501 AAGAATTACAAGCACGTATTAAGAGTCATTTAAGACGTATTTCAAATACAAAAGCGCCA
      TTCTTAATGTTTCGTGCATAATTTTCAGTAAATTCTGCATAAAGTTTATGTTTTTCGCGGT

      I A K S V K D
1561 TAGCAAAAAGTGTAAGATCT ←
      ATCGTTTTTTCACATTTTCTAGC
      pJH16 end
  
```

Figure 4.1: The complete sequence of the downstream regions of *htrA* and the upstream regions of the response regulator, *regX4* identified prior to this project. Above is the predicted amino acid sequence. The position of each gene is shown in a shaded box with arrow. Sequence highlighted by a shaded area identifies the original PCR DOP sequence. Sequence 5' to the shaded area denotes extra sequence identified from a λ ZAPII library. The start and end of sequence contained within pJH16 is given. The # denotes the stop codon of *htrA*. The 3' restriction *Bgl*III site is underlined.

4.2: Results

4.2.1: *Sau3AI* digest of the 1.04kb fragment

Previous analysis of genomic digests of *C. jejuni* strain 81116 DNA by Southern hybridisation indicated that the *htrA* PCRDOF fragment was located on a 2.38kb *Bgl*II fragment (Henderson, 1996). Five micrograms of genomic *C. jejuni* strain 81116 DNA was digested with *Bgl*II overnight (section 2.11.1.), purified by phenol:chloroform/chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). The digested genomic DNA was ligated (section 2.11.3.) in 200µl to create genomic DNA loops. The DNA concentration was kept low to promote the formation of intramolecular loops rather than concatamers of digested DNA. The genomic DNA *Bgl*II loops were purified by phenol:chloroform/chloroform extraction (section 2.9.2.) ethanol precipitated (section 2.9.3.) and subsequently used as template DNA for inverse PCR amplification. Inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) using primers HTRA6 (5' *Bam*HI) and HTRA7 (5' *Bam*HI) (appendix 1) was used to amplify a single 2.38kb product containing the *htrA* PCRDOF gene fragment. This is the same size as the 81116 *Bgl*II chromosome fragment containing the *htrA* PCRDOF fragment identified by Southern hybridisation. The 2.38kb fragment was then digested with *Bam*HI and *Bgl*II in a double digest (section 2.11.1.) and electrophoresed on a 1% agarose gel (section 2.8.). The digestion revealed two products of 1.34 kb and 1.04 kb respectively. The 1.34kb fragment had already been cloned in pUC19 to form pJH16 (Henderson, 1996). The 1.04kb fragment was expected to contain at least some, if not all, of the unknown regions of the *htrA* gene. This region was subsequently analysed further.

IPCR using HTRA6 and HTRA7 to produce the 2.38kb fragment and subsequent double digestion with *Bam*HI and *Bgl*II incorporated *Bam*HI compatible ends to the 1.04kb fragment. The 1.04kb fragment was excised from the gel and the DNA was purified by polyallomer wool gel extraction (section 2.9.1.), phenol:chloroform/chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). One half of the sample was digested with *Sau3AI* and electrophoresed on a 2% agarose gel (section 2.8.) to reveal one fragment of ~ 700bp and at least one other fragment of less than 300bp. 2µg of pUC19 vector was digested with *Bam*HI (section 2.11.1) phosphatase treated (section 2.11.2.) and electrophoresed on a 1% agarose gel (section 2.8.). The linear plasmid DNA was excised from the gel and purified by polyallomer wool gel extraction (section 2.9.1.), phenol:chloroform/chloroform extraction (section 2.9.2.)

and ethanol precipitated (section 2.9.3). 50ng of the linear plasmid DNA was ligated (section 2.11.3.) to the remaining half of the *Sau3AI* digested 1.04kb fragment, and following ethanol precipitation (section 2.9.3.), the ligation was electroporated into DH5 α host cells (section 2.12.1). Recombinant clones were analysed by small-scale plasmid preparation (mini-prep) (section 2.6.1.) and double digestion (section 2.11.1.) with *KpnI* and *PstI* to isolate the insert (figure 4.2A.). The 1.04kb fragment does not contain any *KpnI* or *PstI* sites (data not shown). The products were ordered according to size and 7 different sized fragments were analysed by Southern hybridisation 2.15.) using the complete 1.04kb fragment as a probe. Five different positive clones were identified and were designated A29, A39, A40, B11 and B23 (figure 4.2B.).

4.2.2: Sequence analysis of recombinant clones

Sequencing reactions were performed as outlined in section 2.14. and using primers M13 Forward and M13 Reverse. Examination of the sequence in each clone and looking for internal *Sau3AI* sites revealed that clone A29 and B11 had contaminating foreign DNA, apparent by its differing A-T content. Clones A29 and A39 contained the same *Campylobacter* DNA and B11 was a partial *Sau3AI* clone of *Campylobacter* DNA. In addition, the sequence data from B23 reflects part of that obtained from clone B11. Contaminating, non-*Campylobacter* DNA was not included in any subsequent analysis. Furthermore, revised figures for the clone sizes with respect to *Campylobacter* DNA are given in table 4.1. The sequence data for each clone was used to perform computer based homology searches using a computer database. The results are given in table 4.2.

Table 4.1: Actual insert size and revised insert size of *Sau3AI* clones.

Clone name	Actual clone size (bp)	Revised clone size (bp)
A29	761	82
A39	82	82
A40	234	234
B11	321	245
B23	127	127

Sequence analysis highlighted that B11 and B23 had homology with the partial coding sequence of *C. jejuni* HtrA (Taylor and Hiratsuka, 1990; Henderson, 1996). Importantly, this indicated that the strategy used had identified the correct region. Clones A29 and A39 were

identical and related to new, previously unidentified sequences. Clone A40 contained at one end, the predicted *Sau3AI* site replaced by a *SmaI* site of the polylinker, suggesting that the complete *Sau3AI* fragment from which the DNA originates is unstable in an *E. coli* background. Sequence from this region, revealed homology with *DnaI* and *GbpA* from a variety of eukaryotes and prokaryotes and from this point, the gene will be designated as *dnaI*-like to avoid confusion. Sequence information from clone A40 suggests the sense of the 5' end of the *dnaI*-like gene was obtained and therefore that the *dnaI*-like gene and *htrA* are transcribed divergently from a central promoter region which still remains to be identified. Using the "mini-library" strategy, the amount of sequence obtained was 241 bp. Given that the original fragment size from which the *Sau3AI* library was made covers 1.04kb, there was still approximately 500bp which remained to be identified. This remaining sequence should

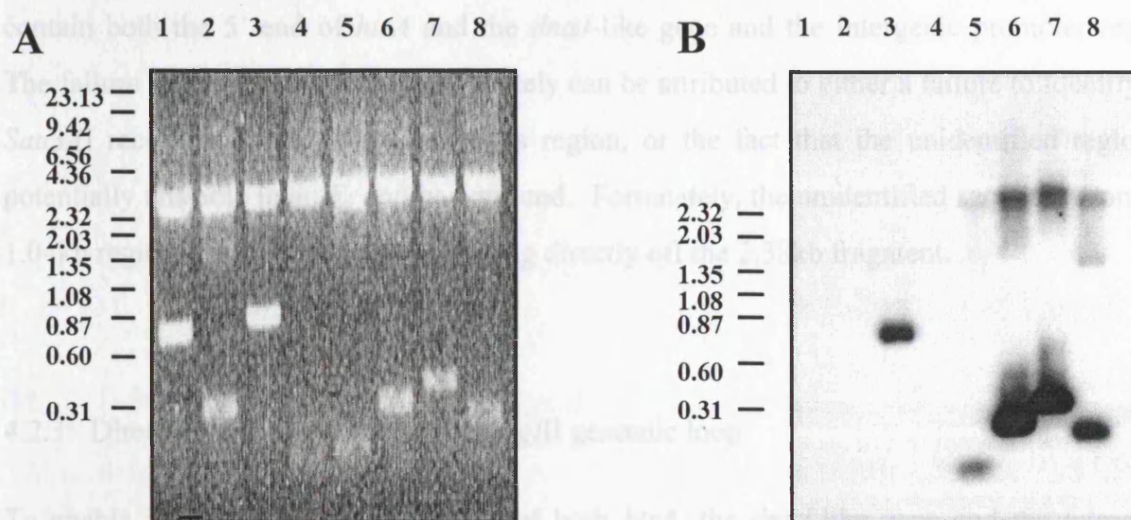


Figure 4.2: A. *KpnI*, *PstI* digest of putative *htrA* *Sau3AI* fragments in pUC19. B. Southern hybridisation of *KpnI*, *PstI* digest of putative *htrA* *Sau3AI* fragments in pUC19. For A and B; Left, λ *HindIII*/ ϕ x *HaeIII* marker (kilobase pairs); Lane1, A7; Lane 2, A28; Lane 3, A29; Lane 4, A36; Lane 5, A39; Lane 6, A40; Lane 7, B11; Lane 8, B23.

The 2.38kb DNA fragment was purified using the Qiagen's PCR purification technology (section 2.9.4.1). Using sequence data already known, sequencing primers HTRAU-SEQ1 and HTRAD-SEQ1 (appendix 13) were devised to anneal to *htrA* sequence and to extend into the unknown region. The strategy is outlined diagrammatically in figure 4.3.

To identify the missing regions of the 2.38kb *AgII* fragment 100ng of the purified PCR product was sequenced as outlined in section 2.14 using primers HTRAU-SEQ1 and HTRAD-SEQ1 (appendix 13). Primer HTRAD-SEQ1 binds at the 3' end of the 2.38kb fragment and amplifies into the *dnaI*-like gene extending past the 3' end *AgII* site. HTRAU-

identical and related to new, previously unidentified sequence. Clone A40 contained at one end, the predicted *Sau3AI* site replaced by a *SmaI* site of the polylinker, suggesting that the complete *Sau3AI* fragment from which the DNA originates is unstable in an *E. coli* background. Sequence from this region, revealed homology with DnaJ and CbpA from a variety of eukaryotes and prokaryotes and from this point, the gene will be designated as *dnaJ*-like to avoid confusion. Sequence information from clone A40 suggests the some of the 5' end of the *dnaJ*-like gene was obtained and therefore that the *dnaJ*-like gene and *htrA* are transcribed divergently from a central promoter region which still remains to be identified. Using the "mini-library" strategy, the amount of sequence obtained was 561 bp. Given that the original fragment size from which the *Sau3AI* library was made covers 1.04kb, there was still approximately 500bp which remained to be identified. This remaining sequence should contain both the 5' end of *htrA* and the *dnaJ*-like gene and the intergenic promoter region. The failure to identify the region completely can be attributed to either a failure to identify the *Sau3AI* recombinant clone covering this region, or the fact that the unidentified region is potentially unstable in an *E. coli* background. Fortunately, the unidentified sequence from the 1.04kb region was obtained by sequencing directly off the 2.38kb fragment.

4.2.3: Direct sequencing of the 2.38kb *BglII* genomic loop

To enable identification of the 5' end of both *htrA*, the *dnaJ*-like gene and the intergenic promoter region, it was proposed that the 2.38kb inverse PCR fragment be sequenced directly. For this purpose *BglII* digested chromosomal loops were prepared as before and inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) using HTRA6 and HTRA7 (appendix 1) amplified a single linear DNA fragment of 2.38kb. The 2.38kb DNA fragment was purified using the Qiaquick PCR purification technique (section 2.9.4.). Using sequence data already known, sequencing primers HTRAU-SEQ1 and HTRAD-SEQ1 (appendix 1) were devised to anneal to *htrA* sequence and to extend into the unknown region. The strategy is outlined diagrammatically in figure 4.3.

To identify the missing regions of the 2.38kb *BglII* fragment 100ng of the purified PCR product was sequenced as outlined in section 2.14. using primers HTRAU-SEQ1 and HTRAD-SEQ1 (appendix 1). Primer HTRAD-SEQ1 binds at the 3' end of the 2.38kb fragment and amplifies into the *dnaJ*-like gene extending past the 3' end *BglII* site. HTRAU-

SEQ1 binds towards the 5' end of known *htrA* sequence and extends towards the 5' end of the *htrA* gene and the putative promoter region. Results of the sequence analysis are given in figure 4.4. and identify the remainder of the 5' coding sequence of the *htrA* gene and the 5' end, of the *C. jejuni dnaJ*-like gene. This confirms the divergent organisation of the two genes and the presence of the putative intergenic promoter region.

Examination of the newly identified sequence revealed the presence of sequence specific to clone A29 and A39. A similar search on the sequence of the *Campylobacter dnaJ*-like gene revealed sequence similarity to clone A40. With specific reference to this region, there was no *Sau3AI* restriction site on the chromosomal sequence at the point at which A40 sequence extends into the *SmaI* site of the polylinker of pUC19. This gives further support to the fact that the region of DNA from which the clone originates is unstable. The position of all of the *Sau3AI* clones in relation to the completed sequence of the 2.38kb region is shown diagrammatically in figure 4.5.

4.2.4: Analysis of the putative open reading frames of the *dnaJ*-like gene and *htrA*

4.2.4.1: Analysis of the *dnaJ*-like gene

4.2.4.1.1: Analysis of the putative promoter region

Visual inspection of the putative promoter region of the *dnaJ*-like gene was performed, looking for either *E. coli* σ^{70} , σ^{32} , σ^{28} , or σ^{54} promoter-like elements. A putative σ^{70} like promoter region was identified, although the -35 region has one base pair change from the consensus, TTGACT as opposed to TTGACA (figure 4.6.). A recent paper by Wösten *et al.*, (1998) describes a newly established consensus sequence for *C. jejuni* promoters. Three conserved regions, located at -10, -16 and -35 upstream of the transcriptional start point were identified. The -10 region is similar to that of a typical σ^{70} *E. coli* promoter although the -35 region is different. The -16 region is typical of that for Gram-positive bacteria. The putative *dnaJ*-like promoter region was both visually inspected and computer aligned to identify any similar regions to the consensus but no specific areas were identified.

Table 4.2: Homology search results using the *Sau3AI* clones of A29, A39, A40, B11 and B23.

Clone	Sequence data	Homology result (top five homology scores), Accession no. (Acc). Protein ID number (PID)
A29/A39	GATCCTTATTTTAAACAATTTTTGATTTTGATTTT CCTCAAAGAAAAGGAAAAAATGATAAAGAAGTGG TGAGTTCCTTTGGGATC	<ul style="list-style-type: none"> No homology
A40	GATCTTTTAAATATCGTCCAAATTTACCCACCA GTATTTCTTGAAAAATCATGAAAACCTTTGTCCACC AAACATAGAATCGCCGTATTGGTCATACTGAGCTC GTTTTTTTTCGTCACTTAAAAATTTTATAAGCAGCA TTAATTTCTTTAAATTTTTCTTCAGCACCTTTTTCT TTGTTGATATCAGGATGATATTTTCTAGCTAGGCG ACGGTAAGCTTTTTTTATTTTCATC- <u>GGGCCC</u> (<i>SmaI</i> site)	<ul style="list-style-type: none"> Curved binding protein A (<i>cbpA</i>) <i>H. pylori</i> (Acc. AE000610; PID. g2314166). Heat shock protein (<i>dnaJ</i>) <i>Lactococcus lactis</i> subsp. <i>lactis</i> (Acc. p35514; PID. g544178). Heat shock protein (<i>dnaJ</i>) <i>Staphylococcus aureus</i>. (Acc. p45555; PID. g1169372). Heat shock protein (<i>dnaJ</i>) <i>Bacillus subtilis</i>. (Acc. p17631; PID. g118716). Heat shock protein (<i>dnaJ</i>) <i>Saccharomyces cerevisiae</i> (Acc. p35191; PID. g462580).
B11	GATCAGGGGTGATTATTTCAAAGATGGTTATATA GTAACAAATAATCACGTTGTAGATGATGCTGATAC GATTACAGTGAATTTACCAGGAAGCGACATAGAA TATAAAGCAAACTTATAGGTAAAGATCCAAAAA CAGATTTGGCTGTTATAAAAAATAGAGGCTAATAAT CTTTCAGCTATTACTTTTACAAATTCTGATGATTTA ATGGAAGGAGATGTTGTTTTTGCACCTGGAAATCC TTGATC	<ul style="list-style-type: none"> High temperature requirement A (<i>htrA</i>) <i>Campylobacter jejuni</i>. (Acc. u27271; PID g881375). Partial coding sequence (Taylor and Hiratsuka, 1990). (<i>htrA</i>) <i>Helicobacter pylori</i>. (Acc AE000610; PID. G2314163). (<i>htrA</i>) homologue (<i>mucD</i>) <i>Pseudomonas aeruginosa</i> (Acc. u32853; PID.g1184684). <i>htrA</i>-like gene (<i>degS</i>) <i>Haemophilus influenzae</i> (Acc. p44947; PID. g1170273). (<i>htrA</i>) <i>Haemophilus influenzae</i> (Acc. AF018151; PID. g2935166).
B23	GATCCAAAAACAGATTTGGCTGTTATAAAAAATAG AGGCTAATAATCTTTCAGCTATTACTTTTACAAAT TCTGATGATTTAATGGAAGGAGATGTTGTTTTTGC ACTTGGAATCCTTGATC	<ul style="list-style-type: none"> (<i>htrA</i>) <i>Campylobacter jejuni</i>. (Acc. u27271; PID g881375). Incomplete ORF (Taylor and Hiratsuka, 1990). (<i>htrA</i>) homologue (<i>algW</i>) <i>Pseudomonas aeruginosa</i> (Acc. u29172; PID.g1184675). (<i>htrA</i>) <i>Helicobacter pylori</i>. (Acc AE000610; PID. G2314163). (orfE0) <i>Rhodobacter capsulatus</i> (Acc. Y11304; PID. g2094850). (<i>htrA</i>) <i>Synechocystis</i>. (Acc. d90905; PID. g1652463).

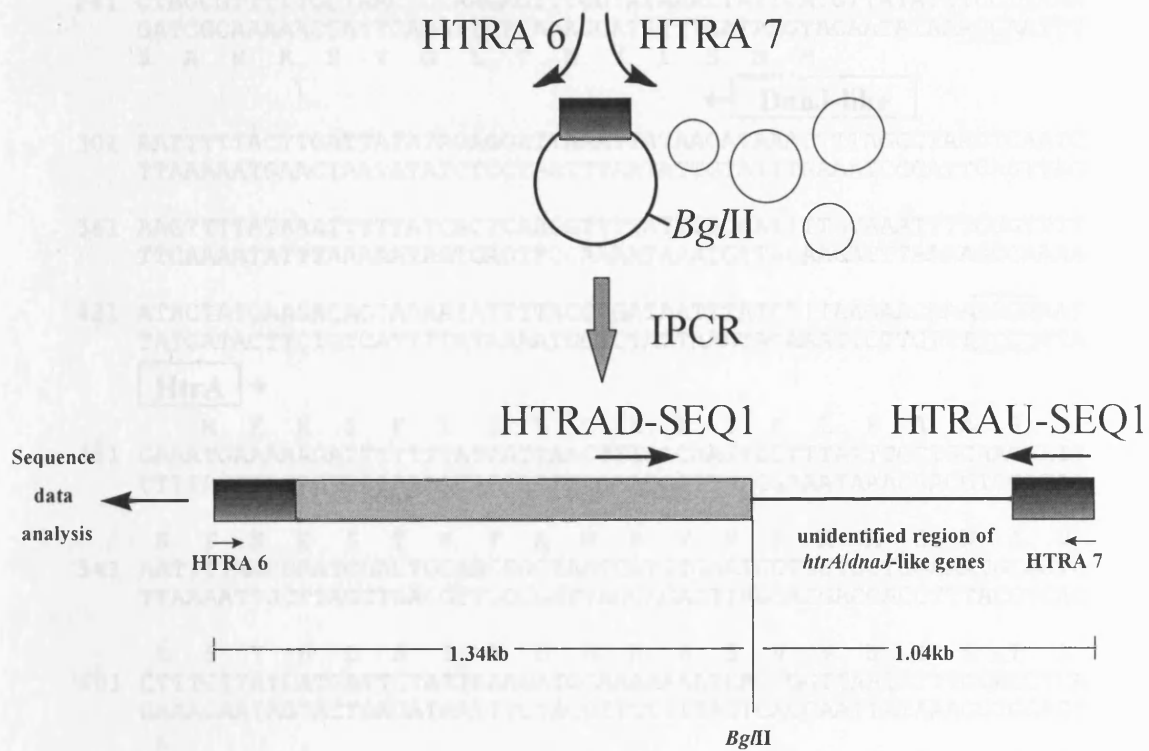


Figure 4.3: Diagram outlining the strategy for sequencing of the upstream region of *htrA* and the *dnaJ*-like gene. IPCR on *Bg*III chromosomal loops using HTRA6 and HTRA7, specific to the original PCR DOP fragment [shaded box] results in production of 2.38kb IPCR fragment. Positioning of sequencing primers, HTRAD-SEQ1 and HTRAU-SEQ1 allowed sequencing into previously unidentified regions of *htrA* and the *dnaJ*-like gene. [hatched box] denotes previously identified sequence.

5' end of 2.38kb *Bg*/II fragment

1 AGATCTTTTAAAATATCGTCCAAATTTACCCCACCAGTATTTCTTGAAAAATCATGAAAA
TCTAGAAAATTTTATAGCAGGTTTAAATGGGGTGGTCATAAAGAACTTTTGTACTTTT
D K L I D D L N V G G T N R S F D H F

61 CTTTGTCCACCAACATAGAATCGCCGATTGGTCATACTGAGCTCGTTTTTTTTTCGTCA
GAAACAGGTGGTTTGTATCTTAGCGGCATAACCAGTATGACTCGAGCAAAAAAAGCAGT
S Q G G F M S D G Y Q D Y Q A R K K E D

121 CTTAAAATTTTATAAGCAGCATTAAATTTCTTTAAATTTTCTTCAGCACCTTTTTCTTTG
GAATTTTAAAGTATTCGTCGTAATTAAGAAATTTAAAAAGAAGTCGTGGAAAAAGAAAC
S L I E Y A A N I E K F K E E A G K E K

181 TTGATATCAGGATGATATTTTCTAGCTAGGCGACGGTAAGCTTTTTTTTATTTTCATCTGCA
AACTATAGTCCTACTATAAAAGATCGATCCGCTGCCATTGCAAAAAAATAAAGTAGACGT
N I D P H Y K R A L R R Y A K K I E D A

241 CTAGCGTTTTTGTAACTCCAAGAGTTTCGTATAAACTATTCATGTTATATTTTCCTTAAA
GATCGCAAAAACGATTGAGGTTCTCAAAGCATATTTGATAAGTACAATATAAAGGAATTT
S A N K S V G L T E Y L S N M

← DnaJ-like

301 AATTTTTTACTTGATTATATAGAGGATTAAATTATAACATAAACTTTAGCCTAAGTCAATC
TTAAAAATGAACATAATATATCTCCTAATTTAATATTGTATTTGAAATCGGATTCAGTTAG

361 AAGTTTTATAAATTTTTATCACTCAAGGTTTTATTTACAATTTTGTAAATTTTCGGTTTT
TTCAAAATATTTAAAAATAGTGAGTTCCAAAATAAATGTAAAACATTTAAAAGCCAAAA

421 ATACTATGAAGACAGTAAAATATTTTACGAGATAATTTATCTTTAAGAACAAAAGGAAT
TATGATACTTCTGTCATTTTATAAAATGCTCTATTAAATAGAAATTCCTGTTTTTCTCTTTA

HtrA →

M K K I F L S L S L A S A L F A A S I

481 GAAATGAAAAAGATTTTTTTTATCATTAAAGTTTAGCAAGTGCTTTATTTGCTGCAAGTATT
CTTTACTTTTTCTAAAAAATAGTAATTCAAATCGTTCACGAAATAAACGACGTTTCATAA

N F N E S T A T A N R V N P A A G N A V

541 AATTTTAACGAATCGACTGCAACGGCTAATCGTGTGAATCCTGCTGCTGGAAATGCAGTC
TTAAAATTGCTTAGCTGACGTTGCCGATTAGCACACTTAGGACGACGACCTTTACGTCAG

L S Y H D S I K D A K K S V V N I S T S

601 CTTTCTTATCATGATTCTATTAAGATGCAAAAAAATCAGTGGTTAATATTTCCACCTCA
GAAAGAATAGTACTAAGATAATTTCTACGTTTTTTTTTAGTCACCAATTATAAAGGTGGAGT

K T I T R A N R P S P L D D F F N D P Y

661 AAACTATTACAAGAGCAAATCGTCCAAGCCCTTTGGATGATTTTTTTAATGATCCTTAT
TTTTGATAATGTTCTCGTTTAGCAGGTTTCGGGAAACCTACTAAAAAATTACTAGGAATA

F K Q F F D F D F P Q R K G K N D K E V

721 TTAAACAATTTTTTGATTTTGATTTTCCTCAAAGAAAAGGAAAAAATGATAAAGAAGTG
AAATTTGTTAAAAAACTAAACTAAAAGGAGTTTCTTTTCCTTTTTTACTATTTCTTCAC

V S S L G S G V I I S K D G Y I V T N N

781 GTGAGTTCTTTGGGATCAGGGGTGATTATTTCAAAGATGGTTATATAGTAACAAATAAT
CACTCAAGAAACCCTAGTCCCCACTAATAAAGTTTCTACCAATATATCATTGTTTATTA

H V V D D A D T I T V N L P G S D I E Y

841 CACGTTGTAGATGATGCTGATACGATTACAGTGAATTTACCAGGAAGCGACATAGAATAT
GTGCAACATCTACTACGACTATGCTAATGTCACTTAAATGGTCCTTCGCTGTATCTTATA

HTRAU-SEQ1

K A K L I G K D P K T D L A V I K I E A

901 AAAGCAAACTTATAGGTAAAGATCCAAAAACAGATTTGGCTGTTATAAAAAATAGAGGCT
TTTCGTTTTGAATATCCATTTCTAGGTTTTTGTCTAAACCGACAATATTTTTATCTCCGA

Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region.

N N L S A I T F T N S D D L M E G D V V
 961 AATAATCTTTCAGCTATTACTTTTACAAATCTGATGATTTAATGGAAGGAGATGTTGTT
 TTATTAGAAAGTCGATAATGAAAATGTTTAAAGACTACTAAATTACCTTCCTCTACAACAA
HTRA7/BamHI
 F A L G N P F G V G F S V T S G I I S A
 1021 TTTGCACTTGGAAATCCTTTTGGAGTTGGTTTTAGTGTTACAAGTGGGATAATATCTGCT
 AAACGTGAACCTTTAGGAAAACCTCAACCAAAATCACAATGTTACCCCTATTATAGACGA
HTRA6/BamHI
 L N K D N I G L N Q Y E N F I Q T D A S
 1081 TTAAATAAAGACAATATAGGTTTAAATCAATATGAAAATTTTATACAAACAGATGCTTCT
 AATTTATTTCTGTTATATCCAAATTTAGTTATACTTTTAAATATGTTTGTCTACGAAGA
 I N P G N S G G A L V D S R G Y L V G I
 1141 ATCAATCCAGGAAATTCAGGTGGAGCTTTGGTGGATAGTCGCGGATATTTAGTAGGTATT
 TAGTTAGGTCCTTTAAGTCCACCTCGAAACCACCTATCAGCGCCTATAAATCATCCATAA
 N S A I L S R G G G N N G I G F A I P S
 1201 AATTCAGCTATTCTTTCTCGTGGTGGTGGAAATAACGGCATAGGTTTTGCCATACCTTCA
 TTAAGTCGATAAGAAAGAGCACCACCACCTTTATTGCCGTATCCAAAACGGTATGGAAGT
 N M V K D I A K K L I E K G K I D R G F
 1261 AATATGGTTAAAGATATAGCTAAAAACTTATTGAAAAGGCAAGATTGATAGAGGATTT
 TTATACCAATTTCTATATCGATTTTTTGAATAACTTTTTCCGTTCTAACTATCTCCTAA
 L G A S I L A L Q G D T K K A Y K N Q E
 1321 CTAGGTGCGTCTATTTTAGCTTTGCAAGGTGATACTAAAAAGCTTACAAAAATCAAGAA
 GATCCACGCAGATAAAATCGAAACGTTCCACTATGATTTTTTTCGAATGTTTTTAGTTCTT
 G A L I T D V Q K G S S A D E A G L K R
 1381 GGAGCTTTAATCACTGATGTTCAAAAAGGTTCAAGTGCTGATGAAGCAGGGCTTAAGCGT
 CCTCGAAATTAGTGACTACAAGTTTTTCCAAGTTCACGACTACTTCGTCCCGAATTCGCA
 G D L V T K V N D K V I K S P I D L K N
 1441 GGAGATTTAGTTACTAAAGTTAATGATAAGGTTATAAAAAGTCCTATTGATCTTAAAAAT
 CCTCTAAATCAATGATTTCAATTACTATTCCAATATTTTTTCAGGATAACTAGAATTTTTA
 Y I G T L E I G Q K I S L S Y E R D G E
 1501 TATATTGGAACCTTTAGAGATTGGTCAAAAATTTTATTAAGTTACGAAAGAGATGGAGAA
 ATATAACCTTGAAATCTCTAACCAGTTTTTTTAAAGTAATTCATGCTTTCTCTACCTCTT
 N K Q A S F I L K G E K E N P K G V Q S
 1561 AATAAGCAAGCAAGTTTTATTCTTAAAGGTGAAAAGAAAATCCTAAAGGCGTACAAAGC
 TTATTGTTTGGTTCAAATAAGAATTTCCACTTTTTCTTTTAGGATTTCCGCATGTTTCG
 D L I D G L S L R N L D P R L K D R L Q
 1621 GATTTGATTGATGGTTTGAGTTTGAGAAATTTAGATCCAAGACTTAAGGATCGTTTGCAA
 CTAAACTAACTACCAAACTCAAACCTTTTAAATCTAGGTTCTGAATTCCTAGCAAACGTT
 I P K D V N G V L V D S V K E K S K G K
 1681 ATTCCAAAAGATGTTAATGGTGTGTTGGTTGATAGTGTTAAAGAAAAGAGCAAAGGGAAA
 TAAGGTTTTCTACAATTACCACAAAACCAACTATCACAATTTCTTTTCTCGTTTCCCTTT
 N S G F Q E G D I I I G V G Q S E I K N
 1741 AATTCTGGTTTCCAAGAGGGTGATATTATCATAGGTGTTGGACAAAGTGAAATTAATAAT
 TTAAGACCAAAGGTTCTCCCACTATAATAGTATCCACAACCTGTTTCACTTTAATTTTTA
 L K D L E Q A L K Q V N K K E F T K V W
 1801 TTAAAAGATTTAGAACAAGCTTTAAACAAGTTAATAAAAAAGAATTTACCAAAGTTTGG
 AATTTTCTAAATCTTGTTGCAAATTTTGTTCAATTATTTTTTCTTAAATGGTTTCAAAC

Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region.

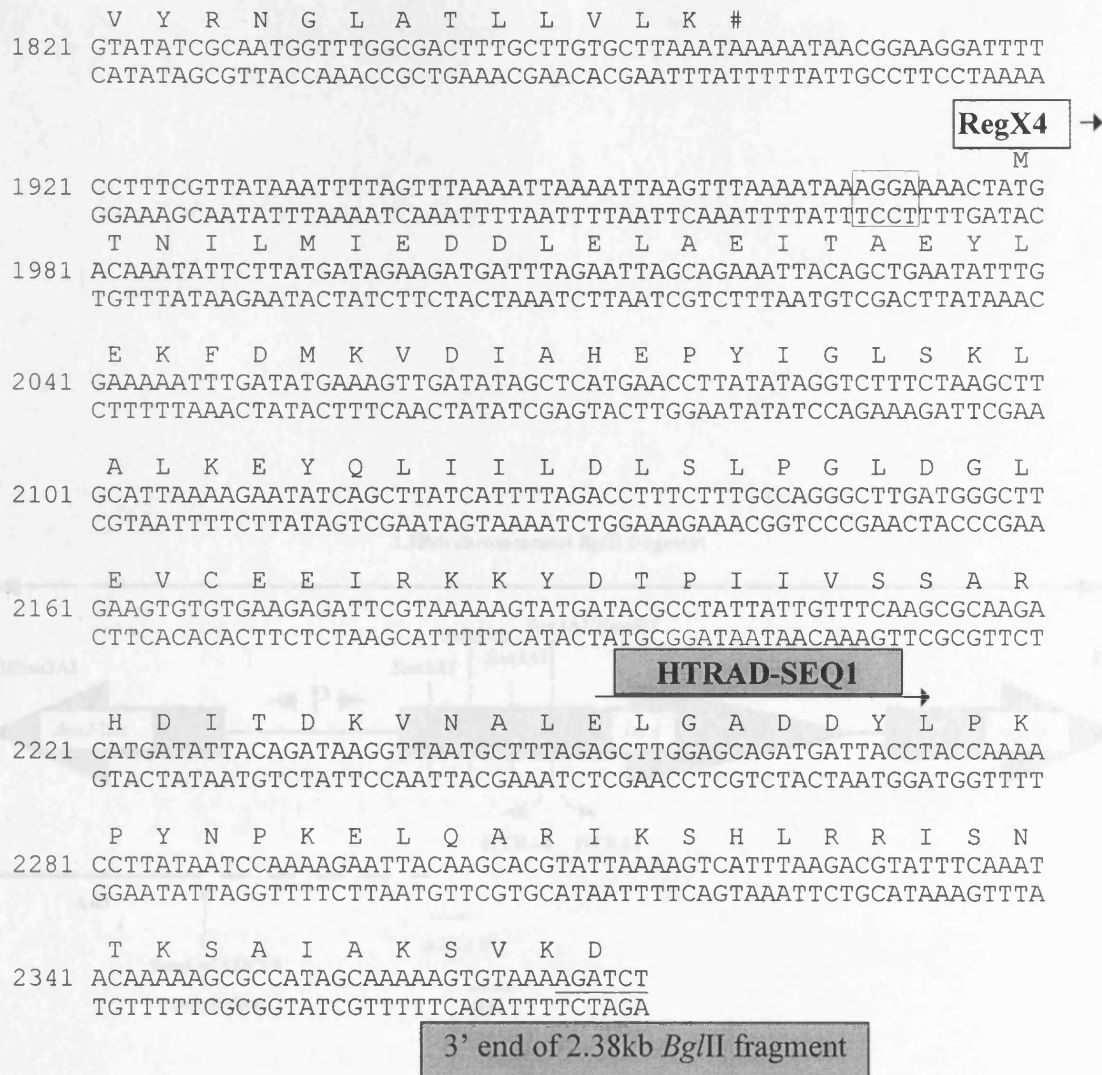


Figure 4.4: Sequence data of the complete 2.38kb *Bgl*III fragment. The primers used to sequence unknown regions of upstream *htrA* were HTRAU-SEQ1 and HTRAD-SEQ1 and are shown in grey boxes with arrows. The *Bgl*III restriction sites are underlined. The position of the primers HTRA6 and HTRA7 used to amplify the 2.38kb IPCR fragment are also highlighted. Predicted open reading frames (starts labelled with clear boxes with arrows) are shown with respective deduced amino acid sequence for DnaJ-like protein, HtrA and RegX4. Putative ribosome binding sites are denoted by unshaded boxes. The *htrA* stop codon is denoted by #.

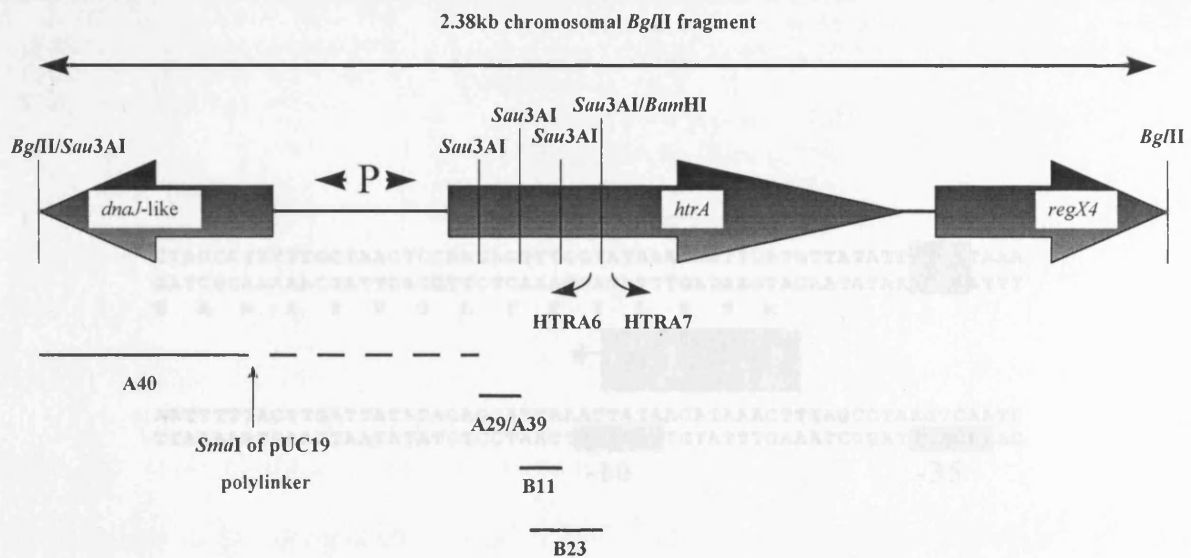


Figure 4.5: Diagram outlining the position of the *Sau*3AI clones in relation to the 2.38kb chromosomal *Bgl*/II fragment. The three open reading frames for the *Bgl*/II fragment are denoted by thick black arrows. Restriction sites are marked. The *Sau*3AI/*Bam*HI site is created by primers HTRA6 and HTRA7 during the IPCR. The intergenic putative promoter region for *htrA* and *dnaJ*-like genes is denoted by the arrowed P. The position of the *Sau*3AI clones is marked. For A40, the continuous line denotes the sequence obtained, the broken line is part of the *Sau*3AI fragment that clone A40 originates from, but terminates on the *Sma*I site of the pUC19 polylinker. The broken line is likely to contain DNA sequence unstable in an *E. coli* background.

4.2.4.2: Computer based analysis of the open reading frame of the *dnaJ*-like gene.

A putative KBS (AGGA) was located 5' up upstream of a possible initiation codon, ATG. The complete open reading frame for the *C. jejuni dnaJ*-like gene consists of 94 amino acids and 357bp and shows considerable homology to a variety of different bacterial examples. The closest 5 are given in table 4.3.

Table 4.3: Five highest homology scores with partial *DnaJ*-like open reading frame.

DnaJ-like open reading frame	Five highest homology scores with <i>C. jejuni</i> <i>dnaJ</i> -like open reading frame
Accession number (Accession number)	Accession number (Accession number)
EF011114 (EF011114)	<i>Helicobacter pylori</i> (chaperone-coupled DNA binding protein) (Acc. AF009910; PID: g2344106) Derived from <i>hsp60</i> gene of <i>E. coli</i> (J95)
EF011115 (EF011115)	<i>Escherichia coli</i> (hsp70) (Acc. F03594; PID: g244113)
EF011116 (EF011116)	<i>Shigella flexneri</i> (hsp70) (Acc. F03594; PID: g244113)
EF011117 (EF011117)	<i>Shigella flexneri</i> (hsp70) (Acc. F03594; PID: g244113)
EF011118 (EF011118)	<i>Shigella flexneri</i> (hsp70) (Acc. F03594; PID: g244113)

CTAGCGTTTTTCTAACTCCAAGAGTTTCGTATAAACTATTCATGTTATATTTCCCTAAA
GATCGCAAAAACGATTGAGGTTCTCAAAGCATATTTGATAAGTACAATATAAAGGAATT
S A N K S V G L T E Y L S N M

← **DnaJ-like**

AATTTTACTTGATTATATAGAGGATTAAATTATAACATAAACTTTAGCCTAAGTCAATC
TTAAAAATGAATAATATATCTCCTAATTTAATATTTGTAATTTGAAATCGGATTCAGTTAG

-10

-35

Figure 4.6: Identification of a putative *E. coli* σ^{70} promoter-like element upstream of the *dnaJ*-like gene. The putative -10 and -35 regions are shown in grey boxes. The putative ribosome binding site is also shown (AGGA).

4.2.4.1.2: Computer based analysis of the open reading frame of the *dnaJ*-like gene.

A putative RBS (AGGA) was located 8 bp upstream of a possible initiation codon, ATG. The incomplete open reading frame for the *C. jejuni dnaJ*-like gene consists of 94 amino acids and 282bp and shows considerable homology to a variety of different bacterial examples. The closest 5 are given in table 4.3.

Table 4.3: Five highest homology scores with partial DnaJ-like open reading frame.

DnaJ-like protein open reading frame.	Five highest homology scores with <i>C. jejuni</i> DnaJ-like protein. Accession number (Acc). Protein identification number (PID).
MNSLYETLGVSKNASADEIKKAYRR LARKYHPDINKEKGAEKFKKEINAA YEILSDEKKRAQYDQYGDSMFGGQS FHDFSRNTGGVNLDDILKDL	<ul style="list-style-type: none"> • <i>Helicobacter pylori</i> co-chaperone-curved DNA binding protein A, <i>cbpA</i>. (Acc, AE000610; PID, g2314166). Derived from similarity with <i>E. coli cbpA</i>. • <i>Lactococcus lactis dnaJ</i> (Acc, P35514; PID, g544178). • <i>Bacillus subtilis dnaJ</i> (Acc, P17631; PID, g118716). • <i>Staphylococcus aureus dnaJ</i> (Acc, P45555; PID, g1169372). • <i>Streptococcus pneumoniae dnaJ</i> (Acc, P95830; PID g3122013).

4.2.4.2: Analysis of *htrA*.

4.2.4.2.1: Analysis of the putative promoter region

Fundamental to the stress response in *E. coli* is the alternative sigma factor, σ^E , which is responsible for the transcription of *rpoE* (encoding σ^E), *rpoH* from its P3 promoter at high temperatures (encodes for the major heat shock factor σ^{32}) and the *htrA* (*degP*) gene (Erickson, 1987; Lipinska *et al.*, 1988). Autoregulation of σ^E homologues has been demonstrated in *Pseudomonas aeruginosa*, (*algU*) (Deretic *et al.*, 1994; Schurr *et al.*, 1995; 1996) and *Salmonella typhimurium* (*rpoE*) (Johnson *et al.*, 1991). Transcription of *rpoH* from *P. aeruginosa* (Martin *et al.*, 1994) and *htrA* from *Salmonella typhimurium* (*cit in* Martin *et al.*, 1994) and *Yersinia enterocolitica* (Li *et al.*, 1996; Yamamoto *et al.*, 1996; 1997) is performed by σ^E homologues. In view of this information, the putative promoter region of *C. jejuni htrA* was analysed specifically for the presence of *E. coli* σ^E -like transcriptional elements. Significantly, no specific -35 and -10 regions were identified. As a result, the putative promoter region was examined further for *E. coli* recognised promoter elements for sigma factors, σ^{70} σ^{28} and σ^{54} but no likely candidates were identified.

As with the *dnaJ*-like gene the putative *htrA* promoter region was both visually inspected and computer aligned with the newly identified promoter consensus given by Wösten *et al.*, (1998) to identify any similar regions. No specific areas were identified.

4.2.4.2.2: Comparative analysis of the open reading frame of HtrA.

The *htrA* gene has a putative RBS (AGGA), 6 bp upstream of a possible initiation codon, ATG. The *htrA* gene consists of 1419 base pairs and the corresponding translated open reading frame of 472 amino acids. The encoded protein has a molecular weight of 51kDa. The newly identified additional *htrA* sequence completes that previously identified (Taylor and Hiratsuka, 1990; Henderson, 1996).

Analysis of important characteristics of the predicted amino acid sequence include an appropriate revision of some information already described (Henderson, 1996). Furthermore, and in light of additional information which relates to this sequence, some new features are described. Other characteristics described relate specifically to the sequence identified in this project.

A computer based homology search using the complete HtrA sequence identified that *C. jejuni* HtrA was similar to other HtrA homologues (table 4.4.). The closest homologue to *C. jejuni* HtrA is that from *Helicobacter pylori* and the two proteins are 50 % identical and 67% similar. The *N*-terminal 100 residues of *C. jejuni* HtrA differs significantly from those from *E. coli* and *S. typhimurium* which are 89% identical to one another (Johnson *et al.*, 1991). An alignment of the ten closest HtrA homologues to *C. jejuni* HtrA is given in figure 4.7.

4.2.4.2.3: Signal peptide cleavage site

Based on data which localises *E. coli* HtrA to the periplasm (Skorko-Glonek, 1997), the assumption is that other HtrA homologues reside in a similar location. In this regard, it appears that *C. jejuni* may be no exception. Fundamental to the process which directs the protein to the cell envelope is the presence of a signal peptide sequence at the *N*-terminus. The translated amino acid sequence is subsequently cleaved into the mature protein once its final destination is reached. Signal peptide cleavage site analysis (Nielsen *et al.*, 1997) was performed on *C. jejuni* HtrA and revealed two putative signal peptidase recognition sequences

at position 16-17, between amino acids (LFA-AS) and/or position 28-29 between amino acids (ATA-NR). The signal peptide cleavage point has been predicted or experimentally determined for a number of HtrA homologues. *E. coli* HtrA is stabilised as a 51kDa precursor that is processed to a 48kDa mature form by removal of a 26 amino acid leader peptide. Cleavage occurs between amino acids ATA-AE (Lipinska *et al.*, 1988; 1989). By extension, *S. typhimurium* HtrA is predicted to have an identical cleavage position (Johnson *et al.*, 1991). *Haemophilus influenzae* *htrA* encodes a protein of 49kDa with a putative 26 amino acid signal sequence and is cleaved between amino acids AQA-TL. The mature protein has a molecular weight of 46kDa mature protein (Loosmore *et al.*, 1998). *Brucella abortus* *htrA* encodes a protein of 53kDa with a putative 25 amino acid signal sequence resulting from cleavage between amino acids AGA-FV. This would result in a 50kDa mature protein (Tatum *et al.*, 1994). The signal peptidase cleavage point for *Yersinia enterocolitica* GsrA has been confirmed experimentally. The gene encodes a 50kDa precursor which is processed to a 47kDa mature after cleavage of a 27 amino acid signal peptide between positions VVA-AE (Yamamoto *et al.*, 1996; 1997). Given these data and the fact that the position of most signal peptidase cleavage points are between 20-40 amino acids from the start of the encoded precursor (Oliver, 1985), then the most likely cleavage point for *C. jejuni* HtrA is at position 28-29 between amino acids ATA-NR. *C. jejuni* *htrA* encodes a protein with a predicted molecular weight of 51kDa. Cleavage at this position would result in a 48kDa mature protein.

4.2.4.2.4: Cell binding properties

At the C-terminus of *H. influenzae* HtrA and HhoA and HtrA homologues of *E. coli*, *S. typhimurium* and *Y. enterocolitica* there is an RGD motif but the corresponding region in *E. coli* HhoA has an RGN motif. Loosmore *et al.*, (1998), examined *htrA* from 13 strains of *H. influenzae* and found that five had an RGN motif but eight presented with an RGD motif. The RGD motif has been identified as a cell attachment motif site for mammalian adhesion proteins (Ruoslahti and Pierschbacher, 1987). HtrA is periplasmic and it is unclear as to what function either the RGD or RGN motif have in *H. influenzae*, *E. coli*, *S. typhimurium* and *Y. enterocolitica* HtrA homologues. Interestingly, examination of the carboxy terminus of the *C. jejuni* HtrA (and the HtrA protein of *H. pylori*) has revealed that neither an RGD nor RGN motif is present, therefore highlighting that any function related to either motif is probably absent from *C. jejuni*.

Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region.

CJHTRA	MKK-IFLSL-----SLASALFAASINFN-----ESTATANR-----VNPAAGN	37
ECHTRA	MKKTTLALS-----ALALSLGLALSPLS-----ATAAETSS-----ATTAQ	36
STHTRA	MKKTTLAMS-----ALALSLGLALSPLS-----ATAAETSS-----S-AMTAQ	37
YEGSRA	MKKTTLVLS-----ALALSIGLAMGPVSS-----VVAETAS-----SSSQ	36
ECDEGQ	MKKQTQLLS-----ALALSVGLTSLASFQ-----AVASIPGQ-----V-ADQAP	38
HIHTRA	MKKTRFVLN-----SIALGLSVLSTSFVA-----QATLP-----S-FVSEQ	35
HIHHA	MKKTRFVLN-----SIALGLSVLSTSFVA-----HVAQATLP-----S-FVSEQ	38
HPHTRA	MKKTLFISL-----ALALSLNAGNIQISMPKVKERVSVPKDDTDLFLPRFVSPKTI	55
BJHTRA	MSKNSSVIKDR---RFAAVLLLCASIAWS-----PVLAQVPD-----IRGG	38
BAHTRA-LIKE	MNWRCLGFAVG---VLAFFSLAAGPVAAQ-----DSPATAVR-----QIPLSRGD	42
BAHTRA	MSRARISNYRKGVAAVALSAALAGAFVVTG----PLGALNEAR-----AEAVHVTPP	48
* . *		
CJHTRA	AVLSYHDSIKDAKKSVMNISTSKTITRANRPSPLDDFFNDPYF-----KQFFDFDF	88
ECHTRA	QMPSLAPMLEKVMPSVVSINVEGSTTVNTPR-----MPRNF-----QFFGDDS	80
STHTRA	QMPSLAPMLEKVMPSVVSINVEGSTTVNTPR-----MPRNF-----QFFGDDS	81
YEGSRA	QLPSLAPMLEKVMPSVVSINVEGSAQVSNAG-----GIPPQF-----QFFGDDS	81
ECDEGQ	-LPSLAPMLEKVLPAVVSVRVEGTASQGQK-----IPEEF-----KFFGDDL	80
HIHTRA	--NSLAPMLEKVQPAVVTLSVEGKAKVDSRSPF--LDDIPEEF-----KFFGDRF	82
HIHHA	--NSLAPMLEKVQPAVVTLSVEGKAKVDSRSPF--LDDIPEEF-----KFFGDRF	85
HPHTRA	RIYSYHDSIKDSIKAVVNISTEKKIKNNFIGG---GVFNDPFF-----QFFGDL	102
BJHTRA	QIPTLAPVVRETTSPSVVNISVHARVKEDNP-----LYRDPVF-----REFF--DL	81
BAHTRA-LIKE	MQLSFAPLVKETAPAVVNVYARQVQARSP-----FEGDPFF-----EQFFGSQF	87
BAHTRA	PQAGFADLVEKVRPAVVSVRVKKDVQETSNRGP--QFFGPPGFDQLPDGHLKRRF--DF	105
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CJHTRA	PQRKGKNDKEV-----VS-SLGSVVIISKD-GYIVTNNHVVDDADTI	128
ECHTRA	PFCQEGSPFQSSPFCQGGQGGNGGG--QQKFMALGSGVIIDADKGYVVTNNHVVDNATVI	139
STHTRA	PFCQDGSPPFQNSPFCQGGGNGGG--QQKFMALGSGVIIDAAKGYVVTNNHVVDNASVI	140
YEGSRA	PFCQDGSPPFQSPMCQGGPGSRGAPSKQDFRALGSGVIIDAAKGYVVTNNHVVDNATKI	141
ECDEGQ	P-DQPAQPFEG-----LGSGVVIINASKGYVLTNNHVINQAQKI	117
HIHTRA	A-EQFGGRGES-----KRNFRLGSGVVIINASKGYVLTNNHVIDEADKI	125
HIHHA	A-EQFGGRGES-----KRNFRLGSGVVIINASKGYVLTNNHVIDGADKI	128
HPHTRA	G---GMIPKER-----MERALGSGVVIISKD-GYIVTNNHVIDGADKI	140
BJHTRA	P---KQLEKEV-----QATGSGVIVDAERGVLTANHVVAQISKA	118
BAHTRA-LIKE	G---ASKPRIQ-----QSLGSGVIVDRS-GIIVTNNHVIKDADEI	123
BAHTRA	GMEPRGDSRSDNRRGKANKPRPG----HERPVAQSGGFVISED-GYVVTNNHVVSDGDAY	160
***: : * : * *		
CJHTRA	TVNLPGSDIEYKAKLIGKDPKTDLAVIKIEA-N-NLSAITFTNSDDLMEGDVVFALGNPF	182
ECHTRA	KVQLS-DGRKFDAKMVGKDPKPRSDIALIQIONPK-NLTAIKMADSDALRVGDYTVAGNPF	197
STHTRA	KVQLS-DGRKFDAKVVGKDPKPRSDIALIQIONPK-NLTAIKLADSDALRVGDYTVAGNPF	198
YEGSRA	NVKLS-DGRSYEAKVIGKDPKPRSDIALQLKDAK-NLTAIKIADSDQLRVGDYTVAGNPF	199
ECDEGQ	SIQLN-DGREFDAKLIGSDQSDIALQLQIONPS-KLTQIAIADSDKLRVGDFAVAVGNPF	175
HIHTRA	TVQLQ-DGREFKAKLVGKDESDIALVQLEKPS-NLTEIKFADSDKLRVGDFTVAGNPF	183
HIHHA	TVQLQ-DGREFKAKLVGKDESDIALVQLEKPS-NLTEIKFADSDKLRVGDFTVAGNPF	182
HPHTRA	KVTIPGSNKEYSATLVGTDSESDLAVIRITK-D-NLPTIKFSDSNDISVGLDLVFAIGNPF	198
BJHTRA	QVTTK-DGRKFDAKLVRDPATDVAVLQQLGRNLKAIPLGDSRIEVDGDFVIAIGNPF	177
BAHTRA-LIKE	KVALLS-DGREFSRILLRDETTDLAVLKIEAKQ-QFPVLALGNSDEVEVGLDLVLAIGNPF	181
BAHTRA	TVVLD-DGTELDKLAGADPRTDLAVLKINAPKRKFVYVAFGDDNKVRVGDVWVAVGNPF	219
: .. : : * : * : : : : * : : : : *		
CJHTRA	GVGFSVTSGIISALNKD-NIGLNQYENFIQTDASINPNSGGALVDSRGYLVGINSAILS	245
ECHTRA	GLGETVTSGIVSALGRS-GLNAENYENFIQTDAAINRNSGGALVNLNGELIGINTAILA	256
STHTRA	GLGETVTSGIVSALGRS-GLNVENYENFIQTDAAINRNSGGALVNLNGELIGINTAILA	257
YEGSRA	GLGETVTSGIVSALGRS-GLNVENYENFIQTDAAINRNSGGALVNLNGELIGINTAILA	258
ECDEGQ	GLGQTATSGIVSALGRS-GLNLEGENFIQTDASINRNSGGALLNLNGELIGINTAILA	234
HIHTRA	GLGQTVTSGIVSALGRSTGSDSGTYENFIQTDAAVNRNSGGALVNLNGELIGINTAIIS	243
HIHHA	GLGQTVTSGIVSALGRSTGSDSGTYENFIQTDAAVNRNSGGALVNLNGELIGINTAIIS	246
HPHTRA	GVGESVTQGIIVSALNKS-GIGINSYENFIQTDASINPNSGGALIDSRGGLVGININTAIIS	257
BJHTRA	GLGQTVTSGIVSALGRS-GLGKQGYEDFIQTDASINPNSGGALINLRGQLVGININTAIIS	236
BAHTRA-LIKE	GVGQTVTSGIVSAQSRT-QVGISDFDFFIQTDAAINPNSGGALIDMRGRIGINTAIIS	240
BAHTRA	GLGQTVTSGIVSARGD--IGAGPYDDFIQIDAANKNSGGPFDLSGEVIGINTAIIS	277
*: : * : * : : : : * : : * : * : : *		
CJHTRA	PDGGNNGIGFAIPSNMVKDIKAKLIE-KGKIDRGFLGASIALQGDTKKAY--KNQEGAL	302
ECHTRA	PDGGNIGIGFAIPSNMVKNLTSQMVE-YGQVKRGELGIMGTENLSELAKAMKVDAQRGAF	315
STHTRA	PDGGNIGIGFAIPSNMVKNLTSQMVE-YGQVKRGELGIMGTENLSELAKAMKVDAQRGAF	316
YEGSRA	PDGGNIGIGFAIPSNMVKNLTSQMVE-FGQVKRGELGIMGTENLSELAKAMKVDAQRGAF	317
ECDEGQ	PDGGSVGIGFAIPSNMARTLAQQLID-FGEIKRGLLGKIGTEMSADIAKAFNLDVQRGAF	293
HIHTRA	PSGGNAGIAFAIPSNQASNLVQILE-FGQVRRGLLGKIGGELNADLAKAFNVSAQQGAF	302
HIHHA	PSGGNAGIAFAIPSNQASNLVQILE-FGQVRRGLLGKIGGELNADLAKAFNVSAQQGAF	305
HPHTRA	KTGGNHGIGFAIPSNMVKDVTQLIK-TGKIERGYLGVLQDLSGDLQNSY--DNKEGAV	314
BJHTRA	PDGGNIGIGFAIPSNMARRVMEQLVQ-YGEVRRGQIGISIRDLGVDLAAK---ESYQAL	292
BAH-LIKE	RSGGSVGIGFAIPSNMRAVVDAAALQGSTRFERPYIGATFQGITPDLAESLGMEKPYGAL	300
BAHTRA	PSGGSVGIAFAIPSTAKQVVDQLIK-KGSVERGWIGVQIQPVTKDIAASLGLAEKGA	336
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Chapter 4: Identification of 5' *htrA* sequence and cloning of the promoter region.

CJHTRA	ITDVQKGS	ADEAGL	KRGDLV	TKVNDK	VIKSPID	LKNYIG	TLEIGQ	KISLSY	ERDGEN	KQ	362				
ECHTRA	VSQVLPN	SSAAKAG	IKAGDV	ITSLNG	KPISSF	AALRAQ	VGTMPV	GSKLTG	LGLLRD	GKQV	375				
STHTRA	VSQVMPN	SSAAKAG	IKAGDV	ITSLNG	KPISSF	AALRAQ	VGTMPV	GSKISL	GLLREG	KAIT	376				
YEGSRA	ISQVLPK	SAAAKAG	IKAGDV	IVSMNG	KAINSF	AGFRAE	IGTLPV	GSKMTL	GLLLRD	GKPV	377				
ECDEGQ	VSEVLPG	SGSAKAG	VKAGDI	ITSLNG	KPLNSF	AELRSRI	ATTEPG	TKVKLG	LGLLRN	GKPLE	353				
HIHTRA	VSEVLPK	SAAEKAG	LKAGDI	ITAMNG	QKISSF	AEIRAKI	ATTGAG	KEISLT	YLRLDG	KSHD	362				
HIHHA	VSEVLPK	SAAEKAG	LKAGDI	ITAMNG	QKISSF	AEIRAKI	ATTGAG	KEISLT	YLRLDG	KSHD	365				
HPHTRA	VISVEKD	SPAKKAG	ILVWDL	ITEVNG	KVKVNT	NELRNLI	GSMLPN	QRVTLK	VIRD-KK	ER	373				
BJHTRA	IAETIAS	GSPAEG	AQLQKG	DIVKAV	DGTPIRS	AQQLRN	LIGLTP	VGSRVEL	RFRNGA	ARS	352				
BAHTRA-LIKE	ITAVVKD	GPAETAG	LKVGDV	LSVQGV	RVVDNQ	DLGYRL	STAGIG	KTISVE	VMRNGK	NLS	360				
BAHTRA	VASPDQD	GPAKAG	IKAGDV	ITAVNG	ETVQDPR	DLARKV	ANIAPE	GKAALT	VWRKNK	AAE	396				
	:	.	:	**:	*::	::.	:	.	:	*					
CJHTRA	ASFILKE	KENENPK	-----	GVQSD	LIDGLS	LRNLDP	RLKDR	LQIPKD	VNGVLV	DSVK	413				
ECHTRA	VNLELQ	QSS-----	QNVDS	SSSIF	NGIEGA	EMSN-K	GKD--	QGVV	VNNVK		417				
STHTRA	VNLELQ	QSS-----	QNVDS	SSSIF	NGIEGA	EMSN-K	QD--	QGVV	VNNVK		418				
YEGSRA	VEVTLE	QSS-----	QNVDS	SSSIF	NGIEGA	EMSN-K	QD--	QGVV	VNNVK		421				
ECDEGQ	VEVTLD	TST-----	SSSAS	AEMIT	PALEGAT	LSDGQL	KDGGK	GKIKID	EVV		398				
HIHTRA	VKMKLQ	ADD-----	SSQLS	SKTELP	ALDGAT	LKDYDA	KG-VK	GIEITK	IQ		406				
HIHHA	VKMKLQ	ADD-----	SSQLS	SKTELP	ALDGAT	LKDYDA	KG-VK	GIEITK	IQ		409				
HPHTRA	AFTLT	LAERKN	PNKKE	TISAQNG	AQQLN-	GLQVED	LQETK	RSMLSD	DDVQGV	LVSV	432				
BJHTRA	ASVEVG	PAK-----	AVPVR	RTADQ-							371				
BAHTRA-LIKE	LPVKLT	KAPKVK	QAEPK	VIE----	GDNPF	DGAAGD	LTA	STA	AKLRL	KRGQ	GVAV	416			
BAHTRA	INVTIA	AMPNDK	GK----	S---	GSQSN	DNDGGQ	GETL	DSYGL	TVPSED	G-KGV	VVDV	448			
CJHTRA	EKSKGK	NSGFQEG	DIIGV	GQSEIK	NKDL	EQALKQ	V--NK	KEFTK	VWVYRN	GLATL	LV	471			
ECHTRA	TGTAAQ	IGLKKGD	VIIIGAN	QQA	VKNIAE	LKRVLD	----	SKPSV	LALNIQ	RGDST	IYLL	473			
STHTRA	ANSPAA	IGLKKGD	VIIIGAN	QQA	VKNIAE	LKRVLD	----	SKPSV	LALNIQ	RGDST	IYLL	474			
YEGSRA	PGTAA	ARIGLKK	GDVIM	GVNQ	QPVQNL	GELRKIL	----	TKPSV	LALSIQ	RGDST	IYLL	477			
ECDEGQ	KGS	PAAQAG	LQKDD	VIIIGV	NRDRV	NSIAEM	RKVL	----	AKPAI	IALQIV	RGNESI	YLL	453		
HIHTRA	PNSLAA	QRLKSG	DIIGIN	RQMIEN	IREL	NKVLE	----	TEPSA	VALN	ILRGDS	NFYLL	462			
HIHHA	PNSLAA	QRLKSG	DIIGIN	RQMIEN	IREL	NKVLE	----	TEPSA	VALN	ILRGDS	NFYLL	465			
HPHTRA	ENSPA	EQAGFR	QGNII	TKIEE	VEVKSV	ADFNH	ALEKY	--	KGKPK	RFLVLD	LNQGYR	ILV	490		
BJHTRA	-----														
BAHTRA-LIKE	SGCPA	ARLGLR	SGDI	IIRSIN	GNQIR	TVD	MTAV	LEA---	GRGLA	WRLEI	ERN	GALL	RQFV	473	
BAHTRA	PDS	DAADR	GIRSG	DVIVS	VNNQT	VKTAG	DINKA	ITAAE	KSGR	KAVLL	QLQ	SNDQ	S	R	508
CJHTRA	K----														472
ECHTRA	Q----														474
STHTRA	Q----														475
YEGSRA	Q----														478
ECDEGQ	-----														
HIHTRA	Q----														463
HIHHA	Q----														466
HPHTRA	K----														491
BJHTRA	-----														
BAHTRA-LIKE	R----														474
BAHTRA	PINQE														513

Figure 4.7: Multiple sequence alignment of homologues of HtrA. Grey boxes denote proposed histidine, aspartate and serine residues of the catalytic triad of serine proteases. The consensus sequence GNSGGAL which surrounds the putative serine active-site residue is boxed. HtrA homologues included in the alignment are as follows: ECHTRA; *E. coli htrA*, STHTRA; *S. typhimurium*, YEGSRA; *Y. enterocolitica*, *gsrA*, ECDEGQ; *E. coli degQ*, HIHTRA; *H. influenzae htrA*, HIHHA; *H. influenzae hhoA*, CJHTRA; *C. jejuni htrA*, HPHTRA; *H. pylori htrA*, BJHTRA; *B. japonicum htrA*, BAHTRA-LIKE; *B. abortus htrA*-like, BAHTRA, *B. abortus htrA*. “*” denotes an identical or conserved residue, “:” indicates a conserved substitution and “.” indicates a semi-conserved substitution.

Table 4.4: Ten highest protein homology scores with *C. jejuni* HtrA.

Highest homology scores with <i>C. jejuni</i> HtrA	Identity to <i>C. jejuni</i> HtrA (%)	Similarity to <i>C. jejuni</i> HtrA (%)
HtrA, <i>Helicobacter pylori</i> . Acc AE000610; PID g2314163.	50	67
HtrA, <i>Bradyrhizobium japonicum</i> . Acc Y13616; PID g2623992.	42	61
HhoA, <i>Escherichia coli</i> . Acc P39099; PID g729711.	39	59
HtrA, <i>Escherichia coli</i> . Acc P09376; PID g547687.	39	57
GsrA, <i>Yersinia enterocolitica</i> . Acc D78376; PID g1526428.	39	55
HtrA, <i>Haemophilus influenzae</i> . Acc AF018151; PID g2935166.	38	58
HhoA, <i>Haemophilus influenzae</i> . Acc P45129; PID g1170411.	38	58
HtrA, <i>Salmonella typhimurium</i> . Acc P26982; PID g123730.	38	56
HtrA-like, <i>Brucella abortus</i> . Acc U07351; PID g497154.	37	57
HtrA, <i>Brucella abortus</i> . Acc U07352; PID g497156.	34	50

4.2.4.2.5: Identification of active site residues

The consensus sequence which surrounds the active serine of serine proteases is GDSGGPK (Brenner, 1988). Examination of *C. jejuni* HtrA revealed a GNSGGAL motif around Ser²²⁵. From the alignment of other members of the HtrA family of serine proteases (figure 4.7.) and similar alignments given in Loosmoore *et al.*, (1998) and Pallen and Wren, (1997) the other members of the catalytic triad were predicted to be His¹²⁰ and Asp¹⁵¹ in *C. jejuni* HtrA.

4.2.4.2.6: Detection of PDZ-like repeats

All bacterial HtrA homologues have PDZ domains at the C-terminus which are thought to play an integral part in mediating a number of protein-protein interactions and assembling proteins into large membrane associated complexes which can initiate signal transduction (Fanning and Anderson, 1996; Ponting, 1997). Therefore, *C. jejuni* HtrA was examined for the presence of similar PDZ domains (table 4.5.). The comparison confirms the presence of two PDZ domains in *C. jejuni* HtrA and implies that the molecule functions in a similar manner to other HtrA proteins.

Table 4.5: Deduced position of PDZ domains in a variety of bacterial HtrA molecules.

Species	PDZ domain location (No. amino acid)
<i>Campylobacter jejuni</i> (HtrA)	• 269-333 and 384-440
<i>Helicobacter pylori</i> (HtrA)	• 281-370 and 390-459
<i>Escherichia coli</i> (HtrA)	• 280-371 and 377-466
<i>Salmonella typhimurium</i> (HtrA)	• 281-372 and 378-467
<i>Yersinia enterocolitica</i> (HtrA/GsrA)	• 282-373 and 379-470

4.3: Cloning the complete *htrA* gene

Work by Henderson, (1996) and construction of the *Sau3AI* library in this study highlight that there are problems cloning specific areas of the 2.38kb fragment, most notably the area which encompasses the *dnaJ*-like gene and *htrA* promoter region. With this in mind, together with knowledge of the complete 2.38kb fragment sequence, primers can be designed to enable cloning of specific regions of *htrA* including the promoter to determine whether the gene would be amenable to further study. Attempts were made to clone three different fragments

of *htrA*; i) a 317bp region surrounding the promoter of *htrA* and the *dnaJ*-like gene, ii) the promoter region plus the translated part of the gene and finally iii) the translated part of *htrA*.

4.3.1: Cloning of *htrA*-specific DNA fragments.

Vector pBluescript plasmid DNA (2µg) was digested overnight with *Bam*HI (section 2.11.1.), phosphatase treated (section 2.11.2.) and electrophoresed on a 1.0% agarose gel (section 2.8.). The linear plasmid DNA was subsequently excised from the gel and purified using polyallomer wool gel extraction (section 2.9.1.) and phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

The 317bp region incorporates at the 5'end, 36 bp of the coding sequence of the *dnaJ*-like gene and extends through the putative promoter region and terminates at the 3'end 87bp past the ATG start codon of *htrA*. This region was amplified (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 30 secs extension) (section 2.13.1.) using primers HTRAP1 (5'*Bam*HI site) and HTRAP2 (5'*Bam*HI site) (appendix 1). The promoter region plus the translated part of the gene (~1.75kb) (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 2 mins extension) (section 2.13.1.) was amplified using primers HTRAP1 (5'*Bam*HI) and R4IU3 (5'*Bam*HI). The amplified product incorporates, at the 5'end, 36 bp of the coding sequence of the *Campylobacter* homologue of the *dnaJ*-like gene and extends through the putative promoter region to the end of the *htrA* gene, terminating past the 3'end of *htrA*. The translated part of the gene (~1.55kb) was amplified (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 2 mins extension) (section 2.13.1.) using HTRA-MET (5'*Bam*HI) and R4IU3 (5'*Bam*HI). Primers with the same restriction site at each end were used to avoid directional cloning. Each of the amplified products was purified using the Qiaquick PCR purification kit (section 2.9.4.) and digested overnight with *Bam*HI (section 2.11.1.) to create cohesive ends. The digested PCR products were electrophoresed on a 1% agarose gel (section 2.8.), excised and purified using polyallomer wool gel extraction (section 2.9.1.) phenol:chloroform/chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). A ligation reaction (section 2.11.3.) was performed between the *Bam*HI digested pBluescript DNA and each individually amplified, *Bam*HI digested *htrA* fragments. A vector only, negative control was also included.

Due to the problems associated with trying to clone this region previously, each ligation was checked by PCR to confirm successful ligation between vector and insert. A positive result from the vector plus insert ligation mix would discount any lack of recombinant colonies being attributed to a failure of the ligation. A 1µl sample from each of the ligations, i.e. vector only and vector plus insert was serially diluted and the PCR was performed. The reactions were as follows; HTRAP1 and P2L on the ligation mix containing the putative promoter region of *htrA* (95°C melting 1 min, 55°C annealing 1 min and 72°C extension 30 secs) (section 2.13.1.). The expected product size was 460bp. HTRAP1 and P2L on the ligation mix containing the *htrA* gene plus putative promoter region of *htrA* (95°C melting 1 min, 55°C annealing 1 min and 72°C extension 2 mins) (section 2.13.1.). The expected product size was 1.9kb. Lastly, HTRA-MET and P2L on the ligation mix of the coding region of *htrA* (95°C melting 1 min, 55°C annealing 1 min and 72°C extension 1 min 30 secs) (section 2.13.1.). The expected product size was 1.7kb. The results are given in figure 4.8. and highlight that each ligation has worked, and the vector only controls are negative. Following ethanol precipitation (section 2.9.3.), each ligation mix was transformed into DH5α by electroporation (section 2.12.1.) and the cells were recovered for an hour at 30°C using SOC and plated out accordingly using ampicillin as selection. Cell recovery and overnight growth were performed at 30°C to limit any problems with instability. All attempts at cloning the *htrA* gene plus the putative promoter and also the translated part of *htrA* on at least three occasions were unsuccessful, but transformants from the promoter region ligation were readily obtained.

Recombinant plasmids were checked for the insertion of the *htrA* putative promoter region by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 30 secs extension) (section 2.13.2.) using primers HTRAP1 and HTRAP2 (appendix 1). A positive result yielded a PCR product of ~320bp. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives, and purified plasmid DNA was digested with *Bam*HI (section 2.11.1).

Recombinants which contained fragment sizes of ~2.9kb (pBluescript) and ~320bp upon *Bam*HI digestion confirmed the presence of the promoter insert (figure 4.9.). The plasmid was designated pME26 and is shown in figure 4.10. To verify the cloned insert was indeed the correct region and that the sequence was the same as that obtained from direct sequencing of the 2.38kb fragment, the plasmid was sequenced as outlined in section 2.14. Plasmid DNA was prepared by small-scale plasmid preparation (Qiagen) (section 2.6.2.) and the primers

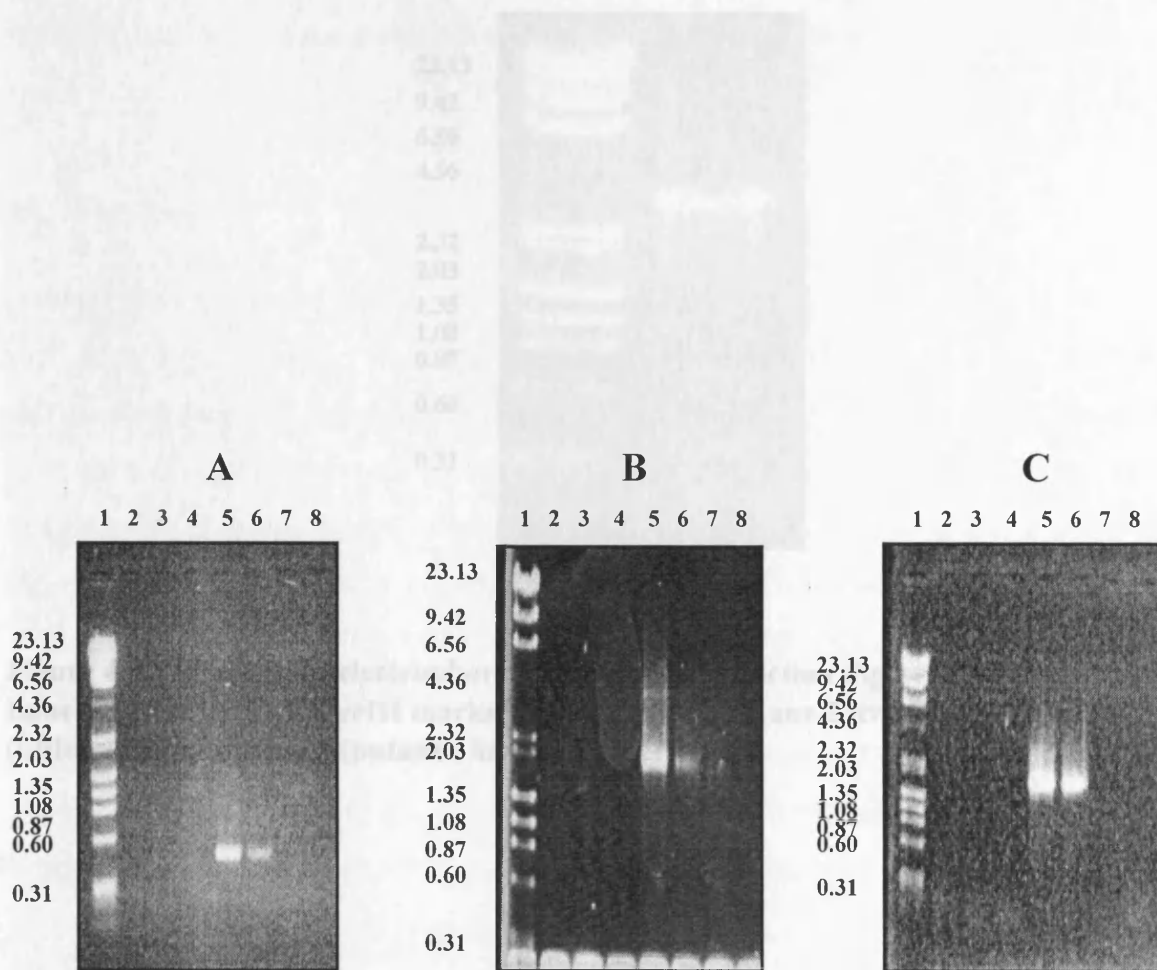


Figure 4.8: PCR amplification of recombinant plasmids from *htrA* gene ligations. **A.** PCR using HTRAP1 and P2L on putative promoter region of *htrA*, expected size ~460bp. **B.** PCR using HTRAP1 and P2L on ligation mix of *htrA* gene, plus putative promoter region, expected size ~1.9kb. **C.** PCR using HTRA-MET and P2L on ligation mix of coding region of *htrA*, expected fragment size ~1.7kb. In each of A, B and C. Lane 1, λ HindIII, ϕ xHaeIII marker (kilobase pairs); Lanes 2-4, negative control PCR on different dilutions of re-ligated pBluescript vector only; Lane 2, 1:10; Lane 3, 1:100; Lane 4, 1: 1000; Lanes 5-7, PCR on different dilutions of insert plus vector; Lane 5, 1:10; lane 6, 1:100; lane 7, 1:1000; Lane 8, dH₂O control.

Figure 4.10: Diagrammatic representation of plasmid pME-4. Black boxes represent local genes. The cloned intergenic region is shown by an open box. The orientation of the putative *dnaJ*-like and *htrA* promoters are shown by a grey arrow.

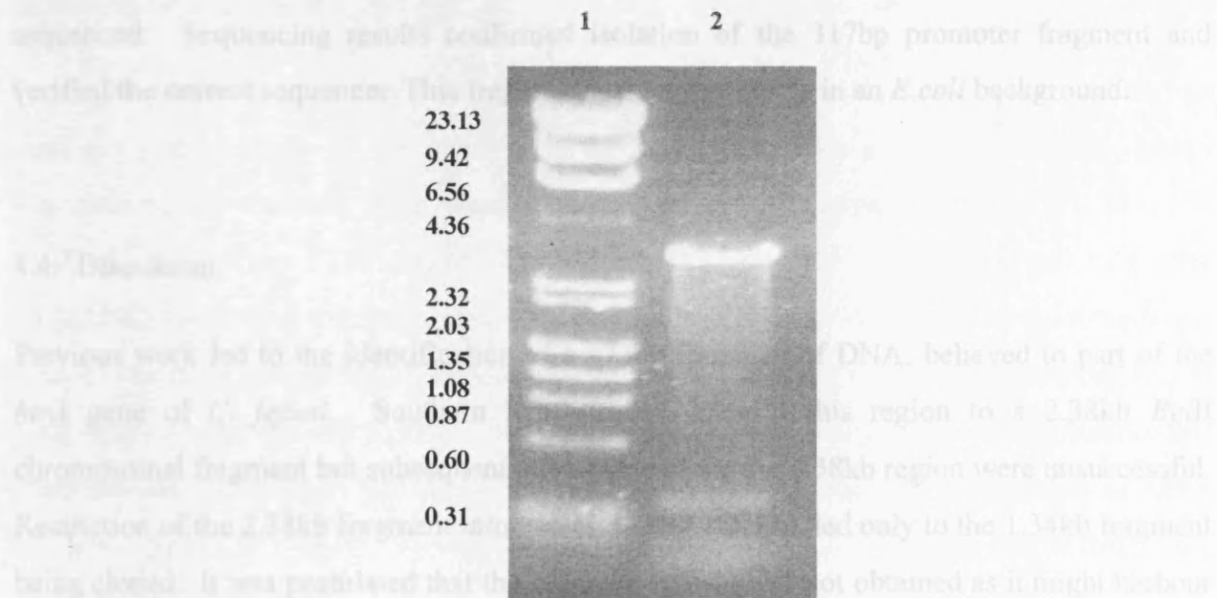


Figure 4.9: Agarose gel electrophoresis of *Bam*HI restriction digest of pME26 (1% gel). Lane 1, λ *Hind*III, ϕ /x *Hae*III marker (kilobase pairs); Lane 2, two bands of sizes ~2.9kb (pBluescript) and 317bp (putative *htrA* promoter)

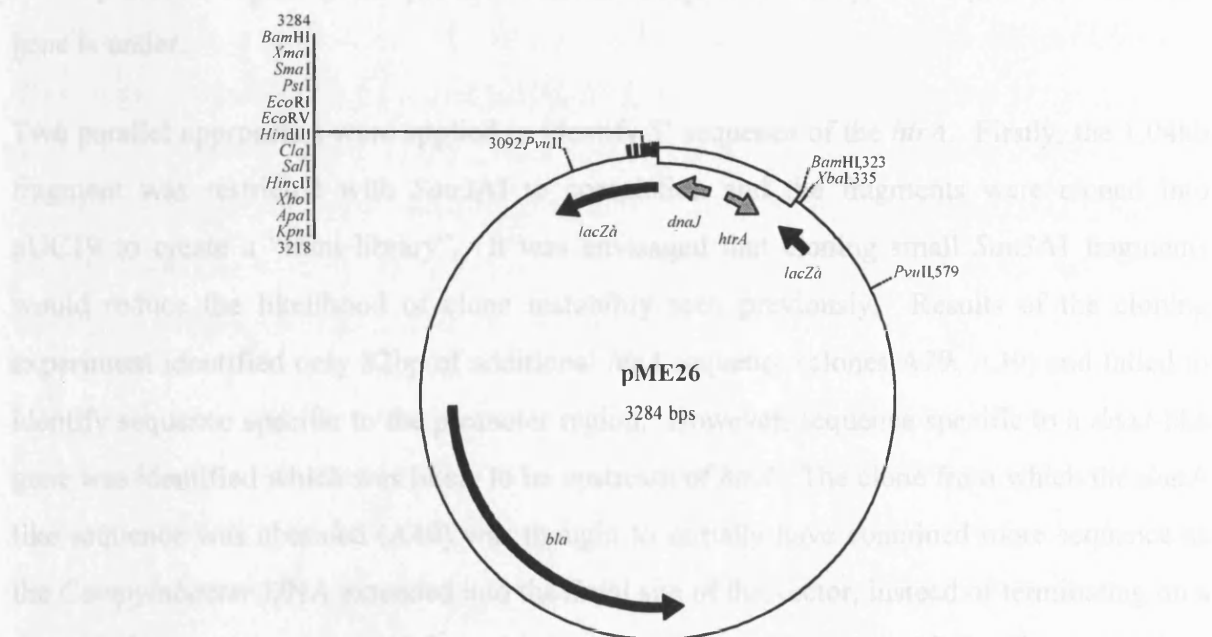


Figure 4.10: Diagrammatic representation of plasmid pME26. Black arrows highlight genes. The cloned intergenic region is shown by an open box. The orientation of the putative *dnaJ*-like and *htrA* promoters are shown by a grey arrow.

used were M13 Forward and M13 Reverse. The samples were sent in duplicate to be sequenced. Sequencing results confirmed isolation of the 317bp promoter fragment and verified the correct sequence. This fragment is therefore stable in an *E.coli* background.

4.4: Discussion

Previous work led to the identification of a 473bp fragment of DNA, believed to part of the *htrA* gene of *C. jejuni*. Southern hybridisation mapped this region to a 2.38kb *Bg/II* chromosomal fragment but subsequent attempts to clone the 2.38kb region were unsuccessful. Restriction of the 2.38kb fragment into two (1.34 and 1.04kb), led only to the 1.34kb fragment being cloned. It was postulated that the 1.04kb fragment was not obtained as it might harbour regions of DNA unstable in an *E. coli* background. Sequence analysis of the 1.34kb fragment identified ~1.0kb of the 3' end of the putative *C. jejuni htrA* gene. By extension, it was postulated that the 1.04kb fragment would most likely contain additional upstream elements specific to the putative *htrA* gene. Therefore, efforts were undertaken to identify DNA specific to this region as this information would complete the open reading frame and confirm whether the putative *htrA* gene was indeed the true *C. jejuni htrA*. Furthermore, identification of the promoter region would possibly offer an insight into the type of regulation which the gene is under.

Two parallel approaches were applied to identify 5' sequence of the *htrA*. Firstly, the 1.04kb fragment was restricted with *Sau3AI* to completion and the fragments were cloned into pUC19 to create a "mini-library". It was envisaged that cloning small *Sau3AI* fragments would reduce the likelihood of clone instability seen previously. Results of the cloning experiment identified only 82bp of additional *htrA* sequence (clones A29, A39) and failed to identify sequence specific to the promoter region. However, sequence specific to a *dnaJ*-like gene was identified which was likely to be upstream of *htrA*. The clone from which the *dnaJ*-like sequence was obtained (A40) was thought to initially have contained more sequence as the *Campylobacter* DNA extended into the *SmaI* site of the vector, instead of terminating on a *Sau3AI* site. As sequence specific to this region still remained unidentified, unknown regions of DNA were sequenced from the 2.38kb IPCR fragment using strategically positioned primers. Results of this sequence analysis highlighted two open reading frames, specific to

htrA and the *dnaJ*-like gene, separated by an intergenic region believed to harbour the promoters of both genes.

Given the identification of new sequence, the 1.04kb fragment could be examined for the presence and position of the *Sau*3AI sites from which clones from the “mini-library” experiment were obtained. This region can be split into four fragments of sizes, 711, 127, 118 and 82bp respectively. The *Sau*3AI boundaries for clones, A29, A39, B11 and B23 were identified. Possibly of greater interest, is that DNA from clone A40 originates from the larger *Sau*3AI fragment of ~ 711bp. This *Sau*3AI fragment covers the putative promoter regions of both *htrA* and the *dnaJ*-like gene. The reason for the discrepancy in the size of the *Sau*3AI fragment and the amount actually obtained in A40 is most likely due to instability of DNA contained within this region. Given the sequence of A40, the unstable area can be tentatively located close to the promoter region, within the 711bp region (refer to figure 4.5.).

With knowledge of the complete *htrA* sequence together with its associated promoter, attempts were made to clone specific regions of the *htrA* gene to see whether the gene would be amenable to further study. This would provide information about whether the promoter region could be used for primer extension analysis and transcriptional assays and whether the HtrA protein could be expressed to allow *N*-terminal sequence analysis, immunogenicity studies and biochemical characterisation. Three different fragments were tried; a 317bp region surrounding the promoter of *htrA* and *dnaJ*, the promoter region plus the translated part of the gene and finally, the translated part of *htrA*. Only clones containing the 317bp region of the promoter were obtained giving further support for difficulties in cloning specific regions of *htrA*. Unfortunately, the inability to clone the other *htrA*-specific fragments limits the number of options with regard to further analysis. The cloned promoter region was used for subsequent expression analysis (see chapter 5).

The identification of new sequence also allows an analysis of the open reading frames of both the *dnaJ*-like gene and *htrA*. Analysis of the *dnaJ*-like gene suggests that it is transcribed in a divergent orientation to the *C. jejuni htrA* gene and is located at the 5' end of the 2.38kb *Bgl*II fragment. Interestingly, this is one of three *dnaJ* or *dnaJ*-like genes in *Campylobacter* (see chapter 9 for more details). The putative promoter region for the *dnaJ*-like gene was compared with the *E. coli* consensus sequence σ^{70} , σ^{32} , σ^{28} and σ^{54} . A putative σ^{70} recognised promoter element was identified. The significance of this is unclear as the identification is based on the *E. coli* consensus. Furthermore, as there is yet no data with regards to the

transcriptional start of the gene, one must regard the designation of this promoter element with care. The putative promoter region was also both visually inspected and computer aligned with the promoter recognition sequence given by Wösten *et al.*, (1998). The analysis failed to identify any similar regions. Once again this may in part be due to a lack of knowledge of the transcriptional start of the gene or may be due to the type of genes from which Wösten's consensus was derived.

With regards to *htrA*, the newly identified promoter region was visually inspected for the presence of the *E. coli* consensus sequence of σ^E promoter elements as HtrA homologues from *E. coli* (Lipinska *et al.*, 1988, 1989 and 1990) *S. typhimurium* (Martin *et al.*, 1994) and *Y. enterocolitica* (Li *et al.*, 1996; Yamamoto *et al.*, 1996) which are regulated by the extracytoplasmic sigma-factor, σ^E . The analysis failed to identify σ^E promoter elements. The lack of σ^E recognised promoter elements for the *C. jejuni htrA* suggests that the gene might not be not regulated in a σ^E -dependent manner, but by another regulatory mechanism(s) which remains to be identified. The failure to identify any σ^E promoter elements may not be surprising. Although many HtrAs have been assigned overlapping functions, the regulation of *htrA* expression in specific bacterial examples is more likely to depend on the phylogenetic relationship between the different bacterial species and not the particular gene in question. The genome of *H. pylori*, a close relative of *C. jejuni* has been sequenced (Tomb *et al.*, 1997) and from these data it appears that there is no *rpoE* gene present in the genome. Given the phylogenetic relationship between *H. pylori* and *C. jejuni* it would not be surprising if the *C. jejuni* genome lacked *rpoE*; this is considered in more detail in chapter 9. A failure to identify any *E. coli* σ^E -like promoter elements led to an examination of the putative promoter region for *E. coli* σ^{70} , σ^{32} , σ^{28} , or σ^{54} promoter elements but no likely candidates were identified. As with the *dnaJ*-like gene the putative *htrA* promoter was aligned with the promoter consensus given by Wösten *et al.*, (1998). Once again the alignment failed to identify a clear relationship with any of the important regions described in the paper. This may be due to the fact that most promoters aligned in the study are situated in front of *C. jejuni* housekeeping genes, and unlike *htrA*, their transcriptional start sites have already been established. The *htrA* gene may not harbour a promoter region specific for genes involved in housekeeping functions and furthermore there is no data available concerning the transcriptional start site for the gene. Primer extension analysis using the putative promoter region would allow this to be determined.

The presence of a putative promoter region upstream of *htrA* together with a recognisable RBS (AGGA) in the non-translated part of the gene suggests that the *htrA* is not a pseudogene. This possibility remained because at the start of this work, the complete open reading frame of *htrA* had not been identified and no phenotype had been determined for the *C. jejuni htrA* mutant. In addition, the complete open reading frame of *htrA* finally confirms the authenticity of the gene. *C. jejuni* HtrA has its highest identity (50%) and similarity (67%) with the HtrA homologue from *H. pylori* (table 4.4.) giving additional support for the close phylogenetic similarity between the two genomes.

HtrA homologues are considered periplasmic and harbour signal peptides to direct them to their extracytoplasmic location. *C. jejuni* HtrA has two putative signal peptidase cleavage sites (16-17aa; LFA-AS and 28-29aa; ATA-NR) derived from Nielsen *et al.*, (1997). Given predictive and experimental data of HtrA molecules from other organisms such as *E. coli* (Lipinska *et al.*, 1988; Lipinska *et al.*, 1989), *S. typhimurium* (Johnson *et al.*, 1991) *H. influenzae* (Loosmore *et al.*, 1998), *B. abortus* (Tatum *et al.*, 1994) and *Y. enterocolitica* (Yamamoto *et al.*, 1996; 1997), the most likely cleavage point for *C. jejuni* HtrA is at position 28-29 between amino acids ATA-NR, resulting in a mature protein of 48kDa. *N*-terminal sequence analysis of *C. jejuni* HtrA would confirm this point. Referring back to polypeptide analysis performed on the *C. jejuni htrA* mutant by Henderson, (1996), there was no missing polypeptide noted around this size when compared to the parent.

C. jejuni HtrA lacks a GXGXXG motif which is apparent in the HtrA proteins from *E. coli*, *S. typhimurium* and *Y. enterocolitica* and has been implicated in dinucleotide binding (Wierenga and Hol, 1983). This might indicate a functional difference between these HtrA homologues and *C. jejuni* HtrA.

An alignment of *C. jejuni* HtrA with other protein sequences shows that there is a consensus sequence of GNSGGAL surrounding the putative Ser²²⁵ residue of the molecule. Using residue Ser²²⁵ as a reference point, an alignment of bacterial HtrA homologues given in figure 4.7. and an alignment of bacterial and mammalian serine proteases given by Loosmore *et al.*, (1998) and Pallen and Wren, (1997), the other members of the catalytic triad were determined as His¹²⁰ and Asp¹⁵¹. The presence of these residues suggests that *C. jejuni* harbours serine protease activity akin to that of other HtrA homologues. Site directed mutagenesis of Ser²¹⁰ and His¹⁰⁵ members of the catalytic triad of the mature HtrA protein of *E. coli* has confirmed their involvement in the proteolytic activity of the molecule (Skorko-Glonek *et al.*, 1995a;

1995b). Furthermore, mutations of His⁹¹, Asp¹²¹, and Ser¹⁹⁷ codons of a recombinant HtrA from *H. influenzae* abolish proteolytic activity. Serine protease activity of *C. jejuni* HtrA and the contribution of individual members of the proposed catalytic triad has yet to be determined.

Unlike HtrA proteins of *E. coli*, *S. typhimurium* *Y. enterocolitica* (Li *et al.*, 1996) and some *H. influenzae* strains (Loosmore *et al.*, 1998), HtrA from *C. jejuni* does not contain a carboxy terminal RGD motif, identified as a cell attachment motif for mammalian adhesion proteins (Ruoslahti and Pierschbacher, 1987). *E. coli* DegQ (Waller and Sauer, 1996) and HtrA from some strains of *H. influenzae* (Loosmore *et al.*, 1998) contain a similar RGN motif, which again is absent from *C. jejuni*. As HtrA is proposed to have a periplasmic location its function as a cell binding motif is unclear. Nevertheless, the fact that it is at least semi-conserved amongst different species suggests a functional significance (Loosmore *et al.*, 1998). The absence of such a motif in *C. jejuni* and other HtrA homologues might indicate a lack of this particular function or a further, different function of the protein.

The finding that bacterial HtrA homologues contain PDZ domains poses the question as to their function in *E. coli*. Formerly, PDZ domains were identified purely in eukaryotic proteins where they are implicated in mediating protein-protein interactions by binding to either C-terminal tetrapeptides, internal sequences or other PDZ domains. Furthermore, they play a role in assembling proteins into large membrane associated complexes that can initiate signal transduction (Fanning and Anderson, 1996; Pallen and Wren, 1997; Ponting, 1997; Songyang *et al.*, 1997; Stricker *et al.*, 1997). It is proposed that PDZ domains of *E. coli* HtrA mediate protein-protein interactions, possibly those which aid assembly of the monomers into oligomeric complexes or those involved in recognition of substrates. As with other HtrA proteins, *C. jejuni* HtrA has two PDZ domains and by comparison, it can be proposed that the PDZ domains of *C. jejuni* HtrA may act in a similar way by aiding assembly of the molecule into oligomeric complexes or help in substrate recognition.

A culmination of data from this study and previous studies has identified the complete open reading frame of *htrA*. Analysis of this open reading frame has confirmed that *C. jejuni htrA* identified is indeed a member of the HtrA family of serine proteases. Unfortunately, examination of the proposed promoter region has given no clue as to the mechanism of regulation of the gene or whether it is actually expressed. Furthermore, the inability to clone specific regions of the gene has hindered further analysis of the molecule. Nevertheless, using

this new information together with the ability to clone the putative promoter region there is now an opportunity investigate regulation as expression of the gene has yet to be demonstrated. Furthermore, expression from the promoter can be examined under different conditions with a view to establishing a role for the gene in *C. jejuni*.

Chapter 5: Investigating expression of *htrA*

5.1: Introduction

Problems associated with cloning specific regions of the *htrA* gene and those which may arise from forced expression of the HtrA protein has hindered recombinant protein analysis such as *N*-terminal sequence analysis, examination of serine protease activity and immunological analysis. Instead, more emphasis was given to investigating expression of the *htrA* gene. The rationale for concentrating efforts in this direction stems from the fact that, to date, mutational analysis of the *htrA* gene has not revealed a phenotype. A reason for the absence of a discernible phenotype could be due to a lack of expression of the protein in *C. jejuni*. However unlikely, it was postulated that the gene may be a pseudogene, and as such was non-functional and therefore not expressed. Recent findings however, have somewhat altered the pseudogene view as the complete *htrA* open reading frame has since been identified, together with a putative ribosome binding site, upstream of the translation initiation codon. Despite this discovery, there is still no conclusive data with regards to expression of the gene. With this in mind, efforts were undertaken to demonstrate this point. As the ability to follow expression of *htrA* is a useful tool for determining the type of regulation that the gene is under, efforts were undertaken to develop a system whereby expression from the gene could be demonstrated and then subsequently be used to monitor expression under different environmental conditions. As a result, the signal which induces expression from *htrA* could be elucidated. Furthermore, subjecting the *C. jejuni htrA* mutant to conditions when HtrA is expressed might provide valuable information about its phenotype. In pursuit of this aim, three different approaches were adopted; i) an immuno-based translational HtrA::c-myc fusion created by “epitope-tagging”, ii) a single copy chromosomal *htrA::cat* transcriptional fusion to measure *htrA* expression as a function of chloramphenicol acetyl transferase (CAT) expression and iii) the putative *htrA* promoter region cloned into pMW10 to investigate LacZ expression as a measure of *htrA* expression.

5.2.: The HtrA::c-myc translational fusion

“Epitope-tagging” is a powerful genetic technique whereby a small characterised epitope is used to label a protein of interest (Munro and Pelham, 1987; Reisdorf *et al.*, 1993; Nakajima and Yaoita, 1997) . Furthermore, a commercially available antibody to the epitope bypasses the lack of antibodies specifically directed towards the protein allowing expression to be

monitored. Clearly, this has an advantage in this instance as forced expression of HtrA, at least in an *E. coli* based system may be problematic, limiting the potential for developing antibodies against the recombinant protein and investigating expression of the gene in this way. In addition to being able to investigate expression of *htrA*, the “epitope-tagging” technique can be used to generate antibodies against the truncated HtrA protein with a view to aiding purification of the native protein from *C. jejuni*. Provided the native HtrA protein can be purified in sufficient quantities many of the functional and biochemical properties of HtrA can be determined. Specific application of the “epitope-tagging” technique to this situation involved creation of an in-frame translational fusion between the c-myc epitope (EQKLISEEDL#) and *C. jejuni htrA* in *E. coli* and introduction of this construct into *C. jejuni* 81116 by homologous recombination. Expression of the protein was detected using the commercial human monoclonal antibody 9E10 (Cambridge Research Biochemicals).

5.3: Results I

5.3.1: Investigating HtrA expression using the c-myc immunological epitope.

5.3.1.1: Creation of plasmid constructs

5.3.1.1.1: Construction of pME3

Prior to using c-myc as a tool to investigate expression of *htrA*, it was firstly necessary to create a plasmid copy of an in-frame translational fusion between HtrA and c-myc. Homologous recombination of the plasmid fusion could be used to construct a chromosomal copy allowing c-myc expression to be monitored as a measure of *htrA* expression.

For this purpose, 2µg of pUC19 plasmid DNA was digested overnight with *Bam*HI and *Pst*I (section 2.11.1.) and electrophoresed on a 1.0% agarose gel (section 2.8.). The linear plasmid DNA was subsequently excised from the gel, purified using polyallomer wool gel extraction (section 2.9.1.) and phenol/chloroform:chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3.). A PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.1.) using primers HTRA17 (5' *Bg*/II) and HTRA18 (5' *Pst*I) (appendix 1) amplified ~1.1kb region of *C. jejuni* 81116 chromosomal DNA specific to the *htrA* gene. The PCR product was electrophoresed on 1% agarose gel (section 2.8.), excised and purified using polyallomer wool gel extraction (section 2.9.1.),

phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). The PCR product was subsequently digested with *Bgl*III and *Pst*I to create cohesive ends (section 2.11.1.). The two digested products were ligated together (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), the DNA was transformed into *E.coli* DH α competent cells by electroporation (section 2.12.1.1.) and plated out accordingly onto plates with appropriate selection. Putative recombinant colonies were examined by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) using primers HTRA17 and HTRA18 (appendix 1) to look for PCR products of ~1.1kb in size. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives and purified plasmid DNA was digested with *Hind*III (section 2.11.1.). The presence of 3 fragment sizes of 98, 456 and 3224bp confirmed putative positives (figure 5.1.). The plasmid was designated pME3 and is shown diagrammatically in figure 5.2. In order to verify plasmid pME3, insert DNA was sequenced as outlined in section 2.14. using primers M13 Forward, M13 Reverse HTRA8, HTRA12 and HTRA13 (appendix 1). Plasmid DNA was prepared by small-scale plasmid preparation (Qiagen) (section 2.6.2.) and the samples were sent in duplicate to be sequenced. The results of the sequence analysis were as expected and confirmed introduction of the correct *htrA* insert.

5.3.1.1.2: Introduction of the c-myc epitope into pME3 to form pME12

Plasmid pME3 was purified using large-scale plasmid preparation (Qiagen) (section 2.6.3.). Inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.1.) was performed on a 1:500 dilution of plasmid pME3 using primers MYC-2 (*Bam*HI) and HTRA7 (*Bam*HI) (appendix 1). MYC-2 is a derivative of HTRA6 which was used in conjunction with HTRA7 in construction of the original isogenic *htrA* mutant, JH1 (Henderson, 1996). The sequence of MYC-2 contains nucleotides which when translated appropriately in a double stranded form will create the c-myc immunological epitope, EQKLISEEDL#, where # represents a stop codon. Use of MYC-2 and HTRA7 in tandem creates an internal *Bam*HI site, a 15bp deletion of *htrA* DNA and an in-frame fusion between HtrA protein sequence and the c-myc epitope. The strategy used in construction of the fusion is shown in figure 5.3.

The resulting PCR product of ~3.8kb was electrophoresed on a 1% agarose gel (section 2.8.) excised and purified using polyallomer wool gel extraction (section 2.9.1.),

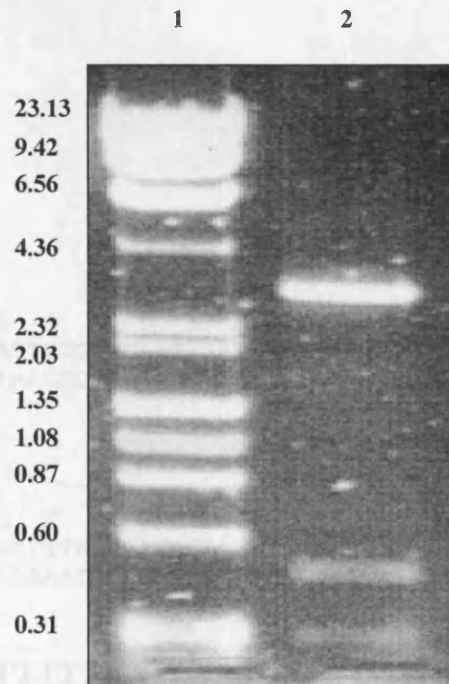


Figure 5.1: Agarose gel electrophoresis of a digest of pME3 with *Hind*III to reveal three fragment sizes of 98, 456 and 3224bp respectively. The presence of the three fragments confirms the presence of the ~1.1kb fragment amplified using HTRA17 and HTRA18.

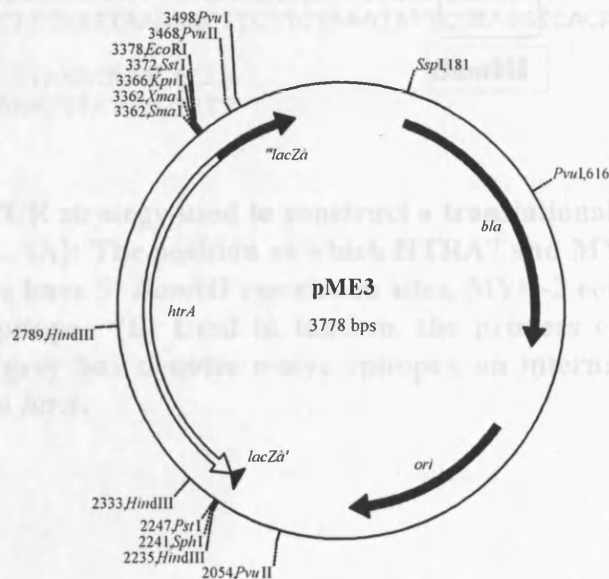


Figure 5.2: Plasmid map of pME3 showing insertion of the amplification product of a fragment of the *htrA* gene using HTRA17 and HTRA18 (white arrow). The product was cloned using *Bam*HI and *Pst*I.

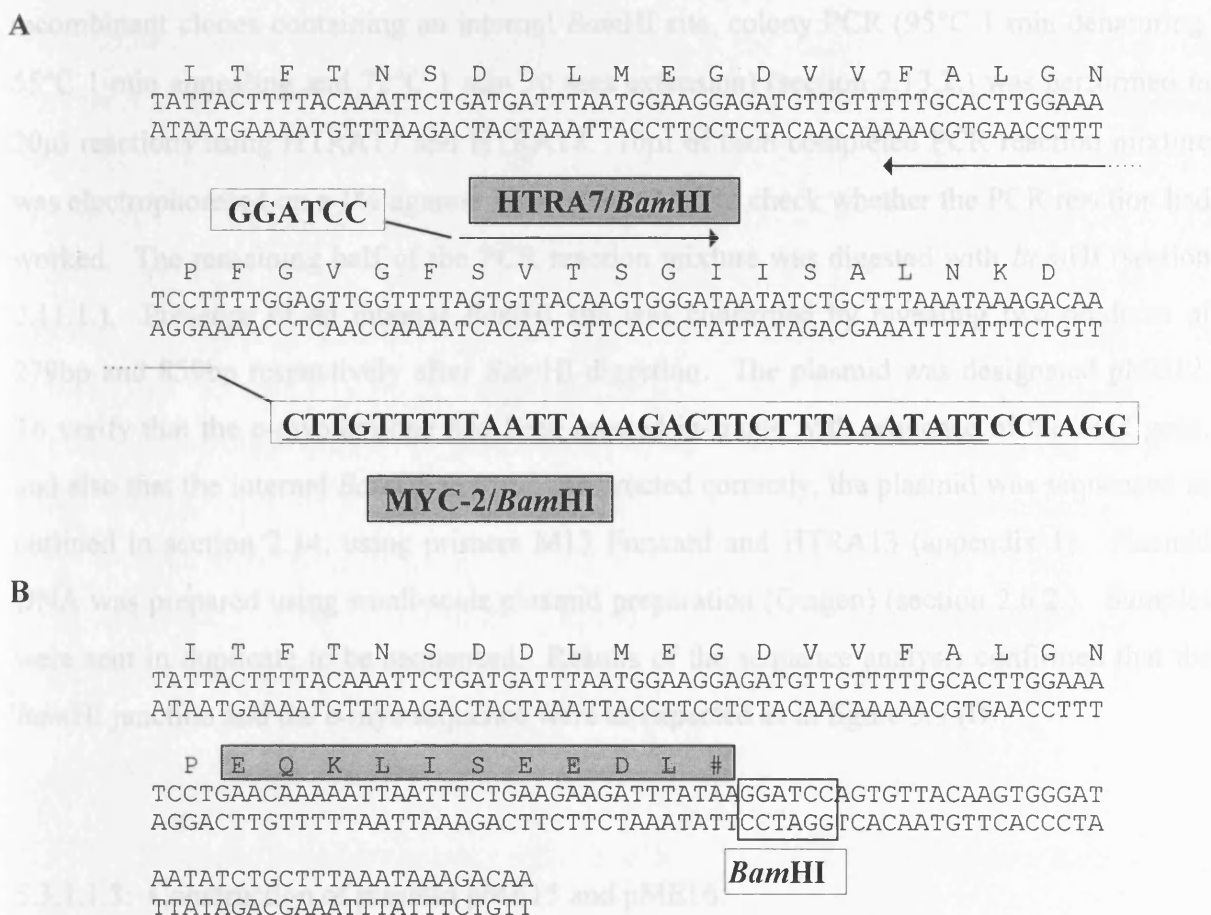


Figure 5.3: Inverse PCR strategy used to construct a translational fusion between the c-myc epitope and *htrA*. (A): The position at which HTRA7 and MYC-2 bind is shown by arrows. Both primers have 5' *Bam*HI restriction sites, MYC-2 contains sequence which encodes the c-myc epitope. (B) Used in tandem, the primers create an HtrA::c-myc translational fusion (grey box denotes c-myc epitope), an internal *Bam*HI site (boxed) and a 15bp deletion in *htrA*.

phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). The product was digested with *Bam*HI (section 2.11.1.), electrophoresed on a 1% agarose gel (section 2.8.) and the purification process was repeated. 100ng of the digested PCR product was self-ligated (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), transformed into *E. coli* DH5 α competent cells by electroporation (section 2.12.1.). Transformants were plated out accordingly using appropriate selection. To check for religated recombinant clones containing an internal *Bam*HI site, colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) was performed in 20 μ l reactions using HTRA17 and HTRA18. 10 μ l of each completed PCR reaction mixture was electrophoresed on a 1% agarose gel (section 2.8.) to check whether the PCR reaction had worked. The remaining half of the PCR reaction mixture was digested with *Bam*HI (section 2.11.1.). Presence of an internal *Bam*HI site was confirmed by revealing two products of 279bp and 859bp respectively after *Bam*HI digestion. The plasmid was designated pME12. To verify that the c-myc epitope had been created in-frame with sequence of the *htrA* gene, and also that the internal *Bam*HI site had constructed correctly, the plasmid was sequenced as outlined in section 2.14. using primers M13 Forward and HTRA13 (appendix 1). Plasmid DNA was prepared using small-scale plasmid preparation (Qiagen) (section 2.6.2.). Samples were sent in duplicate to be sequenced. Results of the sequence analysis confirmed that the *Bam*HI junction and the c-myc sequence were as expected as in figure 5.3 (B).

5.3.1.1.3: Construction of plasmid pME15 and pME16.

In order to introduce the HtrA::c-myc fusion into *C. jejuni* it was necessary to construct plasmid pME15 and pME16. Plasmid pME12 was purified using large-scale plasmid purification (Qiagen) (section 2.6.3.). 2 μ g of plasmid pME12 was digested overnight with *Bam*HI (section 2.11.1.) and subsequently phosphatase treated (section 2.11.2.). The plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear plasmid DNA was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.) and phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

Similarly, 2 μ g of plasmid pAV35 (appendix 2) which contains the *C. coli* chloramphenicol resistance gene was digested overnight with *Bam*HI (section 2.11.1.) to remove the resistance

cassette. The digested plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 850bp chloramphenicol resistance cassette was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). Linear plasmid, pME12, was ligated to the *Bam*HI digested chloramphenicol gene (section 2.11.3.). Following ethanol precipitation, the ligation was transformed into *E. coli* DH5 α by electroporation (section 2.12.1.) and transformants were the plated out accordingly using chloramphenicol as selection. Recombinant clones were checked for the insertion of the chloramphenicol cassette into the internal *Bam*HI site of pME12 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) using primers HTRA17 and HTRA18 (appendix 1). A positive result yielded a PCR product of ~1.99kb in size compared to the positive control of pME12 which was 1.14kb. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives, and purified plasmid DNA was digested with *Hind*III (section 2.11.1.). Two patterns were obtained depending on the orientation of the chloramphenicol cassette. Plasmids which revealed fragment sizes of 3521, 577, 456 and 98 bp upon digestion with *Hind*III confirmed the presence of the chloramphenicol gene in the same orientation as the *htrA* gene. The plasmid was designated pME15. Product sizes of 3199, 899, 456 and 98bp confirmed the opposite orientation of the chloramphenicol gene, (figure 5.4.). Plasmids pME15 and pME16 are shown diagrammatically in figure 5.5.

5.3.1.1.4: Introduction of the c-myc epitope into the *C. jejuni* chromosomal *htrA* locus

Electrotransformation of pME15 and pME16 into *C. jejuni* 81116 was performed as outlined in section 2.12.2. 10 μ g of plasmid DNA was used each time. As electroporation negative and positive controls, 81116 competent cells were electroporated in the absence of DNA and in the presence of 1 μ g of plasmid pTNS#A respectively (appendix 2). Resistant colonies for the positive control could be seen after 2 days. For the pME15, pME16 transformants, colonies could be seen after 3-4 days. All transformants had typical *Campylobacter* colony morphology. Putative positive transformants were sub-cultured onto Mueller-Hinton agar plates supplemented with 20 μ g/ml of chloramphenicol and incubated overnight at 37°C in a microaerophilic atmosphere. For a preliminary examination of the transformants to check for homologous recombination, PCR was performed on chromosomal DNA from 5 colonies from

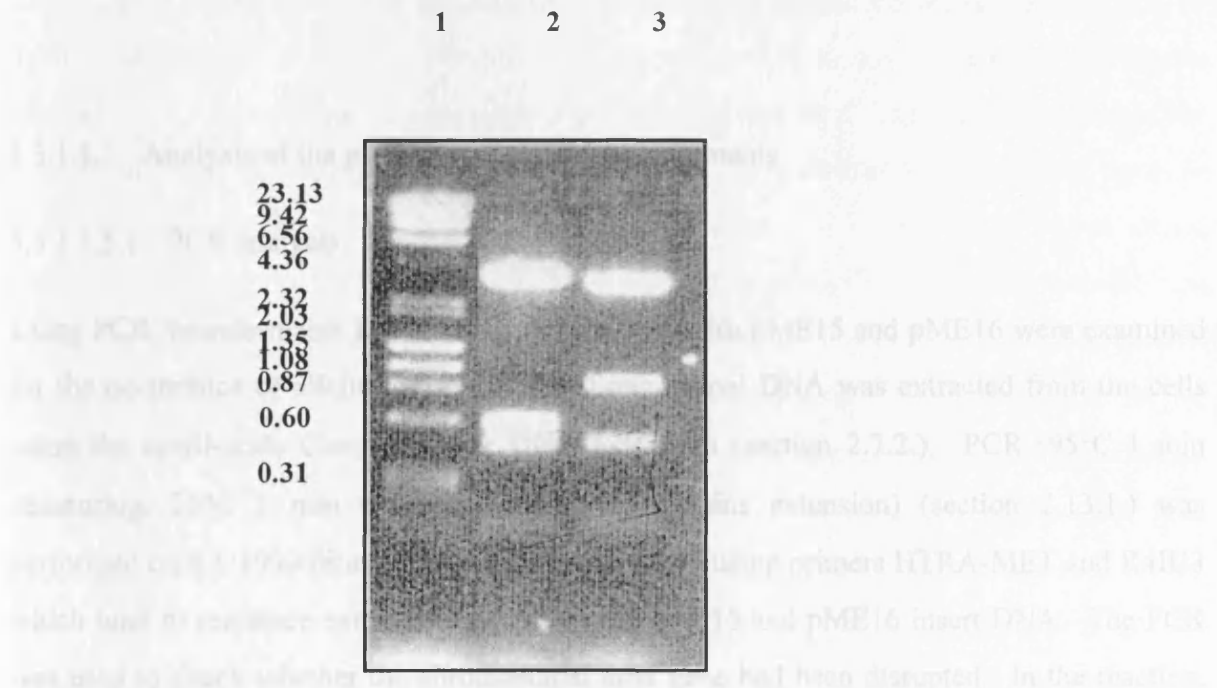


Figure 5.4: Agarose gel electrophoresis of a *Hind*III digest of plasmids pME15 and pME16 highlighting the different orientation of the cloned chloramphenicol gene. Lane 1, λ *Hind*III/ ϕ x *Hae*III marker(kilobase pairs); Lane 2, pME15 (3521, 577, 456 and 98 bp), Lane 3, pME16 (of 3199, 899, 456 and 98bp)

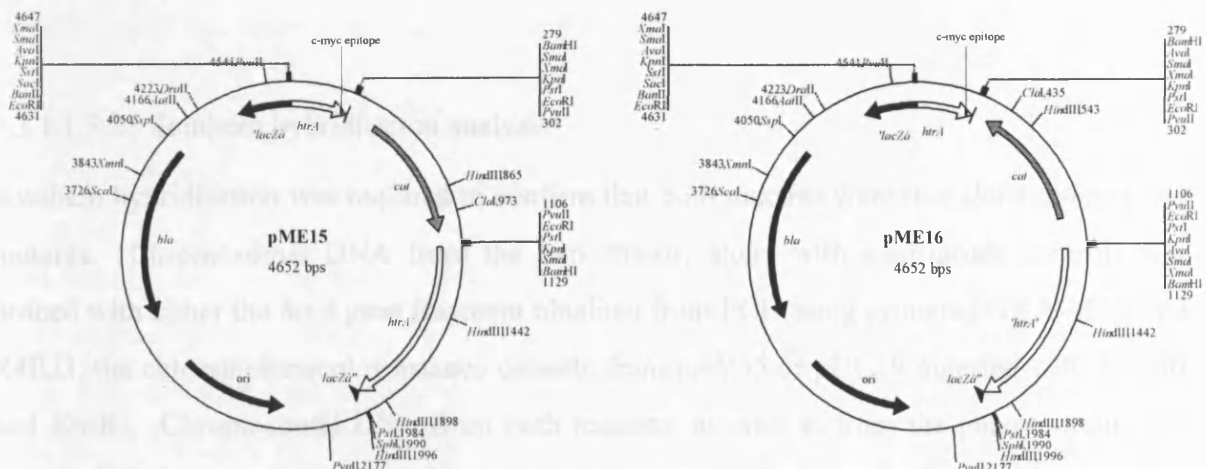


Figure 5.5: Diagrammatic outline of plasmids pME15 and pME16 showing the different orientation of *cat* (grey arrow). The incomplete *htrA* open reading frame (white arrow) is interrupted by *cat*. The position of the c-myc epitope, before the *cat* gene, is given.

each of the transformations of pME15 and pME16 and the results were confirmed by Southern hybridisation analysis.

5.3.1.1.5: Analysis of the pME15 and pME16 transformants

5.3.1.1.5.1: PCR analysis.

Using PCR, transformants from the electroporations with pME15 and pME16 were examined for the occurrence of allelic replacement. Chromosomal DNA was extracted from the cells using the small-scale *Campylobacter* DNA extraction (section 2.7.2.). PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) was performed on a 1:1000 dilution of chromosomal DNA using primers HTRA-MET and R4IU3 which bind to sequence external to that from the pME15 and pME16 insert DNA. The PCR was used to check whether the chromosomal *htrA* gene had been disrupted. In the reaction, two controls were included; a negative control with no DNA template and a positive control of 81116 DNA. The PCR products were separated on a 1% agarose gel. Each transformant tested showed a band of the expected size (~2.3kb), consistent with the introduction of the chloramphenicol cassette into the *htrA* gene (figure 5.6.). One of each potential HtrA::c-myc construct i.e., one pME15 transformant and one pME16 transformant were further analysed by Southern hybridisation.

5.3.1.1.5.2: Southern hybridisation analysis

Southern hybridisation was required to confirm that both mutants were true (*htrA*::c-myc)::cat mutants. Chromosomal DNA from the two strains, along with appropriate controls was probed with either the *htrA* gene fragment obtained from PCR using primers HTRA-MET and R4IU3, the chloramphenicol resistance cassette from pAV35 or pUC19 digested with *Hind*III and *Eco*RI. Chromosomal DNA from both mutants, as well as from the parent strain, was prepared by large-scale *Campylobacter* DNA preparation (section 2.7.2.) and analysed by agarose gel electrophoresis (section 2.8.). To perform each of the three hybridisation experiments, approximately 5µg of chromosomal DNA from each of the strains was restricted with *Bgl*II (section 2.11.1.). Different positive DNA controls were included, according to the hybridisation performed, and were the same as the DNA sample used as a probe. Samples

were separated on a 1.0% agarose gel, together with the controls (section 2.8.). The Southern hybridisation was performed as outlined in section 2.15. The membrane probed with *Hind*III and *Eco*RI digested pUC19 DNA revealed that the probe hybridised to the ~2.7 kb pUC19 band from the pUC19 positive control but did not hybridise to any of the genomic digests (figure 5.7A.). In contrast, the membrane probed with the *htrA* fragment showed that the probe hybridised to all genomic digests (figure 5.7B.). In addition, the highlighted bands in both recombinants underwent a size increase when compared to the 81116 parent strain, indicating that the *htrA* gene was disrupted in both recombinants. The size increase was consistent with the introduction of the chloramphenicol gene. To confirm the introduction of the chloramphenicol gene into the *htrA* gene, the membrane was hybridised to the chloramphenicol gene cassette (figure 5.7C.). The probe did not hybridise to chromosomal DNA from the 81116 parental strain but did so to the DNA from the recombinant strains. The highlighted bands in both recombinants underwent a similar size increase to that seen for the *htrA* gene fragment probe. Again this is consistent with the introduction of the chloramphenicol gene. In summary, the two mutants resulted from the allelic replacement of the wild-type *htrA* gene with the insertionally mutated copy. The insertionally mutated copy should contain an in-frame translational fusion between the *htrA* gene and the c-myc epitope sequence together with the presence of the chloramphenicol gene to provide selection. The two mutants were named ME15 and ME16. Although the presence of the in-frame fusion between *htrA* and c-myc has already been confirmed at the plasmid level with respect to pME15 and pME16, the presence of the in-frame fusion needed to be confirmed at the chromosomal level with respect to ME15 and ME16.

5.3.1.1.5.3: Sequence analysis of the HtrA::c-myc fusion.

To enable expression analysis of the *htrA* gene it was necessary to confirm the presence of the chromosomal in-frame fusion between the coding region of HtrA and c-myc. Chromosomal DNA was extracted from strains ME15 and ME16 using large-scale *Campylobacter* DNA extraction (section 2.7.2.). A PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) using primers HTRAP1 and R4IU3 (appendix 1) was performed on a 1:1000 dilution of chromosomal DNA from ME15 and ME16. These primers were used as they bind outside the insert used to construct the initial mutation and furthermore, HTRAP1 binds upstream of the *htrA* promoter region. This facilitated the examination of the sequence from this region to check that the sequence was as expected and

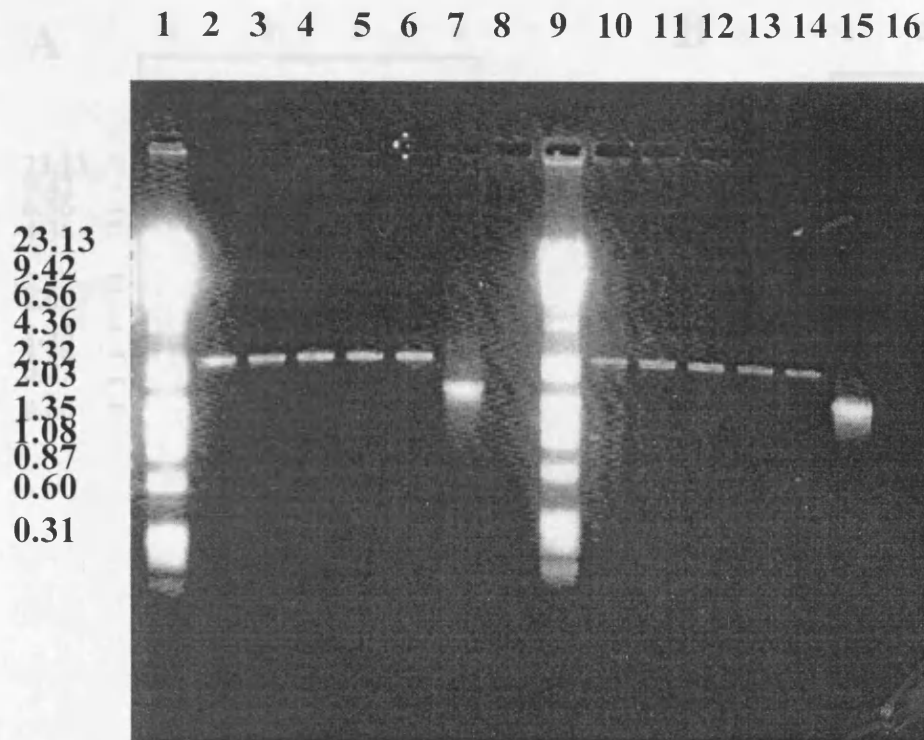


Figure 5.6: PCR on chromosomal DNA from 5 *C. jejuni* transformants after electroporation with pME15 and pME16. The PCR used primers HTRA-MET and R4IU3. Lanes 1 and 9; λ HindIII/ ϕ x HaeIII marker (kilobase pairs), Lanes 2-6; 5 pME15 transformants (~2.3kb), Lane 7 and 15; *C. jejuni* 81116 DNA (~1.7kb), Lanes 10-14; 5 pME16 transformants (~2.3kb), Lanes 10 and 16; negative dH₂O control.

Figure 5.7: Southern hybridisation on ME15 and ME16 mutants to confirm *htrA* replacement on the chromosome. (A) To show absence of vector sequence HindIII and EcoRI digested pUC19 was used as a probe. Left: λ HindIII marker (kilobase pairs); Lane 1; BglII digested ME16 DNA; Lane 2, BglII digested ME15 DNA; Lane 3, BglII digested ME16 DNA; Lane 4, HindIII and EcoRI digested pUC19. (B) To show a change in *htrA* sequence, the HTRA-MET X R4IU3 PCR product was used as a probe. Left: λ HindIII marker (kilobase pairs); Lane 1; BglII digested ME16 DNA; Lane 2, BglII digested ME15 DNA; Lane 3, BglII digested ME16 DNA; Lane 4, HTRA-MET X R4IU3 PCR product. (C) To show insertion of the chloramphenicol gene, BamHI digested chloramphenicol resistance cassette from pAY35 was used as a probe. Left: λ HindIII marker (kilobase pairs); Lane 1; BglII digested ME16 DNA; Lane 2, BglII digested ME15 DNA; Lane 3, BglII digested ME16 DNA; Lane 4, BamHI digested chloramphenicol resistance cassette from pAY35.

that *htrA* was transcribed and translated correctly. The presence of a single PCR product (~2.7kb) was confirmed by electrophoresis of small aliquot of the PCR product on a 1% agarose gel (section 2.8.1). The remainder of the DNA was purified using the Qiequick PCR purification system (Qiagen) and ethanol precipitated (section 2.9.3.). To verify the authenticity of the fusion, 100ng of the purified PCR product was sequenced using primer CA13' or CA13' (depending on the orientation of the chL gene) and the HtrA-MET X R4IU3 PCR product was used as a probe.

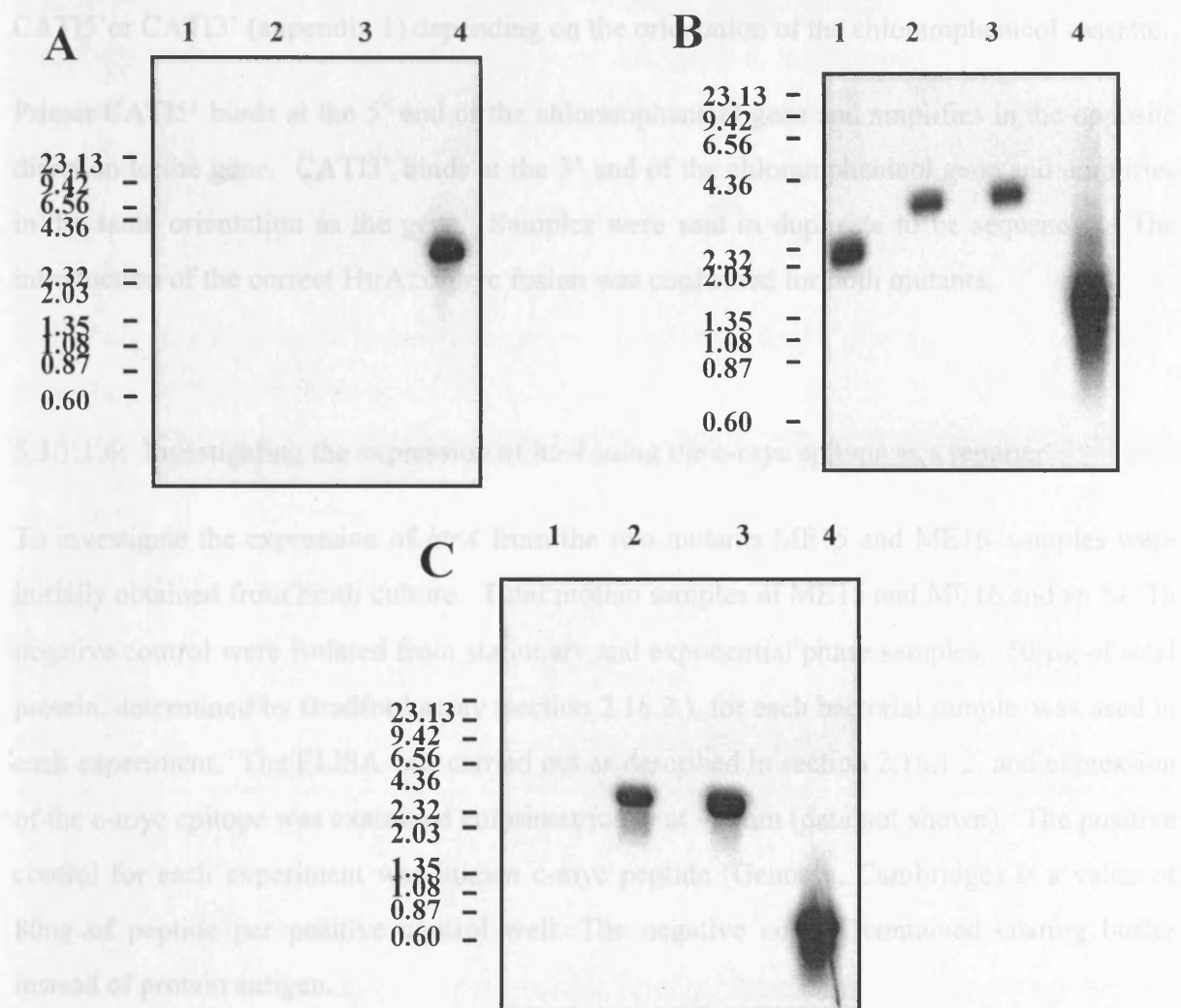


Figure 5.7: Southern hybridisation on ME15 and ME16 mutants to confirm allelic replacement on the chromosome. (A) To show absence of vector sequence *Hind*III and *Eco*RI digested pUC19 was used as a probe. Left, λ *Hind*III marker (kilobase pairs); Lane 1; *Bg*III digested 81116 DNA; Lane 2, *Bg*III digested ME15 DNA; Lane 3, *Bg*III digested ME16 DNA; Lane 4, *Hind*III and *Eco*RI digested pUC19. (B) To show a change in *htrA* sequence, the HTRA-MET X R4IU3 PCR product was used as a probe. Left, λ *Hind*III marker (kilobase pairs); Lane 1; *Bg*III digested 81116 DNA; Lane 2, *Bg*III digested ME15 DNA; Lane 3, *Bg*III digested ME16 DNA; Lane 4, HTRA-MET X R4IU3 PCR product. (C) To show insertion of the chloramphenicol gene, *Bam*HI digested chloramphenicol resistance cassette from pAV35 was used as a probe. Left, λ *Hind*III marker (kilobase pairs); Lane 1; *Bg*III digested 81116 DNA; Lane 2, *Bg*III digested ME15 DNA; Lane 3, *Bg*III digested ME16 DNA; Lane 4, *Bam*HI digested chloramphenicol resistance cassette from pAV35.

that *htrA* was transcribed and translated correctly. The presence of a single PCR product ~2.7kb was confirmed by electrophoresis of small aliquot of the PCR product on a 1% agarose gel (section 2.8.). The remainder of the DNA was purified using the Qiaquick PCR purification system (Qiagen) and ethanol precipitated (section 2.9.3.). To verify the authenticity of the fusion, 100ng of the purified PCR product was sequenced using primer CAT15' or CAT13' (appendix 1) depending on the orientation of the chloramphenicol cassette.

Primer CAT15' binds at the 5' end of the chloramphenicol gene and amplifies in the opposite direction to the gene. CAT13' binds at the 3' end of the chloramphenicol gene and amplifies in the same orientation as the gene. Samples were sent in duplicate to be sequenced. The introduction of the correct HtrA::c-myc fusion was confirmed for both mutants.

5.3.1.1.6: Investigating the expression of *htrA* using the c-myc epitope as a reporter

To investigate the expression of *htrA* from the two mutants ME15 and ME16, samples were initially obtained from broth culture. Total protein samples of ME15 and ME16 and an 81116 negative control were isolated from stationary and exponential phase samples. 50 µg of total protein, determined by Bradford assay (section 2.16.2.), for each bacterial sample, was used in each experiment. The ELISA was carried out as described in section 2.16.1.2. and expression of the c-myc epitope was examined colorimetrically at 490nm (data not shown). The positive control for each experiment was human c-myc peptide (Genosys, Cambridge) at a value of 80ng of peptide per positive control well. The negative control contained coating buffer instead of protein antigen.

The negative and positive controls gave expected values but there was no significant difference between the values under any condition or for any sample (data not shown). It was thought that there may not be enough total protein sample added to the ELISA plate, originating from the difficulty in obtaining large amounts of protein from the shaking broth culture. With this in mind, future bacterial samples were harvested from swab plates as described in section 2.17.1.1.

For the following set of experiments, three different amounts of total protein was added to see whether a protein antigen dose response could be detected. For example, either 300µg, 30µg, or 3µg of total protein from ME15, ME16 or 81116 was used. The total protein concentration

for each sample was again calculated using a standard curve obtained by using a Bradford assay (section 2.16.2.). The ELISA was carried out as described in section 2.17.1.2. Both the negative and positive controls were consistent with those used previously, i.e., coating buffer instead of protein antigen and human c-myc respectively. A further sample was added, which combined together, 81116 total protein samples of either 300µg 30µg or 3µg with 80ng of human c-myc peptide, as for the positive control. The reason for this was to test the sensitivity of the c-myc assay under high-level background contamination, i.e., with varying amounts of 81116 protein. The experiment was repeated twice. Data for the experiment is given in table 5.1.

Table 5.1: Comparison of c-myc expression from 81116, 81116 plus c-myc peptide, ME15 and ME16 at 37 and 42°C.

Sample name	300ug protein	30ug protein	3ug protein
No Antigen	0.0034+/-0.0052		
No Antibody	0.0016+/-0.0018		
Positive (80ng)	0.1377+/-0.0617		
81116 37 spiked	0.0293+/-0.0014	0.0343+/-0.0007	0.0590+/-0.0021
81116 37	0.0260+/-0.0000	0.0260+/-0.0000	0.0267+/-0.0000
81116 42	0.0253+/-0.0014	0.0247+/-0.0000	0.0263+/-0.0007
ME15 37	0.0270+/-0.0007	0.0297+/-0.0007	0.0267+/-0.0000
ME15 42	0.0257+/-0.0007	0.0250+/-0.0007	0.0270+/-0.0007
ME16 37	0.0257+/-0.0007	0.0257+/-0.0007	0.0250+/-0.0007
ME16 42	0.0237+/-0.0007	0.0260+/-0.0000	0.0217+/-0.0007

Examination of the results revealed the same outcome as for the previous experiment. Both the negative and positive controls worked effectively, but expression of the c-myc epitope was not observed from either of the mutants, ME15 or ME16 at any of the total protein values added. In fact, the OD 490nm values for each mutant were comparable to that obtained from 81116. The test sample which combined protein from 81116 and the human c-myc epitope, showed an increase in OD 490nm, as the amount of total protein decreased. In addition, Western blotting was attempted to try and detect expression of the c-myc epitope. The experiments failed to identify any expression of c-myc (data not shown).

5.4: The *htrA*::chloramphenicol acetyl transferase (*cat*) transcriptional fusion.

The *htrA*::*cat* transcriptional fusion is a variation of the HtrA::c-myc translational fusion and retains the characteristic that expression from *htrA* is a result of the gene being in its native, single copy, chromosomal form. A possible advantage over the c-myc-translational fusion is that detection only requires transcription from the *htrA* gene and not a combination of both transcription and translation. To facilitate the use of the *htrA*::*cat* fusion to investigate *htrA* expression it was first necessary to produce a transcriptional fusion between a promoterless chloramphenicol gene and *htrA* in *E. coli*. The construct could be introduced into *C. jejuni* to allow homologous recombination and creation of a transcriptional fusion between *htrA* and the promoterless chloramphenicol gene. To provide selection, the promoterless chloramphenicol gene was linked to the *apha-3* kanamycin resistance gene. Expression of the *htrA* gene was investigated in two ways: i) By examining the sensitivity of the mutant to chloramphenicol in a disc sensitivity assay, and by ii) looking for growth of single colonies on varying amounts of chloramphenicol.

5.5: Results II

5.5.1: Investigating *htrA* expression using the *htrA*::*cat* fusion

5.5.1.1: Creation of plasmid constructs

5.5.1.1.1: Construction of pME21

Plasmid pME3 was purified using large-scale purification (Qiagen) (section 2.6.3.). Inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.1) was performed on a 1:500 dilution of plasmid pME3 using primers HTRA6 (*Bam*HI) and HTRA7 (*Bam*HI) (appendix 1). Use of HTRA6 and HTRA7 in tandem creates an internal *Bam*HI site, and a 14bp deletion (figure 5.8.). The resulting PCR product of 3770bp was electrophoresed on a 1% agarose gel (section 2.8.), excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). The product was digested with *Bam*HI overnight (section 2.11.1.) and the purification was repeated as above. 100ng of the digested PCR product was self-ligated (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), transformed into *E. coli* DH5 α competent cells by electroporation (section 2.12.1.).

Transformants were plated out accordingly using appropriate selection. To check for religated recombinant clones containing an internal *Bam*HI site, colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) was performed in 20µl reactions using HTRA17 and HTRA18 (appendix 1). 10µl of each completed PCR reaction mixture was electrophoresed on a 1% agarose gel (section 2.8.) to check whether the PCR reaction had worked. The remaining half of the PCR reaction mixture was digested with *Bam*HI (section 2.11.1.). Presence of an internal *Bam*HI site was confirmed by revealing two products of 247bp and 859bp respectively after *Bam*HI digestion. The plasmid was designated pME21. In order to verify that the internal *Bam*HI site had been constructed correctly in pME21, insert DNA was sequenced as outlined in section 2.14. using primer M13 Forward (appendix 1). Plasmid DNA was prepared by small-scale plasmid purification (Qiagen) (section 2.6.2.) and the samples were sent in duplicate to be sequenced. Results of the sequence analysis were as expected and confirmed the mutation, as in figure 5.8B.

5.5.1.1.2: Construction of plasmid pME22.

To facilitate the use of the promoterless chloramphenicol cassette as a tool for investigating *htrA* expression in *C. jejuni*, it was first necessary to create plasmid pME22. Plasmid pME22 is a pME21 derivative containing the *C. coli* chloramphenicol resistance gene and the *apha-3* kanamycin gene inserted into the *htrA* gene. 2µg of plasmid pME21 was digested overnight with *Bam*HI (section 2.11.1.) and subsequently phosphatase treated (section 2.11.2.). The plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear plasmid DNA was subsequently excised from the gel and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3.). Similarly, 2µg of plasmid pAV123 which contains the *C. coli* chloramphenicol resistance gene and the *apha-3* kanamycin gene was digested overnight with *Bam*HI to remove the chloramphenicol/kanamycin resistance cassette. The digested plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 2205bp promoterless chloramphenicol gene and the kanamycin gene resistance cassette was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3.). Linear plasmid, pME21, was ligated (section 2.11.3.) to the *Bam*HI digested promoterless chloramphenicol and kanamycin resistance cassette. Following ethanol

precipitation, the ligation was transformed into *E. coli* DH5 α by electroporation (section 2.12.1.3) and transformants were then plated out accordingly using kanamycin as selection. Recombinant clones were checked for the insertion of the joint resistance cassette into the internal *Bam*HI site of pME21 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.12.2.1 using primers HTRA17 and HTRA18 (appendix 1)). A positive result yielded a PCR products of 331bp in size compared to the positive control of pME21 which was ~1.11kb. Small-scale plasmid preparation (Qiagen) (section 2.6.2) was performed on positive positives, and purified plasmid DNA was digested with

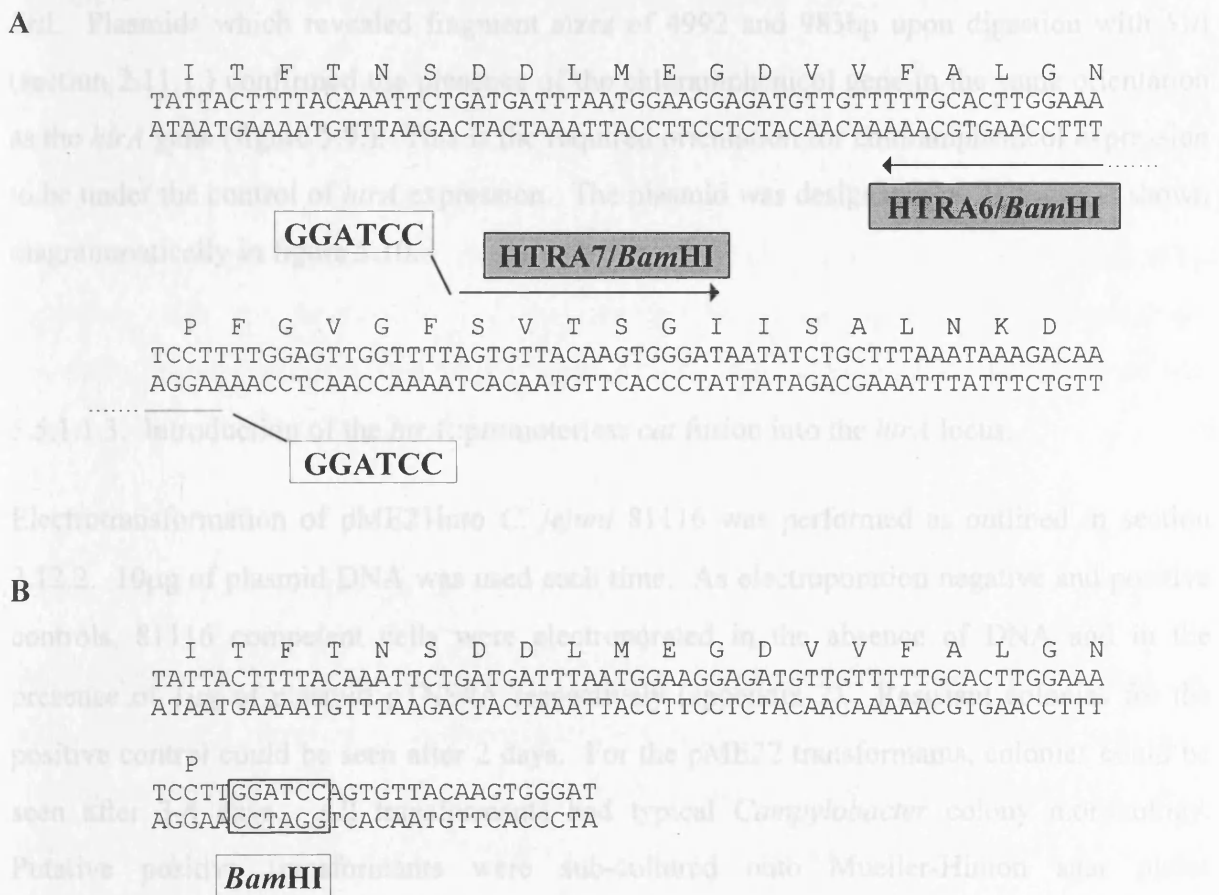


Figure 5.8: Mutagenesis strategy to create plasmid pME21. (A) IPCR on plasmid pME3 using primers HTRA6 and HTRA7. (B) Introduction of a 14bp deletion and an internal *Bam*HI. Primers HTRA6 and HTRA7 are shown in shaded boxes with arrows. Restriction sites are given in unshaded boxes.

precipitation, the ligation was transformed into *E. coli* DH5 α by electroporation (section 2.12.1.) and transformants were plated out accordingly using kanamycin as selection. Recombinant clones were checked for the insertion of the joint resistance cassette into the internal *Bam*HI site of pME21 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.2.) using primers HTRA17 and HTRA18 (appendix 1). A positive result yielded a PCR products of 3311bp in size compared to the positive control of pME21 which was ~1.11kb. Small-scale plasmid preparation (Qiagen) (section 2.6.2.) was performed on putative positives, and purified plasmid DNA was digested with *Sst*I. Plasmids which revealed fragment sizes of 4992 and 983bp upon digestion with *Sst*I (section 2.11.1.) confirmed the presence of the chloramphenicol gene in the same orientation as the *htrA* gene (figure 5.9.). This is the required orientation for chloramphenicol expression to be under the control of *htrA* expression. The plasmid was designated pME22 and is shown diagrammatically in figure 5.10.

5.5.1.1.3. Introduction of the *htrA*::promoterless *cat* fusion into the *htrA* locus.

Electrotransformation of pME21 into *C. jejuni* 81116 was performed as outlined in section 2.12.2. 10 μ g of plasmid DNA was used each time. As electroporation negative and positive controls, 81116 competent cells were electroporated in the absence of DNA and in the presence of 1 μ g of plasmid pTNS#A respectively (appendix 2). Resistant colonies for the positive control could be seen after 2 days. For the pME22 transformants, colonies could be seen after 3-4 days. All transformants had typical *Campylobacter* colony morphology. Putative positive transformants were sub-cultured onto Mueller-Hinton agar plates supplemented with 50 μ g/ml of kanamycin and incubated overnight at 37°C in a microaerophilic atmosphere. For a preliminary examination of the transformants to check for homologous recombination, PCR was performed on chromosomal DNA from 5 colonies from each of the transformations of pME22. The results were confirmed by Southern hybridisation analysis.

5.5.1.1.4: Analysis of the pME22 transformants.

5.5.1.1.4.1: PCR analysis

Transformants from the electroporation using pME22 were examined for the presence of allelic replacement in the *htrA* gene by PCR. Chromosomal DNA was extracted from the cells using the small-scale *Campylobacter* DNA extraction (section 2.7.1.). PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.1.) was performed on a 1:1000 dilution of chromosomal DNA using primers HTRA-MET and R4IU3 (appendix 1) which bind to sequence external to that from the pME22 insert DNA.

The PCR was used to check whether the chromosomal *htrA* gene had been disrupted. In the reaction, two controls were included; a negative control with no DNA template and a positive control of 81116 DNA. The PCR products were separated on a 1% agarose gel (section 2.8.). All 5 chromosomal preparations showed a band of approximately the right size (~3.9kb), consistent with the introduction of the promoterless chloramphenicol and kanamycin resistance cassette into the *htrA* gene (figure 5.11.). One of the pME22 transformants was further analysed by Southern hybridisation.

5.5.1.1.4.2: Southern hybridisation analysis

Southern blotting was required to confirm that the chloramphenicol gene fusion mutant had been constructed correctly. Chromosomal DNA from the recombinant strain, along with appropriate controls, was probed with either the *htrA* gene fragment obtained from PCR using primers HTRA-MET and R4IU3 (appendix 1), the chloramphenicol resistance gene from pAV35 (appendix 2), the kanamycin resistance gene from pJMK30 (appendix 2) or pUC19 digested with *Hind*III and *Eco*RI. Chromosomal DNA from the mutant and the parent strain, was prepared by the small-scale *Campylobacter* DNA preparation (section 2.7.1.) and analysed by agarose gel electrophoresis (section 2.8.). To perform each of the four hybridisation experiments, approximately 5µg of chromosomal DNA from each of the strains was restricted with *Bgl*II (section 2.11.1.). Different positive DNA controls were included, according to the hybridisation performed, and were the same as the DNA sample used as a probe. Samples were separated on a 1.0% agarose gel (section 2.8.), together with the controls. The Southern-hybridisation was performed as outlined in section 2.15.

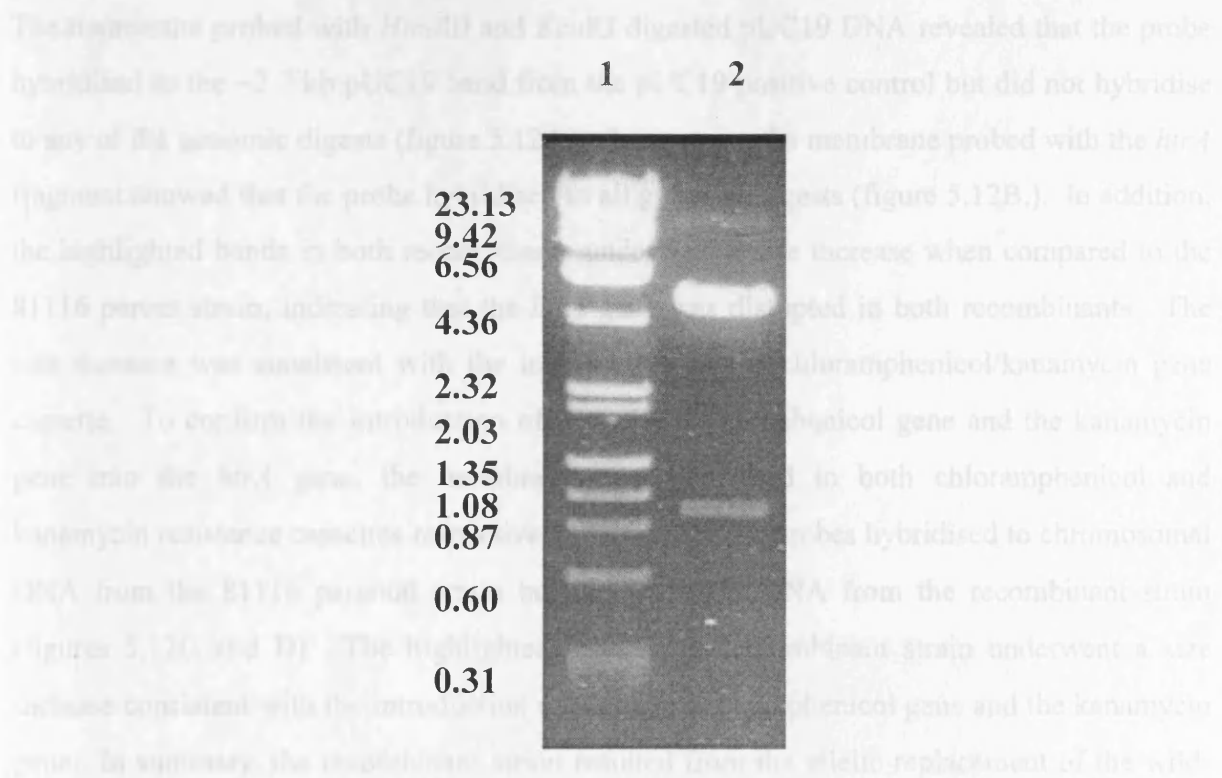


Figure 5.9: Agarose gel electrophoresis of a digest of pME22 with *Sst*I to show the correct orientation of the promoterless chloramphenicol and kanamycin gene cassette in relation to expression from the *htrA* gene. Lane 1; λ HindIII, ϕ xHaeIII markers (kilobase pairs), Lane 2; Plasmid pME22 digested with *Sst*I to reveal fragment sizes of 983 and 4992 bp.

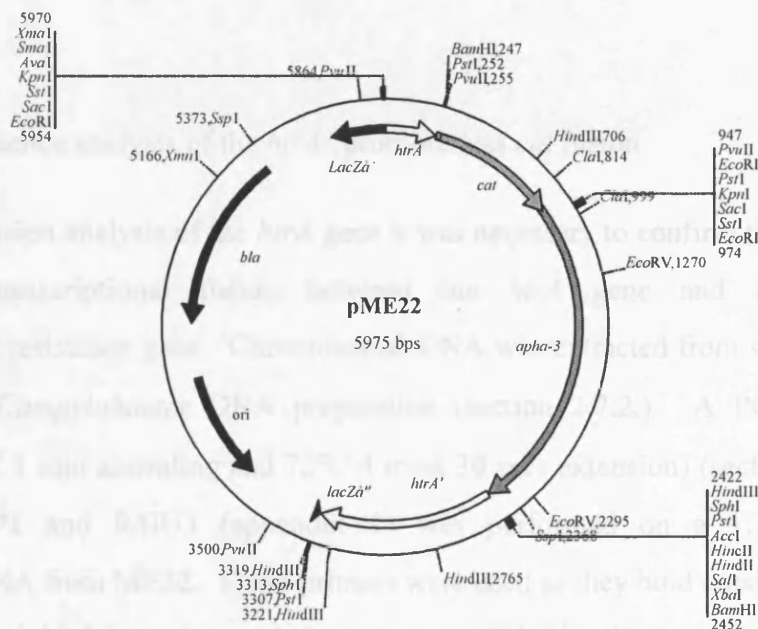


Figure 5.10: Diagrammatic outline of plasmid pME22 containing a *htrA::cat* transcriptional fusion. Plasmid pME22 was used to investigate expression of *htrA* as a function of chloramphenicol expression. The promoterless chloramphenicol gene (*cat*) is coupled with the kanamycin resistance gene (*apha-3*)(grey arrows). The disrupted, incomplete *htrA* open reading frame is highlighted (white arrows).

The membrane probed with *Hind*III and *Eco*RI digested pUC19 DNA revealed that the probe hybridised to the ~2.7 kb pUC19 band from the pUC19 positive control but did not hybridise to any of the genomic digests (figure 5.12A.). In contrast, the membrane probed with the *htrA* fragment showed that the probe hybridised to all genomic digests (figure 5.12B.). In addition, the highlighted bands in both recombinants underwent a size increase when compared to the 81116 parent strain, indicating that the *htrA* gene was disrupted in both recombinants. The size increase was consistent with the introduction of the chloramphenicol/kanamycin gene cassette. To confirm the introduction of both the chloramphenicol gene and the kanamycin gene into the *htrA* gene, the membrane was hybridised to both chloramphenicol and kanamycin resistance cassettes respectively. Neither of the probes hybridised to chromosomal DNA from the 81116 parental strain but did so to the DNA from the recombinant strain (figures 5.12C and D). The highlighted band in the recombinant strain underwent a size increase consistent with the introduction of both the chloramphenicol gene and the kanamycin gene. In summary, the recombinant strain resulted from the allelic replacement of the wild-type *htrA* gene with the insertionally mutated copy. The insertionally mutated copy should contain a transcriptional fusion between the *htrA* gene and the promoterless chloramphenicol gene, together with the kanamycin gene to provide selection. The mutant was named ME22 and the presence of the transcriptional fusion was confirmed at the chromosomal level.

5.5.1.1.4.3: Sequence analysis of the *htrA*::promoterless *cat* fusion

To enable expression analysis of the *htrA* gene it was necessary to confirm the presence of the chromosomal transcriptional fusion between the *htrA* gene and the promoterless chloramphenicol resistance gene. Chromosomal DNA was extracted from strain ME22 using the large-scale *Campylobacter* DNA preparation (section 2.7.2.). A PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins 30 secs extension) (section 2.13.1.) using primers HTRAP1 and R4IU3 (appendix 1) was performed on a 1:1000 dilution of chromosomal DNA from ME22. These primers were used as they bind outside the insert used to construct the initial mutation and furthermore, HTRAP1 binds upstream of the *htrA* promoter region. This facilitated the examination of the sequence from this region to ensure the *htrA* was transcribed appropriately. The presence of a single ~4.1 kb PCR product was confirmed by electrophoresis of small aliquot of the PCR product on a 1% agarose gel. The remainder of the DNA was purified using the Qiaquick PCR purification system (Qiagen)

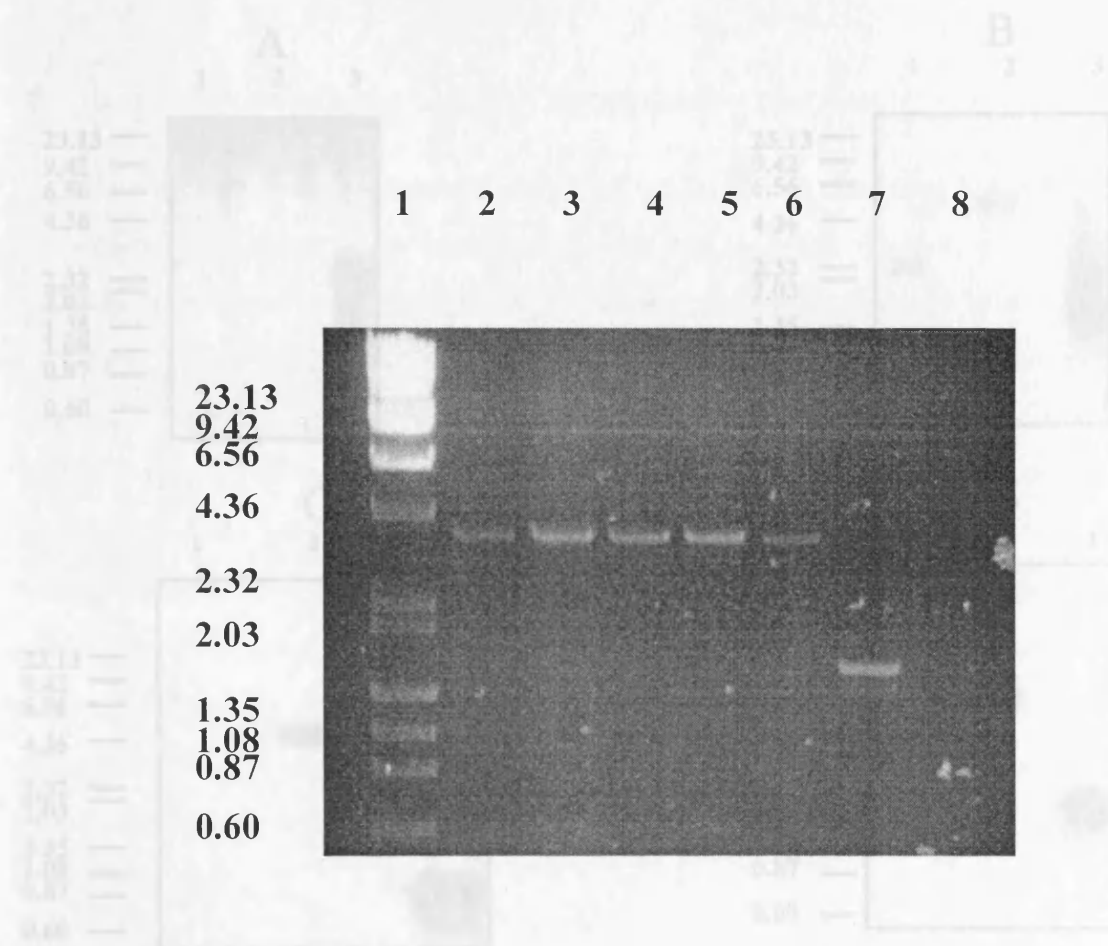


Figure 5.11: Results of PCR analysis on five putative *htrA*::promoterless *cat* fusion mutants. The PCR was performed with primers HTRA-MET and R4IU3 (appendix 1). Lane 1; λ *Hind*III, ϕ x*Hae*III markers (kilobase pairs). Lanes 2-6, five transformants from pME22 electroporation into 81116 showing recombinant band size of ~3.9 kb; Lane 7, 81116 positive control showing wild type band size of ~1.7kb; Lane 8, negative dH₂O control.

product was used as a probe. Left: λ *Hind*III/ ϕ x*Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, HTRA-MET X R4IU3 PCR product. (C) To show insertion of the chloramphenicol gene, *Bam*HI digested chloramphenicol resistance cassette from pAV35 was used as a probe. Left: λ *Hind*III/ ϕ x*Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, *Bam*HI digested chloramphenicol resistance cassette from pAV35. (D) To show insertion of the kanamycin gene, *Bam*HI digested kanamycin resistance cassette from pJMK30 was used as a probe. Left: λ *Hind*III/ ϕ x*Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, *Bam*HI digested kanamycin resistance cassette from pJMK30.

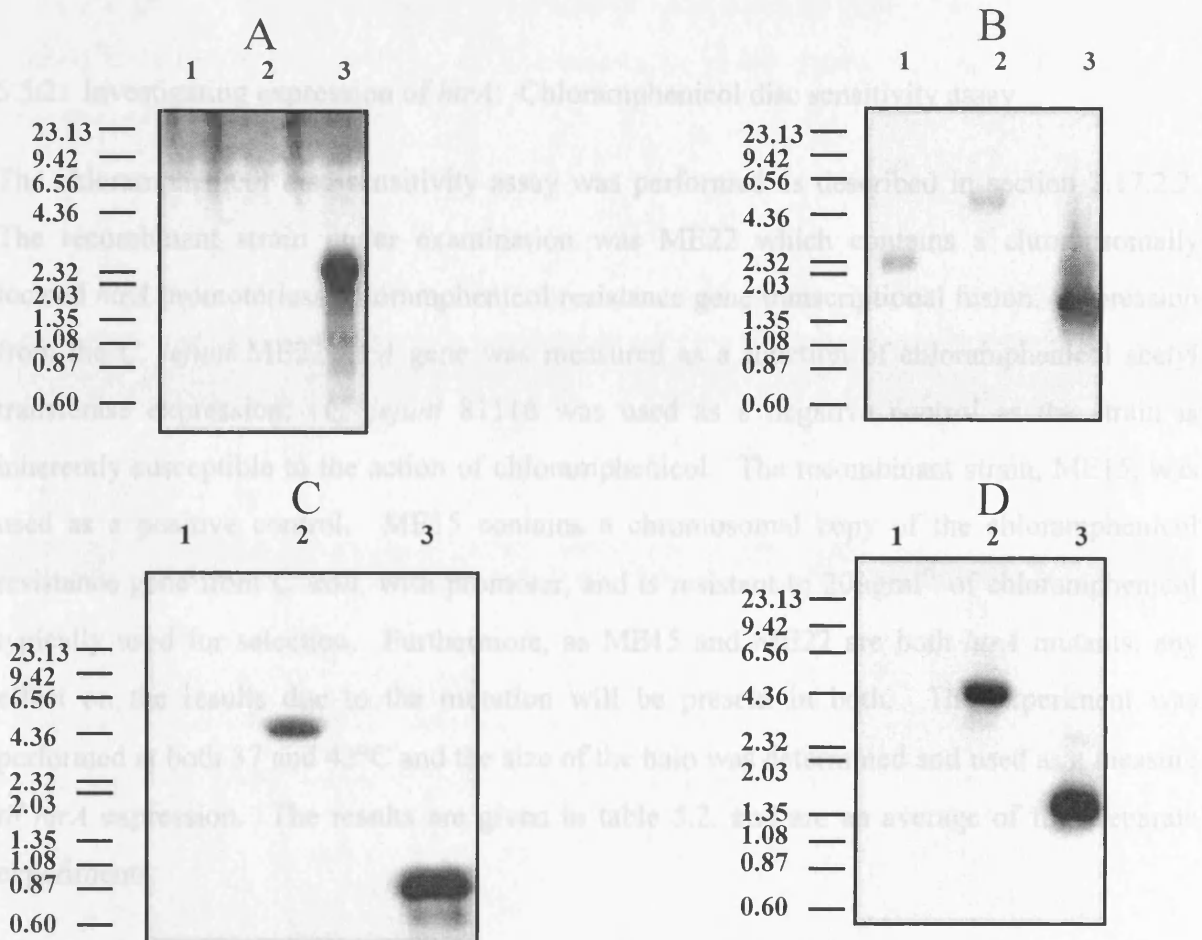


Figure 5.12: Southern hybridisation on ME22 to confirm allelic replacement on the chromosome. (A) To show absence of vector sequence *Hind*III and *Eco*RI digested pUC19 was used as a probe.; Left, λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, *Hind*III and *Eco*RI digested pUC19. (B) To show a change in *htrA* sequence, the HTRA-MET X R4IU3 PCR product was used as a probe. Left λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, HTRA-MET X R4IU3 PCR product. (C) To show insertion of the chloramphenicol gene, *Bam*HI digested chloramphenicol resistance cassette from pAV35 was used as a probe. Left, λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, *Bam*HI digested chloramphenicol resistance cassette from pAV35. (D) To show insertion of the kanamycin gene, *Bam*HI digested kanamycin resistance cassette from pJMK30 was used as a probe. Left, λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 1, *Bgl*II digested 81116 DNA; Lane 2, *Bgl*II digested ME22 DNA; Lane 3, *Bam*HI digested kanamycin resistance cassette from pJMK30.

(section 2.9.4.) and ethanol precipitated (section 2.9.3.). To verify that the transcriptional fusion had recombined appropriately on the chromosome, 100ng of the purified PCR product was sequenced as outlined in section 2.14. using primer CAT15'(appendix 1). The introduction of the correct transcriptional fusion was confirmed for the mutant.

5.5.2: Investigating expression of *htrA*: Chloramphenicol disc sensitivity assay

The chloramphenicol disc sensitivity assay was performed as described in section 2.17.2.2. The recombinant strain under examination was ME22 which contains a chromosomally located *htrA* promoterless chloramphenicol resistance gene transcriptional fusion. Expression from the *C. jejuni* ME22 *htrA* gene was measured as a function of chloramphenicol acetyl transferase expression. *C. jejuni* 81116 was used as a negative control as the strain is inherently susceptible to the action of chloramphenicol. The recombinant strain, ME15, was used as a positive control. ME15 contains a chromosomal copy of the chloramphenicol resistance gene from *C. coli*, with promoter, and is resistant to $20\mu\text{gml}^{-1}$ of chloramphenicol typically used for selection. Furthermore, as ME15 and ME22 are both *htrA* mutants, any effect on the results due to the mutation will be present in both. The experiment was performed at both 37 and 42°C and the size of the halo was determined and used as a measure of *htrA* expression. The results are given in table 5.2. and are an average of four separate experiments.

Table 5.2: Disc sensitivity assay: *htrA* expression as a function of *cat* expression (37 and 42°C) using pME22.

Sample name	Halo size (diameter, mm ^A)
ME15 37	11.3+/-0.6
ME15 42	11.7+/-0.6
81116 37	37.7+/-0.6
81116 42	36.0+/-0.0
ME22 37	17.3+/-1.2
ME22 42	17.0+/-1.0

^Asize of chloramphenicol disc = 8mm.

5.5.3: Investigating expression of *htrA*: Single colony growth

In an effort to quantify the amount of chloramphenicol expression from the *htrA* gene, ME22, together with the positive and negative controls, ME15 and 81116 respectively, were examined for growth of single colonies on increasing concentrations of chloramphenicol, 0, 2, 5, 10, 20 μgml^{-1} . The experiment was performed three times at both 37 and 42°C using the method described in section 2.17.2.1. The results are given in table 5.3. and are a typical of the trend seen on each occasion.

Table 5.3: Single colony growth on differing amounts of chloramphenicol at 37°C and 42°C.

Sample name	Concentration of chloramphenicol per plate ($\mu\text{g/ml}$)					
	0	2	5	10	15	20
ME15	+	+	+	+	+	+
81116	+	-	-	-	-	-
ME22	+	+	+	-	-	-
ME15	+	+	+	+	+	+
81116	+	-	-	-	-	-
ME22	+	+	+	-	-	-

+, represents growth of single colonies, -, represents lack of growth.

5.6: The *htrA*::LacZ reporter system

The LacZ reporter system utilises plasmid pMW10, a promoterless *lacZ* shuttle vector developed to allow screening of promoters by β -galactosidase activity in both *C. jejuni* 480 and *E.coli*. The plasmid replicates in both organisms and contains a promoterless *lacZ* gene 30bp downstream of a small multiple cloning site. Translational stop codons in all three reading frames are present between the multiple cloning site and the *lacZ* gene which is also preceded by a ribosome binding site (RBS) sequence. The advantage of this system is that it requires only simple genetic manipulation and has proved both simple, effective and very sensitive (Wösten *et al.*, 1998). As such it is a valuable alternative to the other systems described. With regards to expression from the *htrA* gene, the plasmid is in multicopy, albeit at a predicted value of 4-5 copies per cell, and increases the chances of detection. Furthermore, as the *htrA* promoter will be in multicopy as opposed to a single chromosomal

copy, it might titrate out any negative regulatory effect or enhance any weak positive regulation which the gene may be under.

To facilitate the use of the promoterless *lacZ* gene to investigate *htrA* expression (and *dnaJ*-like gene expression) it was first necessary clone the putative *htrA* promoter into plasmid pMW10 (in both orientations) and produce a transcriptional fusion between the promoterless *lacZ* gene and the *htrA* promoter in *E. coli*. The newly created shuttle plasmids could then be transformed into *E. coli* MC4100 and subsequently into *C. jejuni* 480 from where β -galactosidase activity can be used as a measure of promoter expression.

5.7: Results III

5.7.1: Investigating *htrA* promoter expression (and *dnaJ*-like gene) using the LacZ reporter system

5.7.1.1: Creation of plasmid constructs

5.7.1.1.1: Construction of pMW10HTRA and pMW10DNAJ

In order to monitor promoter expression from either *htrA* or the *dnaJ*-like gene it was first necessary to sub-clone the putative promoter insert from pME26 (chapter 4) into pMW10. Plasmid pMW10 was purified using large-scale plasmid preparation (Qiagen) (section 2.6.3.) and 2 μ g was digested overnight with *Bam*HI (section 2.11.1.) and phosphatase treated (section 2.11.2.). The plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear plasmid DNA was excised from the gel and purified using polyallomer wool gel extraction (section 2.9.1.) phenol/chloroform:chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3.).

Similarly, 2 μ g of plasmid pME26 (chapter 4) which contains the putative promoter region of *htrA* and the *dnaJ*-like gene was digested overnight with *Bam*HI (section 2.11.1.) to remove the insert. The digested plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 323bp insert was excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3.). Linear plasmid, pMW10, was ligated (section 2.11.3.) to the *Bam*HI digested insert and the ligation was transformed into *E. coli* MC4100 by electroporation (section 2.12.1.). Transformants were the plated out accordingly using kanamycin as selection. Use of

MC4100 means that there is no α -complementation to select for recombinant clones so colonies were checked for the insertion of the putative promoter region by colony PCR (section 2.13.2.). PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min extension) using a primer combination of HTRAP1 which binds 5' to the transcription of *htrA* and HTRAP2 which binds 5' to the transcription of the *dnaJ*-like gene checked for the presence of a single insert and a positive result yielded a product size of ~330bp. This was compared to the positive control of the same size using primers HTRAP1 and HTRAP2 on 1:1000 dilution of 81116 DNA. Again using colony PCR (section 2.13.2.) (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min extension), the orientation of the positives was confirmed by using a combination of HTRAP1 and LACR1 or HTRAP2 and LACR1 (appendix 1). LACR1 binds at the 5' end of the *lacZ* gene. A positive result yielded a PCR product of ~400bp in size. Small-scale plasmid preparation (Qiagen) was performed on putative positives (section 2.6.2.), and purified plasmid DNA was digested with *Bam*HI to reveal two fragment sizes of 323 and 10256bp indicating that the insert had been incorporated successfully (figure 5.13.) The two plasmids were designated pMW10HTRA and pMW10DNAJ depending on the orientation of the insert. PMW10HTRA has transcription of the *htrA* gene in the same direction as *lacZ* and pMW10DNAJ has transcription of *dnaJ* in the same orientation as *lacZ*. As both orientations of the putative intergenic region were obtained it was decided that each of the recombinant clones would be used for comparative analysis. The two plasmids are shown diagrammatically in figure 5.14.

5.7.1.1.2: Transformation of pMW10HTRA and pMW10DNAJ into *C. jejuni* 480

Electrotransformation of pMW10HTRA and pMW10DNAJ into *C. jejuni* 480 was performed as outlined in section 2.12.2. As electroporation negative and positive controls, 50 μ l of competent 480 cells were electroporated in the absence of DNA and in the presence of 1 μ g of plasmid p23E5 respectively. Plasmid p23E5 is a pMW10 derivative, containing a segment of the *metK* housekeeping gene from *C. jejuni* (Wösten *et al.*, 1998) (appendix 2). Resistant colonies for each transformation could be seen after 3 days. Putative positive transformants were sub-cultured onto Mueller-Hinton agar plates supplemented with 50 μ g/ml of kanamycin and incubated overnight at 37°C in a microaerophilic atmosphere. Individual colonies were

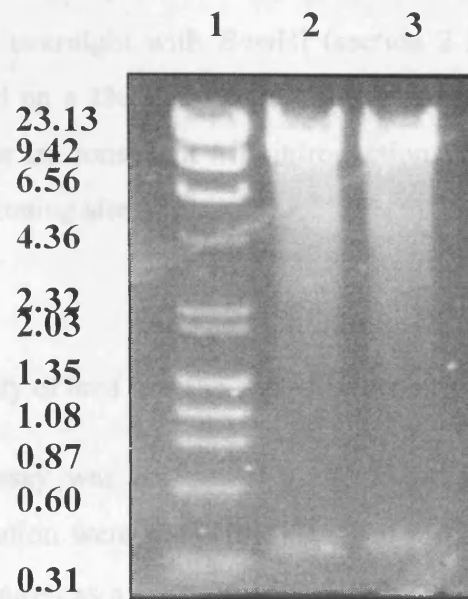


Figure 5.13: Agarose gel electrophoresis to confirm introduction of the putative promoter region of *htrA* and the *dnaJ*-like gene in pMW10. Lane 1, λ HindIII ϕ xHaeIII markers (kilobase pairs); Lane 2, pMW10HTRA digested with *Bam*HI to reveal fragment sizes of 10256 and 323; Lane 3, pMW10DNAJ digested with *Bam*HI to reveal fragment sizes of 10256 and 323.

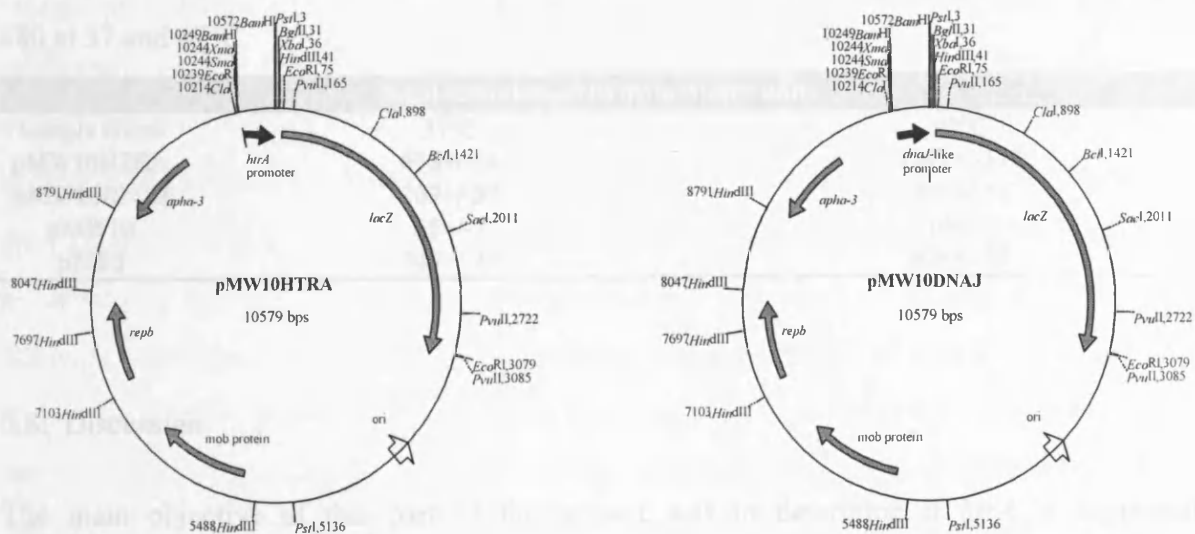


Figure 5.14: Diagrammatic representation of pMW10HTRA and pMW10DNAJ. The orientation of the *htrA* and *dnaJ*-like gene promoters respectively are shown by the black arrows.

grown overnight on Mueller-Hinton swab plates supplemented with 50 μgml^{-1} kanamycin and small-scale plasmid preparation was performed (Qiagen) (section 2.6.2.). 2 μg of transformant plasmid DNA was digested overnight with *Bam*HI (section 2.11.1.) and the digestion was subsequently electrophoresed on a 1% agarose gel to reveal fragment sizes of 10256 kb and 323 bp. These fragment sizes are consistent with introduction of the intergenic region into the *Bam*HI site of the multiple cloning site of pMW10.

5.7.2: β -galactosidase activity of *htrA* and the *dnaJ*-like gene.

The LacZ transcriptional assay was performed as described in section 2.17.3. The two recombinants under examination were pMW10HTRA and pMW10DNAJ. Expression from the two promoters was measured as a function of β -galactosidase activity. Plasmid pMW10 in *C. jejuni* 480 was used as a negative control as there is no endogenous β -galactosidase activity from the promoterless *lacZ* gene. Plasmid p23E5 which contains the promoter of the housekeeping gene *metK* was used as the positive control as it exhibits β -galactosidase activity in *C. jejuni* 480. The experiment was performed in triplicate at both 37 and 42°C. The results are given in table 5.4. and are typical of the trend seen on each occasion the experiment was performed.

Table 5.4: β -galactosidase activity of plasmids pMW10HTRA and pMW10DNAJ in *C. jejuni* 480 at 37 and 42°C.

β -galactosidase activity in Miller units		
Sample Name	37°C	42°C
pMW10HTRA	636 \pm 54	1591 \pm 128
pMW10DNAJ	269 \pm 27	520 \pm 51
pMW10	15 \pm 7	10 \pm 9
p23E5	249 \pm 29	624 \pm 50

5.8: Discussion

The main objective of this part of the project was to determine if *htrA* is expressed. Furthermore, development of a system whereby expression of *htrA* could be demonstrated would be useful to determine the type of regulation that the gene is under and be used to monitor *htrA* expression under different environmental conditions. Together, these data may provide valuable information about the phenotype of an *htrA* mutant. In pursuit of these aims

three methods were developed, i) an immuno-based translational HtrA::c-myc fusion, ii) a single copy chromosomal *htrA::cat* transcriptional fusion to measure *htrA* expression as a function of CAT expression and iii) a pMW10 plasmid based LacZ transcriptional assay system to measure *htrA* expression. Only the latter two techniques successfully demonstrated expression from the *htrA* gene.

Considering the *htrA::cat* transcriptional fusion initially, the promoterless chloramphenicol gene was successfully incorporated into the centre of the 81116 *htrA* gene to make the mutant ME22 and a transcriptional fusion between the two genes. The construct was subsequently used to investigate *htrA* gene expression as a function of chloramphenicol acetyl transferase production gene expression and data obtained from the chloramphenicol disc sensitivity assay successfully demonstrated expression of the *htrA* gene. This information, together with that obtained by examining growth of single colonies on varying amounts of chloramphenicol, has given an indication of the degree of expression, albeit only semi-quantitatively. Analysis of both sets of data indicates that the resistance of the mutant strain, ME22, to chloramphenicol is greater than that of the negative control 81116. The mutant strain, ME22 has an average halo size much less than that of 81116 which is sensitive to the action of chloramphenicol. This is also demonstrated by the experiment looking for growth of single colonies, in which 81116 was unable to grow on $2\mu\text{gml}^{-1}$ of chloramphenicol but, ME22, grows on chloramphenicol at a level between 5 and $10\mu\text{gml}^{-1}$. In contrast, ME22 is less resistant to the action of chloramphenicol than the positive control, ME15, whose expression from the chloramphenicol gene is under the control of its own promoter. The mutant strain, ME22 has an average halo size less than that of ME15, indicating that ME22 is more sensitive to chloramphenicol. It has already been highlighted that ME22 is able to withstand levels of chloramphenicol between 5 and $10\mu\text{gml}^{-1}$, ME15 on the other hand can survive levels of at least $20\mu\text{gml}^{-1}$ of chloramphenicol. Significantly, from these experiments there appears to be no difference between sensitivity to chloramphenicol comparing the results at 37 and 42°C although the experimental system lacks sensitivity and offers more of a qualitative or at most a semi-quantitative analysis. One objective was to correlate the sensitivity to chloramphenicol and level of expression of the promoterless chloramphenicol resistance gene to levels of *htrA* expression for ME22. Moreover, this could be compared to the positive, ME15 and negative 81116 controls which are either resistant or susceptible to the action of chloramphenicol respectively. From both data sets it appears that the *htrA* promoter is not as strong as the *C. coli* chloramphenicol resistance gene promoter and subsequently the gene is not as strongly

expressed under the experimental conditions used. The assay was sufficient to demonstrate that *htrA* is expressed under normal growth conditions. To enable a more qualitative approach, still using the chromosomal transcriptional fusion, there is now the possibility of using a chloramphenicol acetyl transferase (CAT) assay system based on liquid scintillation counting.

The second method to successfully demonstrate expression from *htrA* utilised the pMW10 plasmid based LacZ transcriptional assay system. The intergenic region between the *htrA* and *dnaJ*-like gene was cloned into plasmid pMW10 in both directions to give either plasmid pMW10HTRA or pMW10DNAJ. Plasmid pMW10HTRA has transcription from the putative *htrA* promoter in the same orientation as the promoterless *lacZ* gene. Plasmid pMW10DNAJ on the other hand, has transcription from putative *dnaJ*-like gene promoter in the same orientation as the promoterless *lacZ* gene. As described earlier, the parent plasmid, pMW10, provides a mechanism to investigate expression of already known or putative promoters by measuring the amount of β -galactosidase activity (Wösten *et al.*, 1998). The application of the plasmid to this situation was two fold. Primarily the construct was used to investigate expression of the *htrA* gene by measuring β -galactosidase activity from the transcriptional fusion between the putative *htrA* promoter and the promoterless *lacZ* gene. Furthermore, it was viewed as an alternative method to investigate expression of *htrA* with different qualities to those already described. Although not one of the primary aims, and in addition to investigating *htrA* expression, it was considered that the opposite orientation concerning expression from the putative *dnaJ*-like promoter also be included in the assay for a comparative analysis. Firstly, the activity of β -galactosidase from both pMW10HTRA and pMW10DNAJ in *C. jejuni* 480 was compared to that of the parent, negative control, plasmid pMW10, and plasmid p23E5 used as a positive control. Firstly, analysis of the experimental data indicates that both the positive and negative controls have worked effectively; the level of β -galactosidase activity for plasmid pMW10 is minimal for both temperatures and the activity from plasmid p23E5 is comparable to that given by Wösten, (1998). Plasmid p23E5 also has a documented β -galactosidase activity which is higher at 42° than at 37°C. This is also apparent from these data in which there is approximately a 2.5 fold increase from 37 to 42°C. Analysis of β -galactosidase activity from pMW10HTRA shows that expression of the *htrA* promoter and thus *htrA*, has once again, been successfully established. Interestingly, there is an approximate 2.5 fold increased level of expression at 42°C compared to 37°C. The significance of this is unclear but as there is a similar increase for plasmid p23E5, it may only

represent an increase in metabolic rate of the bacterium which is enhanced at the higher temperature. This assumes that *metK* is not temperature regulated. An alternative suggestion may be that the increase in temperature causes an increase in pMW10 copy number and hence an increase in β -galactosidase activity. Plasmid pMW10DNAJ also demonstrates β -galactosidase activity at both 37 and 42°C and once again the level of transcription at the higher temperature is approximately two fold that of the lower temperature. This gives further evidence to support the fact that the level of transcription of *htrA*, and by extension, the level of transcription of the *dnaJ*-like gene is possibly greater at 42°C because of an increased metabolic rate at this temperature compared to 37°C. Had growth at 42°C instead of 37°C been a stimulus for increased *htrA* expression, and not a result of increased metabolic activity, it is likely that there would have been a significant increase in β -galactosidase activity. This point is drawn from the fact that, two iron regulated promoters of *katA* and *AhpC* show a significant increase in expression of β -galactosidase activity when grown under iron limiting conditions (van Vliet, *pers. comm.*).

Despite successfully demonstrating *htrA* expression using the chromosomal *htrA::cat* transcriptional fusion and the pMW10 plasmid based transcriptional assay, the immunological based translational fusion between the truncated HtrA protein and the human c-myc epitope failed to demonstrate expression. Both *C. jejuni* mutants, ME15 and ME16 were made successfully and given that *htrA* is expressed, albeit at a low level, under normal growth conditions the reasons behind this point are unclear. Without the other data, the failure to express HtrA and therefore detect the HtrA::c-myc truncated product could be attributed to a lack of an appropriate stimulus, a negative regulatory effect or a failure to activate a positive regulatory effect. Given that *htrA* is expressed under normal growth conditions, this is unlikely to be the case unless the level of expression of the fusion may be below the sensitivity threshold of the assay. Therefore, under different experimental conditions there may be increased expression of the HtrA::c-myc truncated product which is subsequently detected. To enable detection of c-myc, a significant percentage of HtrA protein expression compared to total protein may be required, but may not occur in the mutants ME15 and ME16. Perhaps, analysis of cells fractionated into their respective compartments would increase the ratio of truncated HtrA::c-myc to total protein and subsequently increase the chance of detection. There is evidence that the level of detection of lowly expressed c-myc tagged genes may not be sufficiently sensitive (Nakajima and Yaoita, 1997). This paper describes improved sensitivity of detection in a Western blotting procedure by genetically

manipulating the gene of interest to incorporate multiple copies of c-myc. Although the ELISA and Western blotting techniques are not directly comparable, the principle that HtrA::c-myc is not detected because of low expression and sensitivity could apply to both systems. Nevertheless, under the provision that there is expression of the truncated protein, there is no guarantee that the c-myc epitope is in a detectable form. Not having knowledge of how the truncated molecule folds, there is a possibility that the epitope resides deeply within its tertiary structure and as a result, may not be presented appropriately. One option might be to vary the position of the c-myc epitope within the HtrA molecule, possibly at the C-terminus. Again, it is unclear whether the epitope within the folded protein will be present in a detectable form. The original site for the epitope was chosen because it was known that homologous recombination could occur at this position, as in the original isogenic *htrA* mutants (Henderson, 1996). Furthermore, some primers were already available to incorporate the mutation at this site. To incorporate the mutation in a different position may not facilitate homologous recombination and therefore introduction of the epitope. There is a precedent for locating the epitope at one of the protein termini (C-terminus in this instance) as the protein is more likely to fold in a native form. In this experiment, the truncated protein may not fold properly and be recognised as foreign by the cell. Therefore, consistent with any other such molecule, the truncated product, may fall victim to the cell's response to aberrant or misfolded proteins. Following translation of *htrA*, activity by a combination of chaperones and proteases may function to remove the truncated molecule resulting in a failure to detect the molecule in the ELISA based assay. Unfortunately the failure to demonstrate expression using this system limits the possibility of performing any protein analysis of HtrA.

5.9: Future work

In this project, two different methods have been used successfully to demonstrate expression of *htrA*. Consequently, there is now a potential to develop these methods further in order to investigate how the *htrA* gene is actually regulated. A simple investigation into *htrA* expression and regulation could be obtained by subjecting either ME22 or pMW10HTRA in *C. jejuni* 480 to various physical stimuli and observing the response. Such stimuli could include temperature, inorganic salt, bile salts, pH, iron, oxidative stress and air. At a more complex level, the genetics behind regulation of the *htrA* gene could be investigated. Primarily, this would involve mutation of other genes in an effort to show either an increase or a decrease in *htrA* expression from the specific reporter, or alternatively, a failure to respond

to a specific physical stimulus identified. Genetic investigation of other bacteria is commonplace and largely, technically simple. For example, classical genetic techniques such as those involving the use of transposons allow random mutagenesis of the chromosome. In campylobacters many classical genetic approaches are not technically feasible, and even techniques which are available differ greatly in their usefulness depending on the strain used. To a degree, this is true of strain 480. Strain 480 is used with plasmid pMW10 because this strain accepts shuttle vectors isolated from *E. coli* whereas *C. jejuni* 81116 does not (Wassenaar *et al.*, 1993). Unfortunately, *C. jejuni* strain 480, unlike *C. jejuni* 81116, accepts plasmid DNA via electroporation, and chromosomal DNA via natural transformation, only very poorly (Wassenaar *et al.*, 1993). Furthermore, in trying to make a mutant the strain may prove more problematic as it only undergoes single crossover events as opposed to the double crossover events seen with strains like 81116. Despite these drawbacks the possibility of investigating the regulation of *htrA* by using a genetic approach still remains.

In a blanket approach, using a “semi-random” mutagenesis technique chromosomal DNA can be digested with different restriction enzymes and subsequently religated with an antibiotic cassette to create genomic loops. Given the low transformation efficiency of *C. jejuni* 480, this technique could be best applied to ME22. The mutated genomic loops could be electroporated or naturally transformed into ME22 and in a large screening program, a change in CAT expression compared to the parent ME22 could be investigated. The recombinants must be selected initially on the antibiotic used to create the chromosomal loop mutation, for example tetracycline, and subsequently grown on permissive and non-permissive amounts of chloramphenicol with respect to growth of the mutant ME22. This will enable a comparison of *htrA* expression to be made. To analyse pMW10HTRA in *C. jejuni* strain 480, and also ME22, chemical mutagenesis of each of the parents, 480 and 81116 can be performed. Upon introduction of pMW10HTRA into 480 and the *htrA::cat* mutation into 81116 to create ME22, the effect of the chemical mutagenesis on *htrA* expression can be observed. ME22 recombinants can be selected on kanamycin and observed on chloramphenicol as above. For mutants in *C. jejuni* 480 with pMW10HTRA introduced a change in β -galactosidase activity compared with a non-mutagenised control can be monitored to investigate *htrA* expression. For the chemically mutagenised *C. jejuni* 480 with pMW10HTRA there is the possibility of trying to restore wild type levels of *htrA* expression with complementation of 480 chromosomal DNA from a shuttle vector based library.

A further approach which can be applied to both reporter examples takes advantage of newly identified sequence from the *Campylobacter jejuni* genome sequencing project. On a global scale there is now potential to mutate every single open reading frame within the genome. Individual mutations can be incorporated into the reporter strains to detect any change in *htrA* expression or a failure to respond to a specific stimulus. This could prove a massive undertaking and would be a time consuming exercise. Instead, a *Campylobacter* variation on the recent advent of signature tagged mutagenesis might prove more fruitful. An alternative is to adopt a more direct approach. It is widely known that bacteria use a variety of different regulators to control expression of different genes. To this end, it is believed that campylobacters are no different. Therefore to investigate regulation of *htrA* by mutation of various regulators might prove a beneficial exercise. The knowledge that *htrA* is regulated by the two-component regulatory system, *cpxRA* in *E. coli* provides a sound basis for which to begin investigating regulation of *htrA* in *C. jejuni*.

Chapter 6: Two-component regulation

6.1: Introduction

6.2: Environmentally-directed control of gene expression: bacterial regulatory networks

Bacterial survival in a variety of changing ecological niches and micro-environments requires a continuous monitoring of external conditions and the ability to use this information to develop an adaptive response. The bacterium is able to cope with such changing conditions by adjusting its structure, physiology and behaviour, firstly, by sensing a stimulus and then transducing these data to the intracellular apparatus concerned with that particular response. Having the ability to perform such signal transduction places immense demands upon the organism which typically, only has a few thousand genes. Therefore, the only way in which it can control this process is to have a highly co-ordinated regulation of gene expression. Modulation of gene transcription and translation to effect the appropriate response is tightly controlled to guarantee fitness at any stage during its life-cycle.

Pathogenic bacteria, including *C. jejuni* are no exception to the call for a highly adaptive response because they require a strategy for survival that requires multiplication on or within another living organism. Whilst endeavouring to gain a hold within its host the bacterium must employ a variety of mechanisms to establish a suitable niche, to undergo nutrient acquisition and avoid a variety of immune defense mechanisms. In doing so, the dynamic process which the bacterium undertakes means that it will encounter a variety of different environmental conditions such as changes in temperature, osmotic pressure, oxygen status, pH, nutrient availability and iron stress. These conditions are effected as the organism undergoes competitive growth and survival in different locations within the host and the transition to and from an external reservoir (DiRita and Mekalanos, 1989; Miller *et al.*, 1989; Dorman, 1995).

It is clear that when a bacterium adapts to its changing surroundings there is also likely to be a frequent change in the transcriptional profile of the genome. Environmental signals alter gene expression to bring about the necessary cellular adjustments. To this end, some bacteria have evolved a number of precise and finely-tuned regulatory mechanisms that respond to particular stimuli. Examples of these systems include, the AraC family and the LysR family of transcriptional regulators, the Lrp (*leucine responsive regulatory protein*) transcriptional regulator; the Fur (*ferric uptake regulation*) transcriptional regulator and the family of two-component regulators which are considered in more detail below. The AraC family of

transcriptional regulators were initially named after the *E. coli araC* product that regulates transcription of the *araBAD* operon as members of this family share conserved amino acids sequence at the C-terminus. The N-terminus has a carbohydrate-binding domain and the C-terminus has a DNA binding domain with a helix-turn-helix motif. Members of this family are grouped into two classes according to their function; one class is involved in regulating genes involved in carbohydrate metabolism (e.g., rhamnose or cellobiose) and the other class contains regulators of virulence genes. AraC-like proteins bind to specific DNA sequences upstream of genes under their regulatory control (Dorman, 1994a; Finlay and Falkow, 1997). The LysR family of transcriptional regulators have N-terminal amino acid sequence similar to the regulator of the lysine biosynthesis operon. Members of this family contain an N-terminal helix-turn-helix, DNA binding motif which activates transcription of genes downstream. (Dorman, 1994a; Finlay and Falkow, 1997). LysR-like proteins have been implicated in the regulation of amino-acid biosynthesis in *E. coli* (Henikoff *et al.*, 1988), antibiotic resistance in *Citrobacter freundii* (AmpR) (Lindquist *et al.*, 1989) oxidative stress (Dorman, 1994a), iron regulated virulence gene expression in *V. cholerae* (IrgB) (Goldberg *et al.*, 1991) and plasmid encoded virulence gene expression in *S. typhimurium* (SpvR) (Caldwell and Gulig, 1991; Dorman, 1994a). Lrp is a transcriptional regulator which associates with leucine. Together they form a complex which in response to the presence of leucine, regulates either by activation or repression, genes involved in the biosynthesis of some amino acids such as isoleucine, leucine, valine or serine, and the synthesis of porins OmpF and OmpC (Caldwell and Gulig, 1991; Dorman, 1994a). Fur is a transcriptional regulator (DNA-binding) which binds to specific genes (those which have a Fur box in their promoter) only in the presence of iron to repress transcription of downstream genes (Litwin and Calderwood, 1993). In the absence of iron, gene transcription becomes activated as Fur is unable to bind. Fur also regulates genes not directly involved in iron uptake such as the expression of exotoxin A, in *Pseudomonas aeruginosa* and the Shiga-like toxin type I in *E. coli* (Mekalanos, 1992; Gross, 1993) both of which are bacterial virulence determinants.

It is noteworthy that the above examples are of members of bacterial regulatory networks which control expression of specific operons or regulons. Given the low number of genes and the overall low level of complexity of a bacterium it is not surprising that these regulators do not function in isolation but form part of a highly integrated hierarchical organisation in which the proteins are ranked according to their pleiotropy. Regulatory proteins such as AraC, LysR, Lrp and Fur are at the bottom end of the hierarchy scale and are influenced by other, higher ranked regulators called global regulators which regulate transcription from a large set of distinct

operons or regulons (Dorman, 1995). One example is Crp (cyclic AMP receptor protein) which in the presence of the cofactor, cyclic AMP (cAMP), interacts with DNA using its C-terminus to effect activation of genes under its control. Specifically, Crp can antagonise the repressor of the lactose operon, or alternatively, activate expression of the regulator of the maltose operon. Furthermore, cAMP and catabolite repression can regulate certain virulence factors, for example in *S. typhimurium* (Dorman, 1994a; Finlay and Falkow, 1997). As the hierarchical scale ascends, the complexity of interconnections increases such that even global regulators are out-ranked by “universal regulators”. Some universal regulators, for example, HNS, have the capacity to direct gene expression in response to alterations in DNA architecture and not by interacting with specific DNA sequences (Dorman, 1995). Usually, prokaryotic DNA is maintained in a negatively supercoiled form by topoisomerases, DNA gyrase and histone like DNA binding proteins (Finlay and Falkow, 1997). The level of DNA supercoiling is affected by several environmental signals such as temperature and osmolarity which in turn can affect the transcriptional activity of several promoters. Changes in DNA supercoiling affect expression of a number of virulence associated genes. For example, genes involved in invasion in *S. flexneri*, *S. typhimurium* and enteroinvasive *E. coli* (Finlay and Falkow, 1997).

Previously highlighted is the fact that regulation of transcription in response to various environmental parameters can be highly specific and of an ordered, hierarchical nature. The response to certain environmental signals can have additional direction provided by the interaction at specific promoter sites between RNA polymerase and specific σ factors. This level of promoter specificity is achieved as bacteria have evolved a set of alternative sigma factors which reprogram the RNA polymerase to interact with specific promoters. A few examples include σ^{54} (RpoN) dependent promoters which are involved in transcription of genes in response to nitrogen limitation (Sasse-Dwight and Gralla, 1990) σ^{28} dependent promoters which are involved in transcription of genes involved in chemotaxis, motility and flagellar expression (Helmann, 1991; Mirel and Chamberlin, 1989) σ^{32} (RpoH) dependent promoters which leads to transcription of genes in response to heat shock (Erickson, 1987; Nagai *et al.*, 1991) and σ^{38} (RpoS) dependent promoters which regulate several genes which operate during stationary phase and in response to nutrient limitation (Dorman, 1994b; O’Neal *et al.*, 1994). Several bacterial pathogens also use alternate sigma-factors to regulate transcription of virulence genes (Finlay and Falkow, 1997).

It is not within the context of this discussion to examine bacterial regulatory networks in great depth but it is already clear that regulation of gene expression in response to various

environmental parameters is complex and exists in a hierarchical organisation. Two component regulatory systems (TCR systems) are also deeply embedded within the hierarchical structure of bacterial signal transduction and transcriptional regulation and play an integral part in maintaining efficient functioning of the cell. Indeed, for a pathogen they can also be important in the regulating virulence gene expression. TCR systems will now be considered in more detail.

6.3: Two-component regulatory systems

The ability of a bacterium to couple appropriate responses to changing environmental parameters provides a selective advantage. The benefit is conferred on the bacterium by the numerous signal transduction systems which have evolved to permit this type of stimulus-response coupling. Examples of the types of two-component systems can be seen in table 6.1. TCR systems are ubiquitous in nature and at present, around 300 have been identified in approximately 100 different bacterial genera (Goudreau and Stock, 1998). In its simplest form, a bacterial two-component system can be envisaged as being composed of a transmembrane receiver which processes an environmental signal. The signal is subsequently transduced to a target effector protein by a series of phosphotransfer reactions to bring about the necessary response (figure 6.1).

Stimulus-response systems can be described as being modular with specific domains. Once the stimulus is detected, the individual domains become integrated into a network of different pathways whose function varies from system to system. Each two-component regulatory system responds to a highly specific signal but the overall signalling strategy appears conserved (Goudreau and Stock, 1998).

The simplest form of a two-component system consists of two proteins, a histidine protein kinase (HPK-transmembrane receiver) and a response regulator (RR-the effector protein). Both components contain two functional domains which are connected via a linker of variable length and sequence. The specific functional domains have been assigned different nomenclatures. According to that derived by Parkinson and Kofoed, (1992) the HPK contains i) a periplasmic domain located at the *N*-terminus which is responsible for monitoring specific environmental parameters and is referred to as the input domain. ii) a *C*-terminal cytoplasmic domain which transmits the signal to the cognate RR, referred to as the transmitter domain.

Table 6.1: Examples of two-component signalling systems (adapted from Parkinson and Kofoed, (1992)).

Adaptive response	Two-component systems		Organism
	HPK	RR	
Chemotaxis	CheA	CheY, CheB	Bs, Ec, Pa, St
Carbon utilisation	DctB	DctD	Rl, Rm
	PgtB	PgtA	St
	UhpB	UhpA	Ec, St
Nitrogen utilisation	NtrB	NtrC	At, Ec, St, Kp
Phosphate utilisation	PhoR	PhoB	Bs, Ec, Kp. Pa, Sd
	CreC	CreB	Ec
Nitrogen fixation	FixL	FixJ	Rm
Photosynthesis	RegB	RegA	Rc
Redox changes	HydH	HydG	Ec, St
	NarQ	NarX	Ec
	ArcB	ArcA	Ec
Antibiotic stress	RteA	RteB	Bt
Heavy metal stress	CutS	CutR	Sl
Osmolarity stress	EnvZ	OmpR	Ec, St
Starvation stress	AgrB	AgrA	Sa
	DegS	DegU	Bs
Turgor pressure stress	KdpD	KdpE	Ec
Antibiotic synthesis	VanS	VanR	Ef
Capsule synthesis	AlgR2	AlgR1, AlgB	Pa
	RcsC	RcsB	Ec
Fruiting body formation	FrzZ	FrzZ	Mx
Gene transfer	ComP	ComA	Bs
Sporulation	KinA, KinB, KinC	SpoOA, SpoOF	Bs
Virulence	PhoQ	PhoP	St
	Xcc2	Xcc1	Xc
	BvgS	BvgA	Bp
	LemA	not identified	Ps
	VirA	VirG	Atu

Table 6.1: Organism abbreviations: At, *Agrobacterium tumefaciens*; Bp, *Bordetella pertussis*; Bs, *Bacillus subtilis*; Bt, *Bacteroides, thetaiotamicon*; Ec, *Escherichia coli*; Ef, *Enterococcus faecium*; Kp, *Klebsiella pneumoniae*; Mx, *Myxococcus xanthus*; Pa, *Pseudomonas aeruginosa*; Ps, *Pseudomonas syringae* pv. *syringae*; Rc, *Rhodobacter capsulata*; Rm, *Rhizobium meliloti*; Sa, *Staphylococcus aureus*; Sd, *Shigella dysenteriae*; Sl, *Streptomyces lividans*; St, *S. typhimurium*; Xc, *Xanthomonas campestris* pv. *campestris*.

The RR contains i) an N-terminal domain which receives the signal from the HPK's transmitter domain and is therefore referred to as the receiver and ii) a C-terminal domain which elicits the adaptive response and known as the output domain. The transmitter domain of HPKs and receiver domain RRs contain characteristic conserved amino acids. Particularly these conserved amino acids are important for their function. The input domain of HPKs and the output domain of RRs are more variable and relate specifically to the type of signal monitored and the type of response given. Indeed, the sequence of output domains is used to group RRs into sub-families. The interaction in bacteria is initiated by a stimulus that can be summarised as follows. A specific signal is detected by the sensing apparatus (the

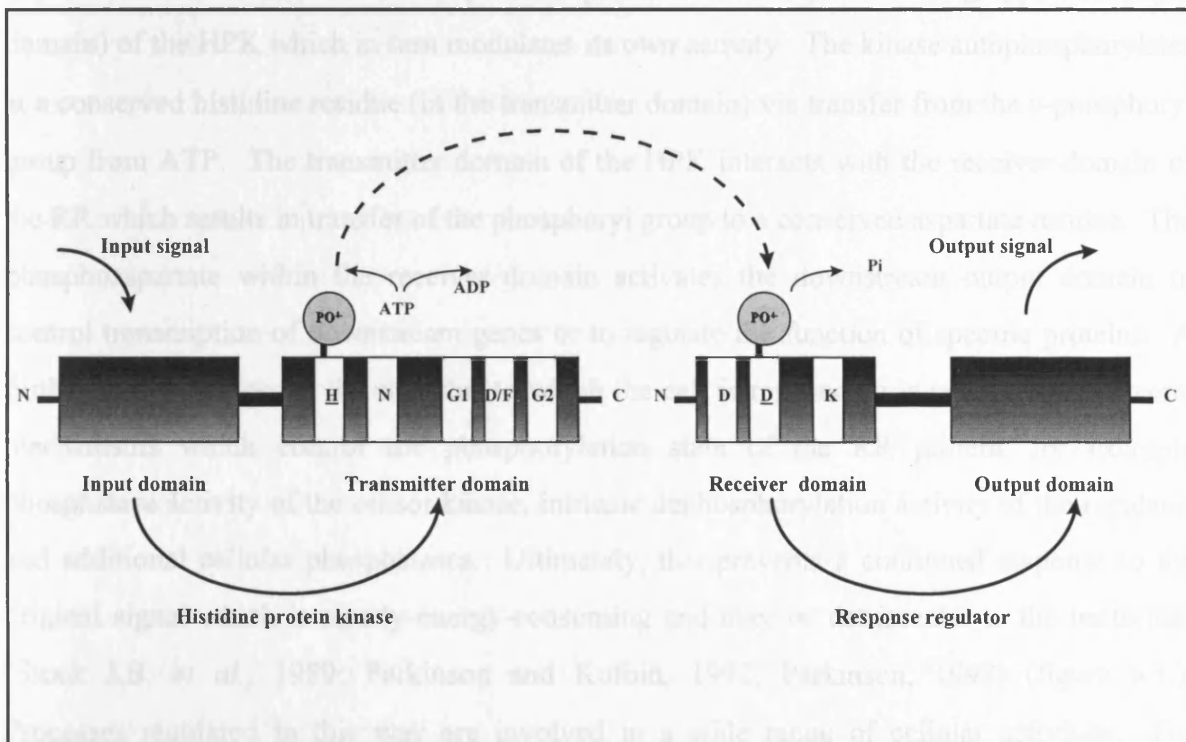


Figure 6.1: General scheme for signal transduction in two-component regulatory systems. A specific environmental stimulus is detected by the histidine protein kinase input domain and the signal is communicated to the transmitter domain of the histidine protein kinase which subsequently undergoes autophosphorylation at the conserved histidine residue (**H**) to produce a high energy phosphohistidine intermediate. The transmitter transfers this information to the cognate response regulator receiver domain by a phosphotransfer reaction to a conserved aspartate residue (**D**). The response regulator is activated and the response regulator's output domain triggers the cellular response. Information transfer is signified by arrows, including phosphotransfer denoted by the dotted arrow. The conserved motifs in the transmitter H, N, D/F, G1 and G2 and in the receiver D, D, K play an integral part in the communication process and are explained later (adapted from Parkinson and Kofoid, (1992)).

The RR contains i) an *N*-terminal domain which receives the signal from the HPK's transmitter domain and is therefore referred to as the receiver and ii) a *C*-terminal domain which elicits the adaptive response and known as the output domain. The transmitter domain of HPKs and receiver domain RRs contain characteristic conserved amino acids. Furthermore these conserved amino acids are important for their function. The input domain of HPKs and the output domains of RRs are more variable and relate specifically to the type of signal monitored and the type of response given. Indeed, the sequence of output domains is used to group RRs into sub-families. The interaction of different domains to bring about a response can be summarised as follows. A specific signal is detected by the sensing apparatus (input domain) of the HPK which in turn modulates its own activity. The kinase autophosphorylates at a conserved histidine residue (in the transmitter domain) via transfer from the γ -phosphoryl group from ATP. The transmitter domain of the HPK interacts with the receiver domain of the RR which results in transfer of the phosphoryl group to a conserved aspartate residue. The phosphoaspartate within the receiver domain activates the downstream output domain to control transcription of downstream genes or to regulate the function of specific proteins. A further change or loss in the stimulus to which the cell is responding is processed by several mechanisms which control the phosphorylation state of the RR protein, for example phosphatase activity of the sensor kinase, intrinsic dephosphorylation activity of the regulator and additional cellular phosphatases. Ultimately, this prevents a continued response to the original signal which is clearly energy-consuming and may be detrimental to the bacterium (Stock J.B. *et al.*, 1989; Parkinson and Kofoid, 1992; Parkinson, 1993) (figure 6.1.). Processes regulated in this way are involved in a wide range of cellular activities. For example, in sporulation, transformation competence, pathogenicity, virulence, chemotaxis, membrane transport and metabolism (Stock J.B. *et al.*, 1990) (table 6.1.).

6.3.1: Histidine protein kinases

A large number of histidine protein kinases have been identified although knowledge about their structure-function relationships is limited. One of the best characterised kinases is CheA which is involved in chemotaxis (Zhou *et al.*, 1996; Zhou and Dahlquist, 1997). The domain structure of CheA is somewhat atypical and varies from most other kinases. Nevertheless, NMR, genetic and biochemical analysis of the protein has given an insight into how members of the family of histidine protein kinases function. HPKs generally function as dimers and the

control of dimerisation may play a role in regulation of some kinases, especially CheA. All of the determinants for dimerisation of CheA are in the catalytic domain which contains the ATP binding site. Histidine protein kinases also fall in two groups with respect to their kinase activity i.e., the kinase reaction is readily reversible or the molecule can act as a phosphatase. CheA falls into the former group.

The structural organisation of most histidine protein kinases (figure 6.2.) can be envisaged as an *N*-terminal sensory input domain and the *C*-terminal transmitter domain separated by a linker region. The *N*-terminus is extremely variable and imparts a recognition specificity to reflect the wide range of inputs which the kinases sense. Most HPKs are anchored in the cell membrane with variable numbers of hydrophobic transmembrane segments. Most commonly there are two, designated TM1 and TM2 (Parkinson and Kofoed, 1992). The configuration of transmembrane segments within a molecule directs the sensory domain to the external surface of the membrane, within the periplasm. Their role as sensors of periplasmic components is thus highlighted although most regulatory ligands have not been identified. The simplest linear organisation for an HPK can be described as TM1-P-TM2-L-C where TM1 and 2 represent transmembrane helices, P represents the periplasm, L represents the cytoplasmic linker and C represents the cytoplasm (Goudreau and Stock, 1998). A few HPKs do not contain membrane spanning components and are cytoplasmic. These molecules rely on accessory factors to “sense” the environment, for example, NtrB of *E. coli* which regulates glutamine synthetase expression in response to available nitrogen by regulating the phosphorylation state of NtrC (Keener and Kustu, 1988; Ninfa *et al.*, 1993) and CheA (Lukat and Stock, 1993). The *C*-terminal cytoplasmic domain (the transmitter domain) consists of approximately two domains of equal size and usually contains about ~240 residues (Stock J.B. *et al.*, 1989; 1995; Parkinson and Kofoed, 1992) have termed these domains T_L and T_R (Parkinson and Kofoed, 1992).

The overall sequence identity between HPKs over the transmitter region is ~25%. Within the primary sequence of the *C*-terminus there are a number of subdomains of highly conserved amino acids, that are given “box” or “block” names, i.e., the H (histidine), N (asparagine), D/F (aspartate/phenylalanine), G1 and G2 (glycine rich) boxes respectively. These regions are so-called due to the relative occurrence of each amino acid therein, across the family of HPKs (figure 6.3). The *C*-terminus most likely contains all of the catalytic functions of the molecule. The most variable of these five regions is the H-box which is located at the

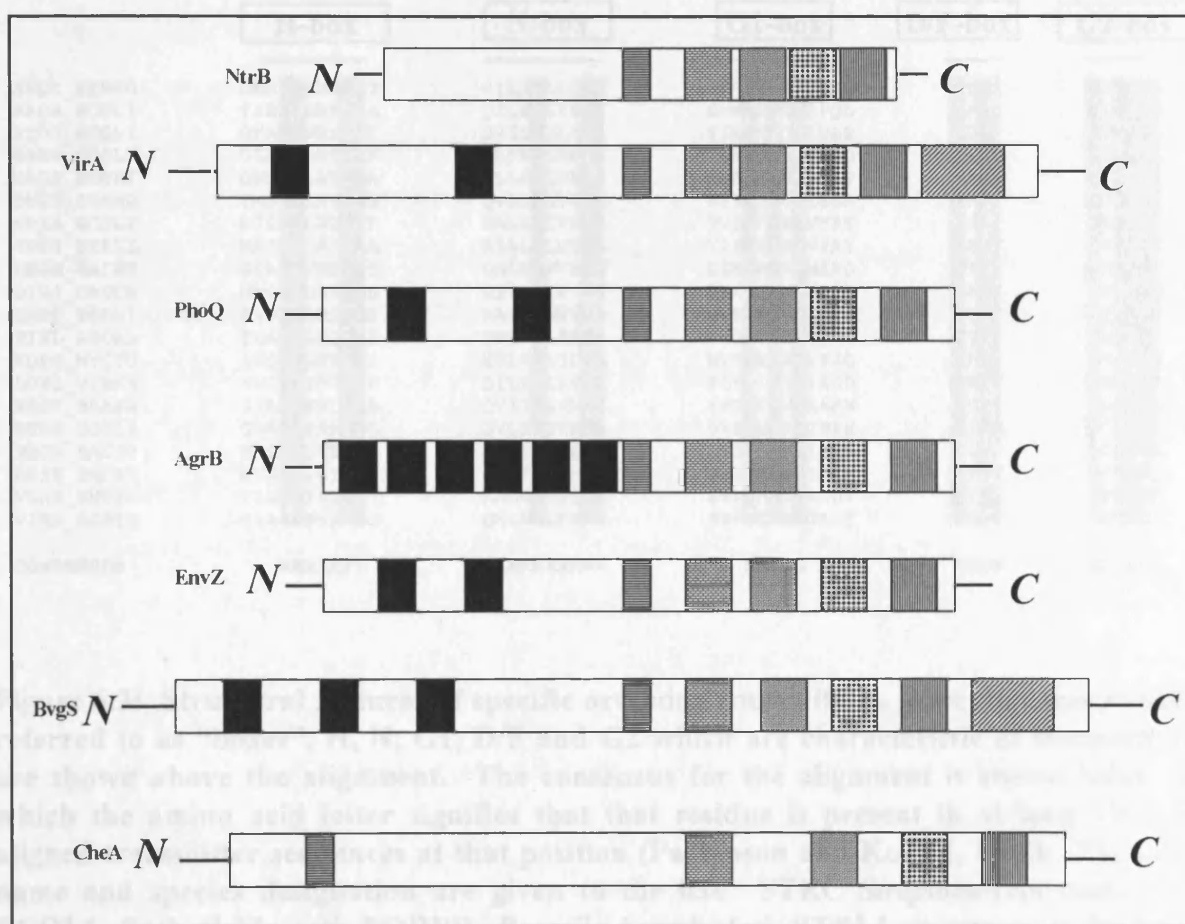


Figure 6.2: Domain organisation of HPKs: ■ denotes membrane spanning regions. ▨ denotes position of H-box. ▩ denotes position of N-box. ▧ denotes G1-box. ▦ denotes D/F box and ▨ denotes G2-box. ▨ corresponds to domains homologous to response regulator receiver domains. Transmitter domains between different HPKs share approximately 25% homology, however, there is considerable variability in the length and amino acid sequence of the linkers between the box-regions. Most HPKs are integral membrane proteins although some are cytoplasmic relying on other molecules to sense the environment, e.g., NtrB and CheA.

N-terminal half of the transmitter about 110 amino acid residues into the C-terminus. The conserved H residue is believed to be the site of autophosphorylation and is directly involved in kinase and phosphatase activities (Hing et al., 1994). Hing and Silhavy (1992) have proposed that the autokinase and phosphatase sites overlap substantially and that ATP and the H-box is required for both functions. Mutational analysis of the H-box in *EnvZ*, considered a paradigm for σ^{54} Ks, has given further insight into its function. Replacement of the H-box histidine with glutamine or aspartate abolishes both kinase and phosphatase

	H-box	N-box	G1-box	D/F-box	G2-box
AFQ2_STRCO	DMSHELRTPLT	VILANLIGNA	RVRDNGPGIPED	FDRF	GSGLGL
ARCB_ECOLI	TISHELRTPLN	QILWNLISNA	EVEDSGIGIPQD	FAMY	ATGIGL
ATOS_ECOLI	GVAHEVRNPLT	QVLLNILINA	SIEDNGCGIDLS	FDPF	GTGLGL
BAES_ECOLI	DISHELRTPLA	QLFNLLLENS	TFADSAFGVSDD	FERF	GSGLGL
BASS_ECOLI	DVAHELRTPLA	MLLRNLVENA	AVEDEGPGIDES	SKAF	GIGLGL
BVGS_BORBR	TMSHEIRTFMN	QVLSNLVGNA	SVSDTGCCISEA	EKPF	GTGLGL
CPXA_ECOLI	DISHELRTPLT	SALENIVRNA	TVDDDGPGVSPE	FRPF	GTGLGL
CUTS_STRLI	NASHELRTPLA	RIALNLVQNA	VVTNTGPPVPAY	FEFF	GVGLGL
DEGM_BACSU	SIAHEVRNPLT	QALMNFVKNG	EINDNGVMSRQ	GTAY	GNGIGT
DIVJ_CAUCR	NMSHELRTPLN	QIVLNLVSNA	VVADTGVGISPE	GRPY	GTGLGL
ENVZ_ECOLI	GVSHDLRTPLT	RAVANMVVNA	QVEDDGPGIAPE	FQPF	GTGLGL
FIXL_AZOAL	TLAHELNQPLS	QVLVNLFRNA	SVCDTGHGISDE	FTPF	GMGVGL
KDPD_MYCTU	AVSHDLRTPLA	RVLANLIDNA	NVIDEGPGVPRG	FAPF	GVGLGM
LUXQ_VIBHA	KMSHEIRTPIN	QILFNLVSNA	ELTDTGIGIESD	FEFF	GSGLGL
NODV_BRAJA	SIAHEITQPLA	QVIINLVMNG	SVTDCGVGIAEN	LDPF	GLGMGL
NTRB_ECOLI	GLAHEIKNPLG	QVLLNIVRNA	DVEDNGPGIPPH	FYPM	GTGLGL
PHOR_BACSU	NVSHELKTPIT	QVFLNLVNNA	EVADSGIGIQKE	FERF	GTGLGL
RESE_BACSU	NVSHELRTPIS	QVFTNLIDNA	DIKDSGSGIPEE	FERF	GTGLGL
VANS_ENTFC	YLAHDIKTPLT	KALSNVILNA	SVLNMGVHIDDT	FIPF	GSGLGL
VIRA_AGRTU	GIAHEFNNTLG	QMLMNLCKNA	SVSDDGEGIAET	FEFF	GTGLGL
CONSENSUS	HEXXXPL	QXXXXXXNA	DXGXG	FXPF	GXGLGL

Figure 6.3: Structural features of specific orthodox transmitters. Five sequence motifs, referred to as “boxes”, H, N, G1, D/F and G2 which are characteristic of transmitters are shown above the alignment. The consensus for the alignment is shown below in which the amino acid letter signifies that that residue is present in at least 70% of aligned transmitter sequences at that position (Parkinson and Kofoid, 1992). The gene name and species designation are given to the left. STRC *Streptomyces coelicolor*; ECOLI, *Escherichia coli*; BORBR, *Borrelia burgdorferi*; STRLI, *streptomyces lividans*; BACSU, *Bacillus subtilis*; CAUCR, *Caulobacter crescentus*; AZOAL, *Azotobacter alcaligenes*; MYCTU, *Mycobacterium tuberculosis*; VIBHA, *Vibrio haemolyticus*; BRAJA, *Bradyrhizobium japonicum*; ENTFC, *Enterococcus faecalis*; AGRTU, *Agrobacterium tumefaciens*.

Rhodospirillum rubrum, the T-box is missing from DegS from *Bacillus subtilis* and the G2-box is missing from NarX and NarX from *E. coli* and NarX from *Haemophilus influenzae* (Stuck et al., 1989, and alignments in this study).

An average of Chou-Fasman prediction scores for 33 orthodox transmitters has established a secondary structure profile for their respective transmitter domains (Parkinson and Kofoid, 1992). The conserved histidine is present within β -structure and is flanked by regions of α -structure. Following the α - β - α motif there is a flexible linker region of 30 residues which is polar but has no discernible secondary structure. This is followed by more variable sequence

N-terminal half of the transmitter about 110 amino acid residues into the *C*-terminus. The conserved H residue is believed to be the site of autophosphorylation and is directly involved in kinase and phosphatase activities (Hsing *et al.*, 1998). Hsing and Silhavy, (1997) have postulated that the autokinase and phosphatase sites overlap substantially and that ATP and the H-box is required for both reactions. Mutational analysis of the H-box in EnvZ, considered a paradigm for HPKs, has given further insight into its function. Replacements at the H-box histidine eliminate autophosphorylation and phosphatase activity (Kanamaru *et al.*, 1990; Parkinson and Kofoed, 1992; Hsing and Silhavy, 1997), although mutations at other H-box residues can affect either kinase activity, phosphatase activity or both, probably by altering the molecule's conformation and shifting the balance of the two reactions (Russo and Silhavy, 1991). The N and G1/2-boxes are thought to form a nucleotide binding surface within the active site and together these particular regions function to chelate Mg^{2+} , bridge the β and γ -phosphates of ATP and position the phosphate moiety waiting to be transferred (Hsing *et al.*, 1998). The N-box is critical for autokinase activity but the phosphatase activity can go unaffected (Kanamaru *et al.*, 1990; Hsing *et al.*, 1998). The D/F box is important for kinase activity but it is also thought to be important in nucleotide binding. The effect of mutations in this region are thus difficult to explain as kinase activity does not require ATP for energy. It is thought that conformational changes may affect presentation of the regulator to the kinase (Hsing *et al.*, 1998). Mutations in G1 and G2 eliminate autokinase activity (Kanamaru *et al.*, 1990). Mutations in G2 also exhibit very low kinase activity and have no phosphatase activity (Hsing *et al.*, 1998). It is not unprecedented for transmitters to lack one of these H, N, G1, D/F and G2 boxes (Parkinson and Kofoed, 1992). For example, CheA from a variety of organisms is missing the H-box but contains a histidine near the *N*-terminus which serves as the site for autophosphorylation (Hess *et al.*, 1988b). The N-box is lacking in FrzZ from *Myxococcus xanthus*, the G1-box is missing in CitA from *Escherichia coli* and *Klebsiella pneumoniae*, the F-box is missing from DegS from *Bacillus subtilis* and the G2-box is missing from NarQ and NarX from *E. coli* and NarQ *Haemophilus influenzae* (Stock J.B. *et al.*, 1989, and alignments in this study).

An average of Chou-Fasman prediction scores for 35 orthodox transmitters has established a secondary structure profile for their respective transmitter domains (Parkinson and Kofoed, 1992). The conserved histidine is present within β -structure and is flanked by regions of α -structure. Following the α - β - α motif there is a flexible linker region of 30 residues which is polar but has no discernible secondary structure. This is followed by more variable sequence

with regions of α - β repeats. Finally, there are regions rich in β -strands interrupted with large areas without obvious secondary structure (Parkinson and Kofoed, 1992).

A newly identified region located between the H box and the N-box has been reported (Hsing *et al.*, 1998). The so-called, X-region, is not generally recognised as a conserved motif but there appears to be scattered but significant homology with this region found in all two-component sensors. The X-region is thought to be important to confer specificity on the sensor-response regulator interaction (Hsing *et al.*, 1998). Mutations in the X-region of EnvZ harbour detectable defects in autokinase, kinase and phosphatase activities. The phosphatase negative phenotype is believed to be indirect and due to a conformational change preventing a productive interaction between EnvZ and OmpR-phosphate. A similar phosphatase negative phenotype was seen with an NtrB mutant, the sensor protein involved in nitrogen sensing (Atkinson and Ninfa, 1992).

Dividing the *N* and *C*-terminal domains of histidine protein kinases is a linker region which has also recently been characterised for EnvZ. (Park and Inouye, 1997). The linker region is located between the second transmembrane domain and the cytoplasmic domain (Park and Inouye, 1997). It has been postulated that this region may have a crucial role in orientating EnvZ dimers and in transmitting the signal from the periplasmic domain to the cytoplasmic signalling domain. The primary sequence similarity between linker regions is low although similar secondary structure, a helix-1 loop-helix-2 configuration, exists across the family of transmembrane receptors. Linker mutations in EnvZ decrease both kinase and phosphatase activities but dimerisation is not affected. The signal sensing mechanism of some kinases is more complex than simply a periplasmic sensing mechanism as the linker region of some systems may be involved sensing cytoplasmic signals. For example, NarX of *E. coli* senses molybdenum levels (Kalman and Gunsalus, 1990), FixL of *Rhizobium meliloti* has an oxygen binding haem moiety and senses oxygen (Monson *et al.*, 1992), the linker region of BvgS of *Bordetella pertussis* senses the sulphate anion and nicotinic acid (Miller *et al.*, 1992) and VirA of *Agrobacterium tumefaciens* senses phenolic compounds and pH (Chang and Winans, 1992). This is not a common mechanism as most transmembrane HPKs are not known to sense cytoplasmic signals (Park and Inouye, 1997).

6.3.2: Mechanisms of sensing and signalling in transmembrane receptors

6.3.2.1: Sensing

Sensory input domains of transmitters differ broadly in structure, which is not surprising given the great diversity in the types of physical and chemical stimuli that bacteria sense (Goudreau and Stock, 1998). A few that have been characterised include the periplasmic domain of PhoQ, involved in virulence in *S. typhimurium* (Soncini and Groisman, 1996) which senses divalent cations such as Mg^{2+} and Ca^{2+} (Vescovi *et al.*, 1996; 1997). Aer is a recently identified sensor of intracellular energy levels and mediates aerotaxis in *Escherichia coli* (Bibikov *et al.*, 1997). The protein has two transmembrane domains in the *N*-terminus but no intervening periplasmic domain. Instead, a cytoplasmic sensing domain containing a PAS motif senses the oxygen status of the cell (Zhulin *et al.*, 1997). Transmembrane domains are also known to sense certain stimuli. For example, alterations in membrane lipid composition can directly affect signalling of the Cpx two-component regulatory system (Mileykovskaya and Dowhan, 1997). Indeed, the periplasmic domain of EnvZ is not required to sense the levels of osmolarity, indicating that the signal may propagate through membrane perturbations instead. The periplasmic domain may therefore sense other signals (Leonardo and Forst, 1996). Therefore, it appears that receptor sensing can be mediated by any domain: periplasmic, transmembrane and cytoplasmic.

6.3.2.2: Signalling

The process of transmembrane signalling is fundamental to many sensory systems. There is no simple model which describes the mechanism of signalling for a histidine protein kinase. Generally, signalling occurs through dimerisation of two histidine protein kinases or formation of a complex between a histidine protein kinase and a second, different, interaction domain from another protein (Goudreau and Stock, 1998). The best characterised signalling system is that of the bacterial chemotaxis receptors which belong to the latter class (Le Moual and Koshland, 1996). Formation of a bacterial receptor dimer is required for the kinase, CheA, to become activated and the signal is believed to be modulated by the orientation of the monomers (Cochran and Kim, 1996; Surette and Stock, 1996). In engineered dimers, in which chemoreceptor heterodimers contain only one cytoplasmic domain, signalling can still occur (Gardina and Manson, 1996). It is postulated that signalling occurs through interactions

between dimers (Gardina and Manson, 1996). Importantly, in the truncated monomer, the linker is essential for signalling to occur (Gardina and Manson, 1996). The contribution of the linker region to signalling has also been demonstrated for EnvZ (Park and Inouye, 1997). Transmembrane regions are thought to have a significant contribution to signalling. Molecular modelling has hinted that these regions are α -helical (Milburn *et al.*, 1991). Upon receiving a stimulus, movement of TM1 and TM2 relative to each other within a monomer results in translation of one helix perpendicular to the plane of the membrane linked with rotation of the helix about its axis. TM regions originally appeared to be essential for signalling (Baumgartner and Hazelbauer, 1996; Hughson and Hazelbauer, 1996). Although recently, an engineered soluble chemoreceptor lacking transmembrane regions, in which the sensing and signalling domains are coupled together, was still capable of functioning (Ottemann and Koshland, 1997).

In the signalling process, it is not unprecedented for dimers to form interdimer interactions. This provides a further level of complexity in receptor regulation. An example is seen with bacterial chemoreceptors which are a family of homologous transmembrane molecules with different ligand specificities. CheR catalyses the reversible methylation of glutamate residues in the cytoplasmic signalling domains by recognising a specific five amino acid sequence (Wu *et al.*, 1996). Some members of the chemoreceptor family do not harbour these specific sequences therefore it is suggested that CheR is tethered onto one receptor dimer and from this position is able to methylate an adjacent receptor dimer in an interdimer complex to propagate the signal (Djordjevic and Stock, 1997; Le Moual *et al.*, 1997; Li *et al.*, 1997). Overall, there appears to be a complex sensing and signalling mechanism which enables transmission of information to bring about the desired response.

6.3.3: Response regulators

Response regulators constitute a superfamily of proteins which harbour a conserved *N*-terminus which is separated from a specific *C*-terminus by a flexible linker region (figure 6.4). The *N*-terminal, receiver domain (~125 amino acids) interacts with its respective histidine protein kinase in order to receive a phosphoryl group from the phosphohistidine and to effect the appropriate response. Sequence alignments of individual response regulator receiver domains show approximately 20-30% identity which from an evolutionary standpoint suggests their underlying processes of communication are relatively conserved

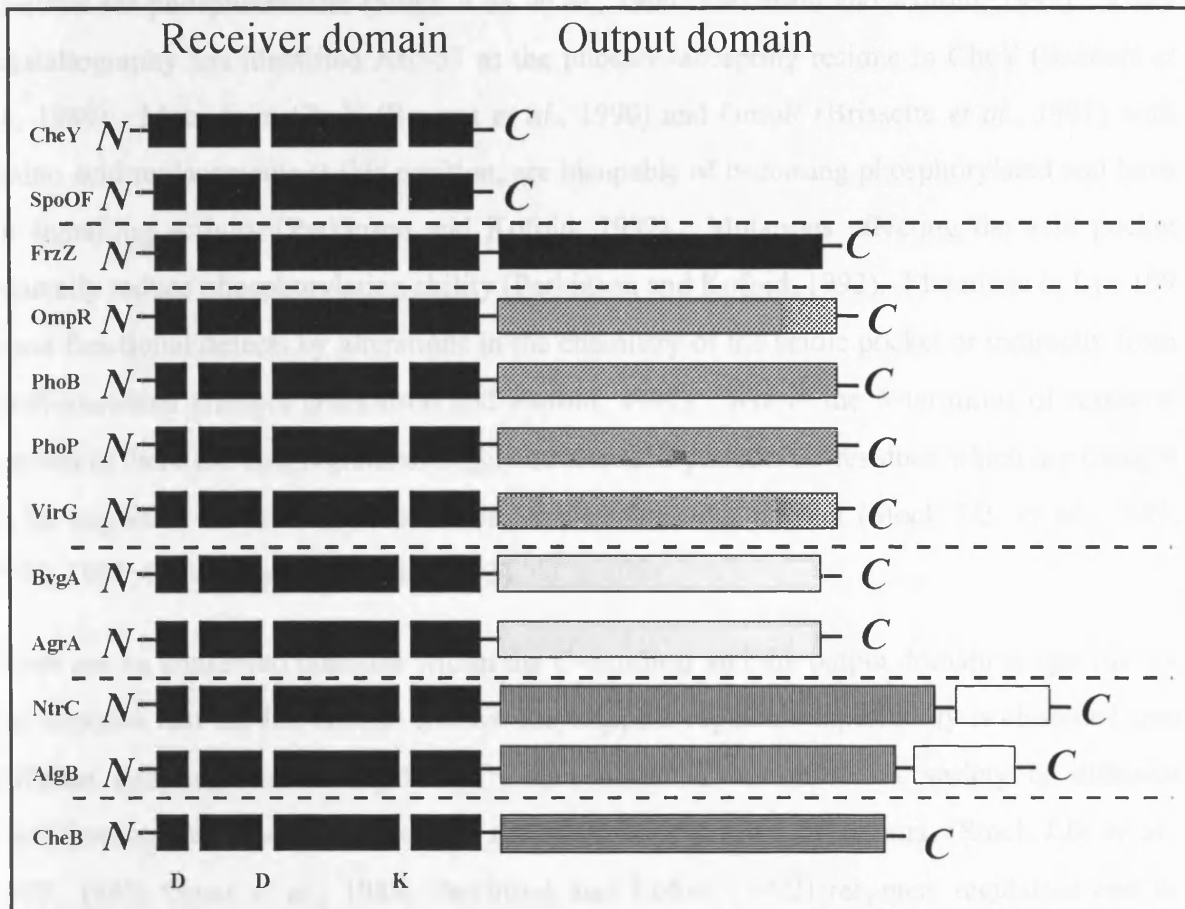


Figure 6.4: Domain organisation of RRs. The *N*-terminal receiver domains are represented by black boxes. These regions are very conserved among RRs and contain the highly conserved amino acids D, D, and K. The *C*-terminal output domains are more variable but RRs which respond to similar signals share a considerable degree of sequence similarity. On the basis of this similarity the RRs are organised into sub-families. Some RRs contain two receiver domains in tandem, e.g., *FrzZ*

(Stock J.B. *et al.*, 1989; 1990; 1995; Parkinson and Kofoed, 1992). One of the best characterised response regulators is probably CheY from *E. coli* which is involved in chemotaxis. The receiver domain of CheY contains a number of very highly conserved amino acids, Asp-12, Asp-13, Asp-57 and Lys-109 which appear to be conserved amongst all receiver sequences. The conserved aspartates are postulated to form an acidic pocket within which Asp-57 is centrally located and in which the side chain Lys-109 protrudes (Parkinson and Kofoed, 1992). The remaining conserved residues function to bind Mg^{2+} which is essential for phosphotransfer (Stock A.M. *et al.*, 1990; Parkinson and Kofoed, 1992). X-ray crystallography has identified Asp-57 as the phospho-accepting residue in CheY (Sanders *et al.*, 1989). Mutants in CheY (Bourret *et al.*, 1990) and OmpR (Brissette *et al.*, 1991) with amino acid replacements at this position, are incapable of becoming phosphorylated and have no signalling activity (Parkinson and Kofoed, 1992). Mutations affecting the acid pocket generally reduce phosphorylation ability (Parkinson and Kofoed, 1992). Mutations in Lys-109 cause functional defects by alterations in the chemistry of the acidic pocket or indirectly from conformational changes (Parkinson and Kofoed, 1992). Within the *N*-terminus of response regulators there are also regions of highly conserved hydrophobic residues which are thought to be important in protein dimerisation or intramolecular contact (Stock J.B. *et al.*, 1989, 1990, 1995; Parkinson and Kofoed, 1992).

There are no conserved domains within the *C*-terminus and the output domain is specific for the response that the RR elicits. Indeed, the response regulator superfamily is classified into different subgroups according to the nature of the *C*-terminus. A variety of different classifications have been derived, but according to a number of authors, (Stock J.B. *et al.*, 1989; 1995; Gross *et al.*, 1989; Parkinson and Kofoed, 1992) response regulators can be grouped as follows. The first group can be characterised by a lack of output domain. The receiver domain may be present alone as with CheY or SpoOF or in tandem as with FrzZ. The second group are DNA-binding proteins which bind to specific target sequences upstream of the genes they regulate. This group can be further subdivided by the respective homology between their output domains and the way they interact with genes under their control. For example the NtrC sub-family which contains NtrC (nitrogen assimilation) and DctD (dicarboxylate transport) may be involved in an interaction with genes harbouring σ^{54} promoter elements (Debruijn and Ausubel, 1993). The OmpR-PhoB sub-family contains OmpR (osmolarity, virulence), PhoB (phosphate regulation), ArcB (oxygen regulation), VirG (virulence), PhoP (virulence). Members of this family are thought to regulate expression of

genes whose promoters contain σ^{70} recognition elements. A third family, the FixJ family, includes FixJ (nitrogen fixation), UhpA (uptake of hexose phosphates), ComA (competence), NarL (nitrate reductase) and DegU (degradative enzymes) which are transcriptional regulators. The final group contains unique output domains which fail to resemble output domains of any other known response regulators. This group includes CheB which is a methylesterase involved in chemotaxis and is not involved in DNA transcription (Gross *et al.*, 1989; Stock J.B. *et al.*, 1989; Parkinson and Kofoed, 1992).

One member of the receiver domain superfamily has been extensively studied, the bacterial chemotaxis factor, CheY (Stock A.M. *et al.*, 1989; 1993; 1995; Bellolell *et al.*, 1994; 1996; Lowry *et al.*, 1994). These efforts have proved useful in determining the 3D structure of CheY which comprises a 5 strand β -sheet flanked by α -helices (Stock A.M. *et al.*, 1989; Stock J.B. *et al.*, 1989). Recently, a number of other response regulator structures have been characterised; the *Bacillus subtilis* sporulation factor, SpoOF, (Madhusudan *et al.*, 1996; Feher *et al.*, 1997; Goudreau and Stock, 1998), the *N*-terminal regulatory domain of *S. typhimurium* NtrC, (Volkman *et al.*, 1995; Goudreau and Stock, 1998), the *N*-terminal regulatory domain of the *E. coli* nitrate-dependent regulator, NarL (Baikalov *et al.*, 1996; Goudreau and Stock, 1998b), and the *S. typhimurium* chemotaxis regulator CheB (Djordjevic *et al.*, 1998; Goudreau and Stock, 1998). All these proteins share the same structure as CheY but with variation in the loops connecting the secondary structural elements resulting in similar folding patterns (Goudreau and Stock, 1998).

Further major developments within the response regulator field include the newly determined structures of response regulator DNA-binding domains of *E. coli* NarL and OmpR (Baikalov *et al.*, 1996; Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997a,b). Together, they provide a structural framework with which to examine response regulator sub-families. For NarL, the *C*-terminal DNA binding domain consists of 62 amino acids and is comprised of 4 α -helices. The middle two form a helix-turn-helix motif (HTH motif) that is representative of HTH motifs in the FixJ/LuxR sub-family of transcriptional regulators and other DNA binding proteins (Baikalov *et al.*, 1996). The *C*-terminal domain is separated from the *N*-terminal domain by an α -helix of 10 amino acids and a 13 residue linker (Baikalov *et al.*, 1996). The crystal structure of the OmpR DNA-binding domain has recently been independently determined by two groups (Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997a,b). These authors report a newly defined sub-family of transcriptional activators containing a “winged” helix-turn-helix motif whose function is to direct binding of the output domain to specific

DNA sequences and to facilitate interactions with RNA polymerase. The 98 amino acid C-terminal domain of OmpR (OmpRc) has a domain topology of $\beta 1$ - $\beta 2$ - $\beta 3$ - $\beta 4$ - $\alpha 1$ - $\beta 5$ - $\alpha 2$ - $\alpha 3$ - $\beta 6$ - $\beta 7$ in which $\alpha 2$ -loop- $\alpha 3$ region forms the helix-turn-helix motif. The $\alpha 3$ region is the recognition helix which binds to the major groove of DNA and its positioning is aided by the $\alpha 2$ -helix. As with NarL and many other response regulators that serve as transcriptional activators, the C-terminus of OmpR is separated from the N-terminus by a flexible linker, shared by many response regulators. The OmpR family of response regulators have linker lengths ranging from 5-21 residues (Martinez-Hackert and Stock, 1997a and b). OmpR has a linker of approximately 12 amino acids, VanR has 11, VirG has 9 and PhoB has a linker of 6 residues (Martinez-Hackert and Stock, 1997a; 1997b). The linker contributes greatly to the conformation and function of multidomain response regulators which is affected by the phosphorylation state of the molecule.

6.4: Phosphorylation and dephosphorylation reactions of transmitters and receivers

Histidine protein kinases and response regulators can be split into modules which communicate by a series of reversible phosphorylation and dephosphorylation reactions and which are regulated in response to sensory inputs. The resultant effect is the modulation of transmitter activities that regulate the flux of phosphoryl groups to and from target receivers. In many cases, multiple transfers occur in systems which are described as phosphorelay pathways. The most widespread is the two-step phosphorelay mechanism. The ArcB/A system of *E. coli* comprises individual histidine and aspartate modules which have the ability to fold on their own and function within a phosphorelay pathway with or without linkage of contiguous domains (Georgellis *et al.*, 1997). A four step phosphorelay system exists for the BvgA/S system in *Bordetella* (Uhl and Miller, 1996a; Uhl and Miller, 1996b) and an even more complex system is present in the filamentous cyanobacterium *Fremyella diplosiphon* (Kehoe and Grossman, 1997). The phosphorelay mechanism in its simplest form is described below.

The nature of an HPK's input domain is to sense a stimulus and to consequently process the information. This information is propagated through the molecule by a conformational change on the sensor protein and an associated alteration in the HPKs phosphorylation state. Autokinase activity of the HPK results in the formation of a high energy phosphohistidine in a readily reversible reaction (Hess *et al.*, 1988a; Ninfa and Bennett, 1991). The

phosphohistidine is an intermediate for the subsequent transfer of phosphoryl groups to their cognate response regulator (Sanders *et al.*, 1989) and is most likely the preferred substrate for this reaction (Hess *et al.*, 1988a; Sanders *et al.*, 1989). The phosphotransfer reaction is believed to be catalysed by the response regulator (Parkinson and Kofoed, 1992; Parkinson, 1993). The interaction between the two proteins is both reversible and specific which is very important to avoid unnecessary cross-talk. Autophosphorylation of the HPK transmitter in response to a signal is a major check point in the flow of phosphates throughout the system and consequently to the activation of the response regulator receiver. The mechanism behind the autokinase activity is not well established although Parkinson and Kofoed, (1992) have proposed a model based on sensors of the chemotaxis system. As discussed earlier, Parkinson and Kofoed, (1992) have defined two transmitter domains for sensor kinases, T_L (containing the H-box) and T_R (containing the N, D/F and G1/G2 boxes). These domains interact to create either an active or inactive autokinase. Prior to stimulation, the HPK's input domain exists in a relaxed conformation whereas T_L and T_R are tightly bound. In this conformation, the kinase remains inactive. Once stimulated, the input domain acquires a tense conformation, resulting in T_L and T_R becoming more relaxed and exposing the molecule to ATP. The role of ATP is unclear but it is believed to function as an allosteric effector and destabilise the T_L/T_R interaction. Ultimately this provides a molecular surface upon which the transmitter and cognate response regulator receiver can interact. According to the model proposed by Parkinson and Kofoed, (1992) and Parkinson, (1993), transmitters which remain in a relaxed conformation would have their autokinase activity switched ON thereby phosphorylating the molecule. In contrast, a tense conformation would render the molecule's autokinase activity OFF. However, in OFF mode, the molecule is far from redundant as it harbours phosphatase activity which is important for the various phosphorelay mechanisms to function effectively (discussed below). It is generally accepted that autophosphorylation is a *trans* reaction within a dimer among histidine protein kinases, e.g., NtrB (Ninfa *et al.*, 1993), CheA (Swanson *et al.*, 1993; Tawa and Stewart, 1994; Surette *et al.*, 1996), BvgS (Uhl and Miller, 1996b) and EnvZ (Hidaka *et al.*, 1997).

Not only are response regulators capable of accepting phosphoryl groups from cognate HPKs they can also utilise small phosphodonors such as phosphoramidate, acetyl phosphate and carbomyl phosphate as substrates. In contrast, they are unable to utilise ATP directly (Feng *et al.*, 1992; Lukat *et al.*, 1992; Parkinson and Kofoed, 1992; Parkinson, 1993; 1995; Stock J.B. *et al.*, 1995; Appleby *et al.*, 1996). Nevertheless, the affinity of receivers towards their cognate HPK is much greater than that for small phosphodonors (Stock J.B. *et al.*, 1995) a

finding that may be attributed to receivers recognising the local structure of the HPK between the H-box and the N-box, in the vicinity of the X-region. The phosphotransfer reaction involves stereospecific interactions between exposed surfaces on different modules. Despite this specificity, phosphotransfer to receiver modules can also occur from non-partner histidine protein kinases. As for the situation with phosphotransfer from small phosphodonors, phosphorylation of the receiver by its cognate HPK is much more efficient than that from of non-cognate HPK. The ability of non-partner HPKs to phosphorylate receivers is given the term, “cross-talk” (Albright *et al.*, 1989; Stock J.B. *et al.*, 1989; Bourret *et al.*, 1991; Parkinson and Kofoed, 1992; Wanner, 1992). The result of cross-talk may be non-specific background noise which may affect normal cellular responses (Bourret *et al.*, 1991). In contrast, cross-talk may be a method to modulate a response to numerous environmental stimuli at any given point by linking together different two-component regulatory systems. The concept of linked TCRs is not unprecedented as the Pho-regulatory network in *Bacillus subtilis* consists of three two-component regulatory networks (Goudreau and Stock, 1998). Nevertheless, given that there are more than 50 two-component regulatory networks in *E. coli* the specificity of the kinase-response regulator reaction is crucial to avoid unwanted cross-talk.

Once the receiver becomes phosphorylated, the conformation becomes affected. The conformational changes exhibited by response regulators have recently come to light as the structure of a few response regulators has been determined, for example CheB and NarL (Baikalov *et al.*, 1996; Djordjevic *et al.*, 1998). The flexible linker enables response regulator domains to interact in a way that depends on the phosphorylation state of the molecule. In CheB, whose C-terminal methylesterase activity is inhibited by the N-terminal regulatory domain, the structure of intact, unphosphorylated CheB shows the N-terminal regulatory domain packed against the C-terminal catalytic domain (Djordjevic *et al.*, 1998). In this conformation access of the substrate chemotaxis receptor to the methylesterase active site is prevented. In response to becoming phosphorylated, the N-terminus becomes activated and undergoes a conformational change which perturbs the interface between the two interacting domains so that they reposition with respect one another. This leaves the active site exposed and relieves the inhibitory effect (Djordjevic *et al.*, 1998; Goudreau and Stock, 1998). This is akin to the situation observed with NarL which in its unphosphorylated state has the two domains interacting to prevent DNA binding. Phosphorylation confers a conformational change on the molecule and disables the interaction so that the C-terminus can bind DNA (Baikalov *et al.*, 1996). The influence of N-terminal regulatory domains on the function of

response regulators is not limited solely to an inhibitory effect on catalytic domain activation. The phosphorylated form of CheB provides additional surfaces for its intermolecular reaction with chemoreceptors (Djordjevic *et al.*, 1998). Phosphorylated CheY is believed to have different but overlapping surfaces which interact with CheA, FliM and CheZ (Zhu *et al.*, 1997b). Phosphorylated *N*-terminal domains also serve to facilitate oligomerisation in some response regulators such as PhoB (Haldimann *et al.*, 1996), NtrC (Wyman *et al.*, 1997), OmpR (Huang and Igo, 1996; Huang and Lan, 1997) and UhpA (Webber and Kadner, 1997). NtrC phosphorylation results in formation of large oligomeric complexes which act as transcriptional activators. For OmpR, formation of homologous protein interactions are stimulated at neighbouring sites on the DNA duplex. Interestingly, UhpA, a homologue of NarL binds DNA perfectly well without being phosphorylated, but upon phosphorylation, oligomerisation of UhpA on target promoters allows binding of the molecule at low efficiency sites. The conformational changes induced by phosphorylation changes of response regulators may hold the key to a better understanding of signal transduction in two-component regulatory systems (Goudreau and Stock, 1998).

As long as the response regulator remains phosphorylated, the cellular response continues even if it is no longer required. Clearly this is an energy-consuming exercise and likely to be a disadvantage to the bacterium. Therefore, to prevent continual stimulation, the receiver becomes dephosphorylated restoring the regulator to its unresponsive unphosphorylated state and nullifying the original sensory data (Bourret *et al.*, 1991). Dephosphorylation of the regulator is controlled by a number of different mechanisms. When transmitters are in a tense conformation, they exhibit a lack of autokinase activity (OFF). However as highlighted previously, this conformation is not a redundant state as many transmitters have catalytic phosphatase activity. Phosphatase activity of the sensor kinases requires divalent metal ions such as Mg^{2+} , ATP/ADP or non-hydrolysable analogues of ATP which acts as a co-factor in stabilising the conformation (Parkinson, 1993). Transmitters which are known to exhibit phosphatase activity include EnvZ, PhoR, DegU, and NtrB (Hess *et al.*, 1988a; Parkinson and Kofoed, 1992; Parkinson, 1993; Hsing *et al.*, 1998). The failure to show phosphatase activity for many more kinases may not indicate their lack of ability, but may imply that the conditions under which this property is exhibited has not been identified or that a phosphorylated transcriptional regulator needs to be maintained for a lengthy period (Parkinson and Kofoed, 1992). Dephosphorylation of receivers is autocatalytic and is able to take place in the absence of other proteins. The half-life of phosphorylated receivers varies from milliseconds to minutes and possibly hours depending on the system. Regulators of

chemotaxis such CheY and CheB have half-lives of milliseconds to enable simultaneous responses to a rapidly changing environment, whereas those involved in sporulation, a much lengthier process, are likely to be much longer to avoid premature decision making (Bourret *et al.*, 1991). The importance of half-life characteristics has been demonstrated *in vivo* as response regulator mutants exhibit irregular behaviour (Aiba *et al.*, 1989; Bourret *et al.*, 1990). Intrinsic phosphatase activity of response regulators raises questions as to the nature of the phosphatase activity ascribed to the sensor. This contribution may enhance the receiver's own intrinsic phosphatase behaviour by an allosteric mechanism and by manipulating the local conformation of receiver phosphorylation site. The receiver's phosphatase activity is modulated to control the levels of receiver dephosphorylation. For example, CheZ which is not an HPK accelerates the dephosphorylation of CheY in an allosteric manner as opposed to being a true phosphatase. Further to the phosphatase activities of both the receiver and the transmitter, additional auxiliary phosphatase activity is provided by aspartate phosphatases which can accelerate the dephosphorylation process (Stock J.B. *et al.*, 1989; Perego *et al.*, 1996). These might be required in a highly complex integrated signalling pathway in which the number of two-component systems being employed at any given time cannot be regulated simply by the input kinase. This may occur in *B. subtilis* where sporulation is also linked to bacterial competence and secretion of degradative enzymes (Stock J.B. *et al.*, 1989; Perego *et al.*, 1996). The interaction between sensor kinases and response regulators is a crucial step in the signal transduction pathway. The reaction depends on the rate at which the HPK autophosphorylates in response to a signal, the rate and specific nature of phosphotransfer and the balance of phosphorylation and dephosphorylation of the regulator. The sum of the opposing kinase and phosphatase activities from various sources determines the amount of intracellular phosphorylated regulator which in turn regulates the relative level of transcription of genes under the regulators control.

6.5: Two component regulatory systems involved in pathogenesis

In general, two-component regulatory systems enable transformation of an environmental stimulus into a cellular response which is necessary for the bacterium to adapt to its surroundings, survive and multiply. For a pathogenic organism, adaptation to a host with all of the associated micro-environments therein, is simply an extension of this process and the appearance of disease within the host is largely an inadvertent consequence of bacteria

successfully achieving these goals. During the infection process two-component regulatory systems regulate factors required for motility, adherence, invasion, toxin production and components involved in intracellular survival and evasion of the host immune response. Many of these virulence determinants are co-ordinately expressed via common regulatory cascades to ensure that those genes required at a particular stage during infection or under certain conditions are optimally expressed. This avoids wasteful expression of factors not specifically required. During the infection process, virulence determinants are produced in response to more than one stimulus although there are still a number of common signals detected such as changes in temperature, nutrient limitation and osmolarity. The ability of the bacterium to respond to different signals suggests that a number of different regulatory circuits are involved. The involvement of multiple networks is an advantage as it increases the sensitivity to the response. During infection, two component regulatory systems do not function in isolation as they are integrated into other regulatory circuits and as a result they are subject to cross-regulation (DiRita and Mekalanos, 1989; Mekalanos, 1992; Wanner, 1992; Gross, 1993).

There are many examples of bacterial virulence factors regulated by two component systems (Gross, 1993; Finlay and Falkow, 1997). Possibly the best characterised examples are BvgA and BvgS in *Bordetella pertussis*, VirA/VirG in *Agrobacterium tumefaciens* and the *Salmonella* EnvZ/OmpR and PhoP/PhoQ systems. These highlight some of the differences between two-component regulatory systems of pathogenic organisms which result in a similar outcome of disease.

Bordetella pertussis is a human pathogen in which the BvgA/S system regulates its own expression and expression of a variety of virulence determinants such as FHA, fimbriae and several toxins in a multistep phosphorelay process (Appleby *et al.*, 1996). BvgS is an atypical transmembrane sensor kinase in that its transmitter domain is fused to a receiver domain analogous to the receiver domain of a response regulator. BvgS responds to a variety of different stimuli including temperature, magnesium sulphate and nicotinic acid although the factors which control expression of the BvgA/S system *in vivo* remain unidentified. Transcription from BvgA is initiated at three independent promoters, two of which are BvgA dependent, P1 and P3 and the latter, P2 which is BvgA independent. Temperature supposedly initiates transcription from promoter P1, which results in the shut-down of P2. This switch is regulated by the active form of BvgA. An increase in the intracellular concentration of activated BvgA, results in the BvgA dependent activation of virulence determinants (Dorman,

1994c; Uhl and Miller, 1995; Uhl and Miller, 1996b).

Agrobacterium tumefaciens is a plant pathogen in which the VirA/VirG system is unusually plasmid borne and regulates *vir* genes involved in virulence of the organism. The *vir* genes are present on the Ti (tumour inducing) plasmid and code for genes which sense plant wound metabolites, in addition to genes involved in transfer and integration of the Ti plasmid within the plant cell's chromosome. The *vir* genes are not transferred but are essential for crown gall tumour formation. The sensor component, VirA, is somewhat atypical in that it is a transmembrane protein with an input domain and transmitter domain fused to a receiver domain, homologous to the N-terminal region of VirG. The role of this additional receiver domain is unclear but its phosphorylation allows access of VirG to VirA (Chang and Winans, 1992; Endoh and Oka, 1993). VirA autophosphorylates in response to plant signalling molecules, phenolic compounds and monosaccharides (plus ChvE). VirA also responds to phosphate starvation and acid pH. Monosaccharides complexed with ChvE are sensed by the periplasmic domain but unusually, phenolic compounds and acidity are sensed by the cytoplasmic domain (Chang and Winans, 1992). The phosphorelay circuit which encompasses VirA and VirG comprises autophosphorylation of VirA at the histidine residue in response to a specific stimulus and transfer of this phosphate group onto the receiver domain of VirA. A conformational change is induced in VirA allowing access of VirG to VirA. VirA once again autophosphorylates at the C-terminal histidine residue and the phosphate is transferred onto the conserved aspartate of the response regulator, VirG, activating the molecule. A dual promoter system, P1 and P2 affects transcription of VirG. P1 is induced by phenolic compounds in a VirA/VirG dependent manner, but also phosphate starvation in VirA/VirG independent manner. P2 is possibly induced by a low pH. In its natural environment, the bacterium probably senses plant wound metabolites, utilising the VirA/VirG system and detects a favourable environment for plant colonisation. The result is transcription of *vir* genes in a VirA/VirG dependent manner (Lee *et al.*, 1992; 1995; Heath *et al.*, 1995)

In *Salmonella*, the EnvZ/OmpR and PhoP/PhoQ two component systems are essential requirements for *Salmonella* virulence (Groisman and Heffron, 1995). The EnvZ/OmpR system, is encoded by the *ompB* locus. Mutations in *ompB* render *S. typhimurium* avirulent for mice (Dorman, 1994c). EnvZ/OmpR control expression of several products including the outer membrane proteins, OmpC and OmpF in response to changes in osmolarity, although other components contribute to the virulence phenotype (Groisman and Heffron, 1995). The

PhoP/PhoQ system highlights that a large number of genes can be under the control of one two-component system. PhoP/PhoQ regulates a number of virulence factors and is affected by starvation for phosphate and carbon, low pH and the intracellular environment of macrophages and appears to be directly regulated by Mg^{2+} . The mechanism of expression of PhoP/PhoQ regulated virulence genes is highly co-ordinated during infection, for example, during the process of invasion and intracellular survival. Once the bacterium enters phagocytic cells, the low pH is sensed and expression of genes essential for survival within acidified macrophages (*pag* PhoP activated genes) are positively regulated. In contrast, there is repression of transcription of genes essential for the initial invasion process (*prp* PhoP repressed genes) (Dorman, 1994c; Groisman and Heffron, 1995; Finlay and Falkow, 1997).

6.6: Does a two component system regulate *htrA* expression in *C. jejuni*?

A previous section has already highlighted that in response to a disruption in normal protein trafficking in the *Escherichia coli* cell envelope (inner membrane, periplasm and outer membrane, OM), expression of a number of genes, including *htrA*, is stimulated whose products function to fold or degrade misfolded or aberrant proteins. Expression of a number of these factors, specifically *htrA*, is governed by two distinct overlapping pathways in response to insults to the bacterial envelope (Danese *et al.*, 1995; Pogliano *et al.*, 1997). The first pathway, which has been discussed in more detail elsewhere uses the alternative sigma factor, σ^E . The second system, which overlaps that of σ^E , is the Cpx pathway which also regulates protein trafficking in the cell envelope in response to stress. The type of signal which each pathway responds to may differ implying that despite the two systems overlapping each may carry out a separate function (Danese *et al.*, 1995). Activation of the Cpx pathway can be stimulated in a number of ways. For example, by overproduction of NlpE, a novel outer membrane protein (Snyder *et al.*, 1995), alterations in membrane phospholipid composition in *E. coli* cells which lack the major phospholipid phosphatidylethanolamine (PE) (Mileykovskaya and Dowhan, 1997) and elevated pH (Nakayama and Watanabe, 1995; Danese, 1996; Danese and Silhavy, 1998). Further, production of unfolded P pilus subunits, which aggregate and localise to the outer surface of the inner membrane can induce either the Cpx pathway and/or the σ^E pathway (Jones *et al.*, 1997). The result of activation of the Cpx signal transduction pathway is elevated expression of various envelope stress-combative proteins such as DsbA, FkpA, RotA, PpiA, CpxP (Danese *et al.*, 1995; Pogliano *et al.*, 1997;

Danese and Silhavy, 1997; 1998) and more importantly in this study, *htrA*. In the case of *htrA*, the Cpx pathway functions in concert with σ^E to stimulate transcription from this locus, although other members of the Cpx regulon (e.g. *dsbA* and *rotA*) are not co-regulated by σ^E . This suggests the presence of at least two different classes of Cpx regulated loci.

Characterisation of this pathway at the molecular level has led to the discovery that the Cpx two-component signal transduction system plays an integral part. The *cpx* locus encodes CpxR and CpxA and the genes are contiguous on the chromosome. The *cpxR* gene is predicted to encode the response regulator of this system (Dong *et al.*, 1993) and shows extensive homology with members of the response regulator family, particularly those of the OmpR sub-family. The *cpxA* gene encodes the membrane sensor component of the system, the histidine protein kinase (McEwen and Silverman, 1980a; 1980b; 1980c; Silverman, 1982; Albin and Silverman, 1984). Like other sensor kinases, CpxA contains a number of conserved regions throughout its amino acid structure. Further, the protein is localised to the inner membrane by two transmembrane helices and contains both periplasmic and cytoplasmic domains (Weber and Silverman, 1988). Point mutations in *cpxA* are pleiotropic. One of the primary functions regulated by the Cpx system is conjugative plasmid transfer but point mutations in CpxA render the bacterium defective in this process (Silverman *et al.*, 1993). Other phenotypes include aberrant cell division (Pogliano *et al.*, 1998), decreased stability of inner and outer membrane proteins (McEwen and Silverman, 1982), acquisition of low-level resistance to aminoglycosides, impairment of ion-driven lactose and proline transport, inability to grow on non-fermentable carbon sources such as succinate (Rainwater and Silverman, 1990), ability to utilise L-serine as a carbon source (Su *et al.*, 1989) and isoleucine-valine auxotrophy (McEwen and Silverman, 1982) although some of these phenotypes are dependent on the genetic background in which the mutants are made (McEwen and Silverman, 1980b). Also, it is thought that the phenotypes of point mutations in the Cpx operon are gain-of-function mutations which mirror the stimulus responsive state of the system (Parkinson, 1995; Mileykovskaya and Dowhan, 1997). This can be attributed to the fact that deletion or null mutants in the whole *cpx* operon exhibit wild type phenotypes, with restoration of all properties altered by *cpxA* point mutations.

The fact that the CpxR/A system regulates expression of *htrA* in *E. coli* has major implications as to the role of the *htrA* protease in bacterial pathogenesis. For example, *S. typhimurium htrA* mutants are avirulent or highly attenuated when given orally to mice (Johnson *et al.*, 1990; Chatfield *et al.*, 1992; Everest *et al.*, 1995). Furthermore, expression of

htrA is increased when *Salmonellae* enter eukaryotic cells (Everest *et al.*, 1995). The HtrA protease has also been shown to be involved in the virulence of *Y. enterocolitica* (Li *et al.*, 1996) and *B. abortus* (Elzer *et al.*, 1994). Recently, the *cpxR/A* system has been shown to be involved in a pH-dependent regulation of expression of the *Shigella sonnei virF* gene which positively regulates the *ipaBCD* invasion genes (Nakayama and Watanabe, 1995). In contrast, the *cpxA* gene of *S. typhi* is osmoregulated suggesting that different environmental signals control the expression of virulence factors. These data suggest that the CpxA/CpxR regulon may play a role in *S. typhi* virulence as well as other pathogens, by modulating the expression of the HtrA protease (Leclerc *et al.*, 1998).

These data should be taken into consideration with respect to further characterising the *htrA* gene of *C. jejuni*. The importance of the Cpx pathway in the context of this project concerns the effect of positive regulation of *htrA* in *E. coli* (Danese *et al.*, 1995; Snyder *et al.*, 1995). Indeed, *cpxA* point mutations show constitutive stimulus-independent production of *htrA* (Danese and Silhavy, 1997). The knowledge that expression of *htrA* in *E. coli* is controlled by the two component regulatory system CpxR/A is extremely pertinent because downstream of *htrA* in *C. jejuni*, there is a partial open reading frame, designated RegX4, which bears homology to members of the OmpR-family of response regulators of which CpxR from *E. coli* is a member. Although not previously determined, it is proposed that the cognate HPK is possibly downstream and the two components function together in response to a specific stimulus. The relevance to the *htrA* situation stems from the fact that two component regulatory systems are known to regulate genes within their immediate vicinity.

To date, information concerning the role of *htrA* in *C. jejuni* has been hampered due to problems associated with cloning the gene and assigning a phenotype to the *htrA* mutant. These problems have been exacerbated by the fact that the gene appears to be expressed at low levels when in single copy on the chromosome under normal growth conditions. It is only now, using a combination of different systems, that expression from the gene can be investigated under different environmental conditions with a view to identifying a possible environmental stimulus. There is also a need to identify how *htrA* is regulated at the genetic level and to identify if there are other members of the regulon. The fact that a response regulator lies downstream of *htrA*, response regulators often have their cognate histidine protein kinase contiguous on the chromosome and finally, that *htrA* in *E. coli* is regulated by a two component regulatory system provides the first opportunity to investigate how *htrA* is regulated. To this end, it is necessary to establish whether a putative two-component

regulatory system is present downstream of *htrA* and whether the response regulator and histidine kinase components form a cognate pair. Furthermore, the individual genes can be mutated to help in their characterisation. With this information, any relationship with the *htrA* gene of *C. jejuni* can be investigated.

Chapter 7: Identification of 3' *regX4* sequence and mutation of the gene.

7.1: Introduction

The *E. coli htrA* gene is regulated, in part, by the two component regulatory system, *cpxR/cpxA*, which is a member of the OmpR-like two-component regulatory family. In the partnership, *cpxR*, is the response regulator component and *cpxA* is its cognate histidine protein kinase sensor. With reference to the *C. jejuni htrA* gene, (Taylor and Hiratsuka, 1990; Henderson, 1996) there is a partial open reading frame of a putative response regulator downstream, designated *regX4*. Preliminary information regarding *regX4* suggests that the gene may also be a member of the same OmpR-like family. Often closely associated with the response regulator, either upstream or downstream is the cognate histidine protein kinase. The interaction of the two components in response to a specific stimulus brings about expression or suppression of particular genes under their control. As two component regulators often control the transcription of nearby genes and the fact that there is a putative two-component system downstream of *htrA* the logical step was to investigate the response regulator further and to examine the presence of the cognate histidine protein kinase. As a consequence, two major aims were identified for this section of the project. As only partial 5' coding sequence of the gene had been identified (Taylor and Hiratsuka, 1990; Henderson, 1996) the first aim was to obtain DNA sequence to complete sequence of the gene and to look further downstream to see whether the HPK was present. The completed gene sequence of *regX4* would then enable further insight into the identification of the gene and enable additional characterisation. The second aim was to create an isogenic mutant in the gene using sequence previously identified. This would enable phenotypic analysis of the mutant, and furthermore, facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*.

In order to identify the 3' end of the response regulator gene and additional sequence downstream, a number of approaches were feasible. The first possibility involved creating a plasmid based genomic library which could subsequently be probed with sequence specific to *regX4*. This was decided against given the problems of instability which surround this locus. The second possibility would have taken advantage of a cosmid based genomic library constructed in this laboratory and described by Karlyshev *et al.*, (1998). A probe of the library with sequence specific to *htrA* and other genes in the laboratory, identified that the number of positive clones with respect to *htrA* were significantly less than for other genes suggesting further problems surrounding this region of DNA. Additionally, the library was also known to be

unstable. Therefore, given these problems it was decided that an alternative, more direct approach, be used.

7.2: Strategy to identify 3' *regX4* and additional sequence downstream

The 2.38kb fragment identified by Henderson (Henderson, 1996) contains 399 bp of the putative response regulator *regX4*, downstream of the *htrA* gene. As the gene was found to be located on a *Bgl*II chromosomal fragment, the sequence terminates on a *Bgl*II restriction enzyme site. In contrast, the sequence given by Taylor and Hiratsuka (1990) was identified differently and extends past the internal *Bgl*II site of *regX4* and terminates at the 3' end, on an *Eco*RI restriction site (figure 7.1.). This relates to a further 195bp of *regX4* sequence giving the total response regulator sequence identified as 594bp. As response regulators are typically between 660 and 720bp the sequence left to identify is minimal. The extra sequence identified by Taylor and Hiratsuka (1990), was fundamental to the strategy used to identify the complete response regulator gene and further sequence downstream. This additional sequence could be amplified by PCR and used to probe *Bgl*II digested *C. jejuni* 81116 DNA by Southern hybridisation. The possibility of using IPCR technology on chromosomal loops could allow cloning of the downstream *Bgl*II fragment and allow sequencing analysis on the plasmid. This could enable identification of the 3' end of *regX4* gene and the putative cognate histidine protein kinase downstream.

7.3: Results

7.3.1: Identification of the size of the *Bgl*II fragment downstream of the *htrA* *Bgl*II fragment.

To facilitate the use of IPCR on chromosomal loops instead of probing a genomic library, it was first necessary to establish the size of the *Bgl*II fragment downstream of the *htrA* *Bgl*II fragment by Southern hybridisation. For this purpose, *Bgl*II digested *C. jejuni* 81116 chromosomal DNA was probed with part of the DNA specific to Taylor's sequence downstream of the 3' *Bgl*II site. A PCR reaction (95°C melting 1 min, 55°C annealing 1 min, and 72°C extension 30 secs) (section 2.13.1.) using primers R4ID2 and R4U1 (appendix 1) (figure 7.1.) and a 1/1000 dilution of 81116 chromosomal DNA amplified a product of a size consistent with the 136bp fragment size predicted from the sequence. The resultant product was electrophoresed on a 1% agarose

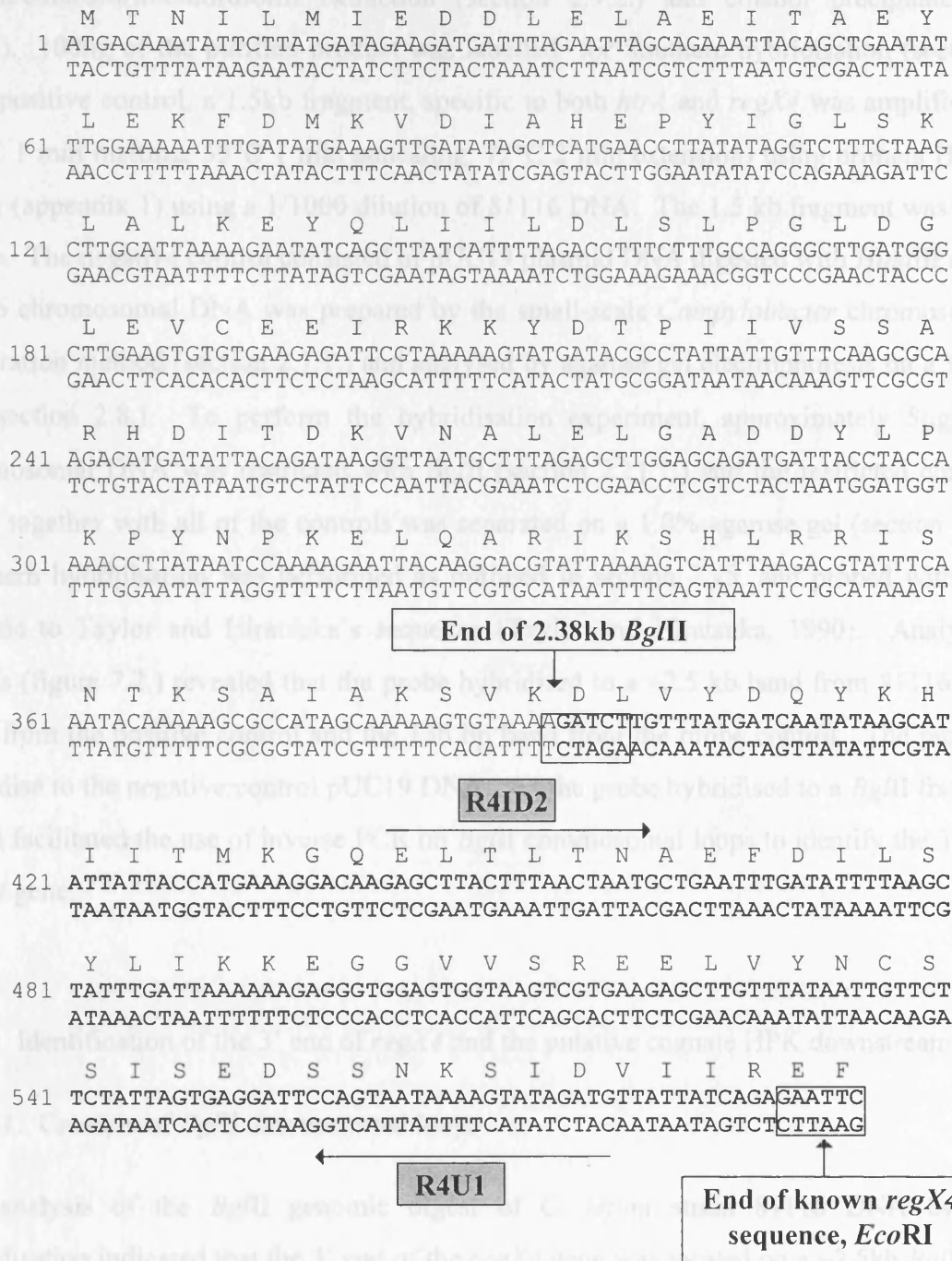


Figure 7.1: Diagram outlining how the sequence of Taylor and Hiratsuka, (1990) and Henderson, (1996) relate to one another with respect to *regX4*. Normal typeface relates to sequence identified by both groups. The sequence identified by Henderson, (1996) terminates at a *Bgl*II site, shown in the labelled box. The sequence in bold typeface relates only to the extra sequence identified by Taylor and Hiratsuka and terminates at an *Eco*RI site, shown in a labelled box. Primers R4ID2 and R4U1 highlighted in grey boxes were used to amplify a probe to identify downstream *Bgl*II fragment.

gel (section 2.8.), excised, purified by polyallomer wool gel extraction (section 2.9.1.) and phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). 100ng of the purified product was labelled for Southern hybridisation (section 2.15.). As a positive control, a 1.5kb fragment, specific to both *htrA* and *regX4* was amplified by PCR (95°C 1 min melting, 55°C 1 min annealing, 72°C 2 min extension) using primers HTRA7 and R4U1 (appendix 1) using a 1/1000 dilution of 81116 DNA. The 1.5 kb fragment was purified as above. The negative control consisted of pUC19 plasmid DNA digested with *HindIII* and *EcoRI*. 81116 chromosomal DNA was prepared by the small-scale *Campylobacter* chromosomal DNA preparation method (section 2.7.1.) and analysed by agarose gel electrophoresis on a 1% agarose gel (section 2.8.). To perform the hybridisation experiment, approximately 5µg of 81116 chromosomal DNA was restricted with *Bgl/II* (section 2.11.1.) and the restricted chromosomal DNA together with all of the controls was separated on a 1.0% agarose gel (section 2.8.). The Southern hybridisation was performed as outlined in section 2.15. and probed with the DNA specific to Taylor and Hiratsuka's sequence (Taylor and Hiratsuka, 1990). Analysis of the results (figure 7.2.) revealed that the probe hybridised to a ~2.5 kb band from 81116, the 1.5kb band from the positive control and the 136 bp band from the probe control. The probe did not hybridise to the negative control pUC19 DNA. As the probe hybridised to a *Bgl/II* fragment of < 4kb it facilitated the use of inverse PCR on *Bgl/II* chromosomal loops to identify the 3' end of the *regX4* gene.

7.3.2: Identification of the 3' end of *regX4* and the putative cognate HPK downstream

7.3.2.1: Creation of *Bgl/II* chromosomal loops

The analysis of the *Bgl/II* genomic digest of *C. jejuni* strain 81116 DNA by Southern hybridisation indicated that the 3' end of the *regX4* gene was located on a ~2.5kb *Bgl/II* fragment downstream of the previously identified 2.38kb *Bgl/II* fragment. 5µg of genomic *C. jejuni* strain 81116 DNA was digested with *Bgl/II* overnight (section 2.11.1.) and subsequently purified using phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). The digested genomic DNA was then ligated in a volume of 200µl to create genomic DNA loops. The DNA was ligated at low concentration to promote the formation of intramolecular loops rather than concatamers of digested DNA. The genomic DNA *Bgl/II* loops were then purified by phenol/chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.) and used as template DNA for subsequent inverse PCR amplification.

1.3.2.2: Inverse PCR on *Bgl*II chromosomal loops

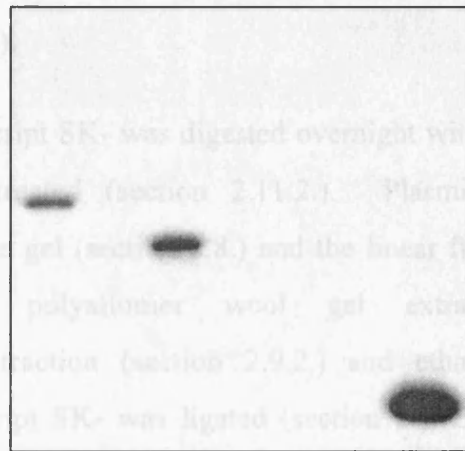
Inverse PCR (95°C, 1 min melting, 55°C, 1 min annealing, 72°C, 3 min extension) using primers R4U2 (5' *Bam*HI) and R4ID2 (5' *Bgl*II) (appendix 1) was performed on chromosomal loops (typical concentration of total DNA 100µg/µl to 500µg/µl) and amplified a single ~2.5kb product. The strategy is outlined diagrammatically in figure 7.3. The size of the amplified product was consistent with the size of the product obtained from the Southern hybridisation analysis. The 2.5kb PCR fragment was purified using the Qiaquick PCR purification kit (section 2.9.4) and digested overnight with *Bgl*II (section 2.11.1). The digested product was electrophoresed on a 1% agarose gel (section 2.9.1) and purified using polyallomer wool gel extraction (section 2.9.1), phenol/chloroform/chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3).

Similarly, 2µg of plasmid pBlueScript SK- was digested overnight with *Bam*HI (section 2.11.1) and subsequently phenol/chloroform extracted (section 2.9.2). Plasmid pBlueScript SK- was electrophoresed on a 1% agarose gel (section 2.9.1) and the linear fragment was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1), phenol/chloroform/chloroform extraction (section 2.9.2) and ethanol precipitated (section 2.9.3). Linear plasmid, pBlueScript SK- was ligated (section 2.12.1) to the digested ~2.5kb PCR product overnight. The ligation was transformed into *E. coli* DH5α by electroporation (section 2.12.1) and transformants were plated out. Recombinant clones were checked for the

Figure 7.2: Southern hybridisation to identify the next *Bgl*II fragment downstream of the original *htrA* containing 2.38kb *Bgl*II fragment. Left, λ *Hind*III/ ϕ x*Hae*III marker (kilobase pairs), Lane 1; *Bgl*II digested 81116 DNA, Lane 2; positive control PCR using HTRA7 and R4U1, Lane 3; negative control pUC19 DNA digested with *Hind*III and *Eco*RI, Lane 4; Probe DNA from PCR using R4ID2 and R4U1.

23.13 =
9.42 =
6.56 =
4.36 =

2.32 =
2.03 =
1.35 =
1.08 =
0.87 =
0.60 =
0.31 =



7.3.2.2: Inverse PCR on *Bgl*II chromosomal loops

Inverse PCR (95°C, 1 min melting, 55°C 1 min annealing, 72°C 3 mins extension) using primers R4IU2 (5' *Bam*HI) and R4ID2 (5' *Bgl*II) (appendix 1) was performed on chromosomal loops (typical concentration of total DNA 100ng/μl to 500ng/μl) and amplified a single ~2.5kb product. The strategy is outlined diagrammatically in figure 7.3. The size of the amplified product was consistent with the size of the product obtained from the Southern hybridisation analysis. The 2.5kb PCR fragment was purified using the Qiaquick PCR purification kit (section 2.9.4.) and digested overnight with *Bgl*II (section 2.11.1.). The digested product was electrophoresed on a 1% agarose gel (section 2.8.), excised, and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

Similarly, 2μg of plasmid pBluescript SK- was digested overnight with *Bam*HI (section 2.11.1.) and subsequently phosphatase treated (section 2.11.2.). Plasmid pBluescript SK- was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear fragment was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). Linear plasmid, pBluescript SK- was ligated (section 2.11.3.) to the digested ~2.5kb PCR product overnight. The ligation was transformed into *E. coli* DH5α by electroporation (section 2.12.1.) and transformants were plated out. Recombinant clones were checked for the insertion of the ~2.5 kb fragment by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.2.) using a combination of primers; either M13 Forward and R4ID2 or M13 Reverse and R4ID2 (appendix 1). A positive result for either PCR yielded a product size ~2.5 kb and confirmed a different orientation for the insert. From the results of the PCR, the plasmid was designated either pME23 if the 3' end of *regX4* was inserted in the same orientation as *lacZ* or pME24 if the insert was in the opposite orientation. As the two plasmids contain the same insert DNA but in different orientations it was decided that any subsequent work be performed on plasmid pME23 only. In order to identify the 3' end *regX4* gene and to investigate the presence of the cognate HPK downstream, plasmid pME23 insert DNA was sequenced by progressive primer walking using primers M13 Reverse and M13 Forward, R4D-SEQ1, R4D-SEQ2, R4D-SEQ3 and R4D-SEQ4 (appendix 1). The sequence data identified in this section of the project completes that previously identified (Taylor and Hiratsuka, 1990; Henderson, 1996). The 3' end of *regX4* was identified and is given in figure 7.4. Downstream of *regX4* there is a overlapping reading frame which has homology to

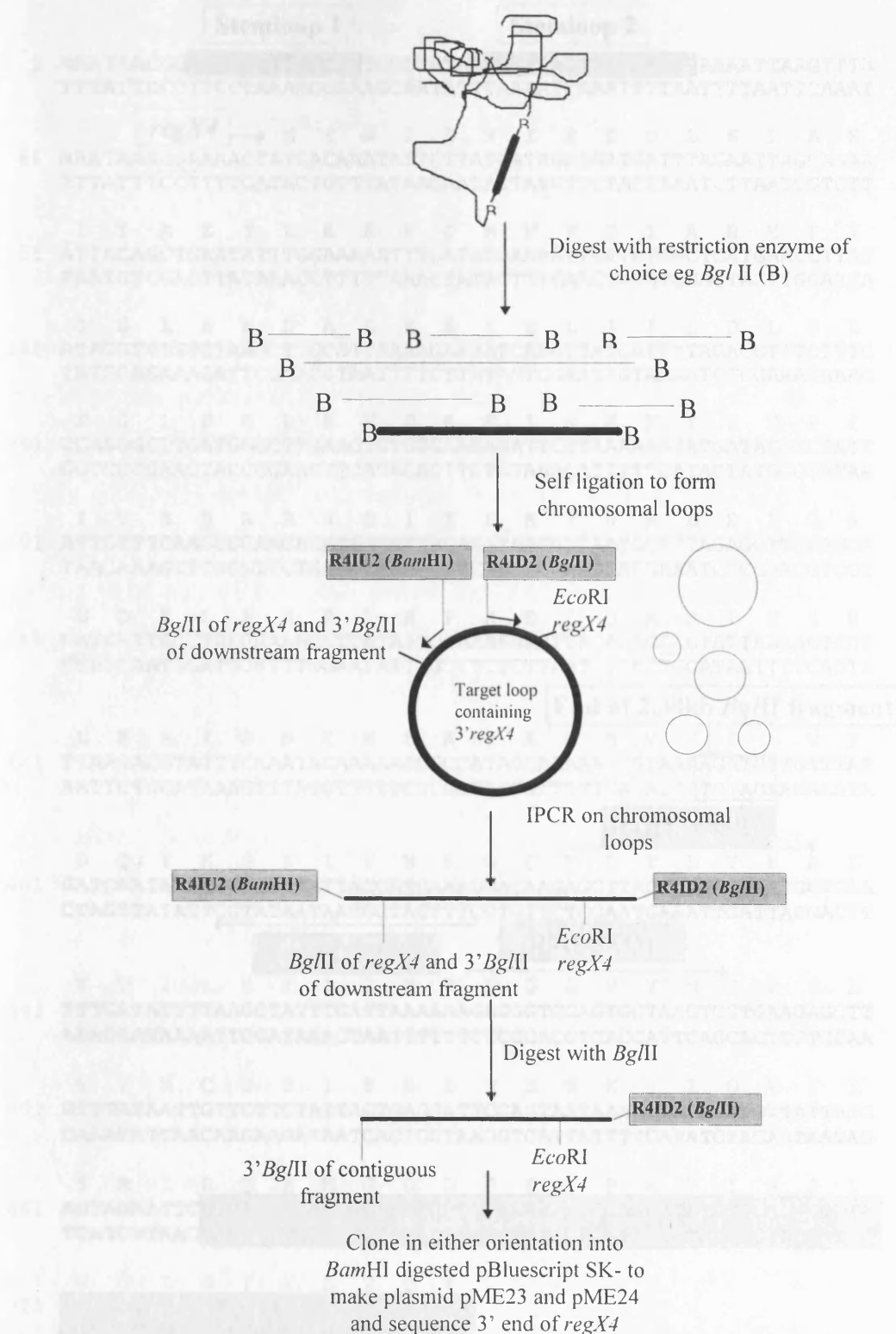


Figure 7.3: Diagrammatic representation of the strategy to identify the 3' end of *regX4*.

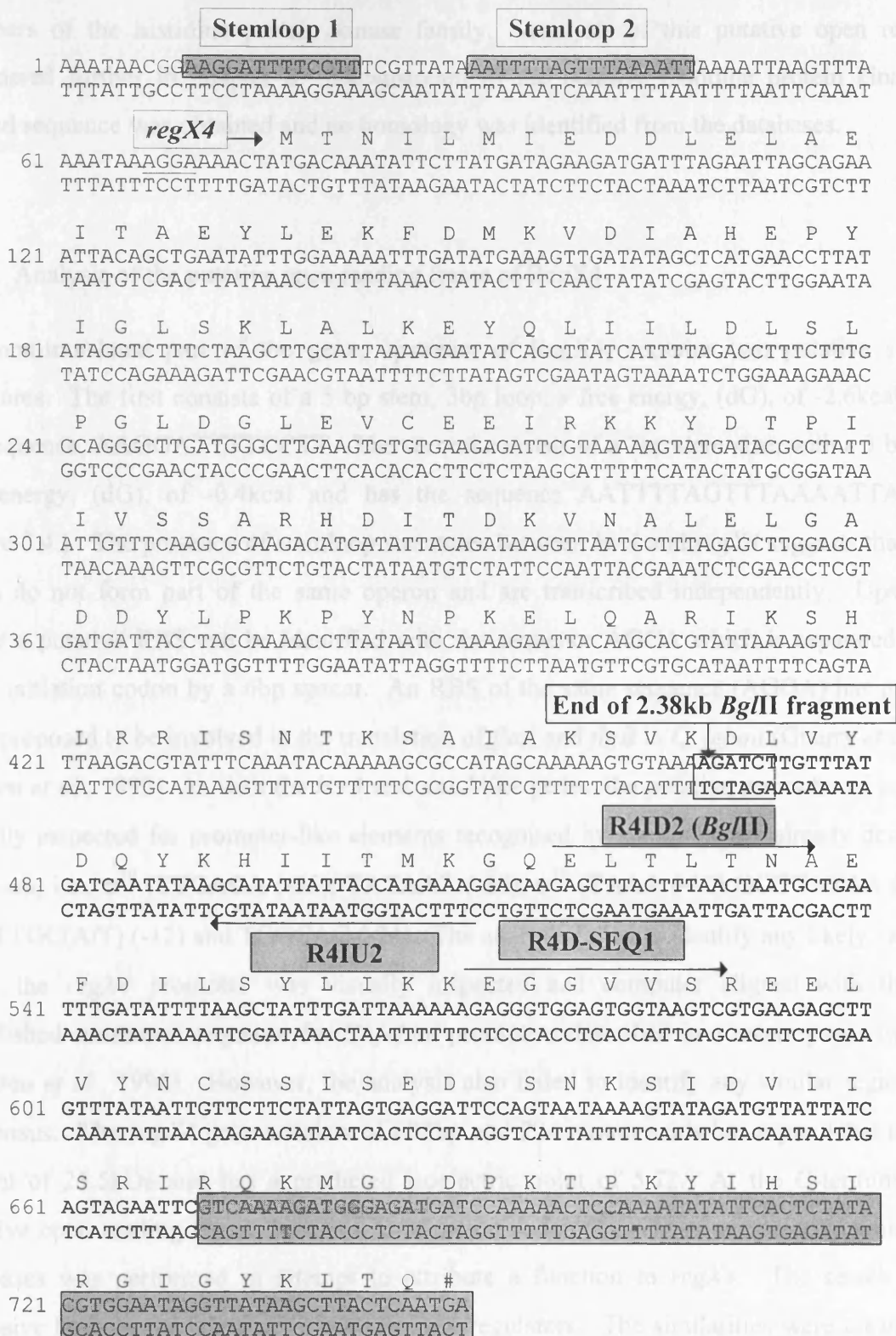


Figure 7.4: The completed sequence of *regX4*. The two stemloop structures in the untranslated region of *regX4* are highlighted by shaded boxes at the top of the figure together with the putative RBS of *regX4* which is underlined. The start of *regX4* is given together with the end of the 2.38kb *Bg/III* fragment and the position at which both R4ID2 and R4IU2 bind. The position of the sequencing primer R4D-SEQ1 is also shown by a labelled box. Bold typeface represents sequence specific to that identified by Taylor. Newly identified sequence in this project is denoted by the shaded box.

members of the histidine protein kinase family. Analysis of this putative open reading is considered further in chapter 8. Downstream of the putative histidine protein kinase, only limited sequence was obtained and no homology was identified from the databases.

7.3.3: Analysis of the putative open reading frame of RegX4

The non-translated part of the gene, upstream of RegX4, contains two putative stem-loop structures. The first consists of a 5 bp stem, 3bp loop, a free energy, (dG), of -2.6kcal and has the sequence, AAGGATTTTCCTT. The second consists of a 7bp stem size, with a 3 bp loop, a free energy, (dG), of -0.4kcal and has the sequence AATTTTAGTTTAAAATTAAAATT (figure 7.4.). The presence of stemloop structures between *htrA* and *regX4* suggests that the two genes do not form part of the same operon and are transcribed independently. Upstream of *regX4* a putative RBS can be identified with the sequence AGGA which is separated from an ATG initiation codon by a 6bp spacer. An RBS of the same sequence (AGGA) has previously been proposed to be involved in the translation of *flaA* and *flaB* in *C. jejuni* (Guerry *et al.*, 1992; Nuijten *et al.*, 1992). As with the *htrA* and *dnaJ*-like genes, the putative promoter of *regX4* was visually inspected for promoter-like elements recognised by sigma-factors already described in *C. jejuni*, i.e., σ^{70} (TTGACA (-35), TATAAT (-10), σ^{28} (TAAA (-10) GCCGATAA (-35) and σ^{54} (TTGC(A/T) (-12) and TGGCAC (-24). The analysis failed to identify any likely candidates. Also, the *regX4* promoter was visually inspected and computer aligned with the newly established consensus sequence for *C. jejuni* promoters described in a recent paper by Wösten (Wösten *et al.*, 1998). However, the analysis also failed to identify any similar regions to the consensus. The *regX4* gene consists of 672bp and 224 amino acids has a predicted molecular weight of 25.5kDa and has a predicted isoelectric point of 5.72. At the C-terminus of the putative open reading frame there is a termination codon (TGA). A comparative search of the databases was performed in attempt to attribute a function to *regX4*. The search revealed extensive homology with many other response regulators. The similarities were greatest at the N-terminus but considerable homology was seen over the whole length of the open reading frame. The 10 most similar proteins over the whole sequence are given in table 7.1., together with their identity and similarity. A computer aided alignment of the protein sequences is given in figure 7.5. The best similarities were observed with RacR, (reduced ability to colonise), a *C. jejuni* response regulator recently identified in this laboratory (50%) (Brás and Ketley, 1996), and an OmpR-like family protein from the *H. pylori* genome sequencing project (51%)(HP0166)

(Tomb *et al.*, 1997). Another 30 response regulator proteins showed 27-38% identity over the whole length of the response regulator protein. The *N*-terminus of response regulator proteins is the most conserved domain. However, there is a certain level of homology between the *C*-terminus domain of response regulators which have the same adaptive responses. Therefore, in light of new *C*-terminus specific sequence data, identified in this project, a putative role for *regX4* was again investigated. To this end, the different databases were searched using the *C*-terminus domain alone. The 10 most similar proteins with respect to the *C*-terminus are given in table 7.2. together with their identity and similarity. A computer aided alignment of the *C*-termini is given in figure 7.6. Once more the best identities were observed with RacR, (55%) (Brás and Ketley, 1996), and the *H. pylori* OmpR-like gene (52%) (HP0166) (Tomb *et al.*, 1997). Other response regulator proteins showed less identity over the *C*-terminus although there are still areas which align very well with *C*-terminal domains of other proteins in the same sub-family. Despite homology with numerous other response regulators the homology scores were generally very low resulting in a failure to establish a function for *regX4*.

Response regulators are grouped into several subfamilies on the basis of the similarity of their *C*-termini. Three of the main subfamilies include the NtrC sub-family, the OmpR sub-family and the FixJ sub-family (Bourret *et al.*, 1989; Stock J.B. *et al.*, 1989; Parkinson, 1993). Given that new sequence data specific for the *C*-terminus has been identified in this project, the regulator was determined and confirmed as being a member of the OmpR sub-family.

Most members of the OmpR sub-family of response regulator proteins contain an *N*-terminal regulatory domain and a *C*-terminal binding domain (Stock J.B. *et al.*, 1989; Bourret *et al.*, 1990). Predictions concerning the protein structure are based on the known three-dimensional structure of the chemotaxis response regulator, CheY (Stock A.M *et al.*, 1989; Stock J.B. *et al.*, 1989) and the recently determined crystal structures of *E. coli* OmpR DNA-binding domain (OmpRc) (Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997a) and NarL (Baikalov *et al.*, 1996). The RegX4 protein, together with CheY, OmpR and the 10 most similar proteins over the whole of the sequence were aligned to establish a framework for examining any conserved features. The alignment is given in figure 7.7A and B. The *N*-terminal regulatory CheY consists of five parallel β strands surrounded by five amphipathic α -helices and in general differences between these structures for response regulators can be seen in the loops connecting the secondary structural elements. For the most part, specific homology is seen with residues that correspond to those residues which form the hydrophobic core of CheY although there is not

Table 7.1: The 10 most similar proteins to RegX4 together with their associated identity and similarity

Top 10 homology scores with *C. jejuni* open reading RegX4

Gene name, Species name, Accession number (Acc.) and Protein identification number (PID)	Identity to RegX4 (%)	Similarity to RegX4 (%)
• <i>ompR</i> -like <i>Helicobacter pylori</i> . Acc AF000537; PID g2313252 (GenEMBL)	• 51	• 68
• <i>racR</i> , <i>Campylobacter jejuni</i> . Acc AF053960; PID g2984736 (GenEMBL)	• 50	• 71
• <i>cpxR</i> homologue, <i>Haemophilus influenzae</i> . Acc P44895; PID g323610 (GenEMBL)	• 38	• 60
• <i>czcR</i> <i>Ralstonia eutropha</i> . Acc Q44006; PID g25000741 (SWISSPROT)	• 37	• 55
• <i>yycF</i> (<i>phoP</i> -like) <i>Bacillus subtilis</i> . Acc P37478; PID g586808 (SWISSPROT)	• 36	• 59
• <i>ycf27</i> (<i>ompR</i> -like) <i>Porphyridium aeruginium</i> . Acc P28835; PID g129159 (SWISSPROT)	• 36	• 54
• <i>RegX3</i> <i>Mycobacterium tuberculosis</i> . Acc Q11156; PID g1731207 (SWISSPROT)	• 35	• 59
• *** <i>yv17</i> (<i>phoP</i> -like) <i>Mycobacterium leprae</i> . Acc P54884; g1731206 (SWISSPROT)	• 35	• 59
• <i>ycf27</i> (<i>ompR</i> -like) <i>Cyanaphora paradoxa</i> . Acc P48259; PID g1351749 (SWISSPROT)	• 35	• 55
• <i>ResD</i> <i>Bacillus subtilis</i> . Acc p35163; PID g466194 (SWISSPROT)	• 32	• 53

***The *yv17* (*phoP*-like) gene entry in GenBank is incomplete because it lacked the *N*-terminal 30 amino acids which almost invariably contain a highly conserved residue corresponding to Asp-13 of CheY, which forms part of the acidic pocket within the active site of response regulators. An analysis by Via *et al.*, (1996) discovered this anomaly and inserted a nucleotide between positions 36589 and 36588 based on the sequence of the response regulator *mtrA* identified in their study. Any subsequent analyses using *yv17* is based on their revised sequence.

Table 7.2: The 10 most similar proteins to RegX4 over the C-terminus (amino acids 136-224) together with their associated identity and similarity

Top 10 homology score with C-terminus of <i>C. jejuni</i> RegX4		
Gene name, Species name, Accession number (Acc.) and Protein identification number (PID)	Identity to RegX4 (%)	Similarity to RegX4 (%)
• <i>racR</i> , <i>Campylobacter jejuni</i> . Acc AF053960; PID g2984736 (GenEMBL)	• 55	• 73
• <i>ompR</i> -like <i>Helicobacter pylori</i> . Acc AF000537; PID g2313252 (GenEMBL)	• 52	• 69
• <i>virG</i> <i>Agrobacterium tumefaciens</i> . Acc P07545; PID g138586 (SWISSPROT)	• 39	• 64
• <i>virG</i> <i>Agrobacterium rhizogenes</i> . Acc P13359; PID g138585 (SWISSPROT)	• 37	• 63
• <i>phoP</i> <i>Bacillus subtilis</i> . Acc P13792; PID g400783 (SWISSPROT)	• 37	• 60
• <i>yv17</i> (<i>phoP</i> -like) <i>Mycobacterium leprae</i> . Acc P54884; g1731206 (SWISSPROT)	• 34	• 61
• <i>RegX3</i> <i>Mycobacterium tuberculosis</i> . Acc Q11156; PID g1731207 (SWISSPROT)	• 34	• 60
• <i>ylcA</i> (<i>copR</i> -like) <i>Escherichia coli</i> . Acc P77380, PID g250749 (SWISSPROT)	• 34	• 57
• <i>yycF</i> (<i>phoP</i> -like) <i>Bacillus subtilis</i> . Acc P37478; PID g586808 (SWISSPROT)	• 32	• 60
• <i>afsQ1</i> <i>Streptomyces coelicolor</i> Acc Q04942; PID g543777 (SWISSPROT)	• 30	• 54

Chapter 7: Identification of 3' *regX4* sequence and mutation of the gene

CJregX4	-----MTNILMIEEDLELAETAEYLEKFDPMKVDAHEPYIGLSKLALKEYQLIIEISL	55
HPompR-like	-----MIEVLMIEEDIELAEFLLSEFLLQHGHIHTNYDEPYTGISAANTQNYDLLLLIELTL	55
CJRacR	-----MINVLMIEEDPDFAQLLSEYLAQFNKITNFENPKSALNVG-IQGYDCLIIIDTL	54
MLYv17	-----MISVLIVIEEESLADPLAFLLRXXGFEPVVDGPAALAEFDRSGADIVLIEIIML	26
HICpxRhom	-----MSKLLLVIEEDIELTELLLELEGFVDVETANNGLEALQKLN-ESYKLVLIIVMM	54
RECzcR	-----MRVLVVEIEEPRTAEYLQKGLSESGFVVDIANNNGDGLHMAEETDYDVIIIEVML	54
BSYycF	MDK-----KILVVIIEKPIADILEFNLRKEGYEVHCAHDGNEAVEMVEELQPDILIIIML	56
PAYcf27	---MQKEKILVIEEASIRRIETRLSIIGYDVISAADGEEALSIFKREHPNLVVIIEIMM	57
MTRegX3	-----MTSVLIVIEEESLADPLAFLLRKEGFVVDGPAALAEFDRAGADIVLIEIIML	55
CPYcf27	-MEINKKKILVVEIEISIRRIETRLSMIGYEVVTAADGEEALTIFQLEHPNLVVIIEVMM	59
BSResD	MDQTNETKILVVEIEAIRIRLLRMYLEENYAIDEAENGDEAIKGLEANYDLIIIEIMM	60
	* * * * *	
CJRegX4	PGLDGLEVCEEIRKKYDTPIIIVSSARHDITDKVNALELGADDYLRKPYNPKEQLQARIKSH	115
HPompR-like	PNLDGLEVCRRISKQKHPIIISSARSDVEDKIKALDYGADDYLRKPYDPKELLARIQSL	115
CJRacR	PGIDGLEVCREIRQKSNIPIIISSARGDLSDKVVGLQIGADDYLRKPYDPKEMYARIMSL	114
MLYv17	PGMSGTDVYKQLRVSSVPVIMVTARDSEIDKVVGLELGADDYVTKPYSAELIARIRAV	86
HICpxRhom	PKLNGIETLKEIRKVSNNVPMMLTARGEDIDRVLGLELGADDCLRKPFNDRELIARIKAI	114
RECzcR	PGMDGWTVIKSIRSKSETPVLFALTALDDVADRVRGFELGADDYLVKPFFAEALLARIRRC	114
BSYycF	PNKDGVEVCREVKKYDMPIMMLTAKDSEIDKVGLEIGADDYVTKPFSTRELLARVKAN	116
PAYcf27	PKLDGYGVCQELRKESDVPIMMLTALSDVSDRITGLELGADDYVTKPFSPKELEARIRSV	117
MTRegX3	PGMSGTDVCKQLRARSSVPVIMVTARDSEIDKVVGLELGADDYVTKPYSAELIARIRAV	115
CPYcf27	PKLDGYGVCQELRKESDIPIMMLTALGDVADRITGLELGADDYVTKPFSPKELEARIRSL	119
BSResD	PGTDGIEVCRQIREKATPIIMMLTAKGEEANRVQGEAGTDDYVTKPFSPREVLRVKAL	120
	* * . . . : * . . . : * . . . : * . . . : *	
CJRegX4	LRR-----ISNTKSAIAKSVKDLV-YDQYKHIIITMKGQELTLTNAEFDILSYLIKKEGG	168
HPompR-like	LRR-----SHKKEEVSEPGDANIFRVDKDSREVYMHKKLDLTRAEEYILSLLISKKG	169
CJRacR	IRR-----TKRVEHTNNENINSAFKIDERREHETIEDKVLTLTPAEFEILEYLIQQHGY	168
MLYv17	LRRGGD---DDSEISDGVLESGPLRMDVERHVSVNGYAITLPLKEFDLLEYLMRNSGR	142
HICpxRhom	LRRSAS---PSNNISNVEILSFDGITLHFHSHGIATYNEENLNLTDYEFKILCLLLKSKGN	171
RECzcR	LR-----QSTSKESERLRIADLDIDVLGRVFRGTTRIELTNQEFSLHLLMRNRGE	166
BSYycF	LRRQLTTAPAE-EPSSNEIHIGSLVIFPDAYVVSKRDETIELTHREFELLHYLAKHIGQ	175
PAYcf27	LRRVDKAS-SNNNLPNSGIINIGFLKIDVNKHQVYKNNERVRLTGMFSLLELLISKAGQ	176
MTRegX3	LRRGGD---DDSEMSDGVLESGPVRMDVERHVSVNGDTITLPLKEFDLLEYLMRNSGR	171
CPYcf27	LRRNTNPT-LSTSTNGENLQIGFLKIDINKRQVFKNGERIRLTGMFSLLELLISKMG	178
BSResD	LRRASQTSYFNANTPTKNVLVFSHLSIDHDAHRVTADGTEVSLTPKVYELLYFLAKTPDK	180
	: * . . . : * . . . : * . . . : *	
CJRegX4	VVSREELVYNCSSESIDS--SNKSIDVIIISRIQKMGD-DPKTPKYIHSIRGIGYKLTQ-	224
HPompR-like	VFSRESIAIESINPES--SNKSIDVIIIGRLRSKIEK-NPKQPQYIISVRGIGYKLEY-	225
CJRacR	SVSREQLVSRCKNLKDKD--S-KSLDVIIIGRLRVKIGD-SSKSPKHIFSVRGIGYKLIG-	223
MLYv17	VLTRGQLIDRVWGVYDVG--DTKTLDVHVKKRLRSKIEA-DPANPVHLVTVRGLGYKLES-	198
HICpxRhom	VVSREELSLEVMEKPLTP--FDRSLDMHISNLRRLPK-RKNKPSWFKTLRGKGYALVT-	227
RECzcR	VLSRTTIASQVWGVNFD--TDTNVVDVAIRRLRSKV-D-DPFDQKLIHTVRGMGYVLDPE	222
BSYycF	VMTREHLLQTWVGYYDFG--DVRTVDVTVRRLREKIED-NPSHPNWIVTRRGVGYLRNP	232
PAYcf27	PFSRATILQEVWGYTAERQVDTRVDVHISRLRAKLED-DPSNPDLILTARGTGFLFQRL	235
MTRegX3	VLTRGQLIDRVWGVYDVG--DTKTLDVHVKKRLRSKIEA-DPANPVHLVTVRGLGYKLEG-	227
CPYcf27	PFSRAQILQEVWGYTPERHIDTRVDVHISRLRSKLEE-NPSNPDLILTARGIGYLFQNT	237
BSResD	VYDREKLLKEVWQYEFFG--DLRTVDTHVKRLREKLNKVSPEAAKIVTVWVGKGYKFEVG	238
	* : . : * : . : * : . : *	
CJRegX4	-----	
HPompR-like	-----	
CJRacR	-----	
MLYv17	-----	
HICpxRhom	-----	
RECzcR	RGR-- 225	
BSYycF	EQD-- 235	
PAYcf27	NDSIV 240	
MTRegX3	-- ---	
CPYcf27	SN--- 239	
BSResD	AE--- 240	

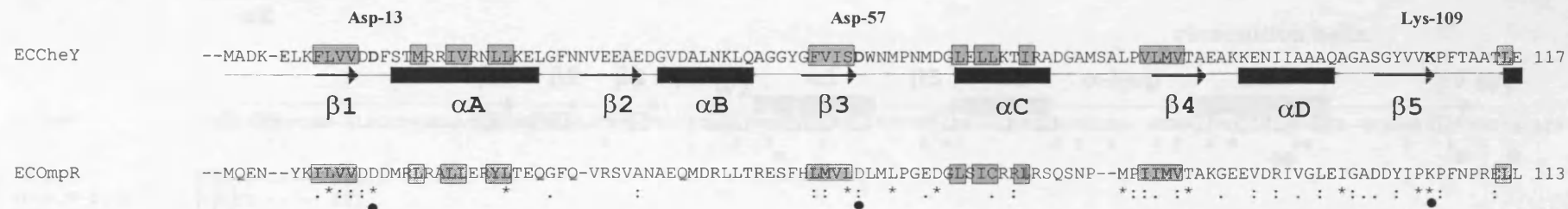
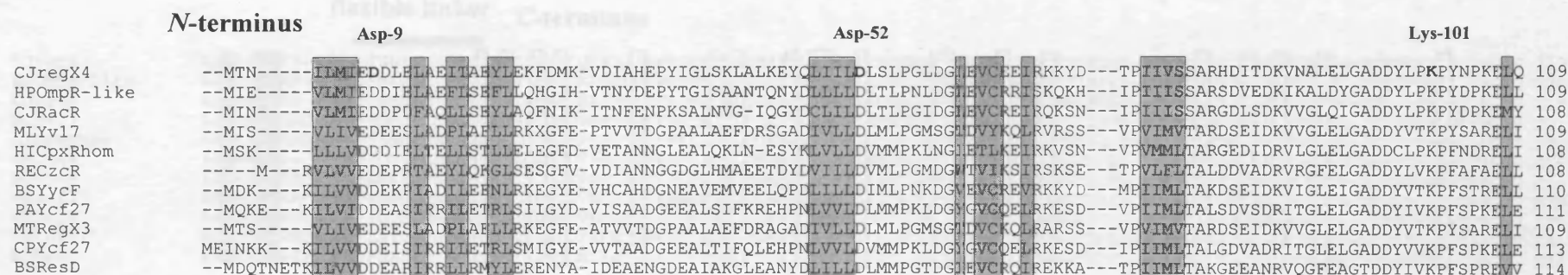
Figure 7.5: Comparative sequence alignment of predicted amino acid sequence of RegX4 with 10 most similar proteins. CJregX4; *C. jejuni* RegX4, HPompR-like; *H. pylori* OmpR-like protein, CJRacR; *C. jejuni* RacR, MLYv17; *M. leprae* YV17 (PhoP-like) HICpxRhom; *H. influenzae* CpxR homologue, RECzcR; *R. eutropha* CzcR, BSYycF; *B. subtilis* YycF (*phoP*-like gene), PAYcf27; *P. aeruginum* Ycf27 (OmpR-like gene), MTRegX3; *M. tuberculosis* RegX3, CPYcf27; Ycf27 (OmpR-like protein) *C. paradoxa*, BSResD; *B. subtilis* ResD. The grey boxes highlight highly conserved amino acids among all response regulators. An “*” denotes conserved residue, “:” denotes conserved substitution and “.” denotes semi-conserved substitution for the alignment given.

CJregX4	DQYKHIITMKGQELTLTNAEFDILSYLIKKEGGVVSREELVYNCSSISEDSSNKSIDVLIISRLRQKMGGDDPKTPKYIHSIRGIGYKLTQ-----	224
CJRacR	DERRHEITYEDKVLTLTPAEFEILEYLIQQHGYSVSREQLVSRCKNLKDKDS-KSLDVIIGRLRVKIGDSSKSPKHIFSVRGIGYKLG-----	223
HPompRlike	DKDSREVMHEKKLDLTRAHEYELSLISKKGYSVSRESIAIESESINPESSNKSIDVLIIGRLRSKIEKNPKQPQYIISVRGIGYKLEY-----	225
ATVirG	LRRRRLISEEGSEVKLTAGEFNLLVAFLEKPRDVLVSREQLLIASRVREEEVYDRSIDVLILRLRRKLEGDPTTPQLIKTARGAGYFFDADVDVSYGGVMAA	253
ARVirG	IRQRRLISEEGGEIKLTAGEFNLLVAFLEKPRDVLVSREQLLIASRVREEEVYDRSIDVLIIFRLRRKLEGDPTSPQLIKTARGAGYFFDADVNVSYGGMMAA	241
BSPHoP	LPDHYEAYFKESQLELTPKEFELLVLRHKGRLVTRDLLLLSAVWNYDFAGDTRIVDVHISHLRDKIENNTKKPIYIKTIRGLGYKLEPKMNE-----	240
MLYv17	DVERHVSVNGYAITLPLKEFDLLEYLMRNSGRVLTRGQLIDRVWGVDYVGDTKTLDVHVKRLRSKIEADPANPVHLVTVRGLGYKLES-----	198
MTRegX3	DVERHVSVNGDTITLPLKEFDLLEYLMRNSGRVLTRGQLIDRVWGADYVGDTKTLDVHVKRLRSKIEADPANPVHLVTVRGLGYKLEG-----	227
ECYlcA	DLVSRKVTRSGTRITLTSKEFTLLEFFLRHQGEVLPRSLIASQVWDMNFDSDTNAIDVAVKRLRGKIDND-FEPKLIQTVRGVGYMLEVDPDQG-----	227
BSYycF	FPDAYVVKRDETIELTHREFELLHYLAKHIGQVMTREHLLQTVWGYDYFGDVRTVDVTVRRLREKIEDNPSPHPNWIVTRRGVGYLLRNPEQD-----	235
SCAfsQ1	DRSAMTVTKNGEDLQLTPTELRLLELSRRPGQALSROQLLRVWEHDYLGDSRLVDACVQRLRAKVEDVPSSPTLIRTVRGVGYRLDPPQ-----	225

:* : . ..* : . . :* : :* * : : *

Figure 7.6: Comparative sequence alignment of predicted amino acid sequence of C- terminus of RegX4 with the 10 most similar proteins. CJregX4; *C. jejuni* RegX4, HPompR-like; *H. pylori* OmpR-like protein, CJRacR; *C. jejuni* RacR, ATVirG; VirG *A. tumefaciens*, ARVirG: VirG *A. rhizogenes*, BSPHoP, PhoP *B. subtilis* MLYv17; *M. leprae* YV17 (PhoP-like protein), MTRegX3; *M. tuberculosis* RegX3, ECYlcA: YlcA (CopR-like) *E. coli*, BSYycF; YycF (PhoP-like protein) *B. subtilis* and SCAfsQ1; AfsQ1 *S. coelicolor*. An “*” denotes conserved residue, “:” denotes conserved substitution and “.” denotes semi-conserved substitution.

Figure 7.7A



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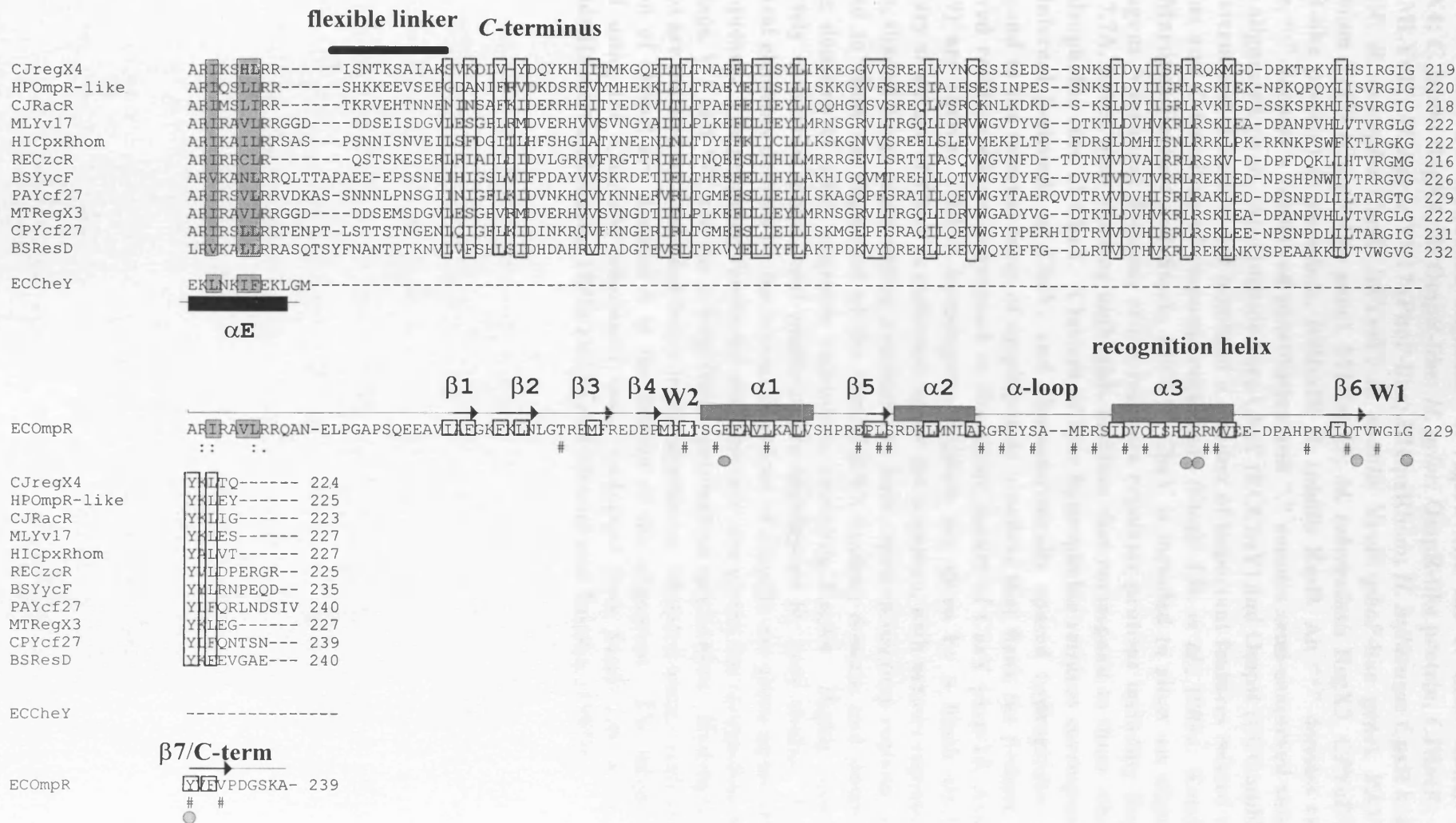


Figure 7.7A and B: Sequence alignment of *regX4* with the 10 most similar proteins CJregX4; *C. jejuni* RegX4, HPompR-like; *H. pylori* OmpR-like protein, CJRacR; *C. jejuni* RacR, MLYv17; *M. leprae* YV17 (PhoP-like) HICpxRhom; *H. influenzae* CpxR homologue, RECzcR; *R. eutropha* CzcR, BSYycF; *B. subtilis* YycF (*phoP*-like gene), PAYcf27; *P. aeruginum* Ycf27 (OmpR-like gene), MTRegX3; *M. tuberculosis* RegX3, CPYcf27; Ycf27 (OmpR-like protein) *C. paradoxa*, BSResD; *B. subtilis* ResD. An “*” denotes conserved residue, “:” denotes a conserved substitution and “.” denotes semi-conserved substitution for the alignment given. Also included are CheY (ECCheY) and OmpR (ECOmpR), which have determined structure and highlight a number of important features related to the *N*-terminus and *C*-terminus of response regulators (Stock J.B. *et al.*, 1989a; Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997b). CheY is included to allow an alignment of homologous *N*-terminal domains of the response regulator proteins including RegX4. In figure 7.7A, boxed grey shading highlights residues that correspond to those which form the hydrophobic core of CheY. Clusters of four hydrophobic residues correspond to the three internal β -strands of CheY, and characteristically spaced hydrophobic residues correspond to the internal faces of amphipathic α -helices that flank the β -sheet. Highly conserved residues which correspond to the acidic pocket of CheY (Asp-13, Asp-57 and Lys-109) are shown. Other homologous residues are given by a black circle. The secondary structure of CheY is indicated below the sequence, with arrows representing β -strands, shaded boxes representing α -helices and lines representing loop regions. OmpR is included to allow an alignment of the OmpR-DNA binding domain and putative DNA binding domains of other response regulators, including RegX4. Highly conserved to completely conserved amino acid positions are highlighted by grey circles. Secondary structural elements defined by the crystal structure of OmpRc are given above the amino acid sequence. The proposed functional elements are also given; the recognition helix, the two wings, W1 and W2 and the α -loop (RNA polymerase interaction). Hydrophobic core residues are marked by unshaded boxes in the alignment. Mutated amino acids that affect function of OmpR are denoted # at the bottom of the alignment. The alignment was created using the ClustalW alignment tool. (Adapted from Stock J.B. *et al.*, (1989), Martinez-Hackert and Stock, (1997a and b) and Mizuno and Tanaka, (1997)).

complete conservation. Further homology for the *N*-terminus can be drawn from three highly conserved residues corresponding to Asp-13, Asp-57 and Lys-109. The second aspartic acid position has been found to be the site of phosphorylation by the cognate histidine protein kinase (Hess *et al.*, 1988a and b; Stock J.B. *et al.*, 1989). For RegX4, the conserved amino acids correspond to residues Asp-9, Asp-52 and Lys-101. The *C*-terminal domain follows the *N*-terminus and they are connected by a flexible linker region which is defined from structural knowledge of the regulatory domain of CheY and the *C*-terminal domain of OmpR. The linker of OmpR is approximately 12 residues in size compared to 11 for VanR, 9 for VirG and 6 for PhoB (*cit in* Martinez-Hackert and Stock, 1997b). The approximate linker size for RegX4 is 10 amino acids.

Using the recently determined crystal structure of the *Escherichia coli* OmpR *C*-terminal domain, some insight can be gained into how RegX4 may function given that RegX4, along with over 50 other transcriptional regulators are members of the same family. The OmpR protein interacts with different components of its secondary structure to regulate numerous inter and intramolecular reactions which bring about transcriptional regulation of members of its regulon. These include regulatory interactions with the *N*-terminal domain, dimerisation or oligomerisation with itself, DNA binding, and interaction with the α -subunit of RNA polymerase. A combination of these data together with genetic studies and *in vitro* binding assays of the *C*-terminal domain of OmpR has been used in this study to identify, by analogy, some putative features of the *C*-terminus of RegX4.

The 98 amino acid OmpRc domain consists of three α -helices flanked on two sides by antiparallel β -sheets, an *N*-terminal four stranded β -sheet and a *C*-terminal hairpin that interacts with a short β -strand, β 5, connecting helices α 1 and α 2 to form a three-stranded β -sheet. The topology of the domain is β 1- β 2- β 3- β 4- α 1- β 5- α 2- α 3- β 6- β 7 (figure 7.7B.). The *N*-terminal β -sheet of the *C*-terminal domain (β 1- β 2- β 3- β 4), apart from the 6 hydrophobic regions, is not very conserved among members of the OmpR sub-family. This can also be seen from the RegX4 alignment. Such a low conservation might reflect the importance of this particular region in determining the particular function of the homologue, for example, the interaction with the *N*-terminal regulatory domain (Martinez-Hackert and Stock, 1997a). The packing of the internal hydrophobic residues is important to stabilise the conformation of the native protein and represents a common fold amongst the transcriptional regulators. This is also likely to be true of RegX4 although there is not complete conservation at each residue, suggesting a slightly

different folded structure. Most of the α -helical DNA-binding proteins have a helix-turn-helix (HTH) motif with the second helix as a recognition helix (Kondo *et al.*, 1997; Mizuno and Tanaka, 1997). The way in which the OmpRc DNA-binding domain folds represents an extreme variation of the HTH motif and OmpRc belongs to the family of “winged-helix-turn-helix” DNA-binding proteins (Brennan, 1993). A survey of the computer databases to identify an HTH candidate for RegX4, common in DNA binding proteins failed to identify an HTH segment. This is not surprising given that previous searches have failed to identify an HTH motif in OmpR (Mizuno and Tanaka, 1997). A classical HTH has only four residues in the turn region. In contrast, the turn region of OmpR and RegX4 (between α 2-loop- α 3) has 11. In OmpRc this loop is thought to have evolved to optimise the contact with RNA polymerase while conserving the hydrophobic core structure as well as the DNA interaction site (Kondo *et al.*, 1997). By comparison with other response regulators (Martinez-Hackert and Stock, 1997a; Mizuno and Tanaka, 1997), the amino acid sequence around the loop region of RegX4 is quite variable.

Many of the secondary structure elements of OmpRc are common to most winged-helix domains and can be assigned functional roles. The α 2-loop- α 3 region forms the helix-turn-helix motif. Helix α 3 corresponds to the recognition helix that interacts with the major groove of DNA and helix α 2 corresponds to the positioning helix. The recognition helix contains 12 amino acids. As with RegX4, those amino acids at positions, 1, 5, 8 and 12 are hydrophobic and well conserved among the OmpR sub-family (Mizuno and Tanaka, 1997). The amino acids at positions 7, 9, and 11 are generally positively charged and are believed to interact with the negatively charged DNA backbone. This may also occur with RegX4 as the protein contains positively charged amino acids, arginine, arginine and lysine at these respective positions. The DNA binding specificity is believed to be determined, at least in part, by the amino acids at positions 3, 6, and 11 which are thought to be in close proximity to specific bases thereby allowing the molecule to recognise specific target DNA (Mizuno and Tanaka, 1997). From the alignment of the closest homologues of RegX4 (figure 7.7B.), the amino acid combination, i.e., Val-3, Ser-6 and Lys-10 is like that of *Cyanophora paradoxa*, a flagellated protozoan and *Porphyridium aeruginum*, a photosynthetic bacterium. The significance of this is unclear.

Mutations in Ser200, Val203 and Met211 in OmpR (Aiba *et al.*, 1994; Russo *et al.*, 1993) and PhoB residues which correspond to OmpR residues Ser200, Asp202, Arg207, and Met211 affect DNA binding (Makino *et al.*, 1996). RegX4 contains residues which correspond to OmpR residues Ser200, Asp202, Val203, Ser206, Arg207 and Arg209 but the effect of mutations in any of these residues for RegX4 remains to be established. The use of mutagenesis data has

identified that the loop which connects $\beta 6$ and $\beta 7$ of the C-terminal hairpin is proposed to interact with the minor groove of DNA. This region is termed the recognition wing, W1. Similarly, adjacent to the recognition helix, and in opposition to W1, is W2 which may also attach the minor groove. Once more, the sequence of RegX4 is seen to fit into this background although once again the affect of mutations in this region of RegX4 remain to be established. The N-terminal β -sheet, $\beta 1$ through $\beta 4$ and the α -loop are distinguishing features of OmpRc and are not present in other winged-helix proteins (Martinez-Hackert and Stock, 1997b). These features are conserved within the OmpR sub-family of which RegX4 is a member. Only preliminary research has been performed on RegX4 and more extensive work is required to determine which of the features described for CheY and OmpRc are important in determining the structure and function of RegX4.

7.3.4: Characterisation of the *regX4* locus of *C. jejuni*

Efforts were undertaken to create an isogenic mutant in *C. jejuni regX4* as it was believed that phenotypic analysis of the mutant could aid characterisation of the role of *regX4* and furthermore, facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*. To perform such a task, inverse PCR (Wren *et al.*, 1994) around a plasmid, containing *regX4* sequence, could be used to create a mutated plasmid copy of the gene. Introduction of this fragment into *C.jejuni* 81116 may facilitate homologous recombination to occur between the plasmid copy of the *regX4* gene fragment and its chromosomal counterpart, resulting in a RegX4 mutant.

7.3.4.1. Creation of Plasmid constructs

7.3.4.1.1. Construction of pME7

In order to create an isogenic mutant to help assign a function to *C. jejuni regX4* and to facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*, it was necessary to construct plasmid pME7. 2 μ g of pUC19 plasmid DNA was digested overnight with *KpnI* and *PstI* (section 2.11.1.) and electrophoresed on a 1.0% agarose gel (section 2.8.). The linear plasmid DNA was subsequently excised from the gel, purified using polyallomer wool gel extraction (section 2.9.1.) and phenol/chloroform:chloroform

extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). A PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.1.) using primers R4D1 (5' *KpnI*) and R4U1 (5' *PstI*) (appendix 1) amplified a single ~1.1kb region of *C. jejuni* 81116 chromosomal DNA specific to the *htrA* gene and the *regX4* gene. This was to provide enough flanking DNA either side of the mutation point to allow homologous recombination. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen) (section 2.9.4.) and was subsequently digested with *KpnI* and *PstI* to create cohesive ends (section 2.11.1.). The two digested products were ligated together (section 2.11.3.) and were subsequently ethanol precipitated (section 2.9.3.). Following the ligation, the DNA was transformed into *E. coli* DH α competent cells by electroporation (section 2.12.1.) and plated out onto plates with appropriate selection. Putative recombinant colonies were examined by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) using primers R4D1 and R4U1 (appendix 1) to look for PCR products of ~1.1kb in size. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives and purified plasmid DNA was digested with *HindIII* (section 2.11.1.). The presence of three fragment sizes 278, 472 and 3019bp respectively confirmed putative positives (figure 7.8.). The plasmid was designated pME7 and is shown diagrammatically in figure 7. 9. In order to verify plasmid pME7, insert DNA was sequenced. The sequencing reactions were performed as outlined in section 2.14. Plasmid DNA was prepared by small-scale plasmid preparation (Qiagen) (section 2.6.2.) and the primers used were M13 Forward, M13 Reverse, HTRA14, HTRA15, R4IU1 and R4IU3 (appendix 1). Results of the sequence analysis were as expected and confirmed introduction of the correct *htrA/regX4* insert.

7.3.4.1.2: Inverse PCR mutagenesis of pME7 to create plasmid pME11

Plasmid pME7 was purified using large-scale plasmid preparation (Qiagen) (section 2.6.3.) and inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.1.) was performed on a 1:500 dilution of plasmid pME7 using primers R4IU1 (*BamHI*) and R4IU3 (*BamHI*) (appendix 1). Use of R4IU1 and R4IU3 in tandem creates an internal *BamHI* site, and a 9bp deletion in *regX4*. The disruption in the coding sequence of *regX4* removes the aspartate residue at position 52. In other response regulators, a similarly positioned conserved aspartate residue becomes phosphorylated by a cognate histidine protein kinase in response to being stimulated. Once phosphorylated a downstream response from the newly activated

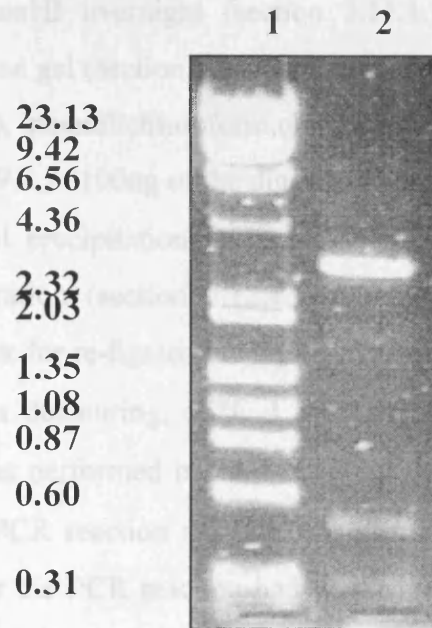


Figure 7.8: Agarose gel electrophoresis of a digest of pME7 with *Hind*III. Lane 1; λ *Hind*III, ϕ /x *Hae*III marker (kilobase pairs), Lane 2; *Hind*III digest of pME7 to reveal three fragment sizes of 278, 472 and 3019bp.

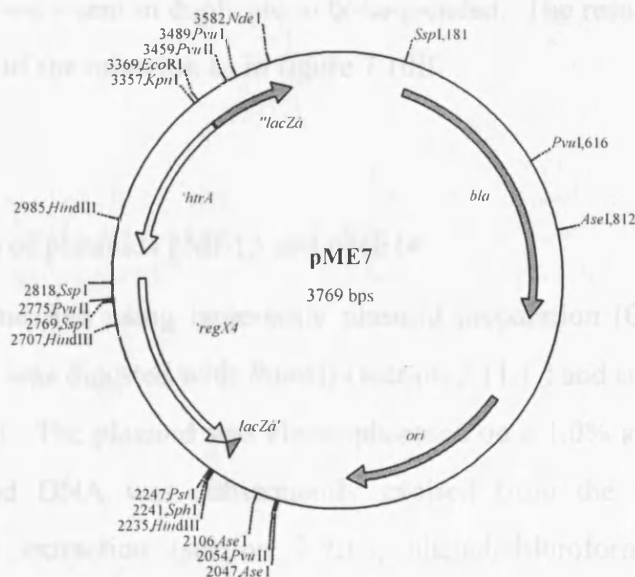


Figure 7.9: Plasmid map of pME7 showing insertion of the R4D1 and R4U1 amplification product (white arrows). The product contains 3' *htrA* sequence and 5' *regX4* sequence and was cloned using *Kpn*I and *Pst*I.

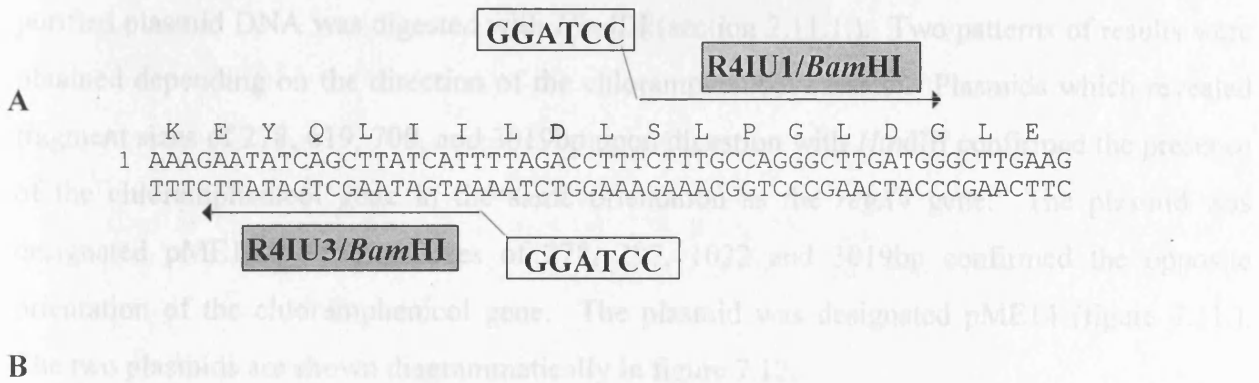
regulator is effected. The construction of the mutation is given in the figure 7.10. The resulting PCR product of ~3.8kb was purified using the Qiaquick PCR purification kit (Qiagen) (section 2.9.4.) and digested with *Bam*HI overnight (section 2.11.1.). The digested product was electrophoresed on a 1% agarose gel (section 2.8.), excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). 100ng of the digested PCR product was self-ligated (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), transformed into *E. coli* DH5 α competent cells by electroporation (section 2.12.1.). Transformants were plated out using appropriate selection. To check for re-ligated recombinant clones containing an internal *Bam*HI site, colony PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) was performed in 20 μ l reactions using R4D1 and R4U1 (appendix 1). 10 μ l of each completed PCR reaction mixture was electrophoresed on a 1% agarose gel (section 2.8.) to check whether the PCR reaction had worked. The remaining half of the PCR reaction mixture was digested with *Bam*HI (section 2.11.1.). The presence of an internal *Bam*HI site was confirmed by revealing two products of 429 and 686bp respectively after *Bam*HI digestion. The plasmid was designated pME11. To verify that the conserved aspartate had been removed and also that the internal *Bam*HI site had been constructed correctly, the plasmid was sequenced as outlined in section 2.14. Plasmid DNA was purified by small-scale plasmid preparation (Qiagen) (section 2.6.2.) and the primers used were M13 Reverse and HTRA14 (appendix 1). Samples were sent in duplicate to be sequenced. The results were as expected and confirmed introduction of the mutation as in figure 7.10B.

7.3.4.1.3: Construction of plasmids pME13 and pME14.

Plasmid pME11 was purified using large-scale plasmid preparation (Qiagen) (section 2.6.3.). 2 μ g of plasmid pME11 was digested with *Bam*HI (section 2.11.1.) and subsequently phosphatase treated (section 2.11.2.). The plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear plasmid DNA was subsequently excised from the gel and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

Similarly, 2 μ g of pAV35 which contains the *C. coli* chloramphenicol resistance gene was digested overnight with *Bam*HI (section 2.11.1.) to remove the resistance cassette. The digested

plasmid was electrophoresed on a 1.0% agarose gel (section 2.8) and the 850bp chloramphenicol resistance cassette was subsequently excised and purified using polyallomer wool-gel extraction (section 2.9.1.), phenol/chloroform/mchloroform extraction (section 2.9.2.) and ethanol precipitation (section 2.9.3.). Linear plasmid, pME11, was ligated to the *Bam*HI digested chloramphenicol gene (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), the ligation was transformed into *E. coli* DH5 α by electroporation (section 2.12.1.). Transformants were then picked out using chloramphenicol as selection. Recombinant clones were checked for the insertion of the chloramphenicol cassette into the natural *Bam*HI site of pME11 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 sec extension) (section 2.13.2.) using primers R4D1 and R4U1 (appendix 1). A positive result yielded a PCR product of 1957bp in size compared to the positive control of pME11 which was 1107bp. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives, and purified plasmid DNA was digested with *Bam*HI (section 2.11.1.). Two patterns of results were



7.10.2: Disruption of the *C. jejuni* 81116 *regX4* locus using pME13 and pME14

Figure 7.10: (A) Inverse PCR strategy used to disrupt the coding sequence of *regX4*. (B) The result is a 9bp deletion to remove the conserved aspartate residue (D) shown in bold and creation of an internal *Bam*HI restriction site. The primers used are shown in grey boxes.

negative and positive controls, 50 μ l of competent 81116 cells were electroporated in the absence of DNA and in the presence of 1 μ g of plasmid pTNS α A respectively (appendix 7). Resistant colonies for the positive control could be seen after 2 days. For the pME13 and pME14 transformants no colonies were observed, even after two weeks. The transformation was repeated three times with 10 μ g of plasmid pME13 and plasmid pME14 DNA, each time no recombinant colonies were observed. The transformation was also repeated several times with 20 μ g of pME13 and pME14 plasmid DNA at 17 and 42°C, and also, using either M13 or CNA recovery plates each time without success. The inability to produce a *regX4* mutant using *C. jejuni* 81116 meant a change in strategy to try and mutate the gene. *C. jejuni* 81116 and other

plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 850bp chloramphenicol resistance cassette was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). Linear plasmid, pME11, was ligated to the *Bam*HI digested chloramphenicol gene (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), the ligation was transformed into *E. coli* DH5 α by electroporation (section 2.12.1.). Transformants were the plated out using chloramphenicol as selection. Recombinant clones were checked for the insertion of the chloramphenicol cassette into the internal *Bam*HI site of pME11 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) using primers R4D1 and R4U1 (appendix 1). A positive result yielded a PCR product of 1957bp in size compared to the positive control of pME11 which was 1107bp. Small-scale plasmid preparation (mini-prep) (section 2.6.1.) was performed on putative positives, and purified plasmid DNA was digested with *Hind*III (section 2.11.1.). Two patterns of results were obtained depending on the direction of the chloramphenicol cassette. Plasmids which revealed fragment sizes of 278, 619, 700, and 3019bp upon digestion with *Hind*III confirmed the presence of the chloramphenicol gene in the same orientation as the *regX4* gene. The plasmid was designated pME13. Product sizes of 278, 297, 1022 and 3019bp confirmed the opposite orientation of the chloramphenicol gene. The plasmid was designated pME14 (figure 7.11.). The two plasmids are shown diagrammatically in figure 7.12.

7.3.4.2: Disruption of the *C. jejuni* 81116 *regX4* locus using pME13 and pME14

Electrotransformation of pME13 and pME14 into *C. jejuni* 81116 was performed as outlined in section 2.12.2. 10 μ g of plasmids pME13 and pME14 DNA were used. As electroporation negative and positive controls, 50 μ l of competent 81116 cells were electroporated in the absence of DNA and in the presence of 1 μ g of plasmid pTNS#A respectively (appendix 2). Resistant colonies for the positive control could be seen after 2 days. For the pME13 and pME14 transformants no colonies were observed, even after two weeks. The transformation was repeated three times with 10 μ g of plasmid pME13 and plasmid pME14 DNA, each time no recombinant colonies were observed. The transformation was also repeated several times with 20 μ g of pME13 and pME14 plasmid DNA at 37 and 42°C, and also, using either MHB or CSA recovery plates each time without success. The inability to produce a *regX4* mutant using *C. jejuni* 81116 meant a change in strategy to try and mutate the gene. *C. jejuni* 81116 and other

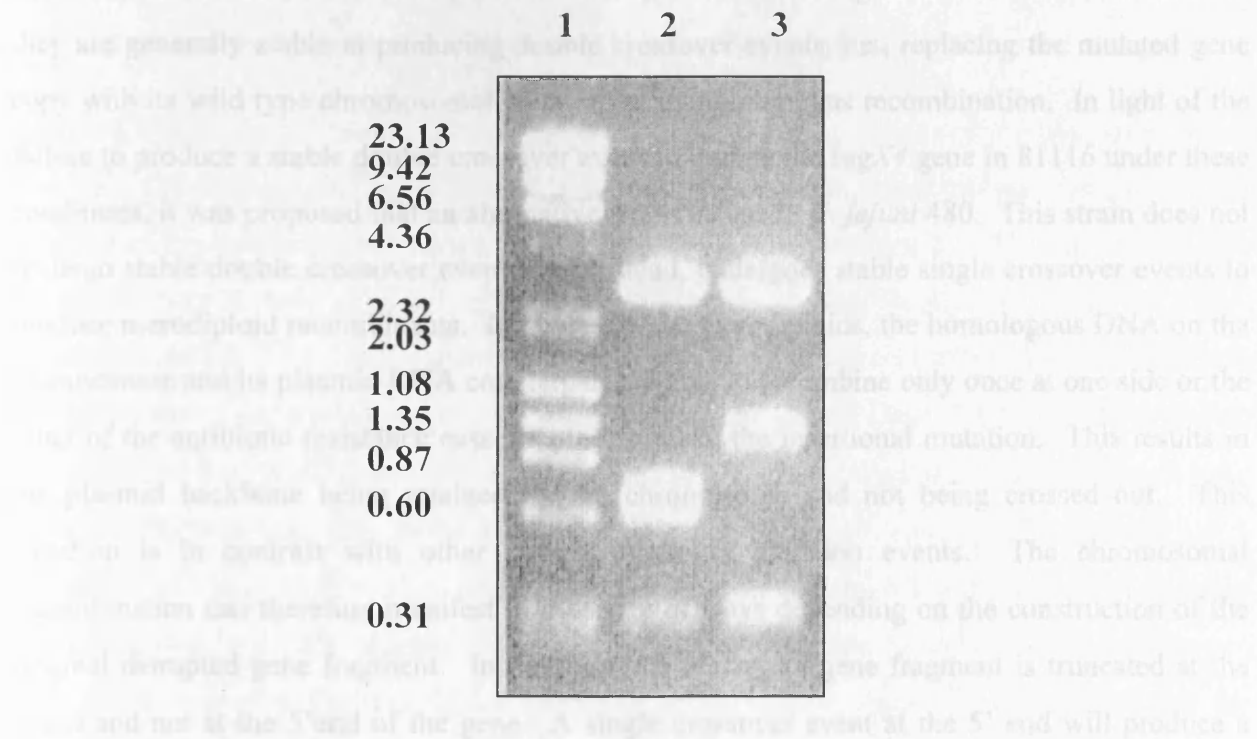


Figure 7.11: Agarose gel electrophoresis of a *Hind*III digest of plasmids pME13 and pME14. Lane 1, λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 2, pME13 digested with *Hind*III to reveal fragment sizes of 278, 619, 700, and 3019bp; Lane 3, pME14 digested with *Hind*III to reveal fragment sizes of 278, 297, 1022 and 3019.

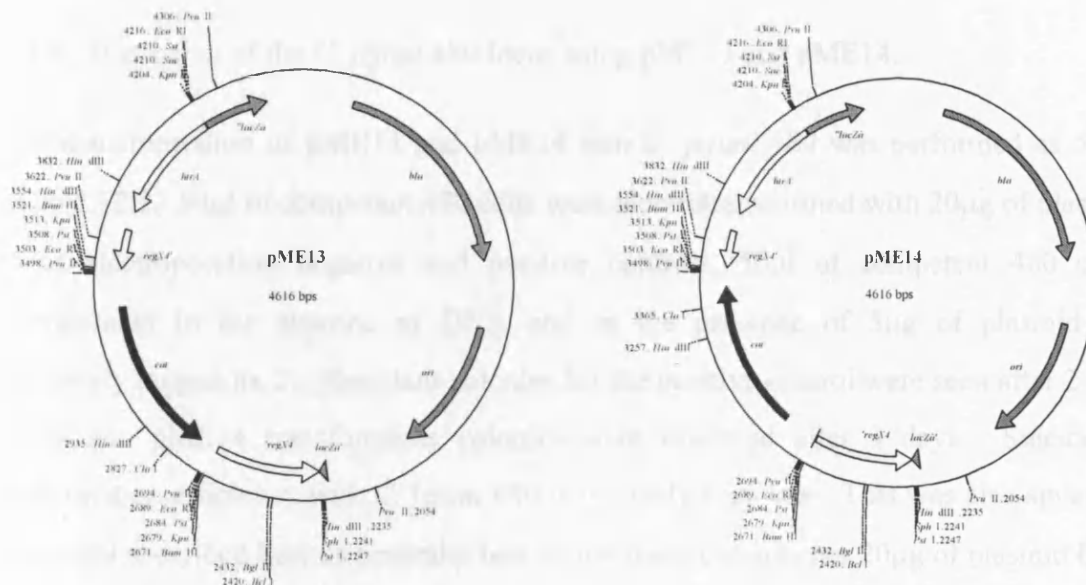


Figure 7.12: Diagrammatic outline of plasmids pME13 and pME14. The interrupted *htrA* and *regX4* fragment (white arrows) has been disrupted by insertion of *cat* (black arrow) in either orientation.

similar strains are used to mutate genes of interest because, amongst other useful characteristics, they are generally stable at producing double crossover events, i.e., replacing the mutated gene copy with its wild type chromosomal counterpart by homologous recombination. In light of the failure to produce a stable double crossover event to mutate the *regX4* gene in 81116 under these conditions, it was proposed that an alternative strain be used, *C. jejuni* 480. This strain does not undergo stable double crossover events, but instead, undergoes stable single crossover events to produce merodiploid recombinants. In *C. jejuni* 480 merodiploids, the homologous DNA on the chromosome and its plasmid DNA counterpart are able to recombine only once at one side or the other of the antibiotic resistance cassette used to make the insertional mutation. This results in the plasmid backbone being retained on the chromosome and not being crossed out. This situation is in contrast with other double crossover mutation events. The chromosomal recombination can therefore manifest in a variety of ways depending on the construction of the original disrupted gene fragment. In this instance the *regX4* gene fragment is truncated at the 3' end and not at the 5' end of the gene. A single crossover event at the 5' end will produce a mutated copy of the gene and a non-mutated functional copy of *regX4* and the recombinants are likely to be phenotypically the same as wild type. On the other hand, a crossover at the 3' end will result in a truncated copy of *regX4* at the C-terminus and a disrupted copy, therefore resulting in a *regX4* mutant (figure 7.13.).

7.3.4.3: Disruption of the *C. jejuni* 480 locus using pME13 and pME14.

Electrotransformation of pME13 and pME14 into *C. jejuni* 480 was performed as outlined in section 2.12.2. 50µl of competent 480 cells were electrotransformed with 20µg of plasmid DNA and as electroporation negative and positive controls, 50µl of competent 480 cells were electroporated in the absence of DNA and in the presence of 5µg of plasmid pTNS#A respectively (appendix 2). Resistant colonies for the positive control were seen after 2 days. For pME13 and pME14 transformants colonies were observed after 3 days. Suicide plasmid transformation efficiency with *C. jejuni* 480 is typically very low. This was also apparent in the experiment described here as generally less than 4 transformants per 20µg of plasmid DNA were observed. Therefore, the same transformation was repeated 5 times. 13 chloramphenicol resistant colonies were obtained for the pME13 transformation and 11 for pME14.

7.3.4.4: Analysis of transformants

7.3.4.4.1: Southern hybridisation analysis of *C. jejuni* 480: presence of Cm^R resistance gene.

Southern hybridisation (section 2.15.) was required to confirm presence of the chloramphenicol cassette. Chromosomal DNA from the 24 putative recombinants was probed with the *Bam*HI digested chloramphenicol resistance cassette from pAV35 (appendix 2). Chromosomal DNA from each strain was prepared using the small-scale *Campylobacter* chromosomal DNA technique (section 2.7.1.) and analysed by agarose gel electrophoresis (section 2.8.). To perform the hybridisation experiment, approximately 5µg of chromosomal DNA from each of the strains was restricted with *Bgl*II (section 2.11.1.). 100ng of the *Bam*HI digested chloramphenicol gene from pAV35 was used as a probe after being labelled. *C. jejuni* strain 81116 and 480 DNA digested with *Bgl*II were used as negative controls for the hybridisation and the DNA samples were separated on a 1.0% agarose gel (section 2.8.). The Southern hybridisation was performed as outlined in section (section 2.15.). Analysis of the results (figure 7.14.) revealed that the probe hybridised to 9/13 of the genomic digests of *C. jejuni* strain 480 transformed with pME13 and only 4/11 from those transformed with pME14. The probe failed to hybridise to the 81116 and 480 genomic digests. The failure of the probe to hybridise to all of the transformants most likely indicates the presence of spontaneous chloramphenicol resistant mutants. With respect to the other genomic digests, a single band of ~3.2 kb hybridises to the chloramphenicol resistance probe indicating the presence of the chloramphenicol resistance gene on the chromosome, and not a recombinant arisen by a spontaneous mutation event.

7.3.4.4.2: PCR analysis of *C. jejuni* 480 to orientate crossover

From figure 7.13 it can be seen that there are two possible outcomes of a single crossover recombination event. A crossover on the left hand side of the chloramphenicol cassette will result in a recombinant most likely to be the same as wild type, phenotypically. The alternative, right hand side crossover event will produce a *regX4* mutant. In order to test which side the crossover event has taken place, those recombinants which produced a positive result for the presence of the chloramphenicol cassette were analysed further by PCR. For pME13 transformants which contain the chloramphenicol cassette in the same orientation as the *regX4* gene, the combination of primers HTRA-MET, CATI5' and REGX4-STOP, CATI3' were used. For pME14 transformants which contain the chloramphenicol cassette in the opposite orientation to the *regX4* gene, the combination of HTRA-MET, CATI3' and REGX4-STOP, CATI5' were

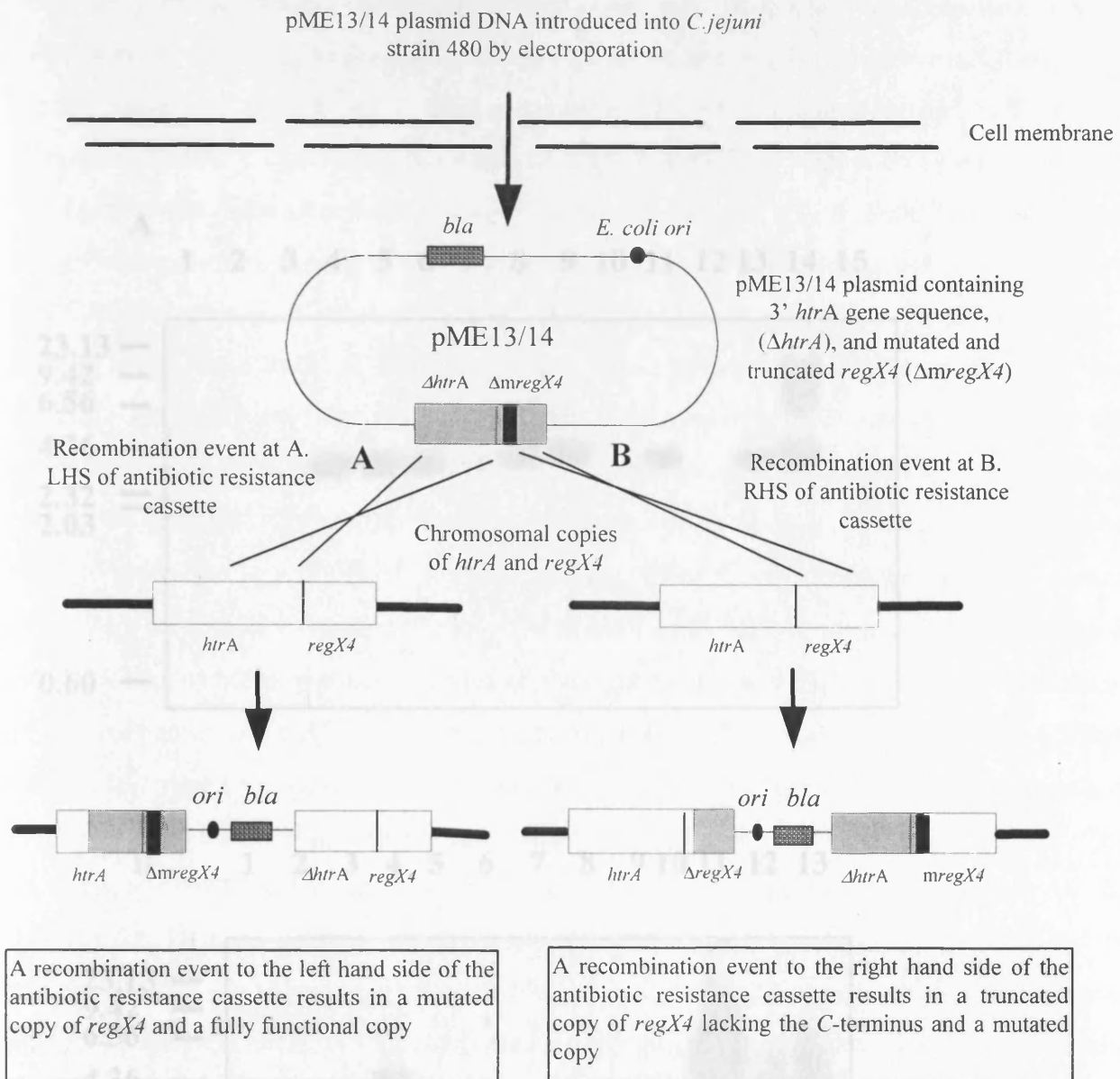


Figure 7.13: An outline of the two possible recombination outcomes after electroporation of *C. jejuni* 480 with either pME13 or pME14. A recombination event to the left hand side of the antibiotic resistance cassette, as in A, results in a mutated copy of *regX4* and a fully functional copy. A recombination event to the right hand side of the antibiotic resistance cassette, as in B, results in a truncated copy of *regX4* lacking the C-terminus and an insertionally mutated copy. This has major implications in concluding whether production of a *regX4* mutant is actually possible given that in theory, the chance for both to occur is possible. The recombination event may be affected by the difference in flanking region either side of the antibiotic resistance cassette.

used (appendix 1). To test which side of the chloramphenicol cassette crossover has occurred for the pME13 transformants, chromosomal DNA from each of the 9 chloramphenicol positive samples, diluted 1:1000, was used to perform a PCR. To test whether crossover had occurred on the left hand side a PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 min extension) (section 2.13.1) using primers HTRA-MET and CAT15* (appendix 1) was performed. To check whether crossover had occurred on the right hand side a PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 min extension) (section 2.13.1) using primers REGX4-STOP and CAT15* was performed. Analysis of the results (Figure 7.15) highlights a

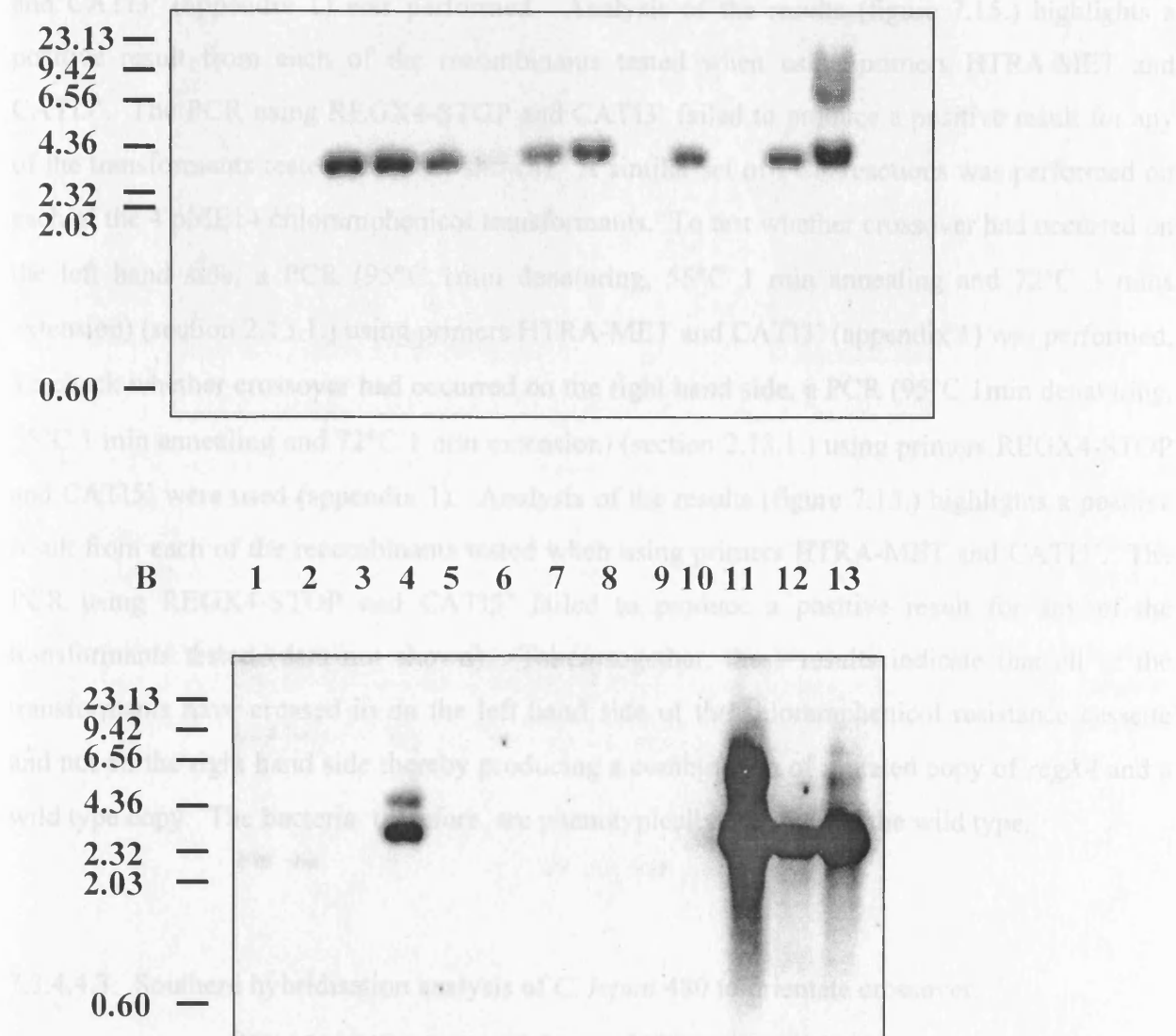


Figure 7.14: Southern hybridisation analysis of putative *regX4* mutants. To show the presence of the chloramphenicol resistance gene used to generate the mutants, *Bam*HI digested chloramphenicol cassette from pAV35 was used as a probe. (A) 13 putative pME13 transformants. Left; λ *Hind*III marker (kilobase pairs); Lane 1, *C. jejuni* 81116; Lane 2, *C. jejuni* 480; Lanes 4-15, 13 transformants (9 positive). (B) 11 putative pME14 transformants Left; λ *Hind*III marker (kilobase pairs); Lane 1, *C. jejuni* 81116; Lane 2, *C. jejuni* 480; Lanes 4-13, 11 transformants (4 positives).

used (appendix 1). To test which side of the chloramphenicol cassette crossover has occurred for the pME13 transformants, chromosomal DNA from each of the 9 chloramphenicol positive samples, diluted 1/1000, was used to perform a PCR. To test whether crossover had occurred on the left hand side a PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) using primers HTRA-MET and CATI5' (appendix 1) was performed. To check whether crossover had occurred on the right hand side, a PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 1 min extension) (section 2.13.1.) using primers REGX4-STOP and CATI3' (appendix 1) was performed. Analysis of the results (figure 7.15.) highlights a positive result from each of the recombinants tested when using primers HTRA-MET and CATI5'. The PCR using REGX4-STOP and CATI3' failed to produce a positive result for any of the transformants tested (data not shown). A similar set of PCR reactions was performed on each of the 4 pME14 chloramphenicol transformants. To test whether crossover had occurred on the left hand side, a PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) using primers HTRA-MET and CATI3' (appendix 1) was performed. To check whether crossover had occurred on the right hand side, a PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 1 min extension) (section 2.13.1.) using primers REGX4-STOP and CATI5' were used (appendix 1). Analysis of the results (figure 7.15.) highlights a positive result from each of the recombinants tested when using primers HTRA-MET and CATI3'. The PCR using REGX4-STOP and CATI5' failed to produce a positive result for any of the transformants tested (data not shown). Taken together, these results indicate that all of the transformants have crossed in on the left hand side of the chloramphenicol resistance cassette and not on the right hand side thereby producing a combination of mutated copy of *regX4* and a wild type copy. The bacteria therefore, are phenotypically the same as the wild type.

7.3.4.4.3: Southern hybridisation analysis of *C. jejuni* 480 to orientate crossover.

Southern hybridisation analysis (section 2.15.) was performed to confirm the PCR results. As all of the transformants had given the same result using the PCR, it was decided that 4 recombinants from each of the transformations using pME13 and pME14 be used, together with 2 additional spontaneous chloramphenicol mutants from each type of transformation to act as internal controls. Chromosomal DNA from the 12 recombinant strains, was hybridised with two different probes; pUC19 digested with *Hind*III and *Eco*RI and a single 0.68 kb DNA fragment obtained from a PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 1min extension)

(section 2.4.1) using primers R4DI and R4DI2 (Appendix 1). The 0.6kb product is positioned to the left hand side of the internal *Bgl*II site within *regX4*. Each of the products had to make a probe was isolated from a 1% agarose gel and purified using polyacrylamide wool gel extraction (section 2.4.1), phenol/chloroform extraction (section 2.4.2) and ethanol precipitation (section 2.4.3). 100ng of each product was labelled. Both *C. jejuni* strains, 8116 and 450 were used as negative controls and probe DNA was as a positive control.

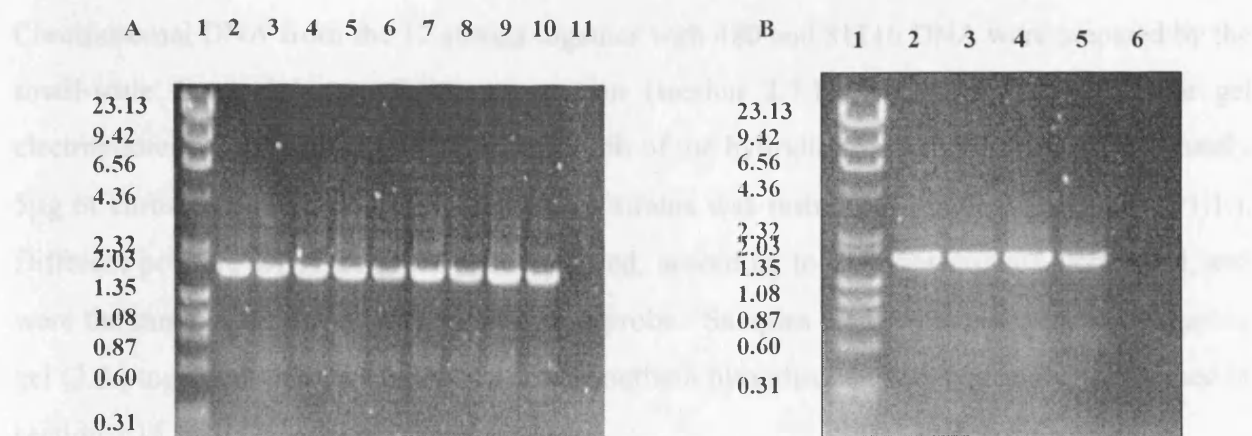


Figure 7.15. A: Diagram showing PCR result using primers HTRA-MET and CATI5' on true chloramphenicol resistant transformants from electroporation with pME13. Lane 1, λ HindIII/ ϕ x HaeIII marker (kilobase pairs); Lanes 2-10, 9 different transformants; Lane 11, dH₂O negative control. Each of the nine transformants has given the same size band of ~1.8kb. **B:** Diagram showing PCR result using HTRA-MET and CATI3' on true chloramphenicol resistant transformants from electroporation with pME14. Lane 1, λ HindIII/ ϕ x HaeIII marker (kilobase pairs); Lanes 2-5, 4 different transformants; Lane 6, dH₂O negative control. Each of the 4 transformants has given the same size band of ~1.8kb.

The membrane probed with the ~0.68 kb product from the PCR using primers R4DI and R4DI2 shows a number of salient features. Firstly, the probe has recognised the positive control. Furthermore, both the 8116 and 450 negative controls, together with the 4 spontaneous mutants have produced a single hybridisation band of ~2.38kb which is consistent with predicted size of a wild type band. Also, there appears to be no apparent difference between the *Bgl*II restriction profile between *C. jejuni* 8116, which is where the DNA originates from, and *C. jejuni* 450, the host strain. The most important features to highlight concern the hybridisation result even with the 9 recombinants not arisen by spontaneous mutation. As predicted before, there are two possible outcomes to the transformation using either pME13 or pME14. A crossover to the left hand side of the chloramphenicol resistance cassette and a crossover to the right. Using PCR, it has been determined that all of the transformants have not arisen by spontaneous mutation

(section 2.13.1.) using primers R4D1 and R4IU3 (appendix 1). The 0.68kb product is positioned to the left hand site of the internal *Bgl*III site within *regX4*. Each of the products used to make a probe was excised from a 1% agarose gel and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). 100ng of each product was labelled. Both *C. jejuni* strains, 81116 and 480 were used as negative controls and probe DNA was as a positive control.

Chromosomal DNA from the 12 strains together with 480 and 81116 DNA were prepared by the small-scale *Campylobacter* DNA preparation (section 2.7.1.) and analysed by agarose gel electrophoresis (section 2.8.). To perform each of the hybridisation experiments, approximately 5µg of chromosomal DNA from each of the strains was restricted with *Bgl*III (section 2.11.1.). Different positive DNA controls were included, according to the hybridisation performed, and were the same as the DNA sample used as a probe. Samples were separated on a 1.0% agarose gel (2.8.) together with the controls and the Southern hybridisation was performed as outlined in section 2.15.

The membrane probed with *Hind*III and *Eco*RI digested pUC19 DNA revealed that the probe hybridised to the ~2.7 kb pUC19 band from the pUC19 positive control and also a ~3.8kb band from each of the 8 recombinants not arisen by spontaneous mutation. The pUC19 probe failed to hybridise to any of the 4 spontaneous mutants and the genomic digests of the 81116 and 480 used as negative controls (figure 7.16A.). From the results it can be seen that the plasmid used to transform *C. jejuni* 480 is present on the chromosome resulting in the production of a single crossover merodiploid.

The membrane probed with the ~0.68 kb product from the PCR using primers R4D1 and R4IU3 shows a number of salient features. Firstly, the probe has recognised the positive control. Furthermore, both the 81116 and 480 negative controls, together with the 4 spontaneous mutants have produced a single hybridisation band of ~2.38kb which is consistent with predicted size of a wild type band. Also, there appears to be no apparent difference between the *Bgl*III restriction profile between *C. jejuni* 81116, which is where the DNA originates from, and *C. jejuni* 480, the host strain. The most important features to highlight concern the hybridisation result seen with the 8 recombinants not arisen by spontaneous mutation. As predicted before, there are two possible outcomes to the transformation using either pME13 or pME14. A crossover, to the left hand side of the chloramphenicol resistance cassette and a crossover to the right. Using PCR, it has been determined that all of the transformants that have not arisen by spontaneous mutation

have originated from a crossover on the left-hand side of the chloramphenicol resistance cassette resulting in a failure to produce a *regX4* mutant. In order to confirm this result, DNA from the 8 recombinants was probed with the 0.68 kb fragment located to the left hand side of the internal *Bgl*III site within *regX4*. The predicted outcome for a crossover on the left hand side of the chloramphenicol resistance cassette would be two positive bands of 3.23 and 3.79 kb respectively. The alternative, right hand crossover event would result in two bands of 2.38 and 3.79 kb respectively. Analysis of the Southern hybridisation (figure 7.16B.) reveals band sizes of ~3.2kb and ~3.8 kb in each example, or at least two bands closely associated with one another suggesting that the bands are much greater than 2.38kb and less than 4.36 kb. This is consistent with the PCR results which suggest that point of crossover is to the left hand side of the chloramphenicol resistance cassette and not the right hand side. This further supports the failure to isolate a mutation in *regX4*. Possibly the most intriguing feature of the Southern hybridisation experiment is the presence of a band of almost identical size, ~2.38kb to the wild type size band seen in 81116, 480 and the spontaneous mutants. Furthermore, the intensity of the 2.38kb band varies with respect to the other bands in the same lane. A band of this size would be apparent in a recombinant which had crossed in at the right hand side of the chloramphenicol resistance cassette. Initial thoughts as to the origin of this band were based on the idea that the chromosomal DNA was made up of a mixture of strains which had crossed in on either side of the chloramphenicol cassette. This conclusion was unlikely as it refuted some of the data obtained from the PCR results. Also, each recombinant originated from a single transformant. An alternative suggestion is based around the fact that the merodiploids maybe relatively unstable for this locus and resolve out to eventually give wild type. The reason behind this may originate from how the strains were grown prior to the isolation of chromosomal DNA and Southern hybridisation analysis such that from the original colony there is a mixed population of both resolved cells which give the same wild type band of 2.38kb and unresolved merodiploids which give the two higher bands of 3.23 and 3.79 kb respectively. The differing intensity of the wild type size band in comparison to the 3.23 and 3.79kb bands for each of the samples might reflect the degree of resolution of the merodiploids back to wild type. The Southern blot shows that some have resolved further than others and some of the examples have not begun to resolve out at all. The fact that there is any resolution back to wild type, suggests that insertion of the plasmid, in either direction around this region has a deleterious effect on *regX4* and gives further support to the inability to produce a mutation in the *regX4* locus.

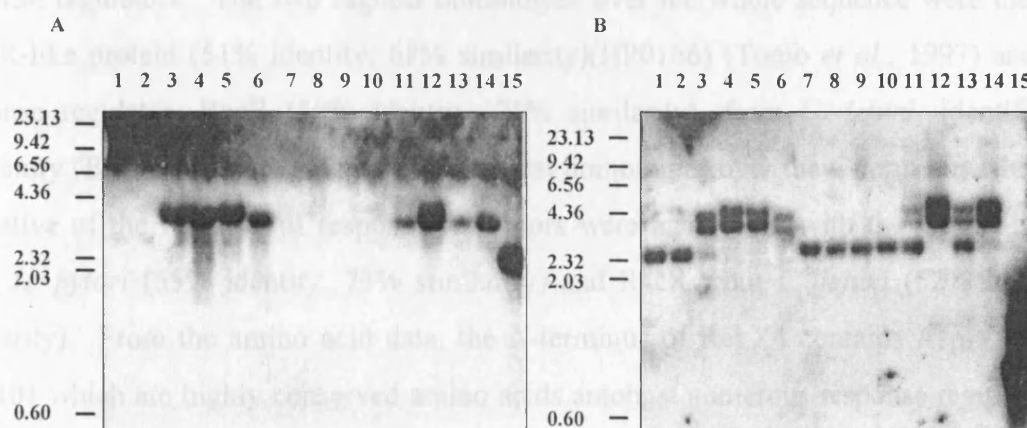


Figure 7.16: Southern hybridisation analysis of pME13 and pME14 transformants. (A) To show the presence of the plasmid backbone on the chromosome, pUC19 digested with *Hind*III and *Eco*RI was used as a probe. Left, λ *Hind*III marker (kilobase pairs); Lane 1, *C. jejuni* 81116; Lane 2, *C. jejuni* 480, Lanes 3-6, 4 true chloramphenicol resistant pME13 transformants; Lanes, 7-10, 4 spontaneous mutants (2 pME13 derived and 2 pME14 derived); Lanes 11-14, 4 true chloramphenicol resistant pME14 transformants. Lane 15, *Hind*III, *Eco*RI pUC19 DNA (~2.7kb positive control-probe DNA). All true chloramphenicol resistant transformants give a similar sized band of ~3.8kb. To confirm the orientation of homologous recombination, the R4D1 X R4IU3 PCR product was used as a probe. Left, λ *Hind*III marker (kilobase pairs); Lane 1, *C. jejuni* 81116 (~2.38kb); Lane 2, *C. jejuni* 480 (~2.38kb), Lanes 3-6, 4 true chloramphenicol resistant pME13 transformants (mixture of ~2.38, ~3.23 and ~3.79kb); Lanes, 7-10, 4 spontaneous mutants (2 pME13, 2 pME14, ~2.38kb); Lanes 11-14, 4 true chloramphenicol resistant pME14 transformants (mixture of ~2.38, ~3.23 and ~3.79kb). Lane 16, positive control.

7.4: Discussion

A ~2.5 kb *Bgl*II fragment downstream of the *htrA* containing 2.38kb *Bgl*II fragment was cloned and sequenced and revealed the 3' end of the response regulator, *regX4*. In addition, downstream of *regX4* there is an overlapping reading frame which has homology to members of the family of histidine protein kinases which form part of two component regulatory systems. The histidine protein kinase is postulated to be the cognate partner of RegX4 and is discussed further in Chapter 8. All attempts at assigning a function to RegX4 based on computer analyses proved difficult, but the findings indicated that RegX4 is most homologous to the OmpR sub-family of response regulators. The two highest homologies over the whole sequence were the *H. pylori* OmpR-like protein (51% identity, 68% similarity)(HP0166) (Tomb *et al.*, 1997) and a further response regulator, RacR (50% identity, 71% similarity), from *C. jejuni*, identified in our laboratory (Brás and Ketley, 1996). The highest homologies over the C-terminus which is more indicative of the function of response regulators were again seen with the OmpR-like protein from *H. pylori* (55% identity, 73% similarity) and RacR from *C. jejuni* (52% identity, 69% similarity). From the amino acid data, the N-terminus of RegX4 contains Asp-9, Asp-52 and Lys-101 which are highly conserved amino acids amongst numerous response regulators. CheY from *E. coli* (Stock A.M. *et al.*, 1989) has the corresponding amino acids, Asp-13, Asp-57 and Lys-109 with the second aspartic being the site of phosphorylation by histidine protein kinases. (Alon *et al.*, 1998). It remains to be established whether Asp-52 of RegX4 is the acceptor site for a phosphoryl group from a cognate histidine protein kinase.

Response regulators such as NarL of *E. coli* (Nohn *et al.*, 1989) and BvgA of *Bordetella pertussis* (Arico *et al.*, 1989) have a classic helix-turn-helix (HTH) DNA binding motif. In contrast, the newly defined crystal structure of the C-terminal DNA binding motif of the *E. coli* osmolarity-dependent regulator, OmpR, defines a new sub-family of transcriptional activators, called “winged-helix-turn-helix” proteins that function to bind both specific DNA sequences and to interact with RNA polymerase (Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997a). An alignment of the putative DNA binding motif of RegX4, together with OmpR, identifies that the RegX4 protein fits into a similar framework of secondary structure. This raises a number of questions as to the how the protein folds and how regulation by RegX4 is achieved. Given the similarity between the two proteins, it can be tentatively put forward that, along with other members of the sub- family of response regulators, the mechanism of folding, DNA binding and interaction with RNA polymerase maybe similar, despite the possibility that the two proteins have very different roles.

In a bacterial cell, the role of various proteins has been identified by making mutants in their respective genes. Therefore, efforts were undertaken to create an isogenic mutant in *C. jejuni* *regX4* with a view to characterise the role of *regX4* and furthermore, facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*. Despite numerous attempts, all efforts failed to isolate a *regX4* mutant in which part of the open reading frame had been replaced by a chloramphenicol resistance gene marker. This was in spite of employing procedures used routinely to generate many disruptions in *Campylobacter* genes, and using two different strains, 81116 and 480. The failure to identify any mutants in 81116 could be due to a number of reasons. Simply, it could be an unidentified technical problem. Alternatively, the amount of flanking DNA either side of the mutation point may not be sufficient to allow homologous recombination to occur. The length of flanking sequence either side of the insertion point is fundamental in facilitating recombination of the mutant gene with its wild-type counterpart (Wassenaar *et al.*, 1993). The plasmid construct used in trying to make the mutation contains 679bp and 424bp of flanking DNA either side of the mutation point which, theoretically, should be sufficient enough to allow homologous recombination to occur. This assumption is primarily based on data provided by Wassenaar *et al.*, (1993) who discovered that recombination was possible with as little as 202 and 348bp of homologous sequence either side of the antibiotic resistance gene when trying to mutate the *C. jejuni* flagellin genes. To add further support, 200bp and 270bp of homologous sequence on each side of the *C. jejuni* *htrA* gene was sufficient to allow it to be mutated (Henderson, 1996). In an effort to resolve the problem, more flanking DNA can be used but is likely to have little effect on the outcome of the transformation. Using *C. jejuni* 480, which undergoes single crossover events, a number of authentic chloramphenicol resistant recombinants were identified. Unfortunately, the point of integration of the transformed plasmid into the chromosome to produce a merodiploid was such that a mutated and non-mutated copy of *regX4* was produced and not two mutated copies. With a non-disrupted *regX4* gene present on the chromosome there is likely to be little or no effect on the resulting phenotype and the bacterium is effectively the same as wild type. As with 81116, the amount of flanking DNA may have influenced the result seen with *C. jejuni* 480. In support of the inability to produce a mutation in 81116, no authentic *regX4* mutants were seen with *C. jejuni* 480 and each of the 13 examples had the point of crossover located to the left hand side of the chloramphenicol cassette. Given the amount of flanking DNA on the left hand side (679bp) compared to that on the right hand side (424bp), there is possibly a greater likelihood that the plasmid will cross in on the left hand side and not the right. If the transformation efficiency for 480 transformations were greater, such that more colonies could be screened, then the potential

for production of recombinants which have undergone crossovers on the right hand side could be examined fully.

A more likely reason for not being able to produce a mutation in *regX4* is that it may be lethal. The *regX4* gene is likely to be a regulatory gene as it is homologous to regulators of the two-component family which often co-ordinate expression of several gene products (Eymann *et al.*, 1996; Mizuno and Mizushima, 1990). Therefore their inactivation usually results in a highly pleiotropic effect. In keeping with this idea, *regX4* might be involved in regulating a number of different genes. As a result, a mutation in *regX4* may not be selected for as it might affect one or more genes of the regulon involved in a process essential for survival of the bacterium. Furthermore, it may be that under the conditions used to isolate the mutation, *regX4* genomic mutants are non-viable or are very slow growing (>2 weeks before colonies observed) and are thus difficult to isolate. The inability to mutate the response regulator component of a two-component regulatory system is not unprecedented. Some examples in the literature include *mtrA* from *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG (Via *et al.*, 1996) *feuP*, from *Rhizobium leguminosarum* (Yeoman *et al.*, 1997) and *chvI* from *Rhizobium* spp., (Østerås *et al.*, 1995; Cheng and Walker, 1998). In conclusion it is likely that *regX4* is essential under the experimental conditions used.

7.5: Future work

Identification of the complete *regX4* DNA sequence has enabled further characterisation of the locus with a view to developing an understanding of the function of the protein in *C. jejuni*. Presently, there is very little information regarding the putative promoter region upstream of the predicted open reading frame of *regX4*. Therefore, the authenticity of the putative promoter can be verified by cloning this region in pMW10 and using transcriptional analysis studies with the *lacZ* reporter system in *C. jejuni* 480. The pMW10 expression vector construct can also be used to investigate expression of the putative promoter under various conditions in an attempt to identify any environmental stimulus. Once the authenticity of the promoter region upstream of *regX4* has been confirmed it is subsequently necessary to identify the transcriptional start to *regX4*. This can be accomplished by performing RNA transcript mapping, by using either primer extension or S1 nuclease protection. In order to confirm that the predicted open reading frame for *regX4* indeed encodes a polypeptide, the *regX4* gene can be cloned into an expression vector and the gene product be visualised in *E. coli* by labelling with [³⁵S] methionine

and [³⁵S] cysteine. The same system can be used to analyse the RegX4 protein further and enable *N*-terminal sequence analysis to verify the predicted open reading frame assignment.

On the basis of its sequence homology, RegX4 can be postulated to function as a response regulator in a two component regulatory system. As many response regulators have been shown to autophosphorylate in the presence of acetylphosphate (Lukat *et al.*, 1992; McCleary *et al.*, 1993; Stock A.M. *et al.*, 1995; Danese *et al.*, 1995) the same distinctive property could be tested for the RegX4 protein. The RegX4 protein could be purified, and subsequently incubated with radiolabelled [³²P] acetylphosphate as the phosphate donor. The stability of phosphorylated RegX4 can be tested under both acidic and alkaline conditions to test whether phosphorylation occurs at an acidic residue. By analogy with other response regulators this is likely to be Asp-52. In addition, the capacity for purified RegX4 to undergo phosphorylation by a typical histidine protein kinase can be tested. Later, similar experiments can be performed with its own putative cognate histidine protein kinase identified downstream. A typical histidine protein kinase such as CheA can be used after being autophosphorylated. Although CheA is obviously not the cognate kinase for RegX4 it has been used successfully on previous occasions in heterologous phosphotransfer reactions (Deretic *et al.*, 1992; Via *et al.*, 1996).

Further analysis of the protein could involve investigation of the molecule's potential DNA binding capacity. RegX4 could be purified and its DNA binding capacity determined with or without prior incubation with a small molecule phosphodonor, such as acetyl phosphate. A similar possibility exists to investigate its capacity to bind DNA and RNA polymerase by analysing various mutagenised protein recombinants. Similar experiments with OmpR and PhoB mutants have determined their effect on DNA binding capacity and interaction with RNA polymerase (Russo *et al.*, 1993; Pratt and Silhavy, 1994; Kato *et al.*, 1995; Makino *et al.*, 1996).

The nature of these techniques is such that they require a background knowledge of individual genes, or at least potential individual genes under the control of the regulator. This is obviously a major drawback with respect to RegX4 because as yet there are no known genes under control of the protein. One way in which members of a regulon under the control of a particular regulator are identified, is by mutational analysis of the gene in question. This is usually followed by from one-dimensional and two-dimensional polypeptide profiling and *N*-terminal sequencing of proteins with altered expression. Unfortunately, with RegX4 this is not possible as all attempts at mutating *regX4* have failed. This is compounded by the fact there is limited information with regards to homology of the protein with other response regulators on the

databases and in particular genes under their control. At present, the only information available is the knowledge that two component regulatory systems often regulate genes in their vicinity. Specifically, this relates to the *dnaJ*-like gene and *htrA* gene upstream of the regulator, as little is known about other genes around the locus. The knowledge that *htrA* in *E. coli* is regulated by a two-component system, namely *cpxR/cpxA* lends further support to the investigation of a putative relationship between the RegX4 and *htrA*, despite scant evidence to link the two. In order to investigate any putative relationship, electrophoretic mobility shift assays and/or DNase I protection assays can be used to identify whether the RegX4 protein can bind the promoter of *htrA* and act as either a transcriptional activator or transcriptional repressor of the gene. Similar techniques have been used successfully before, most notably with ArcA (Lynch and Lin, 1996), OmpR (Rampersaud *et al.*, 1994) and CpxR (Danese *et al.*, 1995; Pogliano *et al.*, 1997) from *E. coli*. These experiments can be coupled with characterisation of a putative relationship between RegX4 and *htrA*, using the pMW10HTRA construct in *C. jejuni* 480. The *regX4* gene can be provided in *trans* on a shuttle vector into the pMW10HTRA, 480 background to observe any change in expression of LacZ. Hypothetically, if any putative relationship between RegX4 and the *htrA* promoter is identified it may be possible that RegX4 is functionally similar to CpxR from *E. coli*. This point can be investigated by using a *cpxR* mutant of *E. coli* coupled with an *htrA-lacZ* fusion (Danese *et al.*, 1995). Introduction of an inducible *regX4* gene in *trans* into an *htrA-lacZ* background could alter expression of LacZ.

Clearly, the main problem associated with assigning a function to the *RegX4* gene in *C. jejuni* and identifying which genes are under the regulatory control of the protein, is the failure to isolate an isogenic *regX4* mutant. This is possibly compounded by the fact that campylobacters are not particularly amenable genetically. An alternative approach to mutating RegX4 other than by insertional mutagenesis, is to examine the effect of short deletions of the C-terminus. This technique has recently been used to good effect with BvgA from *Bordetella pertussis* (Stibitz, 1998). Clearly, this technique may still have a profound effect on the cell. Therefore, more attention should be focused on different ways to alter transcription of the *regX4*. Altering the level of transcription of *regX4* or limiting the amount of available RegX4 protein may affect the production of downstream genes which are members of the same regulatory hierarchy. Any differences in protein profile can be examined by both one-dimensional and two-dimensional protein profiling techniques and N-terminal sequence analysis in an effort to determine the genes involved. Three different methods could potentially be developed and may benefit from having a mechanism whereby expression of RegX4 can be efficiently regulated. This is important given that a complete lack of expression of the protein, as in an isogenic mutant, could potentially be

lethal. That said, the ability to control progression towards lethality using an inducible system may be extremely useful and provide lots of information to help characterise the gene. The first and probably technically easiest method involves cloning *regX4* into a shuttle vector, but in the reverse orientation and transforming the construct into 480. Expression of the *regX4* gene in the reverse orientation will result in the production of antisense RNA which will bind to the sense RNA produced from the chromosomal copy of the gene. The antisense:sense RNA complex may prevent or at least limit translation of sense RNA into protein, thus restricting the amount of RegX4 within the cell. Potentially, this could dramatically alter the level of production of other proteins of the regulon which can be investigated using protein profiling techniques. The second technique takes advantage of the fact that RegX4, may form dimers or oligomers upon binding at the promoter interface to affect transcription of genes under its control. This situation is seen with OmpR (Harlocker *et al.*, 1995; Huang and Igo, 1996; Huang and Lan, 1997). A mutagenised version of RegX4, mutated by site-directed mutagenesis or UV/chemical mutagenesis, can be cloned into a shuttle vector and introduced into *C. jejuni* 480. The effects of such a strategy may vary markedly. Simultaneous expression of the native chromosomal copy of *regX4* and the plasmid located mutated copy of *regX4* may result in different recombinant protein:native protein complexes or recombinant protein:recombinant protein complexes at the promoter interface. In turn, this may affect the binding capacity of the molecule or its interaction with RNA polymerase, resulting in a change in expression of those genes under the control of RegX4. The final technique involves cloning *regX4* on a suicide plasmid together with a large amount of flanking DNA but swapping the promoter of *regX4* with an inducible promoter. Introduction of the suicide plasmid into 81116 together with an antibiotic resistance cassette cloned upstream of the inducible promoter to provide selection will enable homologous recombination to occur. As a result, the chromosomal copy of the promoter of *regX4* will be replaced with the inducible promoter. Selection for recombinants using just antibiotic resistance may not be enough, therefore growth of the recovering transformants must be in conditions whereby the inducible promoter is “on” and *regX4* is expressed. The level of transcription can be altered and the protein profile of the recombinant investigated as previously mentioned. Although in theory each technique seems feasible, the ability to develop such systems in practice together with their ability to produce meaningful results remains to be established.

The problems associated with trying to mutate *regX4* have been noted. These difficulties have hindered the understanding of the function of the protein in *C. jejuni* and the potential for investigating any effect on the regulation of *htrA*. Despite these problems, and in view of the fact that its putative cognate HPK has been identified downstream, there is now an opportunity to

characterise the HPK instead with respect to its function in *C. jejuni*, its putative relationship with the response regulator and whether there is any regulatory effect on *htrA*.

Chapter 8: Identification and mutation of *regY4*

8.1: Introduction

Downstream of RegX4, an overlapping open reading frame of 415 amino acids was identified and revealed homology to histidine protein kinases from the two-component regulatory family. Due to its possible association with *regX4*, the open reading frame was designated *regY4*. Taking these data, the first aim of this part of the project was to describe sequence which relates to the histidine protein kinase, *regY4*, and to fully characterise any important features of the sequence within the framework of other histidine protein kinases. Computer based analyses of the newly identified RegY4, together with other HPKs, can be performed with a view to gaining any insight into its putative function. Not only may this help to assign a function to RegY4, but also to the putative RegX4/RegY4 partnership given that analysis of RegX4 failed to identify any such role.

The inability to obtain a *regX4* mutant led to the suggestion that the gene maybe essential, regulating members of the regulon important for survival of the bacterium. If this is the case, then there are a number of implications relating to the investigation of RegY4. Firstly, given the identification of RegY4, there is now a sound base with which to attempt creation of a mutant in this gene with a view to characterising its role in the cell. As a caveat, similar reasons behind the inability to produce a RegX4 mutant may be also be apparent when trying to obtain a RegY4 mutant, assuming that the two genes function together in a regulatory system to control expression of a distinct set of genes. Nevertheless, the second aim of this part of the project involved creating an isogenic mutant in *regY4*. Production of a *regY4* mutant would facilitate phenotypic analysis with respect to the gene itself and furthermore present the first opportunity to investigate how the putative RegX4/RegY system functions. Phenotypic analysis of the mutant may help to identify other members of the regulon, and additionally, facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*.

8.2: Results

8.2.1: Sequence analysis of pME23 (chapter 7): identification of the putative cognate HPK, *regY4*.

Sequence analysis of plasmid pME23 insert DNA (chapter 7) using primers M13 Forward and M13 Reverse and by progressive primer walking using primers R4D-SEQ1, R4D-SEQ2, R4D-SEQ3 and R4D-SEQ4 showed the presence of an open reading frame believed to be the putative cognate histidine protein kinase of *regX4*. Due to the possible association with RegX4, the protein was designated RegY4 and the sequence is given in figure 8.1. Analysis of important characteristics of the predicted amino sequence of *regY4* are given below. Downstream of *regY4* there is only very limited sequence data obtained from the sequencing reactions using R4D-SEQ4 and M13Forward. A search of the databases using this limited sequence failed to identify any homology with other known data and will not be considered further.

8.2.2: Analysis of the putative open reading frame of RegY4

The newly identified open reading frame of RegY4 was analysed with a view to identifying important features (figure 8.1.). An examination of the non translated region, upstream of the proposed ATG start codon, failed to identify any stem-loop structures. Furthermore, there appears to be a lack of a discernible ribosome binding site (RBS), although, one of two regions at -11 (AAG) and -18 (AGG) relative to the start of the open reading frame may act to position RNA polymerase. As with many coupled response regulator, histidine protein kinase systems, the two genes overlap. The *regX4* gene overlaps the predicted translational start of *regY4* gene by 4 nucleotides, suggesting translational coupling. RegY4 consists of 415 amino acids, has a predicted molecular mass of 48.5kDa and an isoelectric point of 6.66. A computer based analysis revealed RegY4 to be a histidine protein kinase with similarity to over 50 sensor kinases, mostly in the C-terminal region, but there appeared to be no direct homologue. The best score was seen with a histidine protein kinase from *H. pylori* (30% identity 49% similarity) (HP0164-0165), identified from genome sequencing analysis (Tomb *et al.*, 1997). The histidine protein kinase (HP0164-0165) appears to have no known function. RegY4 was also aligned with the complete sequence of RacS, a newly identified histidine protein kinase in our laboratory (Brás and Ketley, unpublished) thought to be the cognate

regX4 →

1 ATGACAAATATTCTTATGATAGAAGATGATTTAGAATTAGCAGAAATTACAGCTGAATAT
 61 TTTGAAAAATTTGATATGAAAGTTGATATAGCTCATGAACCTTATATAGGTCTTTCTAAG
 AACCTTTTTTAACTATACTTTCAACTATATCGAGTACTTGGAAATATATCCAGAAAGATTC
 121 CTTGCATTAAAAGAATATCAGCTTATCATTTTAGACCTTTCTTTGCCAGGGCTTGATGGG
 GAACGTAATTTTCTTATAGTCGAATAGTAAATCTGGAAAGAAACGGTCCCGAACTACCC
 181 CTTGAAGTGTGTGAAGAGATTCTGTAAGATGATACGCCTATTATTGTTTCAAGCGCA
 GAACTTCACACACTTCTCTAAGCATTTTTCATACTATGCGGATAATAACAAAGTTCGCGT
 241 AGACATGATATTACAGATAAGGTTAATGCTTTAGAGCTTGGAGCAGATGATTACCTACCA
 TCTGTACTATAATGTCTATTCCAATTACGAAATCTCGAACCTCGTCTACTAATGGATGGT
 301 AAACCTTATAATCCAAAAGAATTACAAGCACGTATTAAAAGTCATTTAAGACGTATTTCA
 TTTGGAATATTAGGTTTTCTTAATGTTTCGTGCATAATTTTCAGTAAATTCTGCATAAAGT
 361 AATACAAAAGCGCCATAGCAAAAAGTGTAAGATCTTGTATTATGATCAATATAAGCAT
 TTATGTTTTTCGCGGTATCGTTTTTCACATTTCTAGAACAAATACTAGTTATATTCGTA
 421 ATTATTACCATGAAAGGACAAGACTTTAATAATGCTGAATTTGATATTTTAAGC
 TAATAATGGTACTTTTCTGTTCTCGAATGAAATTGATTACGACTTAAACTATAAAATTCG

R4D-SEQ1

481 TATTTGATTAAAAAGAGGGTGGAGTGGAAGTCTGAAGAGCTTGTTTATAATTGTTCT
 ATAACTAATTTTTTCTCCACCTCACCATTACAGCACTTCTCGAACAAATATTAACAAGA
 541 TCTATTAGTGAGGATTCCAGTAATAAAAGTATAGATGTTATTATCAGTAGAATTCGTCAA
 AGATAATCACTCCTAAGGTCATTATTTTCATATCTACAATAATAGTCATCTTAAGCAGTT
 601 AAGATGGGAGATGATCCAAAACTCCAAAATATATTTCACTCTATACGTGGAATAGGTTAT
 TTCTACCCTCTACTAGGTTTTTGAGGTTTTATATAAGTGAGATATGCACCTTATCCAATA

**Overlapping, *regX4* stop
and *regY4* start**

M N K S S I E Y T I T F I F I F
 661 AAGCTTACTCAATGATAATAATCTTCGATTTTTTATACTATAACTTTTATTTTTATTTTG
 TTCGAATGAGTTACTTATTTAGAAGCTAAAAAATATGATATTGAAAAATAAAAAATAAAC

TM1

A G V S V I L G F L W L I E Y D Q Q N Y
 721 CTGGGGTTAGTGTTATACTTGGCTTTTTGTGGCTTATAGAATACGATCAGCAAAATTATA
 GACCCCAATCACAATATGAACCGAAAAACACCGAATATCTTATGCTAGTCGTTTTAATAT
 T R E L N T K Y S L I A N A R L L N F A
 781 CTAGAGAATTAAACACAAAATATTCTTTGATAGCTAATGCAAGGCTCTTAAATTTTGCAG
 GATCTCTTAATTTGTGTTTTATAAGAACTATCGATTACGTTCCGAGAATTTAAACGTC

G V I S E K E F E E Q T K N Y N K M D E
 841 GAGTTATCAGTGAAAAAGAATTTGAAGAGCAGACAAAAAATTATAATAAATGGATGAGA
 CTCAATAGTCACTTTTTCTTAACTTCTCGTCTGTTTTTAATATTATTTTACCTACTCT

R4D-SEQ2

I T E A K Q I R K I L F R G D V L A R V
 901 TAACAGAAGCAAAGCAGATCCGTAAAATTCTTTTAGAGGTGATGTTTAGCTAGGGTTG
 ATTGTCTTCGTTTCGTCTAGGCATTTTAAGAAAAATCTCCACTACAAAATCGATCCCAAC
 E V N N G L I E I I S Y N R Q V Y L N I
 961 AGGTTAACAATGGCTTAATCGAGATTATTTCTTATAATAGACAGGTGATTTAAATATTA
 TCCAATTGTTACCGAATTAGCTCTAATAAAGAATATTATCTGTCCACATAAATTTATAAT
 I Y D G K V Y L Y K D Q D Y Q T Y R Y F
 1021 TTTATGATGGAAAGGTTTATCTTTATAAGGATCAAGATTATCAAACCTATAGGTATTTTA
 AAATACTACCTTTCCAAATAGAAATATTCTAGTTCTAATAGTTTGATATCCATAAAAT

TM2

I I K A I A V A V I C I L V L L Y I Y I
 1081 TTATCAAAGCTATAGCTGTAGCTGTGATTTGTATTCTTGATTGCTTTATATTTATATAT
 AATAGTTTCGATATCGACATCGACACTAAACATAAGAACATAACGAAATATAAATATATA

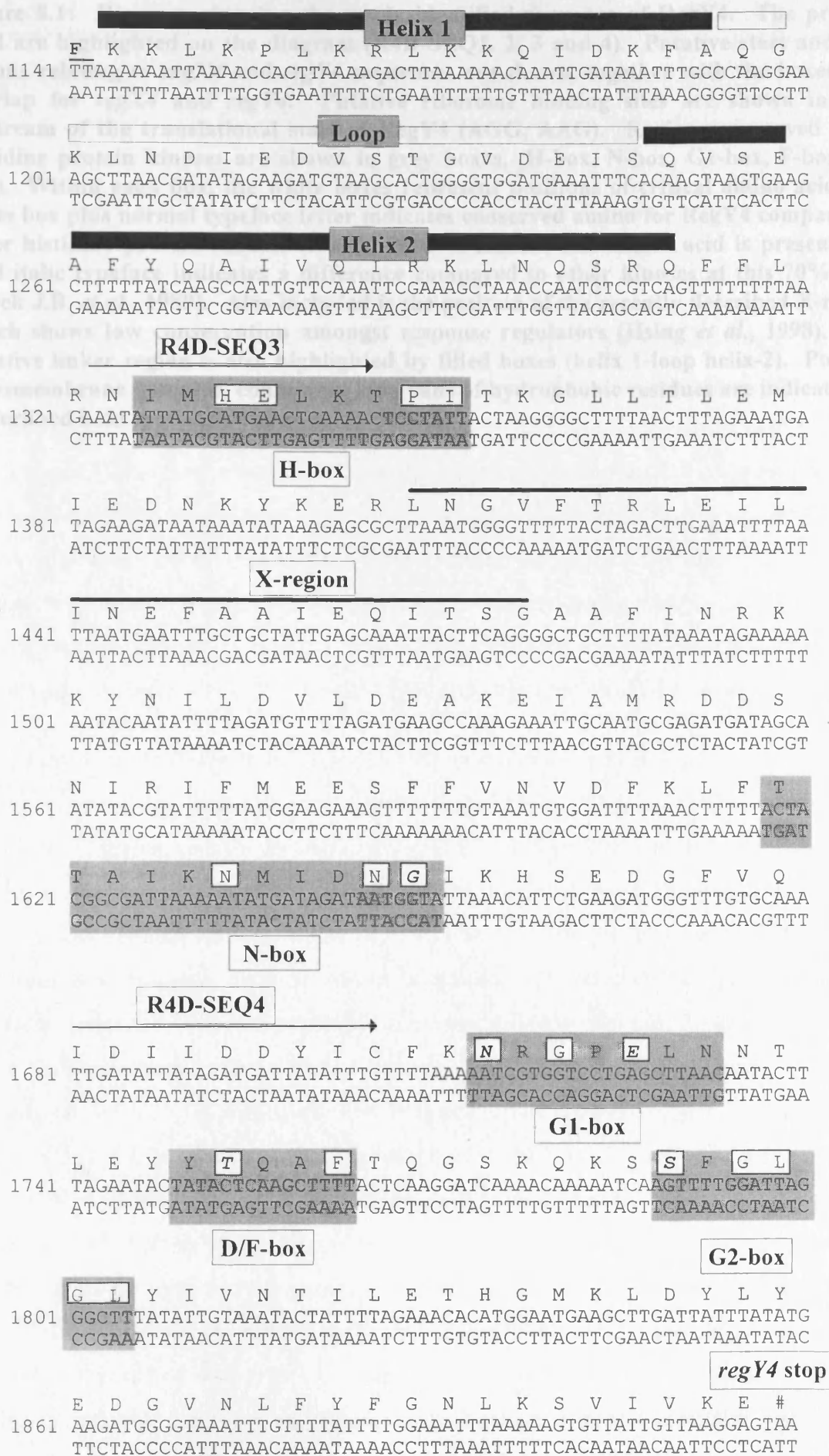


Figure 8.1: Diagram showing the newly identified sequence of RegY4. The primers used are highlighted on the diagram (R4D-SEQ1, 2, 3 and 4). Putative start and stop codons relating to *regX4* and *regY4* sequences are shown, together with the boxed 4bp overlap for *regX4* and *regY4*. Putative ribosome binding sites are shown in bold upstream of the translational start of RegY4 (AGG, AAG). Regions conserved in all histidine protein kinases are shown in grey boxes, (H-box, N-box, G1-box, F-box G2-box). Within each box, the white boxes represent positions of critical amino acids. A white box plus normal typeface letter indicates conserved amino for RegY4 compared to other histidine protein kinases (i. e. in 70% of kinases this amino acid is present). A bold italic typeface indicates a difference compared to other kinases at this 70% level (Stock J.B. *et al.*, 1989). Also included is the position of the recently described X-region which shows low conservation amongst response regulators (Hsing *et al.*, 1998). The putative linker region is also highlighted by filled boxes (helix 1-loop helix-2). Possible transmembrane domains, containing long runs of hydrophobic residues are indicated by underlined bold type-face amino acids.

partner of RacR (chapter 5). The RacR/RacS system appears to be a temperature responsive system which regulates *dnaJ* and *ccp* (Brás and Ketley, unpublished). RacS shows 38% identity and 58% similarity with RegY4. The 10 most similar proteins over the whole sequence identified together with RacS are given in table 8.1. Their associated identity and similarity with RegY4 are highlighted. Many histidine protein kinases contain short blocks of common sequence, arranged in a similar manner but spaced differently. The motifs are referred to by their characteristic residues (blocks “H”, “N”, “G1”, “G2” and D/F) (Stock J.B. *et al.*, 1989; Parkinson and Kofoed, 1992). Despite a recognised consensus for these regions it is not uncommon for non-critical amino acids to vary (Parkinson and Kofoed, 1992). In other histidine protein kinases, whole regions or parts of regions may be absent or vary markedly. For example, CheA from a variety of organisms is missing the H-box but contains a histidine near the *N*-terminus which serves as the site for autophosphorylation (Hess *et al.*, 1988b). The N-box is lacking in FrzE from *Myxococcus xanthus*, the G1-box is missing in CitA from *Escherichia coli* and *Klebsiella pneumoniae*, the F-box is missing from DegS from *Bacillus subtilis* and the G2-box is missing from NarQ and NarX from *E. coli* and NarQ from *Haemophilus influenzae* (Stock J.B. *et al.*, 1989 and alignments made for this study).

An alignment of RegY4 with RacS and the 10 most similar proteins enabled a comparative analysis (figure 8.2.). This alignment highlights that RegY4 fits into a similar framework of other sensor kinases, specifically over the *C*-terminus and exhibits similarities encompassing the previously recognised conserved motifs. In common with other histidine protein kinases the pattern of residues varies. In RegY4, the H-box extends between amino acids 219-227 and contains an invariant histidine residue at position 221, which by analogy is the site of autophosphorylation. The E at position 222 and the proline at position 226 in RegY4 are also very highly conserved amongst sensor kinases. As with other sensor kinases, the N-box precedes the H-box by approximately 100 base pairs. The N-box of RegY4 extends between positions 317-326 and has conserved asparagines at residues 321 and 325. In a very high proportion of histidine protein kinases, an alanine follows the second asparagine but in RegY4 the alanine is substituted for a glycine at position 326. DctS, from *Rhodobacter capsulatus* and PilS from *Pseudomonas aeruginosa* also have glycines in this position. The glycine rich, G1 box is thought to be involved in nucleotide binding and extends between amino acids 348-352. The proposed consensus for G1 is DXGXG (Parkinson and Kofoed, 1992) but RegY4 is particularly variable in this region, harbouring the amino acid sequence NRGPE (or NXGXE). Interestingly, both RacS from *C. jejuni* and HP0164-0165 from *H. pylori* have asparagine at

Table 8.1: Highest homology scores with RegY4

Highest Homology scores with *C. jejuni* open reading frame RegY4

Gene name, Species name, Accession number (Acc.) and Protein identification number (PID)	Identity to RegY4 (%)	Similarity to RegY4 (%)
• <i>racS</i> , <i>Campylobacter jejuni</i> . (Bras and Ketley, unpublished)	• 38	• 58
• ***HP0164/0165, <i>Helicobacter pylori</i> . Acc AE000537; PID g2312351	• 30	• 49
• <i>kdpD</i> , <i>Clostridium acetobutylicum</i> . Acc P94608; PID g3122318	• 25	• 47
• <i>resE</i> , <i>Bacillus subtilis</i> . Acc P35164; PID g466195	• 25	• 46
• <i>phoR</i> , <i>Synechococcus WH7803</i> . Acc U38917; PID g1055348	• 24	• 48
• <i>yclK</i> (<i>phoR</i> -like), <i>Bacillus subtilis</i> . Acc D50453; PID g1805446	• 24	• 46
• <i>phoR</i> (<i>phoR</i> -like), <i>Clostridium acetobutylicum</i> . Acc U58131; PID g3025461	• 24	• 44
• <i>hpkA</i> , <i>Thermotoga maritima</i> . Acc U67196; PID g1575578	• 23	• 47
• <i>cpxA</i> , <i>Escherichia coli</i> . Acc P08336; PID g416838	• 23	• 44
• <i>envZ</i> , <i>Salmonella typhimurium</i> . Acc P08982; PID g119395	• 23	• 44
• <i>phoR</i> <i>Bacillus subtilis</i> . Acc P2354; PID g130130	• 23	• 42

(*** The *H. pylori* gene entry in GenBank is incomplete because it lacked the some of the *N*-terminal amino acids which constitute the input domain. This became apparent in making an alignment of the above proteins. Therefore open reading frames HP0164/0165 were combined to enable a more accurate identity and similarity score together with a better alignment. Please note, the sequence may not be 100% correct. Any subsequent analyses is based on the combination of HP0164/0165).

Chapter 8: Identification and mutation of *regY4*

CJRegY4	-----MKNSSIFYTTITFIFIFAGVSIVLGLFWLIEY	31
CJRacS	-----MTKNYSIHTKLIILFVVVTFFLVCVLFIVLLKI	32
HPHPK	-----MRFSIFFKVVAFMITLEFSFGA	22
ECKdpD(1-420)	ELFSYMGFKDQVNVIMIFILGVIIVYMATKGQIMGIISIAAVLVFNRYFTEPKNSFIVYD	480
BSResE(1-127)	GLS-----DTDTDNERLIVGVPYE---KDGKKGMVFLSQSL LAVKDTTKHTRYIFLAAG	179
SYPhoR	-----	
BSyclK(1-13)	-----HISILILAFVIIIS---LFSHFVKEFAYQNKVEELTSYAVQIANEFQSGQ	60
CAPhoR(1-17)	-----FFVPSSIYLINSSFK---SNLQDEISSGISEKRFSSLIQANLTFFKIKRG	65
TMHpkA	-----MSVFLFVIVA	10
ECCpXa	-----MGSLTARIFAI FWLTLALVLMVLMLPKLDSRQMTLELSD	43
STenvZ	-----MRMRFRSPRSSFARTLLLVTLFLFSVLTLELQV	35
BSPHoR(1-114)	-----TTDN-KLYYGLSLR---SEGEKTYGVLLSAEKSQDLGKELWGLMTASLC	160

CJRegY4	DQQNYIRELNTKYSLIANARLLNFAGVISE---KEFEEQTKNYNKMDEITEAKQIRKILF	88
CJRacS	EGNTYNEEESLKQENLTKNLLISYENTSG--VEIGAYLGNSGFNAIQPNLVKAIRN---	87
HPHK	FAYFYVSSQISHENYQNMRRHYQVFTTINEIL--NNYSYDRAIEDLYIKIGFRETIENTLE	81
EckpD	KSYLVTFPIIMLIVAFIIGSLTKNVKQAEQDSNMREKRTQTLIVSGKLLSAVG-TSEVYS	539
BSResE	IAIVLTTFFAFFLSSRVTYPLRKMREGAQDLA-KGKFDTKIPILTQDEIGELATAFNQMG	238
SYPhoR	-----MSAGTGVVLGLLIGLVQ---GWFLAGRQYQSTTGRSKAN-----	36
BSyclK	VDMMRLYPYQDILSTRKTQFIIFNEEQPYFLPEFGFHHPPREQLLKSEWNKLKGQTVTI	120
CAPhoR	KYDVSYNLTKEELDVFTINTYSLDFDKQKVYIDIVDEKGNVSFVNDLSNKKPKRPELDLNK	125
TMHpKa	VLFVLLFLVFKKRLSEYKILIEKLSDMI GE---KG---VPPLY--LFERLKKY--VD---	57
ECCpxA	RQGLMTEQHVAAELANDPPNDMLMWRRLFRADIKWAPQRRLLVLTTEGRVYTAERSEMG	103
STEnvZ	NFMAILPSQVQENKVLAIEVRMLMDTKLEDGTQLVVPFAFRREIYRELGRISLYTNEAAE	95
BSPhoR	TAFIVIVFYSSMTSRXKYSIESATNVATELS-KGNYDARTYGGYIRRSDKLGHAMNSLA	219

CJRegY4	RGDVLARVEVN---NGLIEIISYNRQVYLNI IID-GKVLYKDQDYQTRYFIKAI---	141
CJRacS	NGQSLFKAGGELCTLSLKYHSNLYFDVQCKDFDGLYEENTSDRVYNLLLIGFFSFS---	144
HPHK	KVLAKRRHQLHHRNICYAEVFTSDMVFILKKD----EHFVLYKDLHSYSRNYFLAIT.	137
EKdpD	IGIKYISRLVNRNIVCYLADTNSKLTSPFYVKDKAKEEIMSKDHNAAAYWTFNLGKE	599
BSResE	RQLNFHINALNQEKEQLSNILSSMADGVITINID-GTILVTNPFAERFLQAWYYEQN---	294
SYPhoR	-LTPSVLSGHALATPQLLAWIDAATQGWLILTPD---LTIGIVNSKAERLLQVSNN---	88
BSyclK	RADGRFDEVSLSVAQPIFVQNEFKGAVLLISPIS-GVEQMVNQVNLMYFAVISTLV---	176
CAPhoR	NQLKYILRDIN--GKTYLFI VSKLKHDSYKFYSIKDVSYDNRKYLLNVLKLN---	180
TMHpKa	-NLKETISRVEVSRDNFLTILNSLSEPIFILDRE-GKITFLNEIARELV-QGRINPE---	111
ECCPxA	IIRNFTQADNADHPQKKKYGLGVGTFVSRVDGEDNYQLYLIRPASSQVDFINLLFDR	163
STEnvZ	EAGLRWAQHYEFLSHQMAQQLGGPTEVRVEVNKSSPVVWLKTWLSPNIWVRPLTEIHQ	155
BSPhoR	ILDMEMTRTQEMORDRLTLVIENIGSGLIMIDGR-G---FINLVNRSYAKQPHINPNHML	275

	Helix-1	Loop	
CJRegY4	---AVAVI---CILVLLYIYIFK KLLKPLKRLKKOTDK FGQGLKNDIEDVSTGVDEIS-Q		193
CJRacS	---LLVVF---MYFSVLKSLPLKRLRQVAEVANGQ- VGLLLILFLFLVLQSSLPLRELRSQVKPSLKGIKAMSCSKQKDEIGDLANEFD-		195
HPHPK	SGCGSTFYRAKGYYIPIKIKNVLGVI-VCSPSGLRPQKAVDVTVTGQAIADLR		189
ECKdpD	---MNIK---EGDNLPEPAEKLFQNAVSTEKEQMIEMTLQGRSWSLLMSPLQYIAESHW-R		658
BSResE	----LLVR---GQALDEVLAVPELEEAIVSVRHQQRQRCWEQQRIPLAIVLPGSD-E		140
SYPhoR	---ITILV---SWLLSKFHVIRIQKLREATDKVASGDYDIHLNSYGDIEIGLASDFN-I		229
BSyclK	-IFIIIILLIIVMLLSKFIKVPINVMIKSTQKIAEGNFERNVSGVRNDEIGLSKNFN-Y		238
CAPHoR	GRPYEYIF---EDYYINEMVEETIKSEPPQEGTLVTYVGNEKKYFHVKVIPVELKSGD-K		167
TMHpKa	PLLLLIVT---MLVSTPLLLWLAWLSLQNRPAKRLKNAADEVAQGNLRQHPLEAGPQEEFLA		220
ECCPxX	DFSPLFRYTLAIMLLAIGGAWLFIIRIQNPLVDLEHAALQVGKGIHPPPLEYGAQSEV-R		215
STEnvZ	RRLYHDAF---EHEEVQLVEDIFMTETKCKLLRLPKIERYFEVDGVPIMGPDDEWK		332
BSPhoR			

	Helix-2	H-box	
CJRegY4	VSEAFYQAIVQIRKLNOSRQ	ELHKTPI	TKGLLTLEMIEDNKYKER----- 245
CJRacS	-----KAFKKNQELIQSRQLFRTIM	ELHKTPI	IGKGRIISEMIKEDRQKER----- 241
HPHPK	-----N--CILKINAMNESRVLFRLRSIM	ELHRTPI	TKGKILSSMLKEELSCKR----- 243
ECKdpD	ILSKEQKSKVEIERERLRNRLRSIS	ELRSP	LAGIKGAASTILENGELIDEKRKQELI 718
BSResE	GAVAVLDRMTEERRLLDKLREDFIANVS	HLRTP	ISMLQGYSEAIVDDIASSSEDRK-EIA 715
SYPhoR	LLVLVLLQNRQSLAQAQQQQRWVSDVA	HELKTP	ITALMLVSDRLLEGAVSEDDGVLVLR-- 198
BSyclK	MAKKLQKSRDEITERLEKRRRRFIADV	HELKTP	ITTINGLVEGLNSHTIPEDKKDK-CFS 288
CAPhoR	MADVIEDKIKELKTTSSDDKQRFIDLL	HEIRTP	ITSIGYADFRLTAKYDEKTLFS--SL 296
TMHpKa	IFVILFHDVTKERKLLDEMRRFETATVS	HLRTP	ITSIHGAETLLLEDLENKELVK-RFL 226
ECCpxA	AGASFNMQMTALERMMTSSQRRLLSDIS	HLRTP	ITRLQGLTALLRRRSGSEKELER---- 276
STEnvZ	VTRAFNMGMAAGVKQLADRTLLMAGVS	HLRTP	ITRIRLATEMMRRGGDGYLAE--- 269
BSPhoR	GLVLVFHDMTETKKLEQMRKDFVANVS	HLKTP	ITSIKGFTETLLDGAMEDKEALS-EFL 391

Chapter 8: Identification and mutation of *regY4* X-region

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CJRegY4  --LNGVFTRLEILINEFAAIEQITSGAA--FINRKKNYILDVLDEAKEIAMRDDSNI-- 303
CJRacS   --LTAIFLRMDSLINEFAKIEIENLFKSNY--NLHFKPSRFSTILEEAKEHLMIDDFNK--V 299
HHPHK    --FSSIFDHLNMLIEQFARIEQLASKNY--GSNKEKFLMSDLIDKIEKMLLIDEDKE--- 290
ECKdpD   NGIYEDTEWLIRLLENLLSMTKFDGENT--KIKKDVELVEEVSEAVQRSSKYFKNH--K 774
BSResE   QIYIDESLRMGRVLNDLLDLARMESGHT--GLHYEKINVNEFLEKIIKRFSGVAKEKNIA 463
SYPhoR   --LQRELRLRLQIMVEDLLELSRLNENILPRDKVDYSALNLEQLVEGAWNIRPLADQRD-V 255
BSyclK   --LISEETKRMLRLVKENLDYEKIRSQOI--TLNKLDPVPLIEVFEIVKEHLQQQAEEKQ-N 344
CAPhoR   NYIYDEGKRLQKLSKLMQLTILRKE---KFEMREESIETLVFRVKRAMLHKLKDK--N 350
TMHpkA   KIIEESARMTRLINDLLDLLEKIEESEEA--NFEMKDVDLCEVIEYVYRIIQPIAEEN--E 282
ECCpxA   --IETEAQRLDSMINDLLVMSRNQOKN---ALVSETIKANQLWSEVLDNAFAEAEQMGKS 331
STEnvZ   --INKDIEECNAIEQFIDYLRGTQEMP-----MEMADLNSVLGEVIAAESGYEREIN-- 320
BSPhoR   SIILKESERLQSLVQDLLDLDSKIEQQNF--TLSIETFEPAKMLGEIETLLKHKADEKGIS 449

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N-box

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CJRegY4  ---IFMEESFFVNVDKFLFTTAIKMIDNGIKHS--E-DGFVQIDIIDDY-----ICFK 347
CJRacS   VKVDIRYD-ALINVDMEIFSVILKLNLDNALKYS--N-NGTCELFCCKEC-----FTIK 345
HHPHK    SPIHVSSSNYIIIEADFELFSIALKINMVDNAIKYS--D-DKQVFLDFIGNN-----LVVS 336
ECKdpD   IKVSVPEDEVIMVSMDSLIEQVIIINLLDNASKFS--PKGSTIEIKVYEKKK-D-VVFEII 830
BSResE   LDHDISLSTEEFMFDEDKMEQVFTNLIDNALRHT--SAGGSVSVISVHSVK--DGLKIDIK 519
SYPhoR   SLTINTNEPGLLLGDQRRHLRAVINLLDNALRYS--PNHGCVEVEILPSGG--WWLLCVR 311
BSyclK   KLMIQVEDHVIVHADYDRFIQILVNITKNSIQFT--Q-NGD--IWLGRMEGYKETIIEIE 399
CAPhoR   IKLKISADKFTHLMDIELITILVTNLIDNALIKAS--KSGDEICFNAYKSSASN-LILEVR 407
TMHpkA   VDLIVECEDVVVRGNKERLIQMLINLVDNAVKYTSLKEKGEKKVWVRAYDTPDWVVVEVE 342
ECCpxA   LTVNFPPGPWPPLYGNPNALESALENIVRNALRYS--HTKIEVGFAVDKDG---ITITVD 385
STEnvZ   --TALQAGSIQVKMHPLSIKRAVANMVVNAARYG---NGWIKVSSGTESH--RAWFQVE 372
BSPhoR   LHLNVPKDPQYVSGDPYRLKQVFNLVNNALTYT--PEGGSVAINVKPREK-D-IQIEVA 505

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G1-box

D/F-box

G2-box

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CJRegY4  NRGPELNN-TLEYTQAFTQGSQKKS-----SFGGLGIYIVNTILETHGMKLDYLYEDGVN 401
CJRacS   NPGKPLAEP-IEHYLEAFTREKHNV-----KMGGLGIYIVSEVCKLHNFDLIYFYDDGKH 400
HHPHK    NKSPLKEDFEKYLQPYFKSSNPQA-----HGGGLGMYIKNNAEAMGLNLSYHSNGRI 387
ECKdpD   DEGGISEDILPNIEQDFFTNGSKIS-DSRRGVLGLAICKSIVEAHGGKIEAVNKGSGG 889
BSResE   DSGSGIPEEDLPFIIEEFYKADKART-RGRAGTGLGLAIVKNIVEAHNGSITVHSRIDKG 578
SYPhoR   DHGPGLSSEDLSNMEQREYRGDPSRARSNRSGSLGLAIVQQIAVNHGGRIQARNHPNGG 371
BSyclK   DTGIGIPKEDIEHIWEREYKADISRTNTAYGEYGLGLSIVRQLVEMHQGTVEIKSEEGK 459
CAPhoR   DTGFGIPKEDVQKVNEPFYMVDKSRE-RANNGAGIGLALCNEIAVLHKGKLYIDSEPKRG 466
TMHpkA   DTGPGIPKEAQSRIDFEKYRVDKARS-RKMGGTGLGLTIVKTIIVDKHGGKIEVESEINQG 401
ECCpxA   DDGPGVSPEDREQIFRPFYRTDEARD-RESGGTGLGLAIVETAIQHRGWVKAEDSPLGG 444
STEnvZ   DDGPGIKPEQRKHLFQPEVRGDSARS---TSGTGLGLAIVQRIIDNHNGMLEIGTSEGG 429
BSPhoR   DSGTGIQKEEIPRIEERFYRVDKDRS-RNSGGTGLGLAIVKHLIEAHEGKIDVTSELGRG 564

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CJRegY4  LFYFGNLKSVIVKE----- 415
CJRacS   CFKIFFGDKKE----- 411
HHPHK    CFTIHDCVFNSFYDLEEDNEELPPPPPKI 427
ECKdpD   AIFKENIPKEL----- 900
BSResE   TTFSFYIPTKR----- 589
SYPhoR   TSMELLLPRGQA----- 383
BSyclK   TKFIIRLPLTAKQQ----- 473
CAPhoR   TNVRVII----- 473
TMHpkA   TLMRVLLPKRR----- 412
ECCpxA   LRLVIWLPLYKRS----- 457
STEnvZ   LSIRAWLPVPVARVQGTTEKA----- 450
BSPhoR   TVFTVTIL-KRAAEKSA----- 579

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Figure 8.2: Alignment of RegY4 together with 10 most similar proteins obtained from SWISSPROT and GENBANK databases. Also included is the sequence of RacS, only recently identified in this laboratory, which is the putative cognate HPK of RacR described in chapter 6. Regions conserved in all histidine protein kinases are shown in grey boxes (H-box, N-box, G1-box, F-box G2-box). The position of the recently identified X-region which shows low conservation amongst response regulators is highlighted (Hsing *et al.*, 1998) together with the putative linker region of RegY4. The proteins used in the alignment are denoted as follows: CJRegY4, *C. jejuni* RegY4 (this study); CJRacS, *C. jejuni* RacS; HPHPK, *H. pylori* histidine protein kinase. (This is a combination of ORFs HP0164 and 0165 from the genome sequence due to a sequencing error); ECKdpD, *E. coli* KdpD; BSResE, *B. subtilis* ResE; SYPhoR, *Synechococcus* WH7803; BSyclK, *B. subtilis*, YclK PhoR-like; CAPhoR, *Clostridium acetobutylicum* PhoR; TMHpka, *Thermotoga maritima* Hpka; ECCpxA, *E. coli* CpxA; STEnvZ, *S. typhimurium* EnvZ; BSPhoR, *B. subtilis* PhoR. Brackets containing numbers for ECKdpD, CAPhoR, BSyclK, BSResE and BSPhoR signify regions of N-terminus not included in alignment. “*” denotes identical or conserved residues in all sequences of the alignment, “:” denotes conserved substitution, “.” denotes semi-conserved substitution.

the beginning of this sequence. Other sensor kinases such as VanS from *Enterococcus faecalis* and CutS from *Streptomyces lividans* have an asparagine in this position. The D/F box commonly contains two phenylalanine residues at specific positions although it is not unprecedented for one or both residues to be absent. This is true of RegY4 in which the D/F box extends from amino acids 360-364 and harbours only one phenylalanine at position 364. A second glycine rich region, called G2, which is also thought to be involved in nucleotide binding contains conserved residues which follow the pattern GXGLGL (Parkinson and Kofoed, 1992). In RegY4 the G2 region extends between amino acids 373 and 378 and has the sequence SFGLGL (or SXGLGL). Again, there is a lack of complete conservation for this region but other kinases exhibit similar properties. For example CzcS from *Alcaligenes eutrophus* and YxdK from *Bacillus subtilis* both contain a serine as the first residue in this region. In addition to such well established motifs, Hsing *et al.*, (1998) have identified a novel motif, the X-region, which is weakly conserved amongst two component regulators. The X-region is thought to confer specificity on the sensor-response regulator interaction. In an attempt to identify a similar region in RegY4, the protein was computer aligned with the *E. coli* sensor kinases used in the paper by Hsing *et al.*, (1998) to show an alignment for the X-region (figure 8.3.). The putative X-region for RegY4 is located between residues 246 and 269 and contains a number of conserved residues which are highlighted. The putative RegY4 X-region can be located close the H-box, but in between the H-box and the N-box.

Parkinson and Kofoed, (1992) have proposed that the C-terminal transmitter domain consists of approximately two equal sub-domains, T_L and T_R, with most of the conserved regions (N, G1, D/F and G2) lying in the T_R domain. In contrast, the T_L domain is quite variable except for the H-box. An average score prediction for α -helix and β -sheet secondary structures for 35 orthodox transmitters has indicated that the two regions have distinctly different secondary structure folding configurations. Their analysis predicts an α - β - α motif surrounding the H-box, followed by a polar region with no apparent structure which resembles a flexible linker. Following the linker, in the T_L domain, there are indications of α - β repeats akin to those found in response regulators. In T_R there are large areas of β -strands interspersed with pockets of sequence without obvious secondary structure. Boxes N and D/F have β -structure, while, boxes G1 and G2 are associated with unstructured segments which correspond to the glycine loops characteristic of many ATP-binding regions. With respect to RegY4, in the proposed T_L there is a great deal of variability surrounding the H-box and the putative linker

RegY4	I	N	G	V	F	T	R	I	E	I	I	N	E	F	A	A	I	E	Q	I	T	S	G	-269	
EnvZ	I	N	K	D	I	E	E	C	N	A	I	I	E	Q	F	I	D	Y	I	R	T	G	Q	E	-293
CpxA	I	E	T	E	A	Q	R	I	D	S	M	I	N	D	L	I	V	M	S	R	N	Q	Q	K	-300
BasS	I	V	A	R	L	D	Q	M	M	E	S	V	S	Q	I	I	Q	L	A	R	A	G	Q	S	-202
CreC	I	L	T	Q	N	A	R	M	Q	A	I	V	E	T	L	I	R	Q	A	R	L	E	N	R	-320
KdpD	I	R	Q	H	V	L	N	T	T	R	I	V	N	N	I	I	D	M	A	R	I	Q	S	G	-730
ArcB	I	H	V	S	A	V	T	I	G	N	I	F	E	N	D	I	I	D	M	D	K	M	E	R	-347
PhoR	M	R	E	Q	T	Q	R	M	E	G	L	V	K	Q	I	I	T	L	S	K	I	E	A	A	-269
RcsC	M	N	N	S	S	S	L	I	L	K	I	I	S	D	I	I	D	F	S	K	I	E	S	E	-518
NtrB	I	I	E	Q	A	D	R	I	R	N	I	V	D	R	I	I	G	P	Q	L	P	G	T	R	-194
NarQ	F	S	Q	A	L	N	D	A	Y	R	Q	I	R	E	L	I	T	T	F	R	I	T	L	Q	-428
NarX	I	R	N	E	L	N	A	S	W	A	Q	I	R	E	L	I	T	T	F	R	I	Q	L	T	-457
CheA	V	E	L	T	L	V	G	S	S	T	E	L	D	K	S	I	I	E	R	I	I	D	P	L	-374

Figure 8.3: (Adapted from Hsing *et al.*, (1998)). Sequence alignment of the X-regions from some *E. coli* sensor kinases together with RegY4 from *C. jejuni* (this study). Sequences of the sensor kinases were obtained from Swissprot. Conserved residues are shaded. Numbers denote final residue number for the alignment.

region, making accurate predictions of the secondary structure very difficult. In contrast, T_R of RegY4, contains the large areas of β -strands interspersed with sequence without discernible secondary structure seen with the analysis by Parkinson and Kofoed, (1992).

Not only does RegY4 appear to be a sensor kinase, but the characteristics of its predicted sequence suggests that it belongs to the subset of sensor kinases that are integral membrane proteins which harbour substantial periplasmic and cytoplasmic domains. Hydrophobicity analysis of RegY4 (figure 8.4.) has located two hydrophobic regions, demarcated by charged residues, in the *N*-terminus between residues 6-29 and 136-159, consistent with their being transmembrane helices (TM1 and TM2, figure 8.4.). Interaction between the two transmembrane domains TM1 and TM2 for other kinases is thought to play an important role in signal transduction (Tatsuno *et al.*, 1994). In this organisation, the domain between residues 30-135 would be exposed to the periplasm. A database search of residues 30-135, which probably form part of the sensor domain, showed no significant homology to any other sensor proteins.

The *N*-terminal periplasmic domain and *C*-terminal cytoplasmic domain arrangement have been found in many membrane histidine kinase sensor proteins (Stock J.B. *et al.*, 1995). Separating the second transmembrane domain and the cytoplasmic domain is the linker region which is also considered to play an important role in propagating the signal from the input domain to the cytoplasmic domain (Ames and Parkinson, 1988) and has been implicated in cytoplasmic signal sensing (Kalman and Gunsalus, 1990; Chang and Winans, 1992; Miller *et al.*, 1992; Monson *et al.*, 1992). Mutations in the linker of EnvZ, (Park and Inouye, 1997) increased the ratio of kinase to phosphatase activity increasing the level of OmpR-phosphate, although had no effect on dimerisation or the enzymatic activities of the truncated cytoplasmic form of EnvZ. This led the authors to postulate that the linker may also have an important role in orientating two EnvZ molecules in a dimer to allow the signal to be transmitted from the extracellular input domain to the cytoplasmic transmitter domain (Park and Inouye, 1997). Secondary structure analysis of the linker regions of histidine kinases which have a topology similar to that of EnvZ in *E. coli* suggests a common helix 1-loop-helix 2 structure (Park and Inouye, 1997). In an alignment, EnvZ and the amino acid sequence of the MCP transmembrane receptors (Tsr, Tar, Trg and Tap) show a high degree of similarity around this region (Park and Inouye, 1997). On this basis, a similar alignment of MCPs, EnvZ and RegY4 was derived to identify a similar linker region in RegY4 (figure

8.5A.). A putative linker region was identified, and was located in accordance with other linkers, in between TM2 and the H-box of the RegY4. The linker region fits within the predicted helix-1 loop helix-2 region of EnvZ, the prototype for orthodox histidine protein kinases, and the MCP transmembrane receptors. To confirm the predicted RegY4 helix-1 loop helix-2 region for the putative linker region, secondary structure predictions were performed using the Chou-Fasman method. The results were as expected and gave an α -helix, turn, α -helix structure (figure 8.5B.).

8.2.3: Characterisation of the *regY4* locus of *C. jejuni*

Creation of a RegY4 mutant should facilitate characterisation of RegY4, help gain further insight in to how the putative RegX4/RegY4 system functions as a pairing, identify other members of the regulon and enable any putative interaction between the RegX4/RegY4 system and *htrA* to be identified. With this in mind, efforts were undertaken to create an isogenic mutant in *C. jejuni regY4*. The strategy involved inverse PCR (Wren *et al.*, 1994) around a plasmid containing *regY4* sequence which could then be used to create a mutated plasmid copy of the gene. Introduction of this fragment into *C. jejuni* 81116 would facilitate homologous recombination to occur between the plasmid copy of the *regY4* gene fragment and its chromosomal counterpart, resulting in construction of a RegY4 mutant.

8.2.3.1: Creation of plasmid constructs

8.2.3.1.1: Construction of pME17

To create an isogenic mutant in *C. jejuni regY4* it was first necessary to subclone the complete RegY4 open reading frame and construct plasmid pME17. 2 μ g of pUC19 plasmid DNA was digested overnight with *Kpn*I and *Pst*I (section 2.11.1.) and electrophoresed on a 1.0% agarose gel (section 2.8.). The linear plasmid DNA was subsequently excised from the gel, purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). A PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.1.) using primers H4-MET (5'*Kpn*I) and H4-STOP (5'*Pst*I) (appendix 1) amplified a single ~1.25kb region of *C. jejuni* 81116 chromosomal DNA specific to the start (ATG) and stop (TAA)

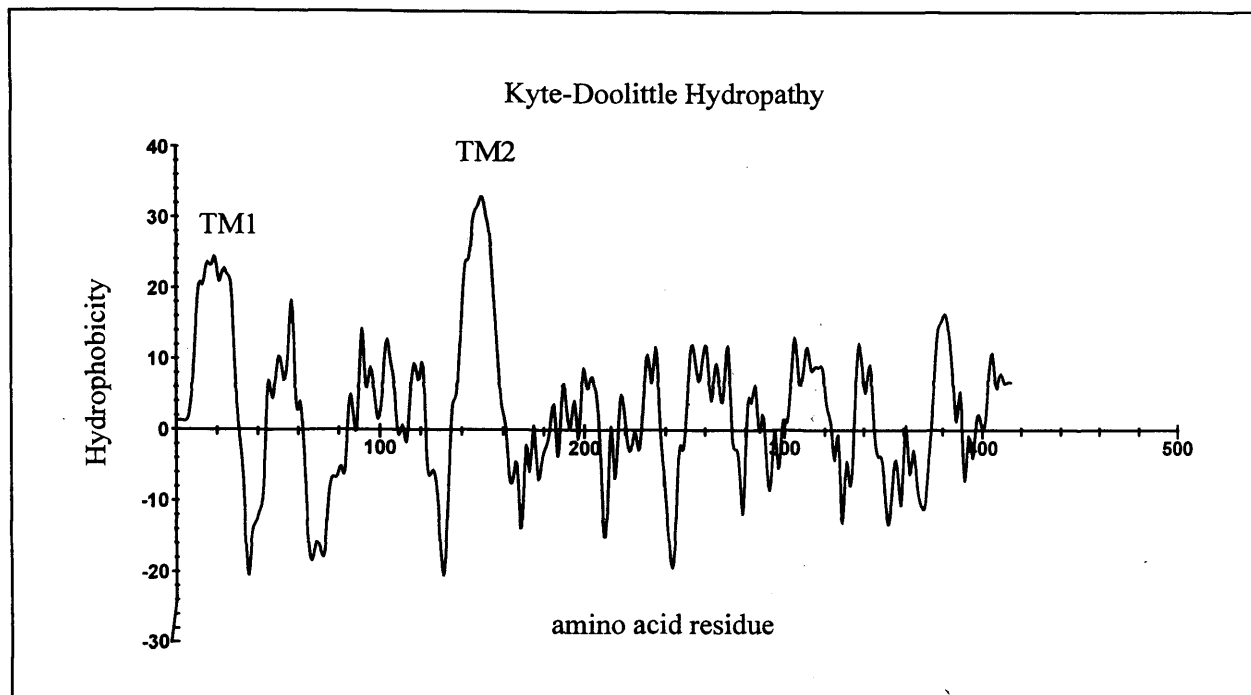


Figure 8.4: Hydrophobicity profile of the predicted amino acid sequence of RegY4. The algorithm of Kyte and Doolittle was used with a window setting of 9. Positive values indicate regions with a hydrophobic secondary structure. Hydrophobic regions of 20 amino acids or greater, which could constitute an α -helical membrane spanning domain are present from amino acid residues 6-29 and 136-159. The two putative transmembrane domains are designated TM1 and TM2 respectively.

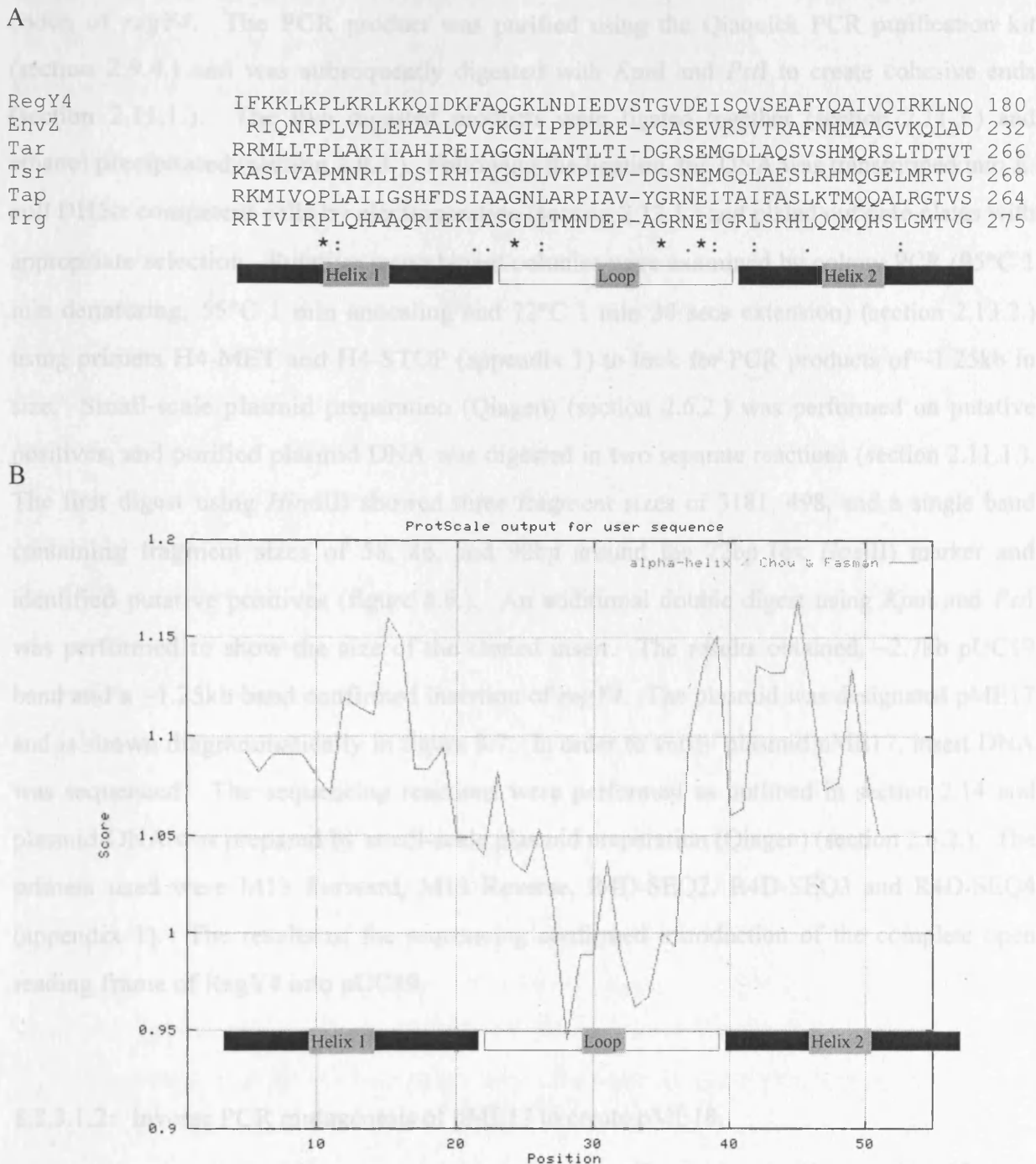


Figure 8.5: A. Alignment of the amino acid sequence of the linker region between RegY4, EnvZ and MCPs, Tar, Tsr, Tap and Trg. The helix-1 loop helix-2 regions predicted for the RegY4 sequence are indicated as bars. The * denotes identical or conserved residues, : indicates conserved substitutions and . indicates semi-conserved substitutions. B Chou-Fasman prediction to confirm the helix-1 loop helix-2 region for the putative linker of RegY4. The higher the score, the greater the probability of an α -helix over that region.

codon of *regY4*. The PCR product was purified using the Qiaquick PCR purification kit (section 2.9.4.) and was subsequently digested with *KpnI* and *PstI* to create cohesive ends (section 2.11.1.). The two digested products were ligated together (section 2.11.3.) and ethanol precipitated (section 2.9.3.). Following the ligation, the DNA was transformed into *E. coli* DH5 α competent cells by electroporation (section 2.12.1.) and plated out onto plates with appropriate selection. Putative recombinant colonies were examined by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) using primers H4-MET and H4-STOP (appendix 1) to look for PCR products of ~1.25kb in size. Small-scale plasmid preparation (Qiagen) (section 2.6.2.) was performed on putative positives, and purified plasmid DNA was digested in two separate reactions (section 2.11.1.). The first digest using *HindIII* showed three fragment sizes of 3181, 498, and a single band containing fragment sizes of 58, 86, and 90bp around the 72bp (ϕ x *HaeIII*) marker and identified putative positives (figure 8.6.). An additional double digest using *KpnI* and *PstI* was performed to show the size of the cloned insert. The results obtained, ~2.7kb pUC19 band and a ~1.25kb band confirmed insertion of *regY4*. The plasmid was designated pME17 and is shown diagrammatically in figure 8.7. In order to verify plasmid pME17, insert DNA was sequenced. The sequencing reactions were performed as outlined in section 2.14 and plasmid DNA was prepared by small-scale plasmid preparation (Qiagen) (section 2.6.2.). The primers used were M13 Forward, M13 Reverse, R4D-SEQ2, R4D-SEQ3 and R4D-SEQ4 (appendix 1). The results of the sequencing confirmed introduction of the complete open reading frame of RegY4 into pUC19.

8.2.3.1.2: Inverse PCR mutagenesis of pME17 to create pME18.

In order to create a RegY4 mutant to help assign a function and to facilitate an identification of any putative relationship between the response regulator gene and the *htrA* gene of *C. jejuni*, it was necessary to construct plasmid pME18 (figure 8.8.). Plasmid pME17 was purified using large-scale plasmid preparation (Qiagen) (section 2.6.3.) and an inverse PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 4 mins extension) (section 2.13.1.) reaction was performed on a 1:500 dilution of plasmid pME17 using primers H4ID1 and H4IU1 (appendix 1). Use of H4ID1 and H4IU1 together creates an internal *BamHI* site, and a 36 bp deletion in *regY4*. The disruption in the coding sequence of *regY4* removes a large proportion of the H-box including the conserved histidine at position 221 which serves as the

site of autophosphorylation. The position of the disruption is approximately midway through the DNA fragment to provide enough flanking DNA either side of the insertion point to facilitate homologous recombination.

The resulting inverse PCR product of ~3.9kb amplified using H4ID1 and H4IU1 was purified using the Qiaquick PCR purification kit (section 2.9.4.) and digested with *Bam*HI overnight (section 2.11.1.). The digested product was electrophoresed on a 1% agarose gel (section 2.8.), excised, and purified using polyallomer wool gel extraction (section 2.9.1.). The DNA was subjected to phenol/chloroform:chloroform extraction (section 2.9.2.) and subsequently ethanol precipitated (section 2.9.3.). 100ng of the digested PCR product was self-ligated (section 2.11.3.) and following ethanol precipitation (section 2.9.3.), transformed into *E. coli* DH5 α competent cells by electroporation (section 2.12.1.). Transformants were plated out using appropriate selection. To check for re-ligated recombinant clones containing an internal *Bam*HI site, colony PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 1 min 30 secs extension) (section 2.13.2.) was performed in 20 μ l reactions using H4D1 and H4U1 (appendix 1). A 10 μ l volume of each completed PCR reaction mixture was electrophoresed on a 1% agarose gel (section 2.8.) to check whether the PCR reaction had worked. The remaining half of the PCR reaction mixture was digested with *Bam*HI (section 2.11.1.). Presence of an internal *Bam*HI site was confirmed by revealing a doublet of ~ 600 bp after *Bam*HI digestion. The plasmid was designated pME18. To verify that the mutagenesis strategy had worked the plasmid was sequenced as outlined in section 2.14 Plasmid DNA was prepared by small-scale plasmid preparation (Qiagen) (section 2.6.2.) and the samples were sent in duplicate to be sequenced. Sequence analysis using R4D-SEQ2 sequencing primer confirmed that the DNA sequence across the deletion was as expected (fig 8.8B).

8.2.3.1.3: Construction of plasmids pME19 and pME20.

In order to facilitate homologous recombination of the mutated plasmid copy of *regY4* with its chromosomal counterpart it was necessary to introduce an antibiotic cassette into plasmid pME18 to provide selection. Plasmid pME18 was purified using large-scale plasmid purification (Qiagen) (section 2.6.3.) and 2 μ g was digested overnight with *Bam*HI (section 2.11.1.) and subsequently phosphatase treated (section 2.11.2.). The plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the linear plasmid DNA was

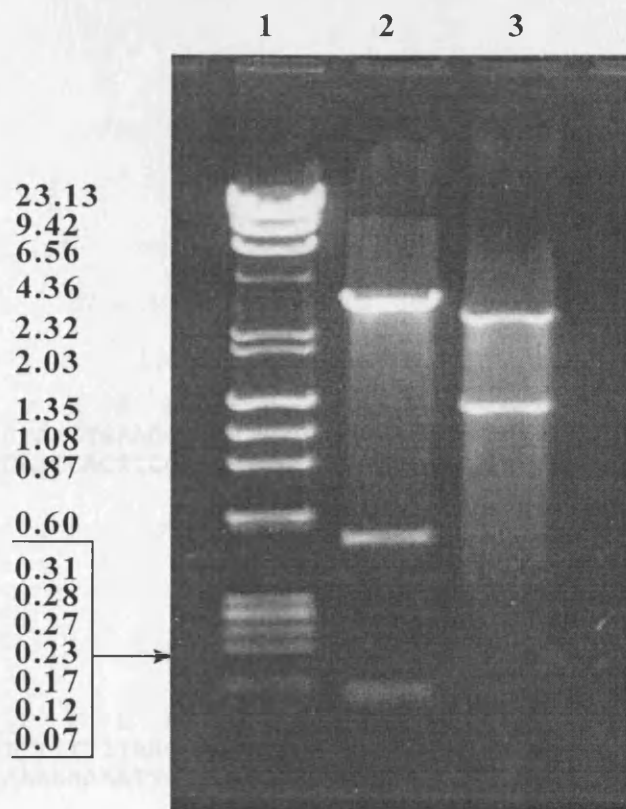


Figure 8.6: Agarose gel electrophoresis of a *Hind*III digest of pME17 (2% gel). Lane 1, λ *Hind*III, ϕ /x *Hae*III marker (kilobase pairs); Lane 2, *Hind*III digest of pME17 to reveal 3 bands of sizes 3181bp, 498bp and a single band ~72bp, (90, 86 and 58bp respectively). Lane 3, *Kpn*I, *Pst*I double digest of pME17 to reveal 2 fragment sizes of ~2.7kb and ~1.25 kb which relate to pUC19 and the amplified *regY4* insert.

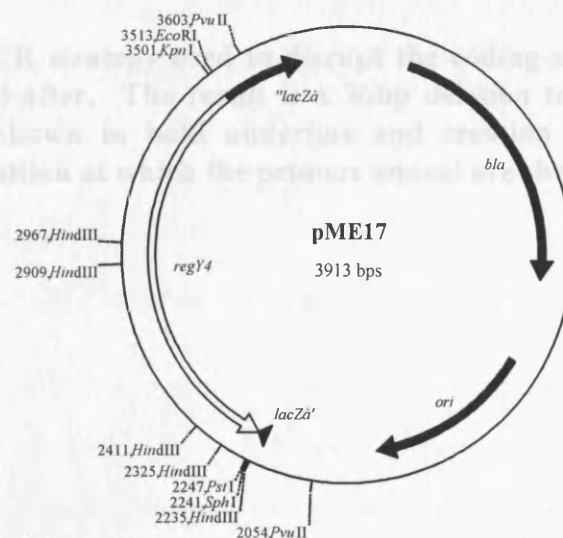


Figure 8.7: Diagrammatic representation of plasmid pME17. The complete open reading frame of RegY4 (white arrow) was cloned using *Kpn*I and *Pst*I.

subsequently excised from the gel (section 2.8.) and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform/chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

Similarly, 2 µg of plasmid pJMK30 which contains the *C. jejuni* kanamycin resistance gene was digested overnight with *Bam*HI (section 2.11.4.) to remove the resistance cassette. The digested plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 1495bp kanamycin resistance cassette was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform/chloroform extraction (section 2.9.2.) and

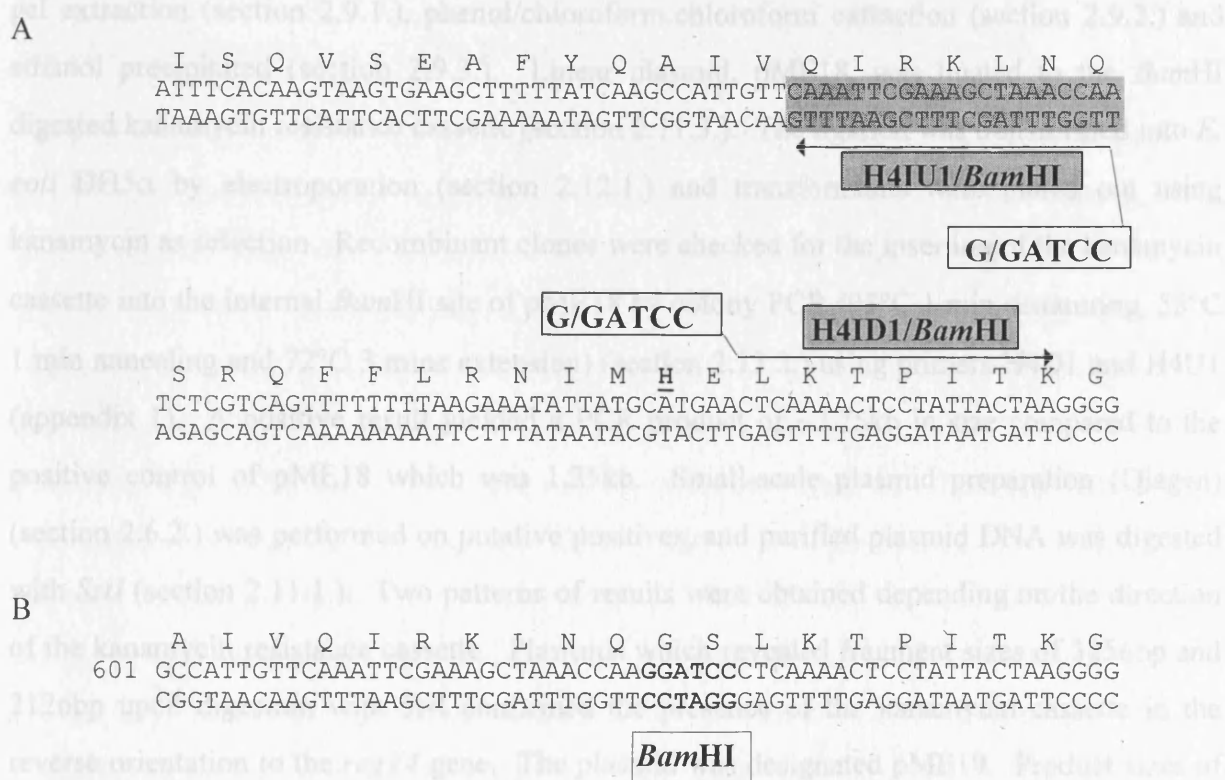


Figure 8.8: Inverse PCR strategy used to disrupt the coding sequence of *regY4*. (A)-before mutagenesis, (B)-after. The result is a 36bp deletion to remove the conserved histidine residue (H) shown in bold underline and creation of an internal *Bam*HI restriction site. The position at which the primers anneal are shown by arrows.

8.2.3.2: Disruption of the *regY4* locus in *C. jejuni* 81116 using pME19 and pME20

Electrotransformation of pME19 and pME20 into *C. jejuni* 81116 was performed as outlined in section 2.12.2.. 50 µl of competent 81116 cells were electrotransformed with 10 µg of pME19 and pME20. As electrotransformation negative and positive controls, 50 µl of competent 81116 cells were electrotransformed in the absence of DNA and in the presence of 1 µg of plasmid pTNSA respectively (appendix 2). Resistant colonies for the positive control could be seen

subsequently excised from the gel (section 2.8.) and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.).

Similarly, 2µg of plasmid pJMK30 which contains the *C. coli* kanamycin resistance gene was digested overnight with *Bam*HI (section 2.11.1.) to remove the resistance cassette. The digested plasmid was electrophoresed on a 1.0% agarose gel (section 2.8.) and the 1499bp kanamycin resistance cassette was subsequently excised and purified using polyallomer wool gel extraction (section 2.9.1.), phenol/chloroform:chloroform extraction (section 2.9.2.) and ethanol precipitated (section 2.9.3.). Linear plasmid, pME18, was ligated to the *Bam*HI digested kanamycin resistance cassette (section 2.11.3.). The ligation was transformed into *E. coli* DH5α by electroporation (section 2.12.1.) and transformants were plated out using kanamycin as selection. Recombinant clones were checked for the insertion of the kanamycin cassette into the internal *Bam*HI site of pME18 by colony PCR (95°C 1 min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.2.) using primers H4D1 and H4U1 (appendix 1). A positive result yielded a PCR product of ~2.75kb in size compared to the positive control of pME18 which was 1.25kb. Small-scale plasmid preparation (Qiagen) (section 2.6.2.) was performed on putative positives, and purified plasmid DNA was digested with *Sst*I (section 2.11.1.). Two patterns of results were obtained depending on the direction of the kanamycin resistance cassette. Plasmids which revealed fragment sizes of 3256bp and 2126bp upon digestion with *Sst*I confirmed the presence of the kanamycin cassette in the reverse orientation to the *regY4* gene. The plasmid was designated pME19. Product sizes of 4725bp and 657bp confirmed cloning of the kanamycin gene in the same orientation as *regY4*. The plasmid was designated pME20 (figure 8.9.). The two plasmids are shown diagrammatically in figure 8.10.

8.2.3.2: Disruption of the *regY4* locus in *C. jejuni* 81116 using pME19 and pME20

Electrotransformation of pME19 and pME20 into *C. jejuni* 81116 was performed as outlined in section 2.12.2. 50µl of competent 81116 cells were electrotransformed with 10µg of pME19 and pME20. As electroporation negative and positive controls, 50µl of competent 81116 cells were electroporated in the absence of DNA and in the presence of 1µg of plasmid pTNS#A respectively (appendix 2). Resistant colonies for the positive control could be seen

after 2 days. For the pME19 and pME20 transformants, colonies could be seen after 3-4 days. All transformants had typical *Campylobacter* colony morphology as observed on the plates. Putative positive transformants were sub-cultured onto Mueller-Hinton agar plates supplemented with 50µg/ml of kanamycin and incubated overnight at 37°C in a microaerophilic atmosphere.

8.2.3.3: Analysis of transformants

8.2.3.3.1: PCR analysis to confirm RegY4 mutation

Transformants of pME19 and pME20 were examined by PCR to determine whether the RegY4 gene had undergone homologous recombination. Chromosomal DNA was extracted from the cells using the small-scale *Campylobacter* DNA extraction technique (section 2.7.1.). PCR (95°C 1min denaturing, 55°C 1 min annealing and 72°C 3 mins extension) (section 2.13.1.) was performed on a 1:1000 dilution of chromosomal DNA using primers R4IU1 and H4-STOP (appendix 1). R4IU1 binds to sequence external to that from the pME19 and pME20 insert DNA and use of the primers together indicates whether the chromosomal *regY4* locus had been disrupted. In the reaction, two controls were included; a negative control with no DNA template and a positive control of 81116 DNA. The PCR products were separated on a 1% agarose gel (section 2.8.). All five chromosomal preparations from each plasmid transformation showed a band of approximately the right size (~3.0kb), consistent with the introduction of the kanamycin cassette into the *regY4* gene (figure 8.11.). One of each potential mutant, i.e., one pME19 transformant and one pME20 transformant was further analysed by Southern hybridisation.

8.2.3.3.2: Southern hybridisation analysis to confirm *regY4* mutation

Southern hybridisation analysis was required to confirm the authenticity of both mutants. Chromosomal DNA from the two strains, along with appropriate controls, was probed with either the *regY4* gene fragment obtained from PCR using primers H4D1 and H4U1 (appendix 1), *Bam*HI digested kanamycin resistance cassette from pJMK30 (appendix 2) or pUC19 digested with *Hind*III and *Eco*RI. Chromosomal DNA from both mutants, as well as from the parent strain, was prepared by the small-scale *Campylobacter* DNA preparation technique (section 2.7.1.) and analysed by agarose gel electrophoresis (section 2.8.). To perform each of

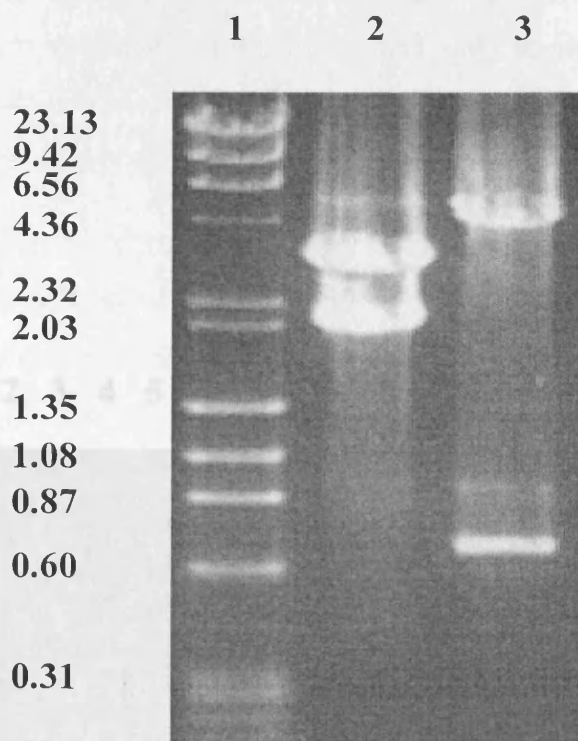


Figure 8.9: Agarose gel electrophoresis of an *Sst*I digest of plasmids pME19 and pME20. Lane 1, λ *Hind*III/ ϕ x *Hae*III marker (kilobase pairs); Lane 2, pME19 digested with *Sst*I to reveal fragment sizes of 3256bp and 2126bp; Lane 3, pME20 digested with *Sst*I to reveal fragment sizes of 4725bp and 657bp.

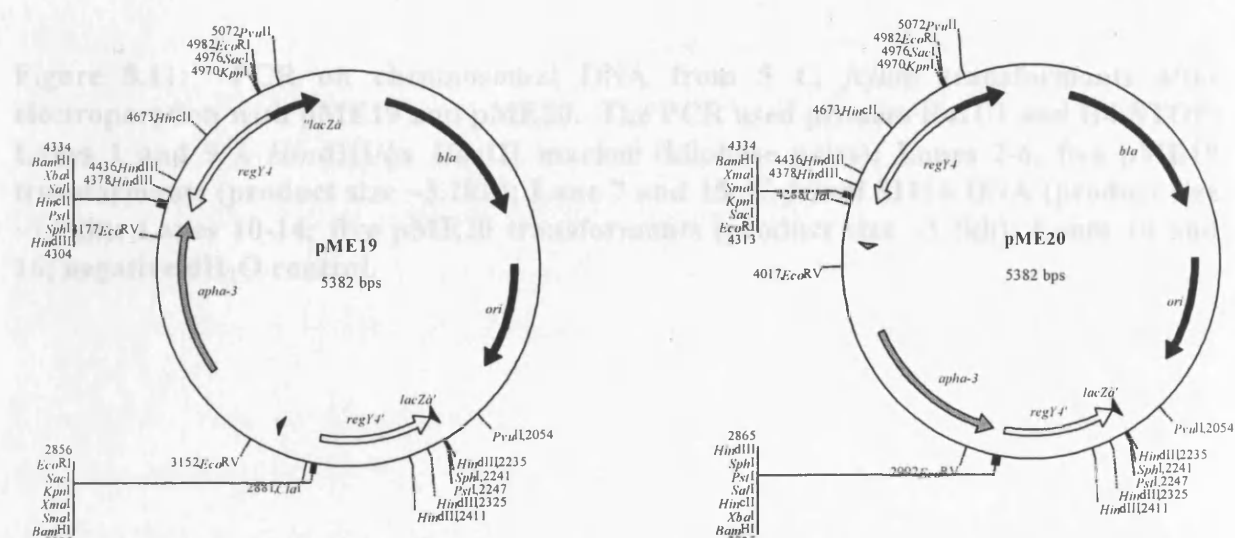


Figure 8.10: Diagrammatic representation of plasmids pME19 and pME20. The *regY4* gene (white arrow) is disrupted with the kanamycin resistance gene (*apha-3*) (grey arrow) in both orientations.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

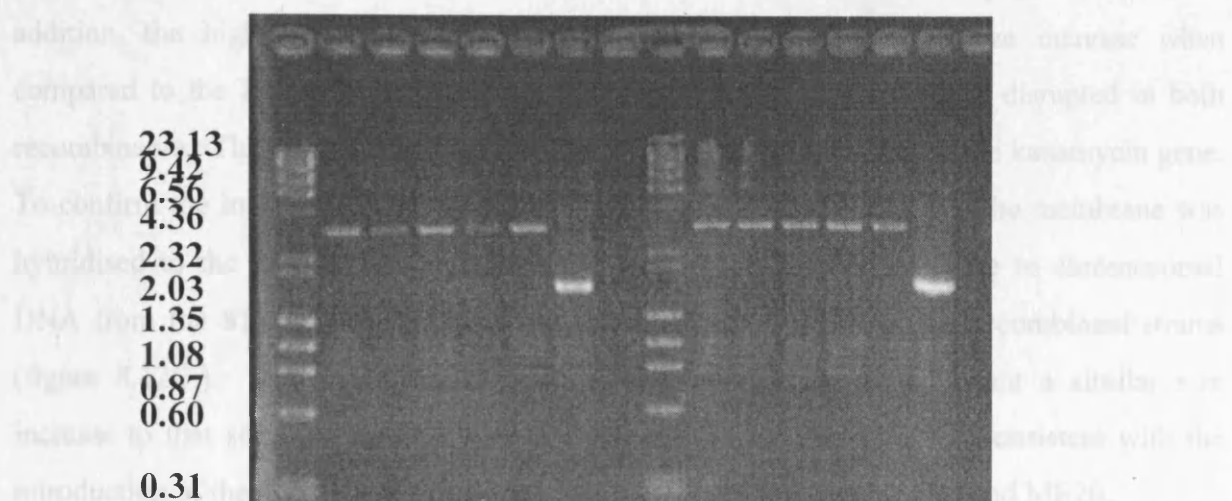


Figure 8.11: PCR on chromosomal DNA from 5 *C. jejuni* transformants after electroporation with pME19 and pME20. The PCR used primers R4IU1 and H4-STOP. Lanes 1 and 9 λ HindIII/ ϕ x HaeIII marker (kilobase pairs); Lanes 2-6, five pME19 transformants (product size ~3.2kb); Lane 7 and 15, *C. jejuni* 81116 DNA (product size ~1.7kb); Lanes 10-14; five pME20 transformants (product size ~3.2kb); Lanes 10 and 16; negative dH₂O control.

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the three hybridisation experiments, approximately 5µg of chromosomal DNA from each of the strains was restricted with *Bgl*III (section 2.11.1.). Different positive DNA controls were included, according to the hybridisation performed, and were the same as the DNA sample used as a probe. Samples were separated on a 1.0% agarose gel, together with the controls (section 2.8.). The Southern hybridisation was performed as outlined in section 2.15.

The membrane probed with *Hind*III and *Eco*RI digested pUC19 DNA revealed that the probe hybridised to the ~2.7 kb pUC19 band from the pUC19 positive control but did not hybridise to any of the genomic digests (figure 8.12A.). In contrast, the membrane probed with the *regY4* fragment showed that the probe hybridised to all genomic digests (figure 8.12B.). In addition, the highlighted bands in both recombinants underwent a size increase when compared to the 81116 parent strain, indicating that the *regY4* gene was disrupted in both recombinants. The size increase was consistent with the introduction of the kanamycin gene. To confirm the introduction of the kanamycin gene into the *regY4* gene, the membrane was hybridised to the kanamycin gene cassette. The probe did not hybridise to chromosomal DNA from the 81116 parental strain but did so to the DNA from the recombinant strains (figure 8.12C.). The highlighted bands in both recombinants underwent a similar size increase to that seen for the *regY4* gene fragment probe. Again this is consistent with the introduction of the kanamycin gene. The two mutants were named ME19 and ME20.

8.2.4: Comparative polypeptide profile analysis of 81116, ME19 and ME20

The ability to construct a *regY4* mutant has presented the prospect of performing phenotypic analysis on ME19 and ME20 with a view to assigning a role for the RegY4 protein within *C. jejuni*. Also, in a more general context, it has provided the first opportunity to investigate the role of the RegX4/RegY4 two-component system in *C. jejuni* in order to identify other members of the regulon. Furthermore, and more specifically, there is now scope to investigate whether the putative RegX4/RegY4 system is involved in the regulation of *htrA*.

It has already been established that RegX4 is a response regulator and as such should play a regulatory function within the cell. Upon a specific stimulus, RegY4, the putative cognate histidine protein kinase, may interact with RegX4 and effect its regulatory role, altering the expression of genes under its control. With this in mind, but given the limited time available, the most rewarding phenotypic analysis of RegY4 proposed was that of one-dimensional

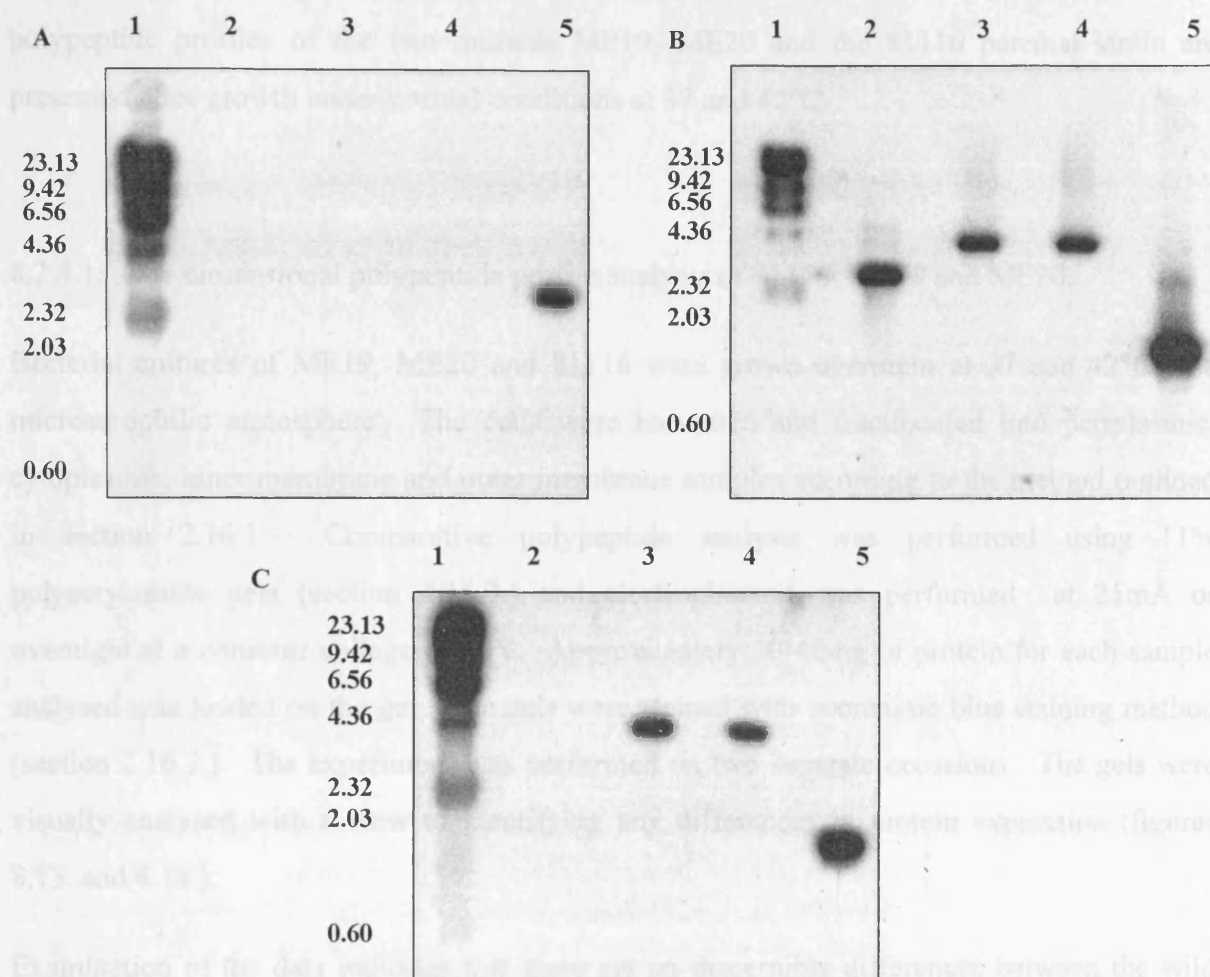


Figure 8.12: Southern hybridisation on ME19 and ME20 to confirm disruption of *RegY4* by homologous recombination. (A) To show the absence of plasmid backbone on the chromosome, *Hind*III and *Eco*RI digested pUC19 was used as a probe. Lane 1, λ *Hind*III marker (kilobase pairs); Lane 2, *Bgl*II digested 81116 DNA; Lane 3, *Bgl*II digested ME19 DNA; Lane 4, *Bgl*II digested ME20 DNA; Lane 5; *Hind*III and *Eco*RI digested pUC19. (B) To show the presence of the disrupted *regY4* gene H4-MET X H4-STOP PCR product was used as a probe. Lane 1, λ *Hind*III marker (kilobase pairs); Lane 2, *Bgl*II digested 81116 DNA; Lane 3, *Bgl*II digested ME19 DNA; Lane 4, *Bgl*II digested ME20 DNA; Lane 5; H4-MET X H4-STOP PCR product. (C) To show the presence of kanamycin resistance gene *Bam*HI digested kanamycin resistance cassette from pJMK30 was used as a probe. Lane 1, λ *Hind*III marker (kilobase pairs); Lane 2, *Bgl*II digested 81116 DNA; Lane 3, *Bgl*II digested ME19 DNA; Lane 4, *Bgl*II digested ME20 DNA; Lane 5, *Bam*HI digested kanamycin resistance cassette from pJMK30.

polypeptide profiling. The value of this technique is that specific proteins which are altered in their expression or are completely absent would be detected, and therefore assumed to be part of the RegX4/RegY4 regulon. As a result, comparative polypeptide profile analysis was performed using ME19, ME20 and 81116. As only preliminary data concerning the putative RegX4/RegY4 pairing is available and the stimulus is unknown, coomassie blue stained polypeptide profiles of the two mutants ME19, ME20 and the 81116 parental strain are presented after growth under normal conditions at 37 and 42°C.

8.2.4.1: One dimensional polypeptide profile analysis of 81116, ME19 and ME20.

Bacterial cultures of ME19, ME20 and 81116 were grown overnight at 37 and 42°C in a microaerophilic atmosphere. The cells were harvested and fractionated into periplasmic, cytoplasmic, inner membrane and outer membrane samples according to the method outlined in section 2.16.1. Comparative polypeptide analysis was performed using 11% polyacrylamide gels (section 2.16.3.) and electrophoresis was performed at 25mA or overnight at a constant voltage of 40V. Approximately 30-40mg of protein for each sample analysed was loaded on the gel. The gels were stained with coomassie blue staining method (section 2.16.3.). The experiment was performed on two separate occasions. The gels were visually analysed with a view to identifying any differences in protein expression (figures 8.13. and 8.14.).

Examination of the data indicates that there are no discernible differences between the wild type and the mutant polypeptide profiles at either of the temperatures for any of the fractionated sample groups. One might expect a difference around the 26kDa region in the cytoplasmic fraction, attributed to the presence or absence of the kanamycin (Apha-3) protein which is constitutively expressed and uniquely present in both mutants. The Apha-3 protein may be present but goes undetected due to the large number of proteins observed around this region. A further difference might be expected in the inner membrane sample around the 48-50kDa region which is the predicted molecular weight of RegY4. The failure to identify any difference in this region might reflect the level of expression of RegY4 related to the number of copies of RegY4 in the inner membrane. The failure to identify any differences between the cells grown at 37°C and 42°C is surprising and might reflect the sensitivity of 1D gel electrophoresis.

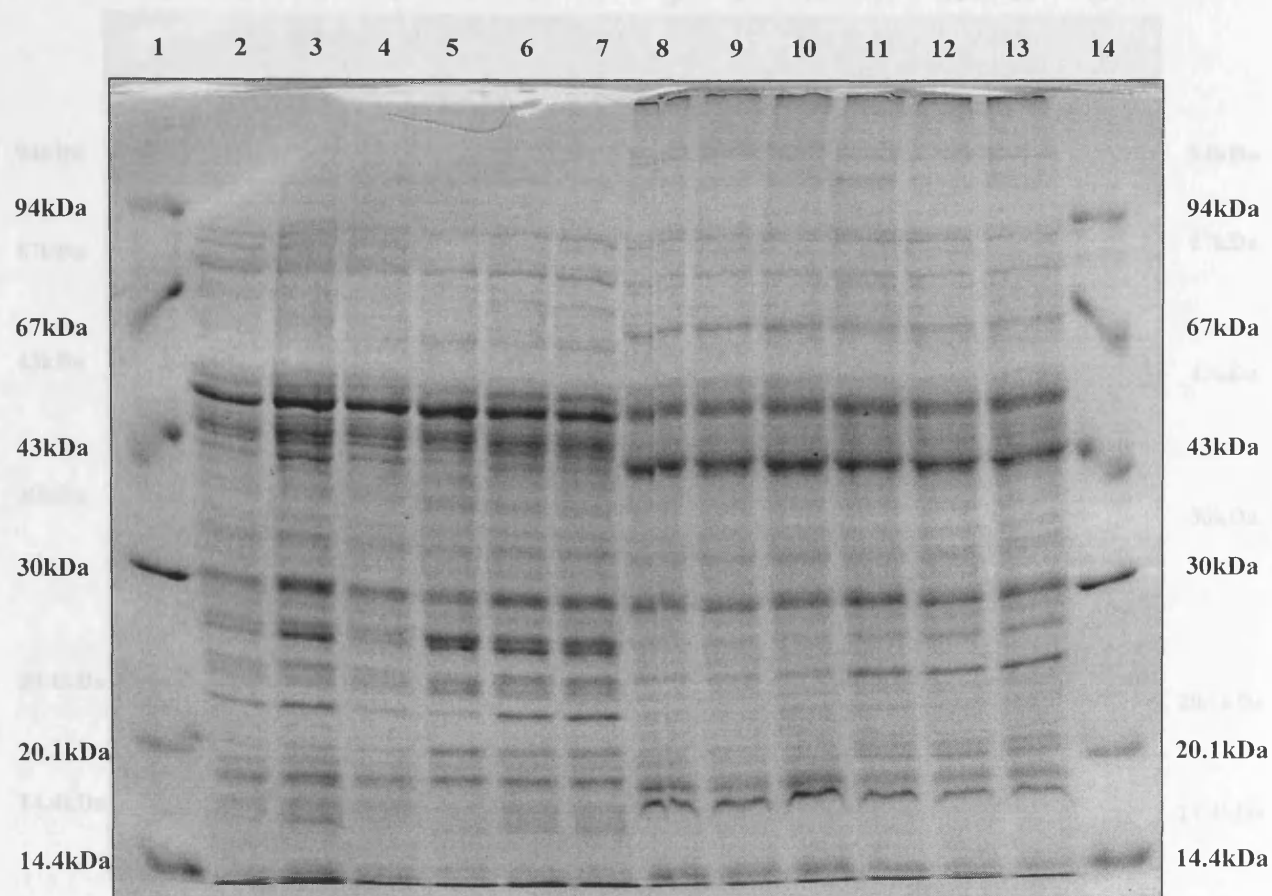


Figure 8.13: Comparison of periplasmic and cytoplasmic polypeptide profiles of 81116, ME19 and ME20. Lanes 1 and 14, low molecular weight marker. Periplasmic profiles: 37°C, lanes 2-4; 42°C lanes 5-7; Lanes 2 and 5, 81116; Lanes 3 and 6, ME19; Lanes 4 and 7, ME20. Cytoplasmic profiles: 37°C, lanes 8-10; 42°C lanes 11-13; Lanes 8 and 11, 81116; Lanes 9 and 12, ME19; Lanes 10 and 13, ME20.

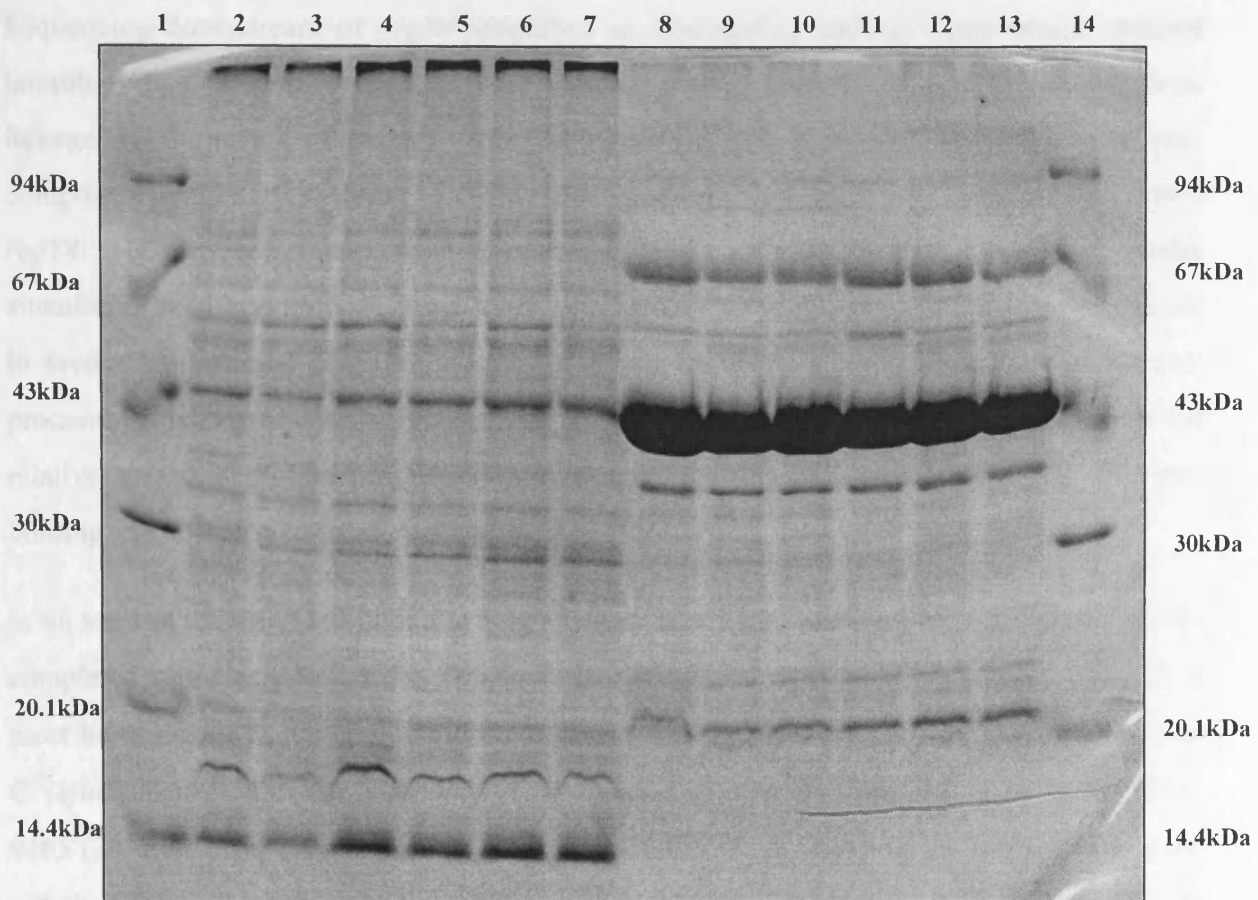


Figure 8.14: Comparison of inner membrane and outer membrane polypeptide profiles of 81116, ME19 and ME20. Lanes 1 and 14, low molecular weight marker. Periplasmic profiles: 37°C, lanes 2-4; 42°C lanes 5-7; Lanes 2 and 5, 81116; Lanes 3 and 6, ME19; Lanes 4 and 7, ME20. Cytoplasmic profiles: 37°C, lanes 8-10; 42°C lanes 11-13; Lanes 8 and 11, 81116; Lanes 9 and 12, ME19; Lanes 10 and 13, ME20.

8.3: Discussion

Sequencing downstream of *regX4* identified an overlapping reading frame which revealed homology to members of the family of histidine protein kinases. Due to the close genetic linkage and the nature of the two genes, their products may represent parts of the same two-component regulatory system. The histidine protein kinase gene was therefore designated *regY4*. The termination and initiation codons in *regX4* and *regY4* overlap by 4bp. A similar situation is seen in *ompR-envZ* and other two-component regulatory systems and is believed to favour translational coupling, thereby allowing an optimal molar ratio between the two proteins. This situation is likely to be true for RegX4/RegY4 with additional control over the relative levels of protein production provided by the potential lack of a strong ribosome binding site, 5' to the translational start of RegY4.

In an attempt to assign a function to RegY4, computer based analyses were performed on the completed amino acid sequence. The findings indicate that over the whole sequence RegY4 is most homologous to the histidine protein kinase, RacS (38% identity, 58% similarity), from *C. jejuni*, identified in our laboratory (Brás and Ketley, unpublished) and *H. pylori* HP0164-0165 (30% identity, 49% similarity) (Tomb *et al.*, 1997). A search of the database using the putative periplasmic component of the sensor domain, residues 30-135, which may be indicative of the nature of the ligand sensed by RegY4, failed to show any significant homology with other sensor kinases. Together, these analyses have hindered identification of a possible function for the regulator.

The prototype structure of sensor kinases consists of an *N*-terminal input domain and the *C*-terminal transmitter domain. This is true of RegY4 where the arrangement of the two domains places the protein within the overall framework of most other "orthodox" sensor kinases, for example EnvZ, PhoR and CpxA (Stock, J.B. *et al.*, 1989; Parkinson and Kofoed, 1992). This is unlike "hybrid" sensor kinases such as VirA and BvgS which account for ~30% of those currently reported (Appleby *et al.*, 1996).

RegY4 shares all the basic architectural features with most other sensor kinases. It belongs to the subset of sensor kinases that are integral membrane proteins which harbour substantial periplasmic and cytoplasmic domains (Stock J. B. *et al.*, 1989; Parkinson and Kofoed, 1992). There are two transmembrane segments, designated TM1 and TM2, which for other sensor kinases are believed to be important for signal transduction (Tatsuno *et al.*, 1994).

Furthermore, the *N*-terminal regulatory or input domain, including the periplasmic domain is separated from the *C*-terminal catalytic or transmitter domain by a linker region. To date, the signal detected by the periplasmic domain has not been identified. The linker region which separates the two domains is also considered to play an important role in propagating the signal from the input domain to the cytoplasmic domain (Ames and Parkinson, 1988) and has been implicated in cytoplasmic signal sensing (Kalman and Gunsalus, 1990; Chang and Winans, 1992; Miller *et al.*, 1992; Monson *et al.*, 1992). Examination of RegY4 led to the identification of a putative linker region, which in accordance with other sensor kinases, was located in-between TM2 and the H-box of the RegY4. Secondary structure predictions of this region confirmed a helix-1 loop helix-2 structure.

The *C*-terminal transmitter domain contains all of the conserved “box” motifs common to the sensor family, H, N, D/F, G1 and G2 including most of the so-called “critical” amino acids. Although in common with other kinases, amino acids within these box regions vary (Parkinson and Kofoed, 1992). Hsing *et al.*, (1998) have recently identified the X-region between the H and N-boxes which is reported to be weakly conserved amongst two-component regulators which is thought to confer specificity on the sensor-response regulator. An alignment of RegY4 together with other characterised sensor kinases located a similar region. A diagrammatic representation of RegY4 is given in figure 8.15.

Secondary structure elements around the conserved histidine in RegY4 were difficult to predict due the variability of the sequence around this region. The *C*-terminus of RegY4, contains large areas of β -elements punctuated with tracts of non-discernible structure which is in accordance with the structural outline described by (Parkinson and Kofoed, 1992).

An examination of the protein structure of RegY4 has identified a number of “conserved” regions, compared to other sensor kinases. This provides a sound base upon which to postulate their contribution to the efficient functioning of the molecule. For other sensor kinases, a combination of physical and biochemical analyses performed on the protein together with genetic analysis of important regions within their structure has meant an increasing amount of information being gathered. This information can be subsequently applied to RegY4 in an effort to characterise the molecule further. Thus far, according to the analyses of the protein structure in this study, RegY4 appears to be a typical member of the “orthodox” family of sensor kinases. As such, the molecule may harbour similar characteristics to those of sensor kinases which have been better characterised. As a caveat, it

is unlikely that the function of any two sensor kinases is exactly the same. Therefore, the actual role of these “conserved” regions in RegY4 will only be established in the event of additional characterisation of the molecule.

Within the context of this study, further characterisation of RegY4 led to efforts to try and mutate the gene. The failure to mutate RegX4 as described in the previous chapter, meant that construction of a RegY4 mutant would provide another approach to investigate how the putative RegX4/RegY4 system functions, provided that the two proteins form part of the same system. In addition, it might aid identification of other members of the regulon and enable any putative interaction between the RegX4/RegY4 system and *htrA* to be identified. In contrast to RegX4, attempts to make a RegY4 mutant proved successful using the same experimental techniques and conditions. The ability to mutate different components of a two component system can vary. There are examples in the literature where the authors have been unable to produce null mutants in both the sensor kinase and the response regulator. For example, the ExoS/ChvG-ChvI systems from *Rhizobium spp.*, (Cheng and Walker, 1998; Østerås *et al.*, 1995). In these instances, the authors indicate the genes may be essential under the conditions used to isolate the mutants. There is also at least one report where construction of a response regulator mutant has failed but construction of a sensor kinase mutant has been successful, the *feuP/feuQ* system in *Rhizobium leguminosarum* (Yeoman *et al.*, 1997). In this case, the author's suggest that the *feuP* mutant may be non-viable or simply slow-growing but do not discuss why they should be able to create a mutant in *feuQ* which is part of the same system (Yeoman *et al.*, 1997). Given these data, it is not surprising that a difference in the ability to produce mutants in the *regX4/regY4* system has been demonstrated. In addition, in two separate systems, *regR/regS* of *Bradyrhizobium japonicum* (Bauer *et al.*, 1998) and *regA/regB* of *rhodobacter capsulatus* (Mosley *et al.*, 1994) both the regulator and sensor kinase have been mutated but only the regulator mutant has given a different phenotype to the wild type. This point might have implications in the ability to determine a phenotype for the *regX4/regY4* system despite construction of a RegY4 mutant.

In the previous chapter, it was intimated that the inability to produce a *regX4* mutant might be because the bacterium is slow-growing or that RegX4 might regulate a particular gene, or a distinct set of genes, which are essential for the continued survival of the organism under the conditions used. Therefore, one might expect a similar inability to produce a RegY4 mutant for the same reasons, provided that they are part of the same two-component regulatory system and function to regulate the same set of genes. Although these data have yet to be

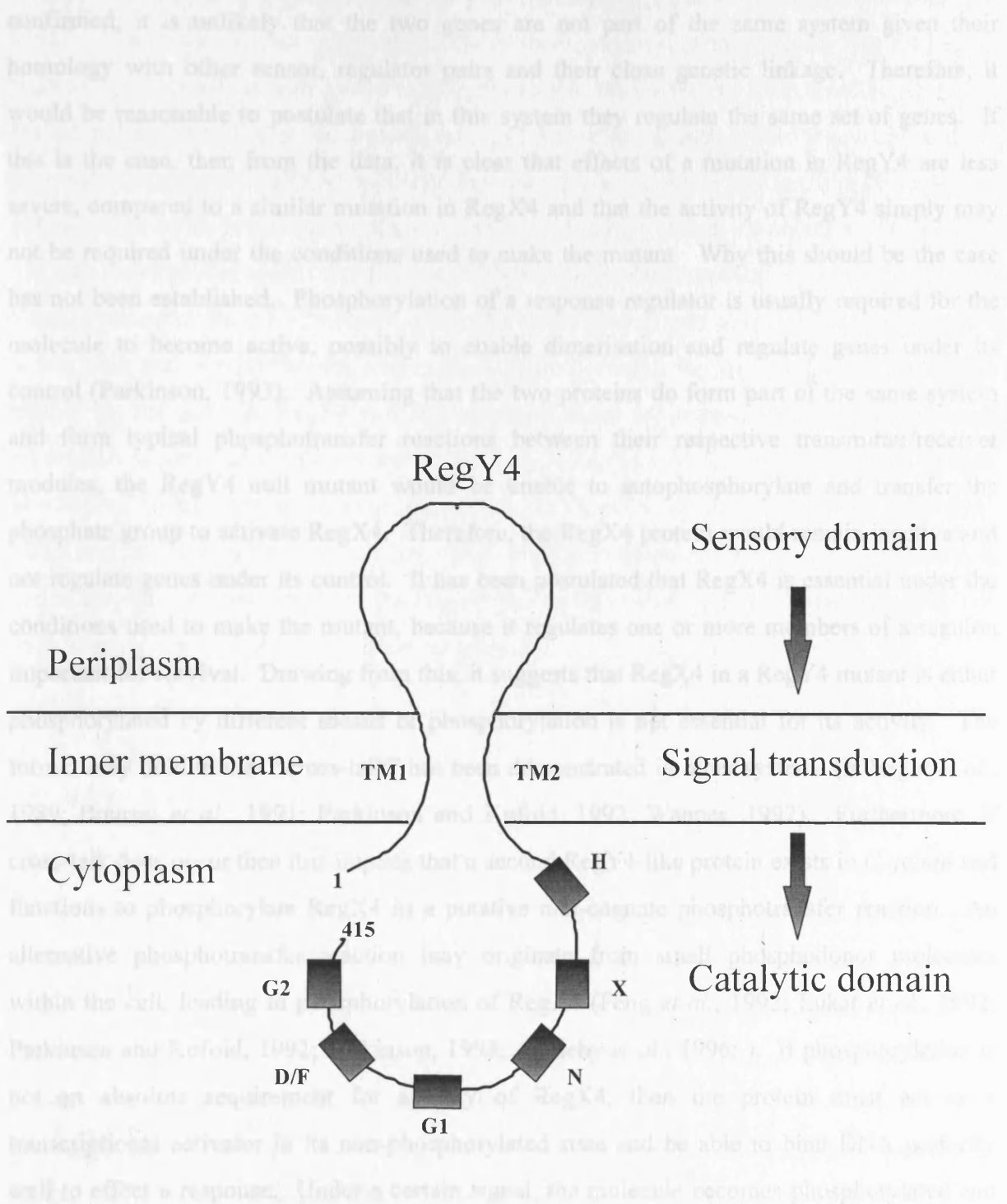


Figure 8.15: Schematic presentation of the structure of RegY4. Conserved regions in the catalytic cytoplasmic domain are indicated with lettered boxes. Arrows denote transfer of sensory information.

confirmed, it is unlikely that the two genes are not part of the same system given their homology with other sensor, regulator pairs and their close genetic linkage. Therefore, it would be reasonable to postulate that in this system they regulate the same set of genes. If this is the case, then from the data, it is clear that effects of a mutation in RegY4 are less severe, compared to a similar mutation in RegX4 and that the activity of RegY4 simply may not be required under the conditions used to make the mutant. Why this should be the case has not been established. Phosphorylation of a response regulator is usually required for the molecule to become active, possibly to enable dimerisation and regulate genes under its control (Parkinson, 1993). Assuming that the two proteins do form part of the same system and form typical phosphotransfer reactions between their respective transmitter/receiver modules, the RegY4 null mutant would be unable to autophosphorylate and transfer the phosphate group to activate RegX4. Therefore, the RegX4 protein would remain inactive and not regulate genes under its control. It has been postulated that RegX4 is essential under the conditions used to make the mutant, because it regulates one or more members of a regulon important for survival. Drawing from this, it suggests that RegX4 in a RegY4 mutant is either phosphorylated by different means or phosphorylation is not essential for its activity. The former may hold true as “cross-talk” has been demonstrated in other systems (Albright *et al.*, 1989; Bourret *et al.*, 1991; Parkinson and Kofoed, 1992; Wanner, 1992). Furthermore, if cross-talk does occur then this implies that a second RegY4-like protein exists in *C. jejuni* and functions to phosphorylate RegX4 in a putative non-cognate phosphotransfer reaction. An alternative phosphotransfer reaction may originate from small phosphodonor molecules within the cell, leading to phosphorylation of RegX4 (Feng *et al.*, 1992; Lukat *et al.*, 1992; Parkinson and Kofoed, 1992; Parkinson, 1993; Appleby *et al.*, 1996;). If phosphorylation is not an absolute requirement for activity of RegX4, then the protein must act as a transcriptional activator in its non-phosphorylated state and be able to bind DNA perfectly well to effect a response. Under a certain signal, the molecule becomes phosphorylated and its activity is increased. The overall effect increases the molecule’s affinity to promoter sites not normally recognised in its unphosphorylated state, thereby directing regulation of a different or additional set of genes. This situation has been described for UhpA (Manson *et al.*, 1998). The affinity of the molecule for different promoter sites may occur as a result of dimerisation or formation of large multimeric complexes which effect the DNA-binding (Manson *et al.*, 1998).

Construction of a *regY4* mutant presented the prospect of performing comparative one-

dimensional polypeptide profiling on the two mutants, ME19 and ME20, and the wild type 81116. The technique was used to enable further members of the putative RegX4/RegY4 regulon to be identified with a view to assigning a role for the RegY4 protein within *C. jejuni*. Unfortunately, examination of the data revealed no additional insight into the components of the putative RegX4/RegY4 regulon as the results identified no discernible difference in polypeptide profile between any of the fractionated samples at any temperature. The reasons for this are multi-factorial. Firstly, any differences maybe concealed by other proteins of similar molecular weight. Secondly, as RegX4 may function perfectly well without RegY4 it is plausible that any difference may go undetected, except for the absence of the RegY4 protein on the gel. If RegY4 is not stimulated under the experimental conditions used, expression of genes under the control of the RegX4/RegY4 system are unlikely to be altered. In addition, the number of copies of RegY4 within the cell may be few making it difficult to detect any loss of RegY4 in the mutant. The inability to detect any differences is obviously a major drawback in characterising the role of the *reg* locus in *C. jejuni*, a situation which will only be remedied by further experimental analysis.

8.4: Future Work

Information regarding RegY4 is limited. To date, it is known that RegY4 is a HPK and can be mutated but there are still a number of other questions regarding the locus which remain to be answered. Biochemical data for RegY4 is scarce and further characterisation will help identify specific properties of the protein. In addition, there is a need to establish whether RegY4 is the cognate partner for RegX4. There is also a lack of knowledge regarding the signal(s) which RegY4 detects and whether the putative RegX4/RegY4 system in response to this signal(s) regulates *htrA* expression. Lastly, it is necessary to identify other members of the regulon to help in assigning a role for the putative RegX4/RegY4 system in the cell.

Biochemical characterisation of the protein using an *in vitro* transcription and translation assays will identify the molecular weight. Overexpression of the molecule to develop antibodies against RegY4 and cell fractionation experiments can pinpoint where it partitions in the cell and confirm whether RegY4 is membrane associated. Furthermore, *N*-terminal sequence analysis can confirm the translational start. RegY4 is likely to autophosphorylate at the conserved H residue in the presence of [$\gamma^{32}\text{P}$] ATP and this can be determined by checking the stability of the phosphorylated protein under different pH conditions.

The putative relationship between RegY4 and RegX4 can be investigated by using *in vitro* phosphotransfer experiments, but taking into account that the phosphotransfer reaction may not be a specific interaction. The relationship can be investigated further by Northern hybridisation analysis to see whether there is transcriptional coupling which will indicate whether the genes are part of the same operon. There is no discernible promoter region upstream of RegY4, apart from that upstream of RegX4. Therefore, to confirm that this promoter controls RegY4 expression, upstream regions of RegY4 can be cloned into the LacZ expression vector pMW10 (Wösten *et al.*, 1998).

Information regarding the environmental signal which controls expression of the *reg* locus can be identified by using data accrued from cloning the *regX4* promoter in pMW10 and investigating expression of LacZ. These data can be coupled with additional information from the *cat::kan* cassette inserted into RegY4, akin to that used with *htrA* in Chapter 5. Northern hybridisation analysis can also be performed under various conditions.

The knowledge that two-component systems often regulate genes in their vicinity, led to the inference that the RegX4/RegY4 system might regulate *htrA*. This putative relationship is enhanced in the knowledge that *htrA* is regulated in *E. coli* by the two-component system *cpxR/cpxA* (Danese *et al.*, 1995). In the previous chapter it was suggested that the relationship could be investigated by DNA-binding assays between RegX4 and *htrA*. This relationship can be further investigated by making a RegY4 mutant in *C. jejuni* 480 and examining transcription from pMW10HTRA. The RegY4 protein can be supplied *in trans* to observe any changes. Mutated versions of the RegY4 protein can also be supplied *in trans* to identify any regions important for the putative RegX4/RegY4 interaction which may bring about transcription of *htrA*. Use of a RegY4 null mutant coupled with a purified RegX4 protein can be used to investigate any putative relationship between this system and *htrA* expression, i.e., does a whole cell protein extract from a RegY4 mutant mixed with the *htrA* promoter cause an electrophoretic mobility shift in the promoter of *htrA*. The RegY4 mutant in 480 can also be used to examine any expression from the RegX4 promoter. This may give an insight into the kind of regulation which *regX4* is under. For example, to determine whether RegX4 is autoregulated.

Most importantly, is analysis of the RegY4 mutant to establish a putative role for the *reg* locus and in addition, identification of other members of the regulon. Obviously, simple phenotypic analysis can be performed such as growth profiles under different conditions,

invasion assays, LPS profiles and motility assays. The analysis using one-dimensional polypeptide profiling unfortunately failed to identify other members of the regulon. As a result, two-dimensional gel electrophoresis would be a better, more sensitive, approach although there is no guarantee that any differences will be apparent. The difficulties in identifying other members of the regulon from polypeptide profile analysis stem from the lack of knowledge of the signal which activates the RegX4/RegY4 system to begin the regulatory cascade. Until this situation is resolved, such difficulties will remain. An approach applied by other researchers which may work in this instance is the creation of chimeric receptors (Turk *et al.*, 1993; Park and Inouye, 1997). With regards to this situation, a potential combination may consist of the periplasmic and transmembrane domains and linker region from an *E. coli* chemoreceptor together with the cytoplasmic signaling domain of RegY4. The result would be controlled regulation of expression of RegY4 in response to a known signal. The feasibility of such an approach with regards to *C. jejuni* remains to be established.

The null RegY4 mutant created may exhibit a lack of both kinase and phosphatase activities, assuming that the molecule functions in a similar way to other sensor kinases of the EnvZ-family. To identify other members of the regulon might require a more subtle approach, or at least an alternative mutation strategy. For example, mutations which are not null mutations have been known to affect phosphatase and kinase activities in different ways, by transforming the sensor into either a constitutively active or inactive sensor kinase. The active form of a sensor is usually a homodimer, for example CheA (Stock A.M and Mowbray, 1995; Stock J.B., 1996), EnvZ (Yang and Inouye, 1991) and NtrB (Ninfa *et al.*, 1993), but the activity can be affected by constructing heterodimers by either UV/Chemical mutagenesis or by removal or alteration of specific segments. The technique has been utilised previously, for example, co-expression of mutant forms of VirA in wild type cells inhibits the function of the VirA protein as a result of inactive heterodimer formation and thus an inactive VirA (Pan *et al.*, 1993). Alternatively, point mutations in the first transmembrane domain of VirA can either result in a constitutively active or inactive sensor kinase (Pazour *et al.*, 1991; Turk *et al.*, 1993; Doty *et al.*, 1996). Replacing either of the two transmembrane domains of VirA with the *E. coli* aspartate sensor, Tar, reduces the ability of the molecule to respond to the inducer (Turk *et al.*, 1993). In the nitrate-nitrite dual sensing two-component regulatory system, substitution of a single amino acid preceding the transmembrane domain made NarX, one of the sensors, constitutively active resulting in up-regulation of relevant genes (Williams and Stewart, 1997). Similarly, an amino acid substitution at the end of the first transmembrane domain of EnvZ rendered the molecule phosphatase negative, although its

kinase activity remained intact (Tokishita and Mizuno, 1994). In addition, a shortened TM1 domain of the ExoS gene (ExoS-ChvI) of *Rhizobium meliloti* caused up-regulation of the *exo* genes due to constitutive kinase activity or diminished phosphatase activity (Cheng and Walker, 1998). Given the success of heterodimer strategies in other systems, it would be reasonable to attempt similar strategies for RegY4 from *C. jejuni* with a view to identifying other members of the regulon.

Chapter 9: General discussion

The aims of this study were to identify the complete open reading of *htrA*, to investigate whether the gene is expressed and to gain an insight into the regulatory mechanisms which the gene is under, in particular, by examining the putative two-component system downstream of *htrA*. The putative sensor-regulator pairing warranted further characterisation because two-component regulatory systems often control genes in their immediate vicinity and in *E. coli*, regulation of *htrA* is controlled by the two component system, CpxR/A. Indeed, by comparison with the *E. coli* situation, *htrA* in *C. jejuni* may be part of a similar regulatory network. It is noteworthy that whilst writing this thesis, genome sequence analysis of *C. jejuni* NCTC 11168 was initiated and is currently progressing towards annotation and publication. Despite having little input into the practical research in this project, it was deemed a worthwhile exercise to consider the results obtained in this study in light of this additional genomic information. Clearly this would provide a more up-to-date analysis and relate results in this study to the genome as a whole.

9.1: Identification of the complete *htrA* ORF, its regulation and expression

Attempts were made to identify the 5' region of *htrA*, including the promoter region, in order to further characterise the gene and give an insight into the type of regulation *htrA* is under. Previously, it was unclear as to whether the proposed *C. jejuni htrA* was indeed a true gene, and not a pseudogene. Only 3' sequence of *htrA* had been identified, phenotypic analysis of the *htrA* mutant revealed none of the overlapping phenotypes for other HtrA homologues and there was no information regarding expression of the gene. Nevertheless, creation of the *Sau3AI* "mini-library" of the 1.04kb fragment together with results obtained from sequencing the 2.38kb IPCR fragment directly identified the remainder of *htrA* including the promoter region. Identification of additional 5' sequence data of *C. jejuni htrA* helped to confirm the protein as a member of the HtrA family of serine proteases.

Examination of the putative promoter region of *htrA* failed to identify any *E. coli* recognised promoter elements for sigma factors, σ^{70} , σ^{28} and σ^{54} , previously identified in *C. jejuni*. Furthermore regulation of *htrA* in *C. jejuni* is not σ^E -dependent as is the case for *htrA* in *E. coli* (Erickson, 1987; Lipinska *et al.*, 1988; Erickson and Gross, 1989; Wang, 1989), *Salmonella typhimurium* (Martin *et al.*, 1994) and *Yersinia enterocolitica* (Li *et al.*, 1996;

Yamamoto *et al.*, 1996). Therefore the gene is regulated by alternative means (see genome sequence analysis, section 9.2.).

With regards to expression of the gene, efforts were undertaken to develop a system which could initially demonstrate that the gene is expressed and then subsequently be used to investigate expression of *htrA* under different environmental conditions. As a result, the signal which induces expression from *htrA* could be elucidated. Furthermore, subjecting the *C. jejuni htrA* mutant to conditions when HtrA is expressed might provide valuable information about its phenotype. Both the *htrA::cat* transcriptional fusion and the LacZ transcriptional fusion successfully demonstrated expression of *htrA*. Furthermore, knowledge that the gene is actually expressed under normal growth conditions, provides a sound base from which to investigate expression under alternative conditions. Identification of the signal upon which *htrA* is activated would clearly help to characterise the role of the *htrA* gene in *C. jejuni* although preliminary results from the *htrA::cat* transcriptional fusion and the LacZ transcriptional fusion indicate that *htrA* may not be regulated by temperature, at least under the experimental conditions used.

9.2: Genome sequence analysis of *htrA* homologues and suppressors and *dnaJ* homologues.

9.2.1: *htrA* homologues and suppressors

Sequence analysis of the complete *htrA* sequence suggests that it is indeed the true *htrA* homologue. Nevertheless, the failure to identify a phenotype, with particular reference to the lack of thermal and/or oxidative stress sensitivity (Henderson, 1996) and also the failure to show temperature regulation (this study) suggests that the *C. jejuni htrA* may have a novel role. The lack of an obvious phenotype also may also suggest that it may not have been the true *htrA* but an homologous member of the family with or without a similar function. It is not unprecedented for bacteria to possess homologues of HtrA as *B. abortus* contains both an *htrA* and *htrA*-like gene (Tatum *et al.*, 1994) *E. coli* has two homologues, *degQ* and *degS* (Bass *et al.*, 1996; Waller and Sauer, 1996) and *H. influenzae* also contains two *htrA* homologues. Therefore, the genome sequence of *C. jejuni* NCTC 11168 was examined for the presence of homologues of HtrA, but failed to identify any such proteins.

In addition, the differential stress response for *C. jejuni htrA* could be attributed to complementation by either secondary mutations in other genes or the function of additional

loci as examples of these points have been noted elsewhere. In *E. coli*, the double mutant *lpp⁻ htrA⁻*, *lpp* denoting a lipoprotein gene, has reduced thermal sensitivity (Strauch *et al.*, 1989). With reference to additional genes being able to complement the *htrA* mutation, *degQ*, the *htrA* homologue, is able to rescue the temperature sensitive phenotype of *htrA* null mutants together with overexpression of SohB (Baird *et al.*, 1991) and SohA (Baird and Georgopoulos, 1990). Analysis of the genome sequence highlights the lack of a *C. jejuni* *sohA* homologue although using SohB from *E. coli*, a putative ORF was identified. A search of the databases using this ORF showed that it related specifically to the *C. jejuni* PspA protease required for pilus synthesis (Doig *et al.*, 1996). Interestingly, other bacterial endopeptidases of the protease IV family of which SohB is a member were also highlighted. The significance of this in relation to *htrA* is unclear, but PspA of *E. coli* is a member of the heat inducible proteins of *E. coli* (Yamamori, 1982). Taking these findings together it is conceivable that similar compensatory effects might occur for the *C. jejuni* *htrA* mutation although this will only be determined upon further analysis.

9.2.2: Homologues of *dnaJ*

Upstream of *htrA*, in this study, there is a *dnaJ* or *dnaJ*-like gene (designated for this purpose as DNAJ1). Konkel *et al.*, (1998) have recently reported identification of a second *dnaJ* or *dnaJ*-like gene (designated for this purpose as DNAJ2). Moreover, the same *dnaJ* or *dnaJ*-like gene has also been identified in this laboratory, upstream of the two-component regulatory system RacR/S (Brás and Ketley, 1996; unpublished). Possibly more intriguing is the fact that genome sequence analysis using the complete sequence of the *dnaJ*-like gene identified in this project actually identified a third *dnaJ* or *dnaJ*-like gene (designated as DNAJ3). Their relationship to one another is as follows; DNAJ 1 has 57% identity and 79% similarity to DNAJ2 and 36% identity and 53% similarity to DNAJ3. DNAJ2 has 40% identity and 58% similarity to DNAJ3. The idea that bacteria have multiple *dnaJ*-like genes is not unusual as there are at least three *dnaJ* or *dnaJ*-like genes in *E. coli* (*dnaJ*, Georgopoulos *et al.*, 1980; *cbpA*, Ueguchi, 1994; and *djlA*, Clarke *et al.*, 1996) which have a range of overlapping functions. Therefore, it can be postulated that the three *dnaJ* or *dnaJ*-like genes in *Campylobacter* may have similar, overlapping roles, as those of *E. coli*.

9.2.3: Does *C. jejuni* possess *rpoH* and *rpoE* regulons ?

Given the similarity to other HtrAs, *C. jejuni* HtrA is likely to be involved in the bacterial stress response although its mode of action has not been determined. Furthermore the response of *C. jejuni* to stress in general is unclear. For example, the general stress response gene, *rpoS*, (Espinosa-Urgel *et al.*, 1996) which is involved in regulating genes required for stationary phase survival, exposure to osmotic shock and acid stress is absent from the *C. jejuni* chromosome. This is also true of *rpoH* which regulates genes involved in combating stresses in the cytoplasm. Interestingly, despite there not being an *E. coli* *rpoH* homologue, examination of the *C. jejuni* genome has identified homologues of many of the components of the *rpoH* (σ^{32}) regulon which are involved in the cytoplasmic heat-shock response. For example, various proteases such as Lon, ClpP, ClpX, HflB (FtsH) and HslU, chaperones such as DnaK, DnaJ, HtpG, GroEL and GroES, the nucleotide exchange factor, GrpE and the dehydrogenase, GapA all appear to have homologues in *C. jejuni*. As a caveat, many of these heat-shock genes also have house-keeping functions which suggests a reason for their presence. Nevertheless, it is possible that the heat shock-response is present, or at least semi-conserved but is just regulated in a different manner (Konkel *et al.*, 1998).

As with *rpoS* and *rpoH*, there is no *rpoE* homologue on the *C. jejuni* chromosome which supports the failure to identify a σ^E -promoter element upstream of *htrA*. This raises questions about how *htrA* is actually regulated in *C. jejuni*. As before with *rpoH*, despite the lack of an *rpoE* on the chromosome, the genome was examined for other *E. coli* σ^E -regulated genes in addition to members of the Cpx pathway. Together, the two systems are responsible for relieving extracytoplasmic stress in *E. coli*. The purpose behind the analysis was to identify whether similar regulatory networks, which include *htrA*, were also present in *C. jejuni* despite there being no regulatory input from σ^E . In *E. coli*, σ^E mediates transcription of at least ten different genes which function to maintain envelope integrity under stressful conditions (Raina *et al.*, 1995; Rouviere *et al.*, 1995b). To date, only four have been identified, *htrA*, *rpoH*, *fpkA* (member of the FKBP-type peptidyl, prolyl isomerase family, PPIase; Missiakas *et al.*, 1996) and *rpoE* itself (Erickson, 1987; Lipinska *et al.*, 1988; Erickson and Gross, 1989; Wang, 1989; Raina *et al.*, 1995; Rouviere *et al.*, 1995; Danese and Silhavy, 1997). The Cpx pathway consists of CpxR (response regulator) and CpxA (histidine protein kinase) in a two-component signal transduction system induces expression of *htrA* together with *dsbA* (thiol:disulphide oxidoreductase), *ppiA* (PPIase of the cyclophilin family; Danese and Silhavy, 1997; Pogliano *et al.*, 1997) and the recently identified, *ppiD* (PPIase of

the parvulin family (Dartigalongue and Raina, 1998).

Members of the σ^E -regulon and the Cpx pathway, together with a number of folding catalysts/chaperones and sensors of misfolded proteins present in the periplasm of *E. coli* known to be involved in combating stress in the extracytoplasmic environment have been highlighted in a recent paper by Missiakas and Raina, (1997). Predictions about the presence of similar genes in *C. jejuni* are presented in table 9.1. From the table, it can be seen that many of the proteins involved in relieving extracytoplasmic stress in *E. coli* are either absent, or their *C. jejuni* homologues are difficult to assign. Based on these data, the regulatory network that responds to extracytoplasmic insult maybe functionally different from that in *E. coli* although clearly this needs to be confirmed.

Making predictions about the nature of the response in *C. jejuni* compared to *E. coli* is hindered firstly, by the amount of data concerning regulation in *C. jejuni per se* and furthermore, by the fact that many of the members of the σ^E regulon and Cpx pathway which could be involved in this process have yet to be identified in *E. coli*. Therefore, both the regulation of *htrA*, and the proposed involvement of *htrA* in a response network that deals with relieving extracytoplasmic stress is still a matter of conjecture in *C. jejuni*. The fact that *htrA* is not regulated in *C. jejuni* in a σ^E -dependent manner suggests an alternative mechanism, for example an homologous system to the *E. coli* Cpx two-component pathway (Danese *et al.*, 1995). The presence of a putative two-component signal transduction system downstream of *htrA* has been identified. In view of this information, the mechanisms behind *htrA* regulation becomes far more pertinent, especially as two-component systems are also known to regulate genes in their vicinity. These points are considered in more detail below.

9.3: Identification and characterisation of the RegX4/RegY4 TCR system downstream of *htrA*

A putative two-component regulatory system has been identified downstream of *htrA* and has been designated RegX4/RegY4. RegX4 is the response regulator and RegY4, the histidine protein kinase. RegX4 is a member of the OmpR sub-family and as such contains all of the characteristic features of this family of response regulators including *N*-terminal conserved residues of the acidic pocket, hydrophobic residues important for the conformation of the molecule and the *C*-terminal winged-helix-turn-helix formation, which for OmpR, is thought

Table 9.1: *E. coli* folding catalysts/chaperones and sensors of misfolded proteins in *C. jejuni*?

Folding catalysts/chaperones and sensors of misfolded proteins in the periplasm of <i>E. coli</i> and <i>C. jejuni</i>		
Family name	Function/relevant characteristics	Presence / Absence
<u>Peptidyl, prolyl isomerases (PPIs)</u>		
• RotA	• Belongs to cyclophilin-type PPI family (no known substrate)	• ? (At least one rotamase present)
• SurA	• Belongs to the PPIc/parvulin type PPI family involved in the folding of OMPs	• ? (Homology to OMP, Peb4A)
• FpkA	• Belongs to the FKBP-type PPI family	• ✕
• PpiA	• Belongs to cyclophilin-type PPI family	• ✓
• PpiD	• Belongs to the PPIc/parvulin type PPI family	• ?
<u>Protein disulphide isomerases (PDIs)</u>		
• DsbA	• Thiol:disulphide oxidase involved in the folding of many transported proteins	• ✓ • ✓
• DsbB	• Thiol:disulphide oxidase. Responsible for recycling DsbA	• ✓
• DsbC	• Thiol:disulphide oxidase and isomerase	• ✓
• DsbD	• Thiol:disulphide reductase	• ?
• DsbE	• Thiol:disulphide reductase	• ✕
<u>Protease/Chaperones</u>		
• HtrA/DegP	• Serine protease	• ✓
• Skp/OmpH	• Involved in the folding of OMPs	• ✕
• EcpD	• <i>E. coli</i> K12 PapD-like chaperone, assembly of pili	• ✕
<u>Sensors</u>		
• CpxA	• Histidine kinase sensing protein misfolding in the cell envelope	• ?
• RcsC	• Histidine kinase involved in capsular polysaccharide synthesis	• ?
• RseB	• Sensor/chaperone (?). Interacts with RseA	• ✕
• RseC	• Positive regulator of σ^E . Anchored in inner membrane	• ✕
<u>Regulators</u>		
• σ^E	• Sigma factor transcribing its own gene, <i>htrA</i> and <i>rpoH</i>	• ✕
• RseA	• Anti- σ -factor in the inner membrane	• ✕
• CpxR	• Response regulator activated upon phosphorylation by CpxA	• ?
• PrpA	• Phosphoprotein phosphatase modulating CpxR:CpxA phosphorylated status	• ✕
• PrpB	• Phosphoprotein phosphatase homologous to PrpA	• ✕

Key to table: ✓ -present; ✕ -absent; ? -difficult to assign.

to enable binding both to specific DNA sequences and to allow interaction with RNA polymerase (Kondo *et al.*, 1997; Martinez-Hackert and Stock, 1997a). Given the similarity between the two proteins, it can be tentatively put forward that, along with other members of the OmpR family of response regulators, the mechanism of folding, DNA binding and interaction with RNA polymerase maybe similar, despite the proteins probably having very different roles. RegX4 has the highest homology to the *H. pylori* OmpR-like protein and RacR from *C. jejuni* but the result of homology searches failed to identify a probable function for the regulator.

The histidine kinase, RegY4, fits within the overall framework of most other “orthodox” sensor kinases, for example EnvZ, PhoR and CpxA (Stock *et al.*, 1989a; Parkinson and Kofoed, 1992). RegY4 shares all the basic architectural features with most other sensor kinases, including integral membrane components (TM1 and TM2) and substantial *N*-terminal periplasmic and *C*-terminal cytoplasmic domains separated by a putative linker (Stock J.B. *et al.*, 1989; Parkinson and Kofoed, 1992; Park and Inouye, 1997). The *C*-terminal transmitter domain contains all of the conserved “box” motifs common to the sensor family, H, N, D/F, G1 and G2 including most of the so-called “critical” amino acids. Although in common with other kinases, amino acids within these box regions vary (Parkinson and Kofoed, 1992). There is also a putative X-region between the H and N-boxes which is reported to be weakly conserved amongst two-component regulators (Hsing *et al.*, 1998). A search of the databases revealed that RegY4 has most similarity to the histidine protein kinase, RacS from *C. jejuni*, identified in our laboratory (Brás and Ketley, unpublished) and *H. pylori* HP0164-0165 but failed to identify a function or a putative ligand sensed by the protein. RegY4 is very similar to other sensor kinases but there is still no hint as to the function of the molecule.

9.4: Does the putative RegX4/RegY4 system regulate expression of *htrA*?

At the beginning of the project, partial sequence data downstream of *htrA* hinted at the presence of a putative two-component regulatory system. The two-component system, RegX4/RegY4, was subsequently investigated on the basis that there may be putative relationship between the two-component system and *htrA*. Unfortunately, the information that alluded to this relationship relied heavily on circumstantial evidence. For example, *htrA*

in *E. coli* is regulated by a two-component system, there is a two component system downstream of *htrA* in *C. jejuni* and that two-component systems often regulate genes in their vicinity. Nevertheless, it was felt that the putative relationship warranted further study. Efforts to resolve the relationship between *htrA* and the RegX4/RegY4 system are only at a preliminary stage and further investigation is required. To this end, experimental analysis can take the form of DNA binding assays using the *htrA* promoter and purified RegX4 and expression from the *htrA* promoter in a RegY4 mutant (pMW10HTRA in a *C. jejuni* 480 RegY4 mutant). Furthermore, a particularly informative assay may be in the determination of the signal(s) which induces expression of either *htrA* and *regX4* promoters, in a pMW10 based assay, to identify whether they are the same.

9.5: Do RegX4 and RegY4 form a cognate pair?

The identification of RegX4 and RegY4 raises questions about whether they actually form a cognate two-component partnership. The genetic linkage of the *regX4/regY4* genes, i.e. the overlapping reading frames, together with the pronounced similarity of RegX4 and RegY4 to other bacterial response regulators and histidine protein kinases, respectively, would suggest that they are a cognate system although experimental evidence is required to confirm this. Obviously, there is a necessity to identify the function of the two proteins within *C. jejuni*. An examination of the databases failed to identify any putative function for the pair although this kind of analysis can be inherently difficult as there are numerous examples of response regulators and histidine kinases on the databases with very similar degrees of homology to one another. To predict a function based solely on these is problematic. Therefore, there is more of a reliance on data from studies on mutational analyses of the proteins involved. All attempts to mutate RegX4 failed although a RegY4 mutant was readily obtained. Why there should be a difference in the ability to produce a RegX4 mutant and RegY4 mutant can only be speculated upon. As suggested before, RegX4 might regulate a particular gene, or a distinct set of genes, which are essential for the continued survival of the organism under the conditions used. Therefore, it follows that RegY4 is involved in regulating the same set of genes, assuming that the two genes form part of the same two-component regulatory system. Clearly, the effects of a mutation in RegY4 are far less severe than a similar mutation in RegX4 suggesting a mechanism of suppression. The ability to produce a RegY4 mutant could also be due to the fact that RegX4 acts as transcriptional activator in its non-phosphorylated

form and does not require phosphorylation by RegY4 to adapt to conditions used in the study. An alternative scenario is that phosphorylation of RegX4 is required but that in a RegY4 mutant, the phosphorylation occurs via cross-talk by an alternative protein kinase. Cross-talk among two-component systems is well documented *in vivo* and *in vitro* (Wanner, 1992) and in this instance it implies the presence of a second RegY4-like protein, able to interact with RegX4. Given the overall similarity to RegY4, the most likely candidate is RacS, although a further kinase may be involved. The ability to produce RegY4/RacS double mutant may aid identification of the relationship.

The inability to produce a RegX4 mutant and the apparent non-pleiotropic nature of the RegY4 mutant has hindered identification of other members of the regulon. This situation may be alleviated to a degree should the signal controlling the system be identified. For example, it may allow formation of a RegX4 mutant and be used to investigate altered expression of proteins in the RegY4 mutant under the proposed signal. As suggested before in chapters 7 and 8, a change in mutational strategy may be a more rewarding exercise to identify other members of the regulon. For RegX4, this could mean altering the level of expression of the protein instead of abolishing it completely as is predicted in a RegX4 null mutant. For the kinase, a null mutant will have both kinase and any phosphatase activity completely abrogated. A mutation rendering the molecule constitutively active or inactive due to an alteration in these properties and possibly a change in conformation may alter expression of genes under the putative system's control.

9.6: Genome sequence analysis of TCR systems in *C. jejuni*

Data concerning the two-component system identified in this study can now be compared with the new *C. jejuni* NCTC 11168 genomic sequence to bring into context, information regarding the presence of other two-component regulators in campylobacters.

Following on from work performed by Marchant, J. E., (1998), who examined the genome for genes encoding factors involved in chemotaxis, it appears that *C. jejuni* NCTC 11168 contains only one isolated CheY regulatory gene. Additional CheY domains have been found in composite genes, for example, CheAY (*N*-terminal CheA kinase fused to a *C*-terminal CheY domain) and CheV (CheW/Y fusion with an *N*-terminal CheW homologue fused to a *C*-terminal CheY domain). The CheAY composite gene is the only kinase thought to be

involved in chemotaxis.

Furthermore, following on from work performed by Dr Ana Brás (*pers comm*), there are 5 response regulator/histidine protein kinase systems, believed to be cognate partners due to their genetic linkage, two of which are RegX4/RegY4 and RacR/RacS. The others have been termed, RegX5/HPK5, RegX7/HPK7, RegX8/HPK8. The remaining members comprise of 3 uncoupled response regulators, RegX3, RegX9 and Reg10 and one uncoupled histidine protein kinase, HPK6. HPK6, although unpaired may partner one of the uncoupled regulators. Of the 8 response regulators, only RegX9 and RegX10 are not OmpR-like. RegX9 is NtrC-like and RegX10 is difficult to classify. An alignment of the 6 OmpR-like response regulators, including RegX4, *E.coli* CheY and OmpR is given in figure 9.2. The similarity of the OmpR-like regulators is also given in table 9.2.

Table 9.2:

Homology scores of <i>C. jejuni</i> OmpR-like response regulators against RegX4		
Designated gene name	Identity to RegX4 (%)	Similarity to RegX4 (%)
• <i>racR</i>	• 48	• 67
• <i>regX3</i>	• 30	• 52
• <i>regX5</i>	• 29	• 50
• <i>regX7</i>	• 26	• 45
• <i>regX8</i>	• 27	• 50

As with RegX4, the other OmpR-like regulators fit within the organisational framework of both CheY and OmpR suggesting that they may be mechanistically similar although have very different roles. Interestingly, RegX3 has a D to N substitution at the proposed phosphoaccepting position. This is not a sequencing error as this difference has been independently confirmed (Henderson, 1996). The significance of this change has yet to be established. From table 9.2. it can be seen that RegX4 has its highest similarity with RacR (48% identity and 67% similarity). Whether this has any functional significance remains to be established. Of the 6 kinases, RegY4, RacS, HPK5 and HPK7 appear to be “orthodox” integral membrane proteins similar to EnvZ and have the conserved sequence motifs and architectural features of most other sensor kinases. An alignment of the proposed “orthodox” kinases is given in figure 9.3. The other kinases HPK6 and HPK8 have no obvious transmembrane domains which may indicate a cytoplasmic location for the proteins. From

Figure 9.2: Sequence alignment of RegX4 with other OmpR-like response regulators in *C. jejuni*. The proteins are designated; RegX4, RacR, RegX5, RegX7, RegX3, RegX8. The order reflects their relatedness. An * denotes conserved residue, : denotes a conserved residue and . denotes semi-conserved residue for the alignment given. Also included are CheY (ECCHEY) and OmpR (ECOMPR), which have determined structure and highlight a number of important features specific to the *N*-terminus and *C*-terminus of response regulators (Stock J.B. *et al.*, 1989; Martinez-Hackert and Stock, 1997b; Kondo *et al.*, 1997). CheY is included to allow an alignment of homologous *N*-terminal domains of the response regulator proteins. A boxed grey shaded area highlights residues that correspond to those which form the hydrophobic core of CheY. Clusters of four hydrophobic residues correspond to the three internal β -strands of CheY, and characteristically spaced hydrophobic residues correspond to the internal faces of amphipathic α -helices that flank the β -sheet. Highly conserved residues which correspond to the acidic pocket of CheY (Asp-13, Asp-53 and Lys-109) are shown. The secondary structure of CheY is indicated below the sequence, with arrows representing β -strands, shaded boxes representing α -helices and lines representing loop regions. OmpR is included to allow an alignment of the OmpR-DNA binding domain and putative DNA binding domains of the response regulators. Secondary structural elements defined by the crystal structure of OmpRc are given above the amino acid sequence. Highly conserved to completely conserved amino acid positions are highlighted by grey circles. The proposed functional elements are also given; the recognition helix, the two wings, W1 and W2 and the α -loop (RNA polymerase interaction). Hydrophobic core residues specific to OmpR are marked by unshaded boxes in the alignment.

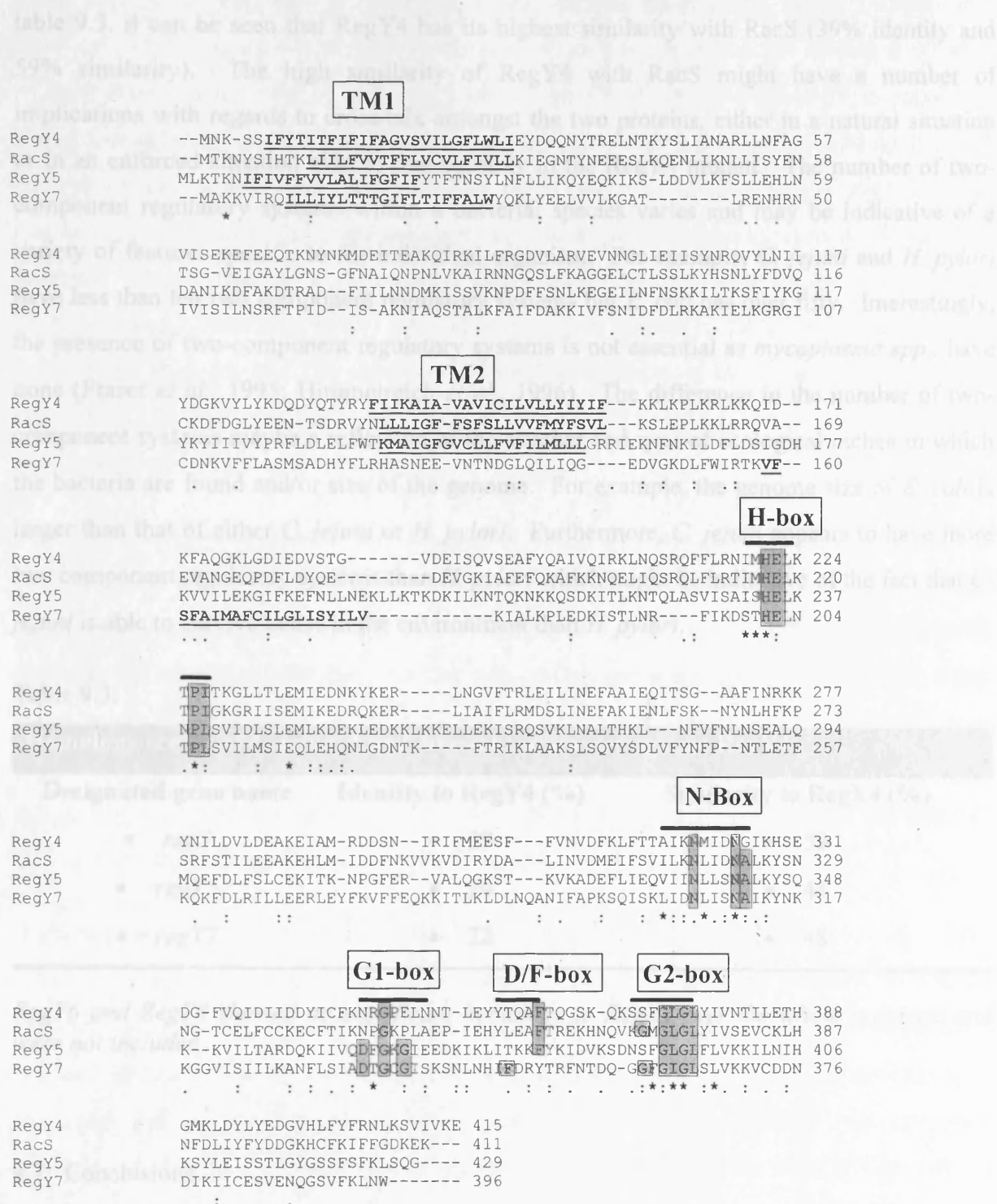


Figure 9.3: Alignment of RegY4 together with similar sensor kinases from *C. jejuni*. Regions conserved in all histidine protein kinases are shown in outlined grey boxes together with individual “box” motifs; H-box, N-box, G1-box, D/F-box G2-box (Stock J.B. *et al.*, 1989; Parkinson and Kofoid, 1992). The proteins used in the alignment are denoted as follows: RegY4, RacS, RegY5 and RegY7. The putative transmembrane regions are shown in underlined, bold type-face, TM1 And TM2. “*” denotes identical or conserved residues in all sequences of the alignment, “:” denotes conserved substitution, “.” denotes semi-conserved substitution.

table 9.3. it can be seen that RegY4 has its highest similarity with RacS (39% identity and 59% similarity). The high similarity of RegY4 with RacS might have a number of implications with regards to cross-talk amongst the two proteins, either in a natural situation or in an enforced situation akin to that possibly in the RegY4 mutant. The number of two-component regulatory systems within a bacterial species varies and may be indicative of a variety of features specific to the individual organism. For example, *C. jejuni* and *H. pylori* have less than ten two component regulatory systems but *E. coli* has over fifty. Interestingly, the presence of two-component regulatory systems is not essential as *mycoplasma spp.*, have none (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). The difference in the number of two-component systems maybe a reflection of the number and type of ecological niches in which the bacteria are found and/or size of the genome. For example, the genome size of *E. coli* is larger than that of either *C. jejuni* or *H. pylori*. Furthermore, *C. jejuni* appears to have more two component regulatory systems than *H. pylori* which might be indicative of the fact that *C. jejuni* is able to survive better in the environment than *H. pylori*.

Table 9.3:

Homology scores of *C. jejuni* EnvZ-like sensor kinases against RegY4

Designated gene name	Identity to RegY4 (%)	Similarity to RegX4 (%)
• <i>racS</i>	• 39	• 59
• <i>regY5</i>	• 24	• 44
• <i>regY7</i>	• 22	• 48

RegY6 and *RegY8* showed no significant homology to *RegX4* over the whole sequence and were not included.

9.7: Conclusions

The aims of the study were to complete the upstream sequence of *htrA*, demonstrate expression from the gene and investigate the type of regulation which the gene is under. For the latter, particular emphasis was placed on characterising the putative two-component regulatory system downstream of *htrA*.

The 5' sequence of *C. jejuni htrA* was identified and aided confirmation that the gene was indeed the true *htrA* gene and that it was a member of the HtrA family of serine proteases. Furthermore, expression of the gene was successfully demonstrated. *C. jejuni* HtrA is likely

to be periplasmic and play a role in relieving stresses encountered in the bacterial cell envelope. In *E. coli*, the periplasmic or extracytoplasmic stress response is mediated in both a σ^E -dependent manner and by the Cpx pathway with both systems involved in regulating expression of HtrA. *C. jejuni* *htrA* is not regulated in a σ^E -dependent manner although within the regulatory network that includes *htrA* there could be similar genes involved. An alternative possibility is that regulation may involve a system homologous to the *E. coli* Cpx pathway. Examination of the *C. jejuni* NCTC 11168 genome sequence for members of either of these systems failed to provide any certainties with regards to the presence of particular genes. This suggests that the overall extracytoplasmic response to stress, that may involve HtrA, may be largely different to the situation seen in *E. coli*. This will only really be determined upon further characterisation.

The involvement of CpxR/A in *htrA* expression and in relieving extracytoplasmic stress in *E. coli* was particularly relevant to this study as a similar two-component system was identified downstream of *htrA* in *C. jejuni*. The possibility that there was a functional relationship between the three genes meant that the two-component system warranted further study. Preliminary characterisation of the two-component system, RegX4/RegY4, was performed but was hampered by the fact that, all efforts to construct a mutation in the regulator, RegX4, failed suggesting that the mutation was lethal. Nevertheless, a RegY4 mutant was obtained allowing preliminary examination of its phenotype.

With respect to the involvement of RegX4/RegY4 in *htrA* regulation, there is yet no data alluding to a relationship, although mechanisms are now in place whereby a putative association can be investigated. A tentative relationship between the respective components is outlined in figure 9.4. If no relationship is identified, it is likely that a different type of regulatory control may be present which could take the form of another two-component regulatory system or a different type of regulation altogether. With the onset of new genome sequence data there is an opportunity to investigate the input from a variety of regulators in control of *htrA* expression and other genes of interest with a view to gaining an insight into the mechanisms of regulation of *C. jejuni* genes.

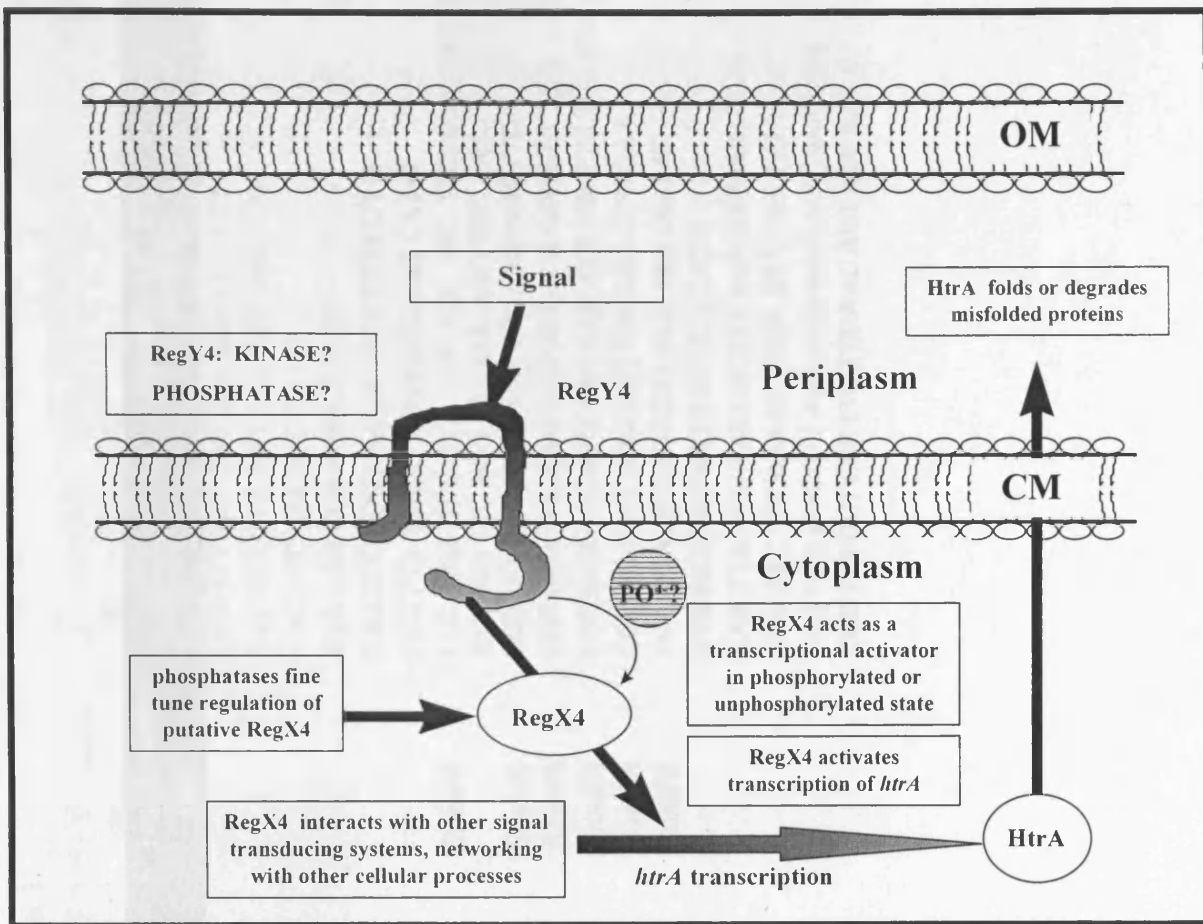


Figure 9.4: Outline of the proposed function of the RegX4/RegY4 system and the putative relationship with *htrA*. OM: Outer membrane, CM: cytoplasmic membrane

Appendix 1

Table A1.1: Primers used in this study.

Primer name	Primer Function	5' restriction site	Sequence (5'-3')
P1L	Sequencing across pUC19/pBluescript MCS region		GGG TTT TCC CAG TCA CGA CGT TGT
P2L	As above		TAT GTT GTG TGG AAT TGT GAG CGG
M13 Forward	Sequencing of cloned inserts in pUC19/pBluescript		AAC AGC TAT GAC CAT G
M13 Reverse	As above		GTA AAA CGA AGG CCA GT
CAT15'	Sequencing out from the Cm ^R gene (5' end)		TAG TGG TCG AAA TAC TCT TTT CGT G
CAT13'	As above (3' end)		CCC TTA TCG ATT CAA GTG CAT CAT G
MYC-2	IPCRM to create HtrA::c-myc fusion in pME12	<i>Bam</i> HI	CGC GGA TCC TTA TAA ATC TTC TTC AGA AAT TAA TTT TTG TTC AGG ATT TCC AAG TGC AAA
HTRAP1	<i>dnaJ</i> -like/ <i>htrA</i> intergenic region to create pME26	<i>Bam</i> HI	CGC GGA TCC GCT AAC TCC AAG AGT TTC G
HTRAP2	As above	<i>Bam</i> HI	CGC GGA TCC TAG CCG GTT GCA GTC GAT TCG
HTRA-MET	5' <i>htrA</i> cloning from methionine start	<i>Bam</i> HI	CGC GGA TCC ATG AAA AAG ATT TTT TTA TCA TTA G
HTRA6	<i>htrA</i> IPCRM and <i>htrA</i> containing <i>Bgl</i> II fragment formation	<i>Bam</i> HI	CGC GGA TCC AAG GAT TTC CAA GTG CAA
HTRA7	As above and creation of HtrA::c-myc fusion in pME12	<i>Bam</i> HI	CGC GGA TCC AGT GTT ACA AGT GGG AT
HTRA8	<i>htrA</i> sequencing		ATG GCA TAG GTT TTG CCA TAC C
HTRA12	As above		AAA TTA TAT TGG AAC TTT AGA GAT TGG
HTRA13	As above		CCA ATC TCT AAA GTT CCA ATA TAA TTT
HTRA14	As above		GGT GAT AGT GTT AAA GAA AAG AGC AAA GGG
HTRA15	As above		CCC TTT GCT CTT TTC TTT AAC ACT ATC AAC C

Primer name	Primer function	5' Restriction site	Sequence (5'-3')
HTRA17	<i>htrA</i> cloning (pME3)	<i>Bgl</i> II	GGA AGA TCT GGG TGA TTA TTT CAA AAG ATG G
HTRA18	<i>htrA</i> cloning (pME3)	<i>Pst</i> I/ <i>Eco</i> RI	AAA ACT GCA GAA TTC TTA TTT AAG CAC AAG CAA AGT CGC C
HTRAD-SEQ1	<i>htrA</i> sequencing from IPCR <i>Bgl</i> II fragment		GAG CTT GGA GCA GAT GAT TAC C
HTRAU-SEQ1	As above		GCT TTA TAT TCT ATG TCG CTT CC
R4U1	cloning of <i>htrA/regY4</i> fragment to create pME7	<i>Pst</i> I	AAC TGC AGC ATC TAT ACT TTT ATT ACT GG
R4D1	As above	<i>Kpn</i> I	GGG GTA CCT TAC TAA AGT TAA TGA TAA GG
R4IU1	IPCRM of pME7 to create pME11	<i>Bam</i> HI	CGG GAT CCT CTT TGC CAG GGC TTG ATG
R4IU3	As above	<i>Bam</i> HI	CGC GGA TCC AAT GAT AAG CTG ATA TTC
R4IU2	IPCR to create next <i>Bgl</i> II fragment downstream of <i>htrA</i> containing <i>Bgl</i> II fragment. creation of pME23/pME24	<i>Bam</i> HI	CGG GAT CCC TTT CAT GGT AAT AAT ATG C
R4ID2	As above	<i>Bgl</i> II	GAA GAT CTG AGC TTA CTT TAA CTA ATG C
R4D-SEQ1	Sequencing of pME23		AGA GGG TGG AGT GGT AAG TCG
R4D-SEQ2	As above	<i>Kpn</i> I	TAA CAG AAG CAA AGC AGA TCC G
R4D-SEQ3	As above		AAA TAT TAT GCA TGA ACT CAA AAC
R4D-SEQ4	As above		TTG ATA TTA TAG ATG ATT ATA TTT G
H4-MET	Cloning of <i>regY4</i> to create pME17	<i>Kpn</i> I	CGG GGT ACC ATG AAT AAA TCT TCG ATT T
H4-STOP1	As above	<i>Pst</i> I	AAA ACT GCA GTT ACT CCT TAA CAA TAA CA
H4ID1	IPCRM of pME17 to create pME18	<i>Bam</i> HI	CGC GGA TCC CTC AAA ACT CCT ATT ACT AAG
H4IU1	As above	<i>Bam</i> HI	CGC GGA TCC TTG GTT TAG CTT TCG AAT TTG
LACRI	Binds to 5' LacZ sequence (used in pMW10 analyses).		AAA GGT ACC GTT GTA AAA CGA CGG CCA GT

Appendix 2.

Table A2.1: Plasmids constructed and utilised during this study.

Plasmid	Derivation	Reference.
PUC19	High copy number cloning vector used in <i>E. coli</i> DH5 α (figure A3.1).	(Yanisch-Perron <i>et al.</i> , 1985)
pBluescript SK-	High copy number cloning vector used in <i>E. coli</i> DH5 α (figure A3.1).	(Short <i>et al.</i> , 1988)
pAV35	pBluescript SK ⁻ derivative containing a <i>Campylobacter</i> Cm ^R gene cassette (Wang and Taylor, 1990a), bracketed by six restriction sites. The primers used in construction were RAA17 and RAA18 (Yao <i>et al.</i> , 1993). The chloramphenicol cassette is inserted into the <i>Bam</i> HI site of the original pBluescript vector (figure A3.2).	(van Vliet, <i>et al.</i> , 1998)
pMW10	LacZ transcriptional assay vector (figure A3.2).	(Wösten <i>et al.</i> , 1998)
pTNS#A	pBluescript vector containing 750bp of the <i>C. jejuni</i> <i>flaA</i> interrupted by the <i>apha-3</i> gene (Trieu-Cuot <i>et al.</i> , 1985) (figure A3.3).	(Wassenaar <i>et al.</i> , 1991)
pJMK30	<i>C. coli</i> <i>apha-3</i> gene (Trieu-Cuot <i>et al.</i> , 1985) inserted between two complete polylinkers (figure A3.3).	(van Vliet <i>et al.</i> , 1998)
pAV110	pBluescript derivative containing a promoterless <i>Campylobacter</i> Cm ^R gene cassette (Δ cat). The primers used in construction were RAA18 (Yao <i>et al.</i> , 1993) and Δ catF2 (ATTATTAGGATCCTGCAGCTGACGGAGGATAAATGATGCAATTC), a variation of the upstream primer described by Dickinson <i>et al.</i> , (1995) (figure A3.4).	(van Vliet, unpublished)
pAV123	<i>C. coli</i> <i>apha-3</i> gene (Km ^R) from pJMK30 inserted into <i>Kpn</i> I site of pAV110 (figure A3.4).	
pME3	pUC19 containing ~1.1kb fragment of <i>htrA</i> . The insert was amplified using HTRA17 and HTRA18.	This study
pME7	pUC19 containing ~ 1.0kb fragment (350bp 3' <i>htrA</i> , 76bp intergenic region, 580bp 5' <i>regX4</i> . The insert was amplified using R4D1 and R4U1.	This study
pME11	pME7 disrupted by IPCRM using R4IU1 and R4IU3. The primer combination introduces a unique <i>Bam</i> HI site and a 9bp deletion to remove the conserved aspartate residue of <i>regX4</i> .	This study
pME12	pME3 disrupted by IPCRM using MYC-2 and HTRA7. The primer combination introduces a unique <i>Bam</i> HI site and the c-myc epitope.	This study
pME13	<i>Campylobacter</i> Cm ^R gene from pAV35 inserted into the <i>Bam</i> HI site of pME11; same orientation as <i>regX4</i> transcription	This study
pME14	As pME13 but <i>Campylobacter</i> Cm ^R gene in opposite direction	This study

Plasmid	Derivation	Reference.
pME15	<i>Campylobacter</i> Cm ^R gene from pAV35 inserted into the <i>Bam</i> HI site of pME12; same orientation as transcription of <i>htrA</i>	This study
pME16	As pME12 but <i>Campylobacter</i> Cm ^R gene in opposite direction	This study
pME17	pBluescript containing the complete ORF (~1.2 kb) of <i>regY4</i> . The insert was amplified by using H4-MET and H4-STOP.	This study
pME18	pME17 disrupted by IPCRM using H4IU1 and H4IU2. The primer combination introduces a unique <i>Bam</i> HI site and a 36 bp deletion to remove the conserved histidine residue of <i>regY4</i> .	This study
pME19	<i>Campylobacter coli apha-3</i> gene (Km ^R) from pJMK30 inserted into <i>Bam</i> HI site of pME18 in the reverse orientation to transcription of <i>regY4</i>	This study
pME20	As pME19 but <i>C. coli apha-3</i> gene (Km ^R) inserted in the same orientation as <i>regY4</i>	This study
pME21	pME3 disrupted by IPCRM using HTRA6 and HTRA7. The primer combination introduces a unique <i>Bam</i> HI site into the <i>htrA</i> gene.	This study
pME22	As pME3 but with a <i>Campylobacter</i> Cm ^R Km ^R gene cassette from pAV123 inserted into <i>Bam</i> HI site; same orientation as <i>htrA</i> transcription to create transcriptional fusion.	This study
pME23	<i>Bgl</i> II fragment downstream of the <i>htrA</i> containing <i>Bgl</i> II fragment cloned into pBluescript. The insert was generated following IPCR on <i>Bgl</i> II chromosomal loops using R4IU2 and R4ID2 and cloned into the <i>Bam</i> HI site of the vector. The fragment contains 3' <i>regX4</i> and the complete ORF of <i>regY4</i> in the same orientation as <i>lacZ</i> .	This study
pME26	pBluescript containing a 323bp DNA fragment of the <i>dnaJ</i> -like and <i>htrA</i> intergenic promoter region. The <i>htrA</i> promoter region is in the reverse orientation to <i>lacZ</i>	This study
pMW10HTRA	pMW10 derivative containing the intergenic insert from pME26 cloned using <i>Bam</i> HI. The putative <i>htrA</i> promoter is in the same orientation as Δ <i>lacZ</i> . β -galactosidase activity was used as a measure of <i>htrA</i> promoter activity	This study
pMW10DNAJ	As above but <i>dnaJ</i> -like gene promoter is in the same orientation as Δ <i>lacZ</i>	This study

Appendix 3: Diagrammatic representation of general plasmids used in this study.

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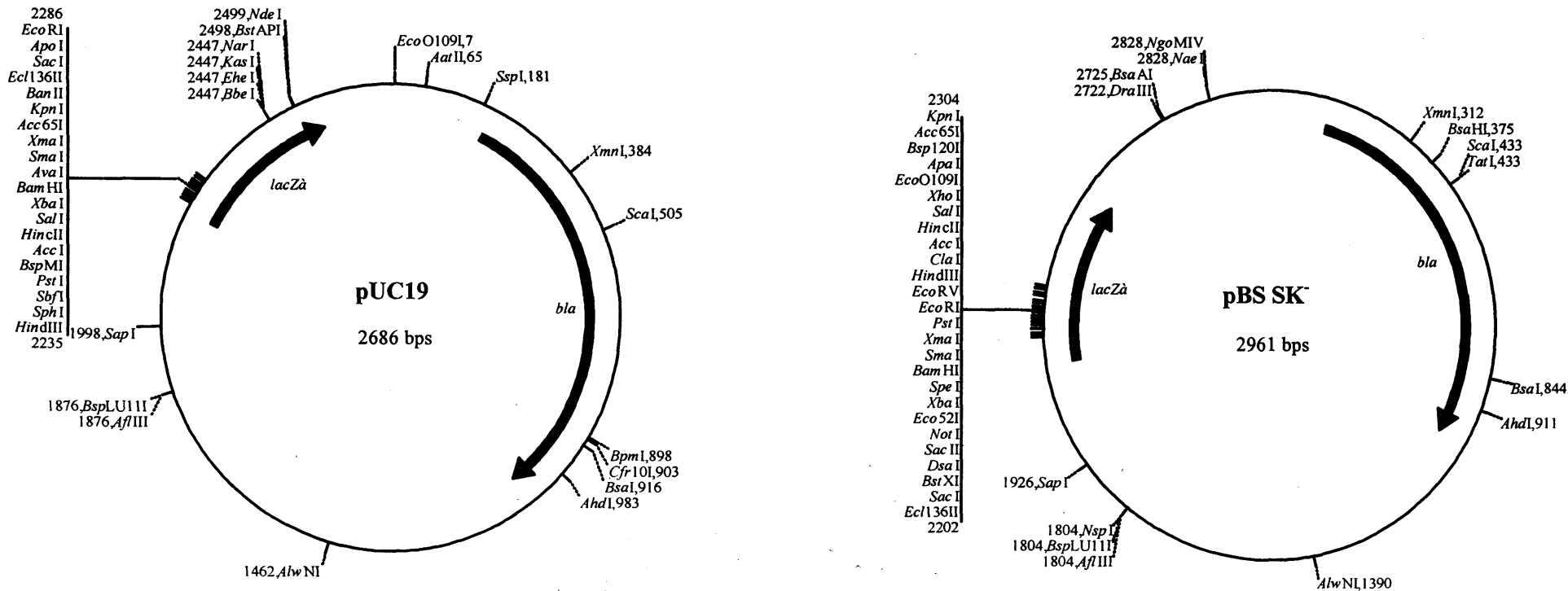


Figure A3.1: Diagrammatic outline of pUC19 and pBluescript SK⁻. Grey arrows represent genes. Restriction sites are indicated.

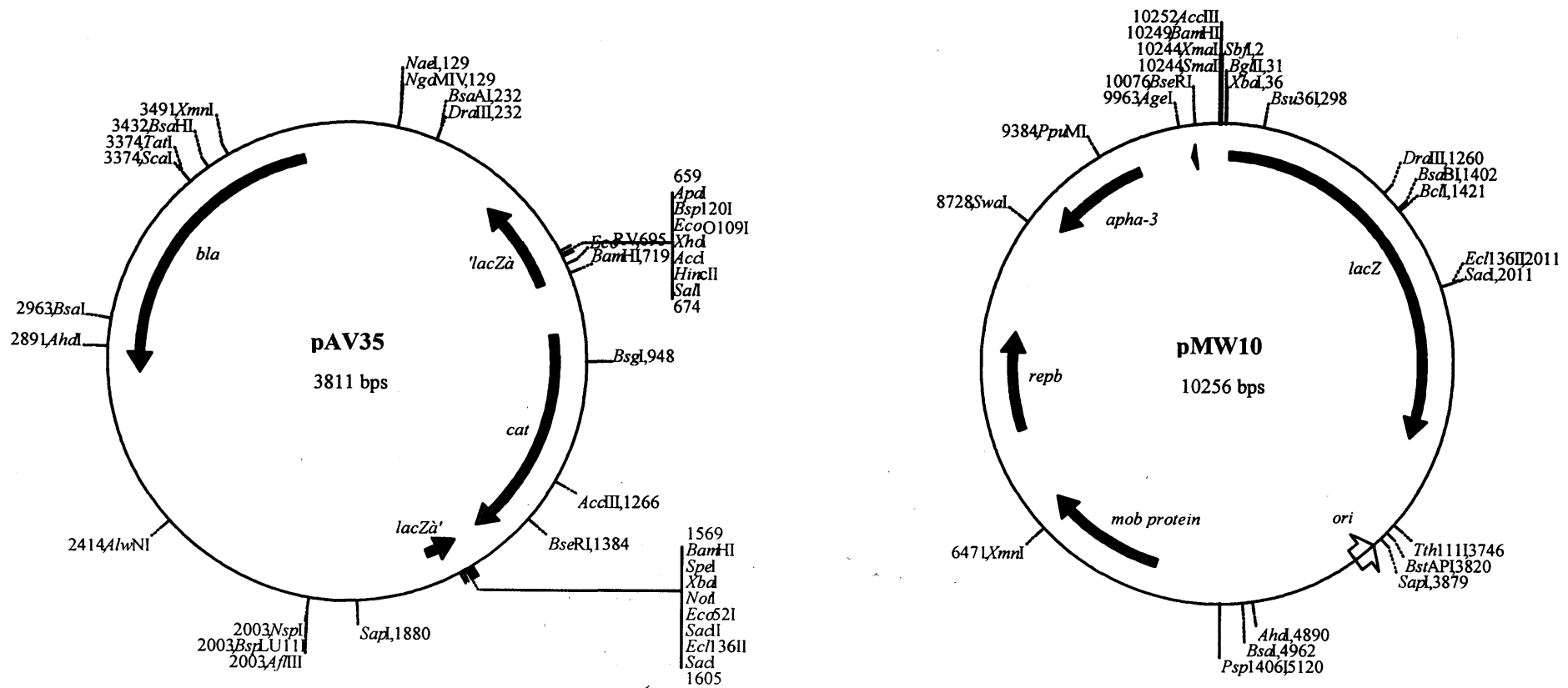


Figure A3.2: Diagrammatic outline of pAV35 and pMW10. Grey arrows represent genes. Restriction sites are indicated.

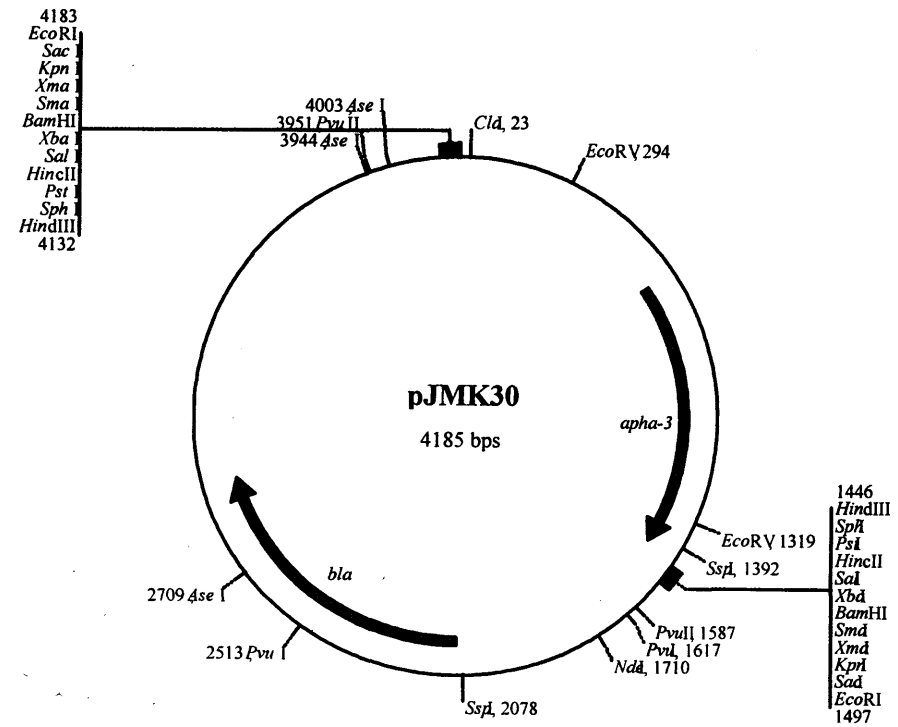
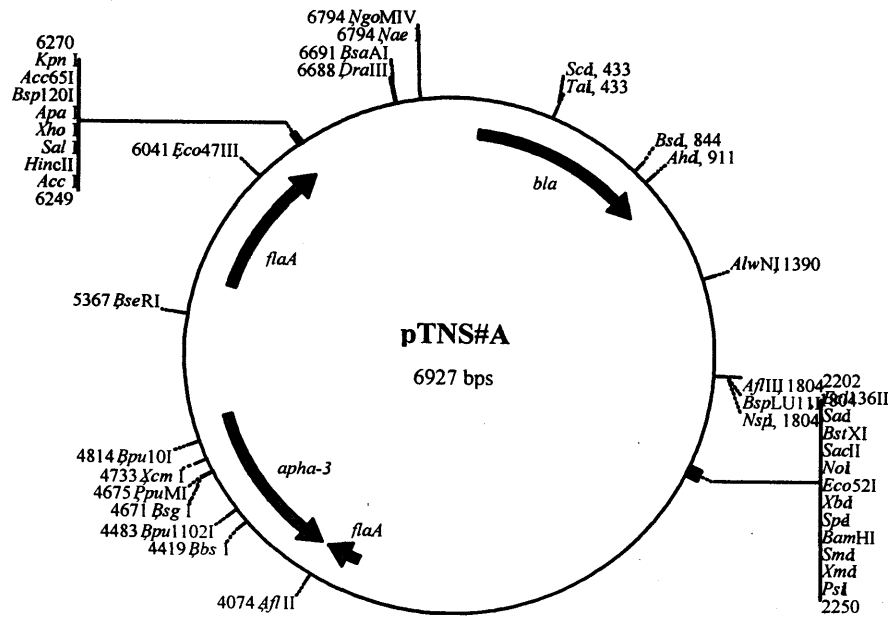


Figure A3.3: Diagrammatic outline of pTNS#A and pJMK30. Grey arrows represent genes. Restriction sites are indicated.

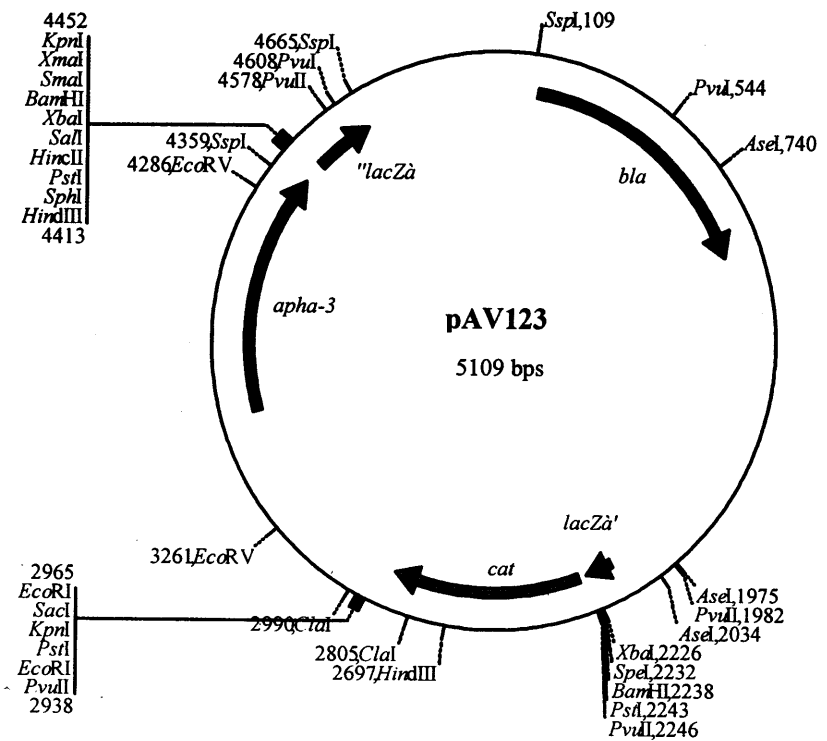
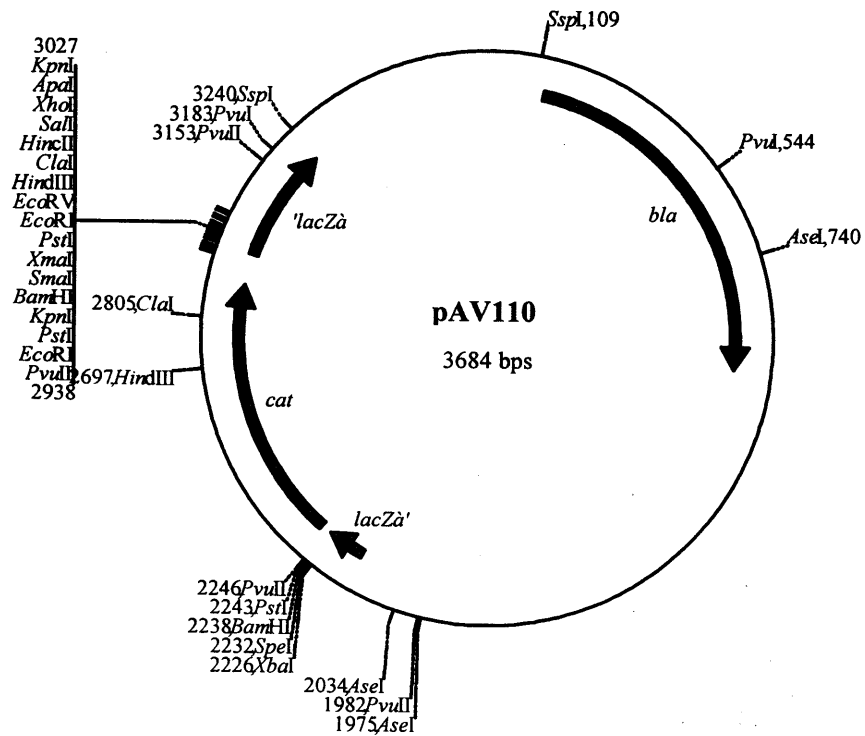


Figure A3.4: Diagrammatic outline of pAV110 and pAV123. Grey arrows represent genes. Restriction sites are indicated.

Appendix 4

Table A4.1: Abbreviations for nucleotide sequence.

Nucleotide	IUPAC code
A or C	M
A or G	R
A or T	W
C or G	S
C or T	Y
G or T	K
C or G or T	B
A or C or T	H
A or C or G	V
Any	N

Table A4.2: Abbreviations for amino acid sequence.

Amino acid	Single letter code
<i>Alanine</i>	A
<i>Arginine</i>	R
<i>Asparagine</i>	N
<i>Aspartic acid</i>	D
<i>Asparagine or aspartic acid</i>	B
<i>Cysteine</i>	C
<i>Glutamine</i>	Q
<i>Glutamic acid</i>	E
<i>Glutamine or glutamic acid</i>	Z
<i>Glycine</i>	G
<i>Histidine</i>	H
<i>Isoleucine</i>	I
<i>Leucine</i>	L
<i>Lysine</i>	K
<i>Methionine</i>	M
<i>Phenylalanine</i>	F
<i>Proline</i>	P
<i>Serine</i>	S
<i>Threonine</i>	T
<i>Tryptophan</i>	W
<i>Tyrosine</i>	Y
<i>Valine</i>	V

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